Dietary folate intake:
is there a need for folic acid fortification in the Netherlands?

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Netherlands?
Thesis Wageningen University, the Netherlands – with summary in Dutch

Abstract

Introduction: Sufficient intake of folate, a B-vitamin, prevents macrocytic anemia and lowers the risk of neural tube defects. This thesis studied whether Dutch diets provide enough folate to meet folate requirements and describes how to deal with folic acid fortification. To be able to study this, we assessed if gender and bioavailability influence folate requirements. In addition, we studied if vitamin B12 fortification, combined with folic acid fortification would be desirable.

Results: In a 3y randomized trial, the erythrocyte folate concentration increased 10% less (143 nmol/L less, [95%CI 46, 241]) in 294 men than in 112 women given 800 μg of folic acid/day. In a second 12w randomized trial with 187 men and 129 women, the erythrocyte folate response to 50-800 μg of folic acid/day was, on average, 5% lower (47nmol/L lower, [95%CI 7, 88]) in men than in women. The larger body size of men largely explained the lower response.

In a 4w dietary trial, we fed parallel groups diets either high in dietary folate (n=30) or low in folate with 100 μg, 200 μg or 300 μg of folic acid as a supplement (n=15 per group). We found that the bioavailability of dietary folate was 82% relative to folic acid from supplements.

In a 12w randomized feeding trial, we fed elderly individuals bread fortified with 138 μg of folic acid and 9.6 μg vitamin B12/day (n=72) or unfortified bread (n=70). Fortified bread improved serum folate concentrations by 45% (mean increase 6.3 nmol/L; 95%CI 4.5-8.1 nmol/L) and serum vitamin B12 concentrations by 49% (102 pmol/L; 95%CI 82-122 pmol/L). The proportion of individuals with marginal serum vitamin B12 status decreased from 8% at the start of the study to 0% after 12 weeks.

Discussion: Men had higher folate requirements than women and thus dietary reference intakes should be higher for men. Overall, folate reference intakes can be lowered because dietary folate is 82% bioavailable instead of 50%, which is currently assumed. We adapted the Dutch dietary reference intakes to these findings and compared published Dutch folate intake with these new references. Folate intake was still too low for 50% of men and 25% of women, but better than previously reported.

Bread fortified with modest amounts of folic acid and vitamin B12 will prevent a considerable proportion of vitamin B12 deficiency in older people. Yet, other possible consequences of increased folic acid intake should be considered, namely that it may protect against the initiation of cancer, but that it may also facilitate the growth of existing lesions. Therefore, folic acid fortification should not be implemented in the Netherlands.

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General
Introduction

Introduction

Folate is a B-vitamin present in our daily diet. Green leafy vegetables, bread, fruits and dairy products are important sources of folate (1, 2). Sufficient intake of folate is necessary to prevent macrocytic anemia. High intake of folic acid by women can reduce the risk of neural tube defects in newborns. Additionally sufficient intake of folate has been linked to the prevention of many age-related diseases, such as vascular disease, dementia and cancer (3, 4).

The Recommended Dietary Allowance (RDA) for folate is defined as the level of intake that is adequate to meet the folate requirement of practically all healthy persons (5, 6). In the Netherlands it is 300 μg of folate from food per day for both adult men and women. However, this recommendation is mainly based on metabolic studies in women, and it is unclear if the requirement for men and women is the same.

In the dietary recommendations for folate, the lower bioavailability of dietary folate relative to folic acid is taken into account. It is assumed that bioavailability of dietary folate is 50% relative to folic acid (5). However, this 50% was chosen somewhat arbitrarily since no solid data exist that can be used to adapt the folate requirements to the lower bioavailability of dietary folate relative to folic acid.

Women who could conceive are recommended to ingest 400 μg of folic acid per day in addition to dietary folate from a healthy diet (5, 6). However, not all women take folic acid periconceptionally. Fortification of a staple food with folic acid can be a solution to ensure sufficient intake of folate of women of childbearing age. However, folic acid fortification may be harmful for older adults with a vitamin B12 deficiency, since high intake of folic acid might mask a vitamin B12 deficiency. This possible harm arrested folic acid fortification in several countries (7, 8). Fortification of food with both folic acid and vitamin B12 may be a strategy to overcome this problem, but the effects on folate and vitamin B12 status of the general population have not yet been studied.

Research questions

The central question of this thesis was whether Dutch diets provide enough folate to meet the folate requirements, and if not how to deal with fortification of food with folic acid.

We formulated several research questions that should be answered before we can answer this question:

- Is the requirement for folate the same for men and women?
- What is the bioavailability of dietary folate relative to folic acid?
- To what extent will consumption of bread fortified with folic acid and vitamin B12 improve the folate and vitamin B12 status of older adults?

Function of folate

Folates are a family of compounds. They include folates naturally occurring in foods, and folic acid, a synthetic form used in food fortification and in supplements. An important cellular role of this B-vitamin is that it accepts and donates one-carbon groups: this makes folate important in DNA synthesis, in methylation of DNA, enzymes and hormones and in the interconversion of amino acids (9), (**Figure 1.1A**). Insufficient intake of folate leads to a decrease in the body's folate levels and to elevated concentrations of the amino acid homocysteine and ultimately to the classic deficiency symptom macrocytic anemia (9). Increased homocysteine status and/or low folate status have been associated with increased risk of age-related diseases such as cardiovascular disease, cancer, cognitive decline and bone fractures. However, the role of folate in the prevention of age-related disease is less convincing than folate's role in the prevention of neural tube defects (3, 10). Long-term folic acid supplementation trials are currently underway to further elucidate the role of folate in age-related disease (11-14).

A

B
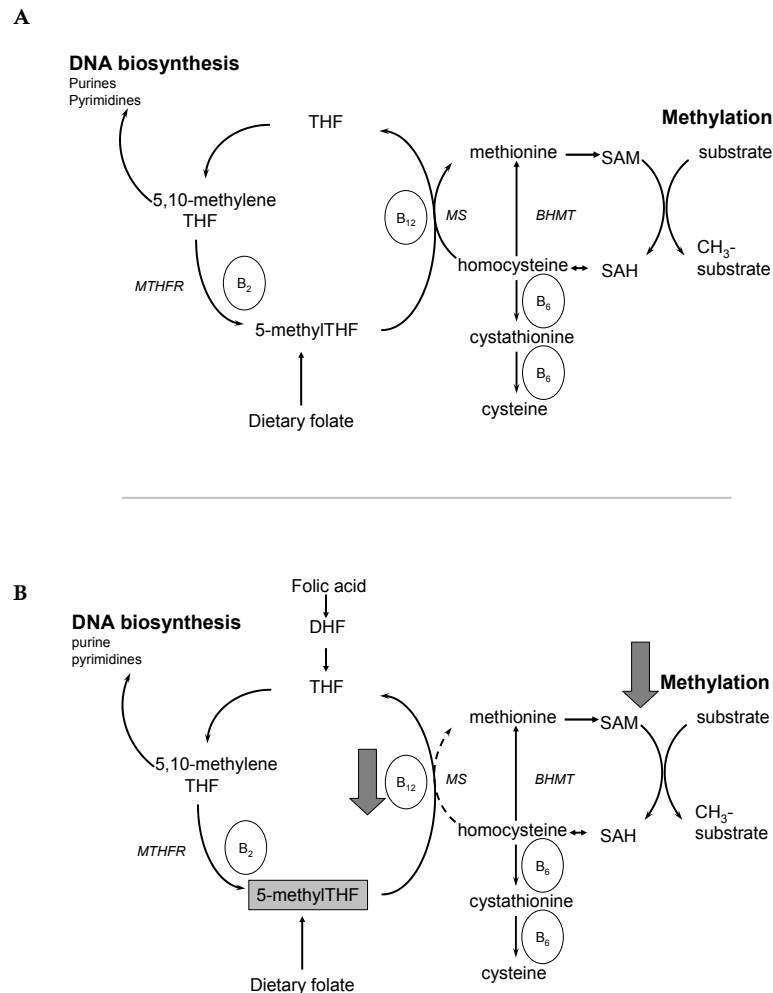
Figure 1.1A <u>Normal folate metabolism</u>. 5-methyltetrahydrofolate from dietary sources is available for the remethylation of homocysteine in a vitamin B12-dependent process. Methionine can be converted to SAM. SAM is a universal methyl group donor for a variety of molecules, e.g. DNA, RNA, enzymes and myelin. THF can be converted into 5,10-methyleneTHF an important factor in DNA-biosynthesis. **1B** <u>Masking of a vitamin B12 deficiency by folic acid</u>. In vitamin B12 deficiency, 5-methylTHF is trapped and cannot function in methylation- or DNA-biosynthesis pathways. Folic acid can partly circumvent this trap and participate in DNA biosynthesis, - thus alleviating anemia-, but not in methylation reactions - thereby maintaining the neurological signs of deficiency. THF = tetrahydrofolate, MTHFR = methylene tetrahydrofolate reductase, B2= vitamin B2, DHF = dihydrofolate, B12 = vitamin B12, MS = methionine synthase, SAM = S-adenosyl methionine, SAH = S-adenosyl homocysteine, CH_3 = methyl-group, BHMT = betaine homocysteine methyltransferase.

Folate requirements

The Recommended Daily Allowance (RDA) for a nutrient is the recommended intake for individuals (5, 15). However, it should not be used to assess the prevalence of inadequate intake for groups; this would lead to a serious overestimation of inadequate intake (16, 17). A better reference intake to evaluate the intake of groups is the Estimated Average Requirement (EAR). This is the nutrient intake estimated to meet the requirement of half of the healthy individuals in a group.

The requirements for folate vary per country. In the Netherlands the estimated average requirement is 200 μg of folate from food per day (5), while in the US it is 320 μg of dietary folate equivalents per day (6). In these two countries, as in most of the world, the requirements for men and women are the same.

The recommendations for folate in the US and Netherlands are expressed as μg folate from food or μg of dietary folate equivalents. In these expressions, the lower bioavailability of naturally occurring food folates relative to folic acid is taken into account.

Folate bioavailability

Folate bioavailability is the proportion of an ingested amount of folate that is absorbed in the gut and that becomes available for metabolic processes (18, 19). In the recommendations for folate, it is assumed that food folate is 50% bioavailable relative to folic acid from supplements. The often cited study by Sauberlich (20) stated that the bioavailability of food folate is no more than 50% of that of folic acid. Unfortunately, the authors did not indicate how they obtained this number and as such it is not definitely known to what extent bioavailability between folic acid and folate differ. Although many factors could influence the bioavailability of dietary folate (21), one important factor could be that about two-third of folate in food is linked to a chain of glutamate residues (2, 21, 22). Enzymes in the brush border of the gut have to cleave this polyglutamate chain from the folate molecule before it can be absorbed (23) (**Figure 1.2**). Inhibition of or limitations to this enzymatic process could influence bioavailability of polyglutamated folates.
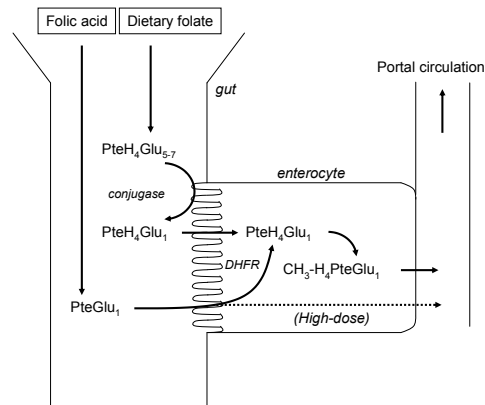
Figure 1.2: intestinal absorption of dietary folate and folic acid. Dietary folates contain a chain of glutamate residues (Glu$_{5-7}$). This glutamate chain is deconjugated by enzymes in the brush border of the gut. The mono-glutamated folate is absorbed and methylated in the enterocyte, if it is not already in the methylated form. Folic acid enters the enterocyte and is reduced by dihydrofolate reductase (DHFR) and methylated. In high doses, folic acid can enter the portal circulation unmetabolized (adapted from (23)).

Strategies to improve folate intake of women of childbearing age

Women of childbearing age are advised to take folic acid supplements in addition to consuming food folate from a varied diet. Several strategies can be used to improve folic acid intake of these women. For example, women in the Netherlands are advised to use folic acid supplements from 4 weeks before to 8 weeks after conception (24). The advantage of this method is that it targets only the women who need the supplements. The disadvantages are that it requires women to take the supplements actively, that not all pregnancies are planned and that not all women know that they should take folic acid. In the Netherlands 20-50% of women who became pregnant reported having taken folic acid in the advised period (25).

Another strategy to improve folic acid intake, used in for example the US, is the fortification of flour products with folic acid. The main advantage of this method is that this intervention reaches almost all women of child-bearing age, since virtually all

people use flour products. An important consequence of fortification is that it is not targeted at these women only: all people will ingest more folic acid, including children and elderly. This is one of the reasons why the Dutch Health Council in 2000 decided against food fortification in the Netherlands (7); excessive intake of folic acid may cure vitamin B12-related anemia and thereby delay the diagnosis of vitamin B12 deficiency, (**Figure 1.1B**). Many elderly have a marginal B12 status and are at risk of developing a vitamin B12 deficiency. As such, an important argument against folic acid fortification is that it could potentially harm a proportion of the elderly population.

However, co-fortification of flour products with modest amounts of both folic acid and vitamin B12 could form a strategy to prevent harm of folic acid fortification to elderly who have a vitamin B12 deficiency.

Outline of this thesis

For this thesis we designed and performed various studies to answer the research questions that we posed. In **chapter two** we studied if the requirement for folate the same for men and women. **Chapter three** describes the validation of a method that can be used to study the bioavailability of dietary folates relative to folic acid. In **Chapter four** we describe the application of the method of chapter three an assessed the bioavailability of dietary folates relative to folic acid.

Chapter five describes an intervention study in which we studied to what extent bread fortified with folic acid and vitamin B12 improves folate and vitamin B12 status of elderly individuals.

In the final chapter (**chapter six**) we will take the findings of the various chapters into account and answer the central question of this thesis whether Dutch diets provide enough folate to meet the folate requirement, and if not, how to deal with fortification.

References

1. Konings EJ, Roomans HH, Dorant E, Goldbohm RA, Saris WH, van den Brandt PA. Folate intake of the Dutch population according to newly established liquid chromatography data for foods. Am J Clin Nutr 2001;73:765-76.
2. Melse-Boonstra A, de Bree A, Verhoef P, Bjorke-Monsen AL, Verschuren WM. Dietary monoglutamate and polyglutamate folate are associated with plasma folate concentrations in Dutch men and women aged 20-65 years. J Nutr 2002;132:1307-12.
3. Durga J, Verhoef P. Let them eat cake. Curr Nutr Food Sci 2005;1:35-40.
4. Lucock M. Is folic acid the ultimate functional food component for disease prevention? BMJ 2004;328:211-4.
5. Health Council of the Netherlands: Dietary reference intakes: Vitamin B6, folic acid and vitamin B12. The Hague: Health Council of the Netherlands, 2003:142.
6. Institute of Medicine. Folate. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. Washington: National Academy Press, 2000:196-305.
7. Health Council of the Netherlands: Risks of folic acid fortification. The Hague: Health Council of the Netherlands, 2000:48.
8. Committee on Medical Aspects of Food and Nutrition Policy (COMA). Folic acid and the prevention of disease. Report on health and social subjects. Department of Health, UK, 2000.
9. Scott JM. Folate and vitamin B12. Proc Nutr Soc 1999;58:441-8.
10. Scientific Advisory Committee on Nutrition. Folate and disease prevention. Norwich, UK: The Stationery Office, 2006:1-211.
11. Clarke R, Armitage J, Lewington S, Collins R, Clarke R. Homocysteine-lowering trials for prevention of vascular disease: Protocol for a collaborative meta-analysis. Clinical Chemistry and Laboratory Medicine 2007;45:1575-1581.
12. Clarke RJ. Homocysteine-lowering trials for prevention of cardiovascular events: A review of the design and power of the large randomized trials. American Heart Journal 2006;151:282-287.
13. Bazzano LA, Reynolds K, Holder KN, He J. Effect of Folic Acid Supplementation on Risk of Cardiovascular Diseases: A Meta-analysis of Randomized Controlled Trials. Jama 2006;296:2720-2726.
14. Wang X, Qin X, Demirtas H, et al. Efficacy of folic acid supplementation in stroke prevention: a meta-analysis. Lancet 2007;369:1876-1882.
15. Institute of Medicine. Definition and applications. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. Washington: National Academy Press, 2000:11-23.
16. Barr SI, Murphy SP, Poos MI. Interpreting and using the Dietary References Intakes in dietary assessment of individuals and groups. J Am Diet Assoc 2002;102:780-788.
17. Carriquiry AL. Assessing the prevalence of nutrient inadequacy. Public Health Nutrition 1999;2:23-33.
18. Gregory III JF. The bioavailability of folate. In: Bailey LB, ed. Folate in health and disease. I ed. Gainesville, Florida: Marcel Dekker, 1995:470.
19. McNulty H, Pentieva K. Folate bioavailability. Proc Nutr Soc 2004;63:529-36.
20. Sauberlich HE, Kretsch MJ, Skala JH, Johnson HL, Taylor PC. Folate requirement and metabolism in nonpregnant women. Am J Clin Nutr 1987;46:1016-28.
21. Brouwer IA, Dusseldorp Mv, West CE, Steegers-Theunissen RPM. Bioavailability and bioefficacy of folate and folic acid in man. Nutr Res Rev 2001;14:267-294.
22. Melse-Boonstra A, West CE, Katan MB, Kok FJ, Verhoef P. Bioavailability of heptaglutamyl relative to monoglutamyl folic acid in healthy adults. Am J Clin Nutr 2004;79:424-9.
23. Smulders YM, Stehouwer CD. Folate metabolism and cardiovascular disease. Semin Vasc Med 2005;5:87-97.
24. Health Council of the Netherlands: Dietary reference values: calcium, vitamin D, thiamin, riboflavin, niacin, panthotenic acid and biotin. The Hague: Health Council of the Netherlands, 2000:180.
25. De Walle HEK, De Jong-Van Den Berg LTW. Growing gap in folic acid intake with respect to level of education in the Netherlands. Community Genetics 2007;10:93-96.

2

Gender and body size affect the response of erythrocyte folate to folic acid treatment

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Abstract

The recommended dietary allowance (RDA) differs between men and women for some vitamins, but not for folate. The RDA for folate is derived mainly from metabolic studies in women. We assessed if men differ from women in their response of erythrocyte folate to folic acid supplementation. We used data from two randomized placebo-controlled trials with folic acid: a three-year trial in which subjects daily ingested 800 μg of folic acid (294 men and 112 women), and a 12-week trial in which 187 men and 129 women daily ingested 0, 50, 100, 200, 400, 600 or 800 μg of folic acid in a parallel design (n=38-42/treatment group). In the three-year trial, the erythrocyte folate concentration increased 10% (143 nmol/L, [95%CI 46, 241]) less in men than in women. In the 12-week trial, regression analysis showed that the response of erythrocyte folate upon folic acid intake for men was 47 nmol/L lower than for women (p for beta for gender is 0.022) : for an intake of 800 μg folic acid per day this resulted in a 5% lower response in men than in women. Differences in lean body size explained 56% of the difference in response of erythrocyte folate between men and women in the three-year trial and 70% in the 12-week trial. Men need more folic acid than women to achieve the same erythrocyte folate concentration, mainly because men have a larger lean body mass. This could be an indication, that the RDA for folate should be higher for men than for women, or that the RDA should be expressed per kilogram of lean body mass.

Introduction

Folate intake in men is generally higher than in women (1, 2). However, folate status is the same or slightly lower in men than in women (3-5).

In most countries, the recommended dietary allowance (RDA) for folate is the same for men and women, but it is derived mainly from metabolic studies in women (6-8). The RDA is 200 μg per day in the United Kingdom (9), 300 μg of folate from food per day in the Netherlands (10) and 400 μg of dietary folate equivalents per day in the US (6). In an earlier version the RDA for folate in the US was higher for men than for women, namely 240 μg/day for men and 190 μg/day for women (11).

For several other vitamins, namely vitamin A, C, K, thiamin, riboflavin, and niacin, and for choline, RDAs are higher for men than for women. For vitamin A, body weight partly determines the RDA (12); thus, the RDA is higher for men than for women since the reference body weight for an average man is higher than for an average woman. For vitamin C, the blood response to vitamin C treatment differs between men and women, partly due to gender differences in body size (13-15). For choline, animal studies suggest that males have a higher choline requirement than (16)females (17). For vitamin K, the RDA is derived from intake data and intake is usually higher in men than in women (18). For thiamin, riboflavin and niacin the gender differences in RDA are not based on data, but on the assumption that men have higher nutritional needs because they have a larger body size (17).

In the current study, we assessed if men and women differ in their change in the concentration of folate in erythrocytes in response to a fixed dose of folic acid and, if so, if differences in body size between men and women explain this difference. The concentration of folate in erythrocytes reflects tissue folate stores and provides the best information about the long-term folate status of humans; it was used as the primary indicator to set the RDA for folate by the US Institute of Medicine in 2000 (6). Our prior hypothesis was that the change in concentration of folate in erythrocytes to folic acid treatment would be inversely associated with lean body mass, since folate is a water-soluble vitamin. In pharmacology, lean body mass is often used to predict the loading dosage of water-soluble drugs (19).

Methods

Study population

We analyzed data from two folic acid supplementation trials: a three-year trial (20, 21) performed between September 2000 and December 2004, and a 12-week trial (22) performed in 2001.

The three-year trial

The effect of folic acid on intima-media thickening, cognitive function and hearing was evaluated in this double-blind, placebo-controlled, randomized trial. A total of 819 men and post-menopausal women ingested placebo or folic acid capsules (800 µg daily) for three years. Important inclusion criteria were that subjects had to be aged between 50 and 70 years and had to have elevated homocysteine concentrations (>13 µmol/L, but <26 µmol/L). Major exclusion criteria were serum vitamin B12 < 200 pmol/L, renal or thyroid diseases, use of B-vitamin supplements and use of medications that influence folate metabolism or atherosclerotic progression (eg lipid-lowering and hormone replacement therapies). A number of 819 out of 4200 subjects met all inclusion criteria and were randomized. For full details of enrollment see Durga et al (20). Subjects were asked to refrain from the use of B-vitamins during the study. Subjects provided fasting blood samples at baseline and after three years of intervention. Erythrocyte folate was analyzed with an immunoassay (Immulite 2000, Diagnostic Products Corporation), intra and interassay variation coefficients were <15% (20). Compliance of the subjects to folic acid treatment was judged by capsule-return counts and by a diary that registered missed capsules. The Medical Ethics Committee of Wageningen University approved the study and subjects gave written informed consent.

The 12-week trial

The effect of various doses of folic acid on homocysteine lowering was studied in this double-blind, placebo-controlled, randomized, parallel trial. Subjects in the folic acid groups daily ingested a capsule with 50, 100, 200, 400, 600 or 800 µg of folic acid;

subjects in the placebo group daily ingested a capsule without folic acid. The subjects had to be aged between 50 and 70 years and had to have a homocysteine concentration below 26 µmol/L and all women were post-menopausal. Major exclusion criteria were renal or thyroid diseases, use of medication that influence folate metabolism and use of dietary supplements containing B-vitamins. Out of 353 applicants, 316 met the inclusion criteria and were included in the trial, for full details of enrollment see van Oort et al (22). Subjects were asked to refrain from the use of B-vitamins during the study. Subjects provided a fasting blood sample at the start of the intervention and after 12 weeks of treatment. Erythrocyte was analyzed in the same laboratory and according to the same method as described above for the three-year trial. Compliance of the subjects was judged by capsule-return counts. All participants gave written informed consent to the protocol that was approved by the Medical Ethical Committee of Wageningen University.

Calculation of body size

Weight and height were measured at baseline in both studies. We derived lean body mass and body surface area with the following formulas (19, 23-25):

For men:

Lean body mass in kg = 1.1013 x weight in kg – 0.0128 x (weight in kg/height in m)^2

For women:

Lean body mass in kg = 1.07 x weight in kg – 0.0148 x (weight in kg/height in m)^2.

For both men and women:

Body surface area in m^2 = weight in kg$^{0.425}$ x (100 x height in m)$^{0.725}$ x 0.007184

Statistical analysis

Effect of gender

We assessed if men and women differ in their response in the concentration of folate in erythrocytes to a fixed dose of folic acid and, if so, if differences in body size between men and women explained this difference. The response in erythrocyte folate is the absolute change in the concentration of folate in erythrocytes from baseline to the end

of the study period. For the three-year trial, we calculated the mean and the 95%CI of the difference between men and women in the response of erythrocyte folate to folic acid. We did not correct for the small changes seen in the placebo group. For the 12-week trial, we constructed the following linear regression model to describe our data:

Response of erythrocyte folate (nmol/L) = intercept + beta$_{dose\ of\ folic\ acid}$ x dose of folic acid in μg/day + beta$_{gender}$ x gender + beta$_{gender\ x\ dose}$ x(gender x dose).

We included gender as an interaction term in the model (gender x dose) since we considered it biologically plausible that any difference in response between men and women would increase with increasing doses of folic acid.

We checked if potentially confounding factors (alcohol intake, smoking, and change in dietary folate intake during the intervention) changed the beta for gender more than 10%. These factors did not change the association, and therefore, we did not include them in the final models.

Effect of body size

We used linear regression to assess if body size influenced the response of erythrocyte folate to folic acid. For the three-year trial, the response of erythrocyte folate was the dependent variable and body size (lean body mass, or height, or weight, or body surface area) was the independent variable:

Response of erythrocyte folate (nmol/L) = intercept + beta$_{body\ size}$ x body size

For the 12-week trial, response of erythrocyte folate was the dependent variable and dose of folic acid (in μg/day), body size and the interaction term (body size x dose) were the independent variables:

Response of erythrocyte folate (nmol/L) = intercept + beta$_{dose\ of\ folic\ acid}$ x dose of folic acid in μg/day + beta$_{body\ size}$ x body size + beta$_{body\ size\ x\ dose}$ x (body size x dose).

We included body size as an interaction term, since we reasoned that any difference in response between subjects with a large and a small body size would increase with increasing intakes of folic acid.

We checked if potentially confounding factors (alcohol intake, smoking, and change in dietary folate intake during the intervention) changed the beta for body size more than

10%. These factors did not change the association, and therefore, we did not include them in the final models.

Combined effect of gender and body size

We used the following method to assess how much of the difference between men and women in response of erythrocyte folate concentrations to folic acid intake was explained by differences in body size (26). We first calculated (beta$_{gender}$)$_{gender}$, which is the beta for gender in a linear regression model in which gender (women =0, men = 1) was the main independent variable that explained differences in response in erythrocyte folate. Next, we included one of the indicators of body size in the model and again assessed the beta for gender: (beta$_{gender}$)$_{gender\&bodysize}$. Finally, we calculated the percentage of the gender difference in erythrocyte folate response that was explained by body size:

Percentage = 100 x [(beta$_{gender}$)$_{gender}$ - (beta$_{gender}$)$_{gender\&bodysize}$] / (beta$_{gender}$)$_{gender}$

For a detailed description of the models, see the appendix.

Results

Both trials included more men than women. Compliance to folic acid treatment was high: 99% as assessed from capsule-returns (20, 22) and did not differ between men and women. All participants were white Caucasians. As expected, men were significantly taller and heavier than women and had a larger lean body mass and body surface area. Erythrocyte folate status did not differ between men and women at baseline, habitual intake of dietary folate was higher in men than in women (**Table 2.1**). During the three-year trial, the concentration of folate in erythrocytes increased by 1391 ± 413 nmol/L in men and by 1534 ± 512 nmol/L in women; the mean difference was 143 nmol/L (95%CI 46, 241) and was statistically significant, **Table 2.2**. Thus, erythrocyte folate increased approximately 10% less in men than in women. In the

Table 2.1: characteristics of men and women who participated in folic acid trials, assessed at the start of these trials. In the three-year trial, subjects ingested 800 µg of folic acid per day for 3 years, in the 12-week trial, subjects ingested 0, 50, 100, 200, 400, 600 or 800 µg of folic acid per day for 12 weeks [1]

		Three-year trial	12-week trial
Number of men/women		294 / 112	187 / 129
Age, years	men	60 ± 6	60 ± 6
	women	61 ± 5	60 ± 6
Height, m	men	1.79 ± 0.06	1.76 ± 0.07
	women	1.66 ± 0.06[2]	1.68 ± 0.08[2]
Weight, kg	men	86 ± 11	84 ± 14
	women	73 ± 13[2]	75 ± 12[2]
Lean body mass, kg	men	65 ± 6	63 ± 7
	women	49 ± 4[2]	50 ± 5[2]
Body surface area, m^2	men	2.05 ± 0.15	2.00 ± 0.18
	women	1.80 ± 0.15[2]	1.84 ± 0.17[2]
Dietary folate intake, µg/day[3]	Men	201 (161, 245)	174 (145, 207)
	Women	180 (150, 216)[2]	160 (136, 197)[2]
Change in dietary folate	Men	15 ± 65	-
intake during intervention[4]	Women	17 ± 80	-
Current smokers	Men	22%	18%
	Women	17%	13%
Alcohol intake, grams/day	Men	15 (6, 25)	12 (3, 23)
	women	7 (1, 17)[2]	6 (1, 18)[2]
Serum folate, nmol/L	men	12.1 ± 4.0	12.2 ± 4.1
	women	12.9 ± 5.0	13.6 ± 4.2[2]
Erythrocyte folate, nmol/L	men	699 ± 270	727 ± 258
	women	659 ± 298	726 ± 249
Total homocysteine, µmol/L	men	13.4 ± 2.6	12.1 ± 2.9
	women	13.0 ± 2.8	10.9 ± 3.0[2]

[1] values are mean ± SD or median (interquartile range)

[2] different from men (P>0.05), Students t-test for normally distributed variables, Mann-Witney U for not-normally distributed variables

[3] assessed with a food frequency questionnaire (27),

[4] daily intake of dietary folate intake reported at the end of the intervention minus the daily intake of dietary folate reported at the start of the intervention; in the 12-week trial, dietary folate intake was only assessed at baseline; in the three-year trial it was assessed at baseline and after three years

Table 2.2: response of erythrocyte folate to placebo or folic acid treatment in men and women in the three-year trial and in the 12-week trial[1]

Dose of folic acid (µg/day)	Number of		Response of erythrocyte folate during intervention, nmol/L		
	Men	Women	Men	Women	Difference
Three year trial					
0	286	120	50 ± 327	61 ± 255	-11 (-77, 55)
800	285	110	1391 ± 413	1534 ± 512	-143 (-241, -46)[2]
12-week trial					
0	28	22	3 ± 126	29 ± 93	-26 (-91, 38)
50	24	18	22 ± 108	96 ± 83	-74 (-136, -12)[2]
100	20	20	130 ± 114	126 ± 137	4 (-77, 85)
200	24	19	252 ± 115	323 ± 129	-71 (-146, 4)
400	29	13	511 ± 186	574 ± 251	-63 (-203, 77)
600	23	16	616 ± 201	751 ± 208	-134 (-269, 0)[2]
800	27	16	825 ± 248	833 ± 294	-8 (-177, 161)

[1] Values are means ± SD or mean difference (95%CI)

[2] Statistically significantly different between men and women (Student's t-test)

placebo group, the erythrocyte folate increased by 50 ± 327 (SD) nmol/L in men and by 61 ± 255 nmol/L in women, this difference was not statistically significant.

The data of the 12-week trial in **Figure 2.1** suggest that in general men have a lower response in erythrocyte folate than women. Statistical analysis showed that the response in erythrocyte folate was significantly lower for men compared with women for two of the dose groups of the 12-week trial, **Table 2.2**. Linear regression analysis with the pooled data from all dose groups showed that the dose-response curve for men was lower than the dose-response curve for women (p=0.022 for the beta for gender, **Table 2.3**), although the slopes of the dose-response curves did not differ for men and women (p=0.674 for the interaction term gender x dose). For men (gender =1) the response in erythrocyte folate was 47 nmol/L lower than in women (**Table 2.4**): *Response of erythrocyte folate (nmol/L) =*

62 + 1.054 x dose of folic acid in µg/day +-47 x gender

At an intake of 800 μg of folic acid/day, which was the dose used in the three-year trial, the response in erythrocyte folate in men would be 5% lower than in women: 858 nmol/L for men and 905 nmol/L for women.

We calculated how the RDA for men and women should differ if we assume that men need more folic acid than women to achieve the same increase in erythrocyte folate. The current RDA for folate is 400 μg of dietary folate equivalents per day or 200 μg of folic acid. At an intake of 200 nmol/L the response in men is 47 nmol/L lower in men than in women; men would have to ingest an additional (47/1.054) = 45 μg of folic acid per day to achieve the same response. In the calculation of the RDA folic acid is assumed to be 2 times more bioavailable than food folate. Based on this assumption, the RDA for men should be 400 +2 x 45 = 490 dietary folate equivalents per day.

In both trials, greater body size -lean body mass, height, weight or body surface area- was associated with a lower response of erythrocyte folate when gender was not included in the linear regression models (**Tables 2.3&2.4**). There was no interaction between body size and dose in the 12-week trial (data not shown).

Differences in lean body size explained 56% of the difference in response of erythrocyte folate between men and women in the three-year trial and 70% in the 12-week trial. Height, weight or body surface area explained less of the difference than lean body mass **(Table 2.5).**

Lean body mass was correlated with gender: the correlation coefficient was 0.81 for the three-year trial and 0.72 in the 12-week trial (**Table 2.5**); the other measures of body size were also associated with gender.

Table 2.3: Linear regression coefficients for the association between the response of erythrocyte folate to folic acid treatment and gender and body size in the three-year trial

Association of response of erythrocyte folate(in nmol/L with[1]:	n=395 (285 men, 110 women) Predictors	Beta estimate	95%CI	p-value[2]
Gender[3]	Intercept	1534	(1451, 1617)	
	gender (women=0, men=1)	-143	(-241, -46)	0.004
Body size[4]	Intercept	1882	(1587, 2177)	
	lean body mass, kg	-7.49	(-12.32, -2.65)	0.002
	Intercept	2828	(1947, 3710)	
	Height, m	-796	(-1297, -295)	0.002
	intercept	1773	(1490, 2055)	
	Weight, kg	-4.16	(-7.56, -0.76)	0.017
	Intercept	2138	(1659, 2616)	
	body surface area, m^2	-357	(-598, -116)	0.004
Gender & body size[5]	Intercept	1778	(1378, 2178)	
	Gender	-63	(-225, 100)	0.448
	lean body mass, kg	-5	(-13, 3)	0.222
	Intercept	2445	(1275, 3615)	
	Gender	-68	(-205, 69)	0.328
	Height, m	-549	(-1254, 155)	0.126
	Intercept	1716	(1430, 2003)	
	Gender	-113	(-221, -5)	0.041
	Weight, kg	-2	(-6, 1)	0.194
	Intercept	1945	(1398, 2491)	
	Gender	-89	(-210, 33)	0.152
	body surface area, m^2	-227	(-526, 72)	0.136

[1] p-value for F-test of the total model was <0.01 for all models.

[2] p-value for the beta estimate

[3] The model is:

Response of erythrocyte folate (nmol/L) = intercept + (beta$_{gender}$)$_{gender}$ x gender

[4] The model is:

Response of erythrocyte folate (nmol/L) = intercept + beta$_{body\ size}$ x body size

[5] The model is:

Response of erythrocyte folate (nmol/L) = intercept + (beta$_{gender}$)$_{gender\&bodysize}$ x gender + beta$_{body\ size}$ x body size

Table 2.4: Linear regression coefficients for the association between the response of erythrocyte folate to folic acid treatment and gender and body size in the 12-week trial

Association of response of erythrocyte folate in nmol/L with[1]:	Predictors	Beta estimate	95%CI	p-value[2]
	n=299 (175 men, 124 women)			
Gender[3]	Intercept	62	(25, 98)	
	Dose, µg folic acid/day	1.054	(0.984, 1.125)	
	Gender (women=0, men=1)	-47	(-88, -7)	0.022
Body size[4]	Intercept	237	(104, 371)	
	Dose, µg of folic acid/day	1.053	(0.984, 1.123)	
	lean body mass, kg	-3.5	(-5.8, -1.26)	0.002
	Intercept	539	(128, 951)	
	Dose, µg of folic acid/day	1.053	(0.982, 1.123)	
	Height, m	-292	(-530, -54)	0.016
	Intercept	177	(53, 302)	
	Dose, µg of folic acid/day	1.048	(0.978, 1.118)	
	Weight, kg	-1.77	(-3.285, -0.257)	0.022
	Intercept	308	(97, 520)	
	Dose, µg of folic acid/day	1.050	(-0.980, 1.120)	
	body surface area, m²	-141	(-250, -33)	0.011
Gender & body size[5]	Intercept	227	(59, 395)	
	Dose, µg of folic acid/day	1.051	(0.984, 1.124)	
	Gender	-6	(-63, 52)	0.842
	lean body mass, kg	-3.3	(-6.6, -0.034)	0.048
	Intercept	407	(-49, 863)	
	Dose, µg of folic acid/day	1.055	(0.985, 1.125)	
	Gender	-31	(-77, 15)	0.188
	Height, m	-206	(-476, 65)	0.136
	Intercept	161	(36, 287)	
	Dose, µg of folic acid/day	1.052	(0.982, 1.122)	
	Gender	-36	(-78, 7)	0.101
	Weight, kg	-1.33	(-2.93, 0.27)	0.103

Table 2.4, continued

	Intercept	258	(34, 481)	
	Dose, μg of folic acid/day	1.053	(0.983, 1.123)	
	Gender	-31	(-75, 14)	0.174
	body surface area, m^2	-107	(-226, 13)	0.082

[1] p-value for F-test of the total model was <0.01 for all models

[2] p-value for the beta estimate

[3] The model is:

Response of erythrocyte folate (nmol/L) = intercept +beta$_{dose}$ x dose of folic acid(μg/day) +
(beta$_{gender}$)$_{gender}$ x gender

[4] The model is:

Response of erythrocyte folate (nmol/L) = intercept +beta$_{dose}$ x dose of folic acid(μg/day) +
beta$_{body size}$ x body size

[5] The model is:

Response of erythrocyte folate (nmol/L) = intercept +beta$_{dose}$ x dose of folic acid(μg/day) +
(beta$_{gender}$)$_{gender&bodysize}$ x gender + beta$_{body size}$ x body size

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Table 2.5: Percentage of the gender difference in the response of erythrocyte folate to folic acid treatment that is explained by body size[1] and the Spearman correlation coefficient between gender and body size in the three-year trial and in the 12-week trial.

Indicator of body size	Three-year trial n=395 (285 men, 110 women)		12-week trial n=249 (147 men, 102 women)	
	Percentage	Correlation coefficient[2]	Percentage	Correlation coefficient[2]
Lean body mass, kg	56	0.81	87	0.72
Height, kg	52	0.71	34	0.48
Weight, kg	21	0.44	23	0.33
Body surface area, m^2	38	0.61	34	0.43

[1] This percentage is derived from the regression coefficients in **table** 2.3 for the three-year trial and in **table 2.4** for the 12-week trial, with the following formula: *Percentage = 100 x [(beta$_{gender}$)$_{gender}$ - (beta$_{gender}$)$_{gender&bodysize}$] / (beta$_{gender}$)$_{gender}$*

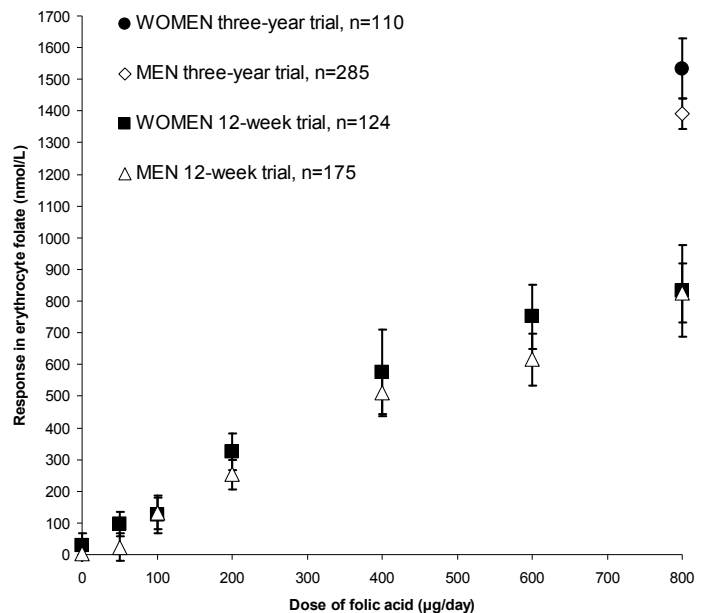[2] Spearman's correlation coefficient between gender and body size

Figure 2.1: Mean response of erythrocyte folate to various doses of folic acid in men and women in the 12-week and in the three-year trial. Error bars represent the 95%CI.

Discussion

The response in erythrocyte folate to folic acid treatment in men was lower than in women in a large three-year trial with folic acid supplements. In a second folic acid intervention that lasted 12 weeks, we showed that the response in erythrocyte folate was lower in men than in women, although we could not show that this difference increased with increasing intakes of folic acid. It is biologically plausible that men would have a lower response to folic acid: in general, men have a larger body size and therefore the dose of folic acid distributes over a larger volume. Our data show that gender differences in body size explain a large part of the gender difference in the response of erythrocyte folate.

Erythrocyte folate concentrations formed the primary indicator to estimate the RDA for folate, sometimes used in conjunction with homocysteine and serum or plasma

folate concentrations (6). The Institute of Medicine considered erythrocyte folate status the best indicator of long-term status and the primary indicator for folate adequacy (6). This means that changes in erythrocyte folate status are informative about changes in folate adequacy. Concurrently, if there is a gender difference in the amount of folic acid that is needed to achieve a certain change in erythrocyte status, this means that there is a gender difference in the amount of folic acid that is needed to achieve folate adequacy. Our data suggest that men need more folic acid to reach folate adequacy, which could suggest that the RDA for folate for men should be higher than for women. However, additional research should first confirm that gender and/or body size difference in folate responses are truly present. A study that includes men and women matched by lean body mass could further elucidate if any gender difference in response to folic acid treatment results from differences in lean body mass.

The men in our studies had higher dietary intake of folate than the women, which was also shown by others (1, 2). However, erythrocyte folate status did not differ between men in women in our studies and those of others (3-5). This supports our findings that men need more folate to achieve the same erythrocyte folate concentration.

We showed that the gender difference in the response of erythrocyte folate to folic acid treatment is largely explained by gender differences in body size. If future studies indeed confirm these findings, this could suggest that the RDA for folate should either be gender-specific or that it should be set on body weight basis, per kg lean body weight. In this paper, we decided to focus on the possibility of a gender-specific RDA, to stay in line with recommendations for other vitamins. The RDA for many other vitamins is higher for men than for women, mainly because men have a larger body size (17).

We assumed linearity in the change in concentration of folates in erythrocytes in response to various doses of folic acid treatment in the 12-week trial. The response of folates in serum to various doses of folic acid is linear (28), however, there are, to our knowledge, no trials that studied the dose-response in folate in erythrocytes. We decided to use the simplest and thus linear model to describe our data, since the data from **Figure 2.1** did not point to clear deviations from linearity.

Two other studies reported the response of folate concentrations in blood to folic acid separately for men and women. Both studies found a lower response in men than in women (29, 30) and are therefore consistent with our findings. Coppen et al (29) found that the plasma folate response to 500 µg of folic acid for 10 weeks was 9.4 nmol/L in men and 19.7 nmol/L in women, but did not statistically test this difference. Van der Griend et al (30) reported that post-treatment plasma folate concentrations were statistically significantly lower in men than in women (median 38 nmol/L in men versus 52 nmol/L in women) after a daily dose of 500 µg of folic acid for 8 weeks, but did not report baseline values separately for men and women.

Gender differences in lean body mass explained at least 50% of the gender difference in response of erythrocyte folate to folic acid, but gender correlated strongly with lean body mass as well. Therefore, we could not completely separate the association with gender from the association with body size. Gender or body size were no longer always significantly associated with the response of erythrocyte folate when gender and body size were simultaneously included in the models, as is shown in **Tables 2.3 and 2.4.** However, the F-test for the total model - including gender and body size simultaneously - was statistically significant: the model with gender and body size predicted response of erythrocyte folate better than the model without gender and body size. We therefore assume that the separate variables no longer significantly contributed to the response because of collinearity. Our data clearly suggest that the response in erythrocyte folate differs between men and women, but our estimate of the contribution of lean body mass to the gender effect may not be entirely correct.

Several factors may limit the generalizability of our findings. First, all participants were aged between 50 and 70 years and all women were post-menopausal. Yet, folic acid absorption does not seem to depend on age (31, 32), but pre-menopausal women monthly lose folate through their menstruation. However, the amount they lose is negligible: menstrual blood loss is about 25-40mL per cycle (33), at an average erythrocyte folate concentration of 1000 nmol/L and an hematocrit value of 0.45 folate loss ranges from 11-18 nmol (or 5-8 ng) per menstrual cycle and will hardly be of influence. Another factor that could limit generalizability is that subjects in the

three-year trial had high homocysteine levels (>13 µmol/L) and therefore, this population was not representative of a normal population. Still, participants in the 12-week trial had normal homocysteine levels, and that trial also suggested gender differences in the response of erythrocyte folate. Moreover, we are not aware of data that suggest that the absorption or bioavailability of folic acid is influenced by homocysteine or folate status (34). Nevertheless, future studies should confirm our findings in a general population.

We calculated that men might need an additional 45 µg of folic acid equivalent to about 90 µg of dietary folate equivalents. This probably slightly overestimates the additional dietary folate that men need, since we recently showed that bioavailability of folic acid relative to dietary folate was 100/80=1.25 (35) instead of the factor 2 used in the calculation of the US dietary folate equivalents. However, we used the factor 2 in our calculations, since it was also used to derive the 400 µg of dietary folate equivalents of the current US RDA.

Do men reach an intake of 490 µg of dietary folate equivalents per day in real life? Although men have a higher dietary folate intake than women, data from the US National Health and Nutrition Examination Survey (NHANES) showed that a large proportion of men does not ingest 490 µg of dietary folate equivalents per day; the median dietary folate intake of men varied with age and ethnicity and ranged from 312 to 552 µg/day (36). Men would have to increase their intake of fortified foods or of supplements to reach this recommendation.

In conclusion, our study suggests that men need more folic acid than women to increase their folate status by the same extent, probably because they have a larger body size. The higher need for folate of larger persons will only partly be met by a higher intake of food and thus of folate. Our findings could indicate that the RDA for folate should be higher for men than for women.

References

1. Dietrich M, Brown CJP, Block G. The Effect of Folate Fortification of Cereal-Grain Products on Blood Folate Status, Dietary Folate Intake, and Dietary Folate Sources among Adult Non-Supplement Users in the United States. J Am Coll Nutr 2005;24:266-274.
2. de Bree A, van Dusseldorp M, Brouwer IA, van het Hof KH, Steegers-Theunissen RP. Folate intake in Europe: recommended, actual and desired intake. Eur J Clin Nutr 1997;51:643-60.
3. Ganji V, Kafai MR. Trends in Serum Folate, RBC Folate, and Circulating Total Homocysteine Concentrations in the United States: Analysis of Data from National Health and Nutrition Examination Surveys, 1988-1994, 1999-2000, and 2001-2002. J Nutr 2006;136:153-158.
4. Verhoef P, Meleady R, Daly LE, Graham IM, Robinson K, Boers GH. Homocysteine, vitamin status and risk of vascular disease; effects of gender and menopausal status. European COMAC Group. Eur Heart J 1999;20:1234-44.
5. Pfeiffer CM, Johnson CL, Jain RB, et al. Trends in blood folate and vitamin B-12 concentrations in the United States, 1988 2004. Am J Clin Nutr 2007;86:718-727.
6. Institute of Medicine. Folate. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. Washington: National Academy Press, 2000:196-305.
7. O'Keefe CA, Bailey LB, Thomas EA, et al. Controlled dietary folate affects folate status in nonpregnant women. J Nutr 1995;125:2717-25.
8. Sauberlich HE, Kretsch MJ, Skala JH, Johnson HL, Taylor PC. Folate requirement and metabolism in nonpregnant women. Am J Clin Nutr 1987;46:1016-28.
9. Committee on Medical Aspects of Food and Nutrition Policy (COMA). Dietary Reference Values for Food Energy and Nutrients for the United Kingdom. Department of Health, UK, 1991.
10. Health Council of the Netherlands: Dietary reference intakes: Vitamin B6, folic acid and vitamin B12. The Hague: Health Council of the Netherlands, 2003:142.
11. Herbert V. Recommended dietary intakes (RDI) of folate in humans. Am J Clin Nutr 1987;45:661-670.
12. Olson JA. Recommended dietary intakes (RDI) of vitamin A in humans. Am J Clin Nutr 1987;45:704-16.
13. Institute of Medicine. Vitamin C. Dietary reference intakes for vitamin C, vitamin E, selenium and carotenoids. Washington: National Academy Press, 2000:35-58.
14. Blanchard J. Effects of gender on vitamin C pharmacokinetics in man. J Am Coll Nutr 1991;10:453-9.
15. Blanchard J. Depletion and repletion kinetics of vitamin C in humans. J Nutr 1991;121:170-6.
16. Health Council of the Netherlands: Risks of folic acid fortification. The Hague: Health Council of the Netherlands, 2000:48.
17. Institute of Medicine. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. Washington: National Academy Press, 2000.
18. Institute of Medicine. Dietary reference intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Solicon, Vanadium, and Zinc. Washington: National Academy Press, 2000.
19. Morgan DJ, Bray KM. Lean body mass as a predictor of drug dosage. Implications for drug therapy. Clinical Pharmacokinetics 1994;26:292-307.
20. Durga J, van Boxtel MPJ, 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;369:208-216.
21. Durga J, Verhoef P, Anteunis LJ, Schouten E, Kok FJ. Effects of folic acid supplementation on hearing in older adults: a randomized, controlled trial. Ann Intern Med 2007;146:1-9.
22. van Oort FV, Melse-Boonstra A, Brouwer IA, et al. Folic acid and reduction of plasma homocysteine concentrations in older adults: a dose-response study. Am J Clin Nutr 2003;77:1318-23.
23. Green B, Duffull S. Caution when lean body weight is used as a size descriptor for obese subjects. Clinical Pharmacology and Therapeutics 2002;72:743-744.
24. Green B, Duffull SB. What is the best size descriptor to use for pharmacokinetic studies in the obese? British Journal of Clinical Pharmacology 2004;58:119-133.

25. Sawyer M, Ratain MJ. Body surface area as a determinant of pharmacokinetics and drug dosing. Investigational New Drugs 2001;19:171-177.
26. Ditlevsen S, Christensen U, Lynch J, Damsgaard MT, Keiding N. The mediation proportion: a structural equation approach for estimating the proportion of exposure effect on outcome explained by an intermediate variable. Epidemiology 2005;16:114-20.
27. van de Rest O, Durga J, Verhoef P, Melse-Boonstra A, Brants HAM. Validation of a food frequency questionnaire to assess folate intake of dutch elderly people. British Journal of Nutrition 2007;98:1014-1020.
28. Wald NJ, Law MR, Morris JK, Wald DS. Quantifying the effect of folic acid. Lancet 2001;358:2069-2073.
29. Coppen A, Bailey J. Enhancement of the antidepressant action of fluoxetine by folic acid: a randomised, placebo controlled trial. Journal of Affective Disorders 2000;60:121-130.
30. van der Griend R, Haas FJ, Biesma DH, Duran M, Meuwissen OJ, Banga JD. Combination of low-dose folic acid and pyridoxine for treatment of hyperhomocysteinaemia in patients with premature arterial disease and their relatives. Atherosclerosis 1999;143:177-83.
31. Bailey LB, Cerda JJ, Bloch BS, et al. Effect of age on poly- and monoglutamyl folacin absorption in human subjects. J Nutr 1984;114:1770-6.
32. Wald DS, Bishop L, Wald NJ, et al. Randomized trial of folic acid supplementation and serum homocysteine levels. Arch Intern Med 2001;161:695-700.
33. Harvey LJ, Armah CN, Dainty JR, et al. Impact of menstrual blood loss and diet on iron deficiency among women in the UK. British Journal of Nutrition 2005;94:557-564.
34. Brouwer IA, Dusseldorp Mv, West CE, Steegers-Theunissen RPM. Bioavailability and bioefficacy of folate and folic acid in man. Nutr Res Rev 2001;14:267-294.
35. Winkels RM, Brouwer IA, Siebelink E, Katan MB, Verhoef P. Bioavailability of food folates is 80% of that of folic acid. Am J Clin Nutr 2007;85:465-473.
36. Bentley TG, Willett WC, Weinstein MC, Kuntz KM. Population-level changes in folate intake by age, gender, and race/ethnicity after folic acid fortification. Am J Public Health 2006;96:2040-7.

Appendix

Description of the linear regression models used to assess the effect of gender and body size on the erythrocyte folate response

We calculated the percentage of the gender difference in erythrocyte folate response to folic acid treatment that was explained by body size with the following formula:

Percentage = 100 x [(beta$_{gender}$)$_{gender}$ - (beta$_{gender}$)$_{gender\&bodysize}$] / (beta$_{gender}$)$_{gender}$

For the three year trial, the betas were estimated with the following models.

(Beta$_{gender}$)$_{gender}$ was the slope for gender in the model with gender as the only independent variable:

Response of erythrocyte folate (nmol/L) = intercept + (beta$_{gender}$)$_{gender}$ x gender

All subjects received the same dose of folic acid in this trial, and thus we did not include the variable 'dose of folic acid' in the analysis of the three-year trial.

(Beta$_{gender}$)$_{gender\&bodysize}$ was the slope for gender in the model that also included body size as independent variable:

Response of erythrocyte folate (nmol/L) =

intercept + (beta$_{gender}$)$_{gender\&bodysize}$ x gender + beta$_{body\ size}$ x body size

We separately included height (in m), weight (in kg) lean body mass (in kg), or body surface area (in m^2) as indicator of body size.

For the 12-week trial, the regression models we used were slightly different, since the variable 'dose of folic acid' was included in the models. Thus, (beta$_{gender}$)$_{gender}$ was derived from the following model:

Response of erythrocyte folate (nmol/L) = intercept + (beta$_{gender}$)$_{gender}$ x gender +beta$_{dose\ of\ folic\ acid}$ x dose of folic acid (µg/day)

And *(beta$_{gender}$)$_{gender\&bodysize}$* was estimated with the following model:

Response of erythrocyte folate (nmol/L) = intercept + (beta$_{gender}$)$_{gender\&body\ size}$ x gender + beta$_{dose\ of\ folic\ acid}$ x dose of folic acid (µg/day)+ beta$_{body\ size}$ x body size

3

Development of a method to study bioavailability of folate from total diets in healthy humans, using daily low doses of ^{13}C-labeled folic acid

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Abstract

Introduction: Stably labeled folic acid has been used to study various aspects of folate uptake and metabolism. We developed a method based on dilution of stably labeled folate concentrations in blood to study the bioavailability of folate from food. As a final step in the validation of this method, we investigated whether the percentage of ^{13}C-labelled folate in the total pool of plasma folate in response to a constant oral dose of ^{13}C-labeled folic acid would decrease with increasing intakes of unlabelled folic acid, and what the shape of the dose-response curve was.

Methods: We performed a double blind, randomized, parallel intervention trial. Fifty-six subjects daily ingested a capsule with 0 µg, 100 µg, 200 µg or 400 µg of unlabeled folic acid, during four weeks. Additionally, all subjects daily ingested a capsule with 55 µg of $[^{13}C_{11}]$-folic acid. The percentage of labeled folate in the total pool of plasma folate after four weeks of intervention was measured by LC-MS/MS.

Results: The percentage of labeled folate in the total pool of plasma folate decreased hyperbolically with increased intakes of unlabelled folic acid: this percentage was 13.0% (SD 2.8) at an intake of 0 µg of unlabeled folic acid, 10.0% (SD 2.2) for 100 µg, 9.3% (1.6) for 200 µg and 6.4% (0.8) for 400 µg. The inverse of this percentage was linearly correlated with the intake of unlabeled folic acid (R^2=0.69).

Conclusion: The inverse of the percentage of labeled folate in plasma folate is linearly associated with the intake of unlabeled folic acid. This enables the application of this stable isotope dilution technique in research on folate bioavailability.

Introduction

Folate is a generic term for the various biochemical forms of the B vitamin pteroyl glutamic acid or folic acid. Sources of folates in our daily diet are fruits, vegetables, liver, bread and dairy products. Folic acid, the synthetic form of this vitamin does not occur naturally in significant amounts (1, 2).

Folate deficiency leads to a decrease in serum and red blood cell folate, to an increase in homocysteine and ultimately to anemia (1, 2). Folates play a role in the prevention of neural tube defects (3).

Bioavailability refers to the proportion of dietary folate that is absorbed and becomes available for metabolic processes in the body. Most studies determined the bioavailability of folates relative to folic acid (4). Estimates for the bioavailability of dietary folates vary substantially since the response to an intervention with folates varies substantially between persons. The current estimates for the bioavailability of dietary folates range from 30% to 98% (5-7), but an accurate figure is lacking. A good estimate of folate bioavailability from the general diet is necessary to construct reliable dietary reference intakes for folate.

Recently we studied the bioavailability of polyglutamyl folic acid relative to monoglutamyl folic acid in humans with a stable isotope method (8). In this method, individuals simultaneously ingested polyglutamyl and monoglutamyl folic acid. Each form carried a different number of labeled ^{13}C-atoms and could therefore be traced separately in the human plasma. This stable isotope method resulted in an estimated bioavailability of poly- relative to monoglutamyl folic acid of 68 to 87% (8), which was comparable to the precision found in a conventional study without stable isotopes (52 to 75%) (9); in that conventional study two parallel groups ingested either monoglutamyl or polyglutamyl folic acid. An important advantage of the stable isotope method compared with the conventional study was that only 20 participants had to be included to obtain the same level of precision as the conventional study, which included 120 participants. Another advantage of the stable isotope method was that it did not disturb normal folate metabolism; subjects were not saturated with high doses of folic acid prior to the study and low doses of labeled folic acid were used.

Our aim is to adapt the earlier described stable isotope method in such a way that it is suitable to study the bioavailability of folates from a mixed diet relative to folic acid. The study described in this paper was a last step in the validation of the method for this application. Aim of the study was to study the amount of labeled folate appearing in the total pool of plasma folate upon ingestion of a fixed low dose of labeled folic acid in combination with various doses of unlabeled folic acid, a so-called 'stable isotope dilution method'. We assessed the dose-response curve of the percentage of labeled folate in plasma folate versus the intake of unlabeled folic acid in a group of healthy volunteers.

Methods

Subjects

The Medical Ethical Committee of Wageningen University approved the study. Subjects were recruited from staff and students of Wageningen University. Subjects gave their written informed consent before the start of the study. Exclusion criteria for the study were: low serum vitamin B12 (<160pmol/L, Immulite 2000, Diagnostic Products Company, Los Angeles, CA), high serum creatinine (>125 µmol/L, DuPont Dimension) or high homocysteine (>26 µmol/L, fluorescence polarization immunoassay (FPIA) on an AxSYM analyzer), use of drugs interfering with folate metabolism, use of B vitamins three months prior to the study, cardiovascular disease, cancer, rheumatoid arthritis, epilepsy or gastro-intestinal disorders. On the basis of these criteria 56 subjects were included in the study. Subjects were stratified by serum folate levels. Within each stratum, randomized blocks assigned the subjects to one of the 4 intervention groups (n=14/group).

Design

The study was a 4-week, double blind, parallel intervention trial. Fasting blood samples were drawn from all subjects at baseline and after two and four weeks of intervention. Subjects daily ingested two capsules. One capsule contained 55 µg [$^{13}C_{11}$]-folic acid. The other capsule contained unlabelled folic acid: 0 µg of folic acid for group

A, 100 μg for group B, 200 μg for group C and 400 μg for group D. Capsules were originally produced for another study and had been stored at 4º C since then (9). The amount of folic acid in the capsules was verified with an HPLC method (10). The analyzed amounts were: group A not detectable, group B 90μg/capsule (range: 86-92), group C 176 μg/capsule (range: 172-183), group D 379 μg/capsule (range: 370-391).

Subjects daily recorded in a diary if they took their capsules. In addition, remaining capsules were counted after the intervention.

The main outcome variable of the study was the percentage of $[^{13}C_{11}]$-labeled folate in the total pool of plasma folate after four weeks of intervention. We constructed a dose-response curve of the percentage of labeled folate in plasma folate versus intake of folic acid from this variable.

Biochemical measurements

Blood samples were drawn into EDTA-containing tubes; these tubes were placed on ice immediately and centrifuged within 30 min at 2600xg for 10 min at 4˚C. Plasma was pipetted of and stored at -80˚C until analysis. We measured the percentage of labeled folate in the total pool of plasma folate with an LC-MS/MS method (8). We limited our analysis to the 5-methyltetrahydrofolate fraction of the plasma, since this is the most abundant folate vitamer in plasma. The percentage of labeled folate is calculated as follows:

$$100 * \frac{[^{13}C_{11}]\text{-}5\text{-}methyltetrahydrofolate}{\{[^{13}C_{11}]\text{-}5\text{-}methyltetrahydrofolate + [^{13}C_{0}]\text{-}5\text{-}methyltetrahydrofolate\}}$$

In this formula the numerator is the area under the curve of the $[^{13}C_{11}]$-5-methyltetrahydrofolate peak and the denominator is the area under the curve of both the $[^{13}C_{11}]$-5-methyltetrahydrofolate and the $[^{13}C_{0}]$-5-methyltetrahydrofolate peak.

Statistics

We calculated means and standard deviations of the percentage of labeled folate in plasma folate after two and four weeks of intervention per treatment group. We created a scatter plot of the percentage of labeled folate in plasma folate after four weeks of intervention against the intake of unlabeled folic acid. We anticipated finding a hyperbolic decrease in the percentage of labeled folate with increased intakes of unlabeled folic acid. Therefore we decided to use an inverse transformation of the Y-variable (1/Y) to try to linearize the hyperbolic association (11). We created a scatter plot of the inverse of the percentage of labeled folate with increased intakes of unlabeled folic acid and calculated the correlation coefficient to assess the strength of this association.

Results

All 56 subjects completed the study. Mean compliance to the study-protocol, assessed from the diaries and from the number of remaining capsules, was 99% (all subjects >82%). Groups were comparable for all characteristics measured at screening (**Table 3.1**).

No $[^{13}C_{11}]$-5-methyltetrahydrofolate was detected in the plasma at baseline. The percentage of labeled folate detected in the plasma folate increased during the course of the intervention (**Table 3.2**). After four weeks of intervention the mean percentage of labeled folate in the plasma folate ranged from 6.4 to 13% (**Table 3.2**); the mean percentage of labeled folate was higher when the intake of unlabeled folic acid was lower.

Figure 3.1 shows the response of the percentage of labeled folate in plasma folate with increasing intakes of folic acid after four weeks of intervention; the R^2 for the relation between the percentage of labeled folate in plasma folate and intake of unlabeled folic acid was 0.55.

In **Figure 3.2** we plotted the inverse of the percentage of labeled folate after 4 weeks of intervention against increased intakes of folic acid; the R^2 of this relation was 0.69.

Figure 3.1: the percentage of labeled folate in plasma folate after four weeks of intervention with various intakes of unlabelled folic acid in combination with a fixed intake of labelled folate. Each circle represents one subject. R^2 of the association is 0.55.
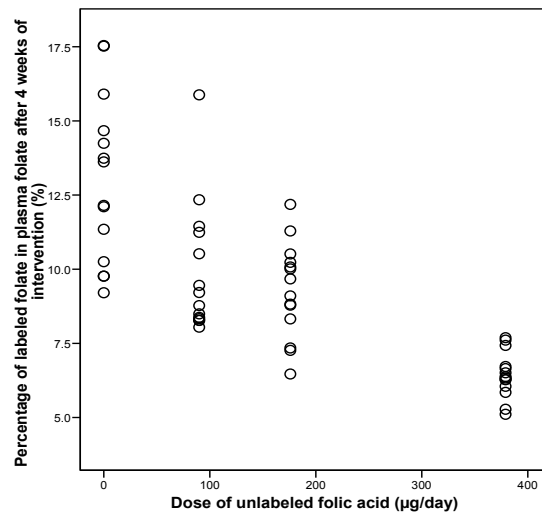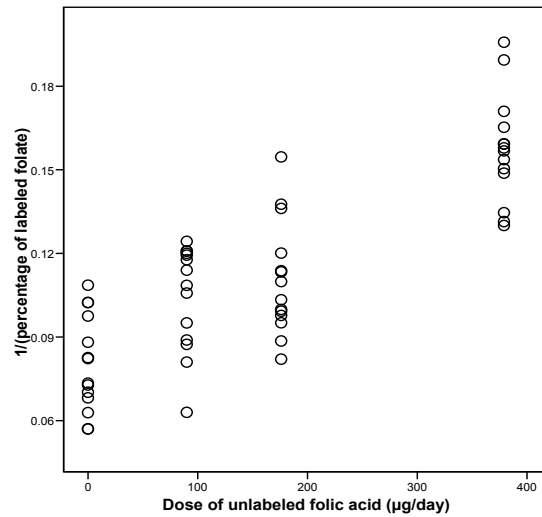

Figure 3.2: the inverse of the percentage of labeled folate in plasma folate after four weeks of intervention with various intakes of unlabelled folic acid in combination with a fixed intake of labelled folate. Each circle represents one subject. R^2 of the association is 0.69.

Table 3.1: characteristics of the subjects assessed two weeks before the start of the 4-wk experimental period

	Group A n=14	Group B n=14	Group C n=14	Group D n=14
Gender, no of men/women	3/11	4/10	2/12	2/12
age, years	23 (19-47)[1]	21 (18-48)	22 (18-26)	22 (18-29)
BMI, kg/m^2	21± 2[2]	22 ± 2	22 ± 2	23 ± 3
serum folate, nmol/L	10.9 ± 4.0	11.1 ± 3.5	11.0 ± 3.7	11.0 ± 3.3
Plasma total homocysteine, µmol/L	9.0 ± 2.0	8.8 ± 2.6	9.1 ± 3.8	8.7 ± 2.7
Creatinine, µmol/L	78 ± 14	77 ± 13	76 ± 9	82 ± 12
serum vitamin B$_{12}$, pmol/L	265 ± 75	265 ± 112	258 ± 106	234 ± 38
MTHFR-genotype, subjects with CC/CT/TT- genotype[3]	8/5/1	7/6/1	7/6/1	10/3/1

[1]Median (range): all such values.

[2]Mean ± standard deviation: all such values.

[3]Genotyping was performed with the method of Frosst *et al.* (13)

Table 3.2: percentages of labeled folate in plasma folate after two and after four weeks of intervention with a daily supplement of 55 µg of labeled folic acid in combination with a variable dose of unlabeled folic acid.

	Group A n=14	Group B n=14	Group C n=14	Group D n=14
Dose of unlabeled folic acid, µg/day	0	100	200	400
week 2	10.9 % ± 2.6[1]	8.2 % ± 3.1	8.2 % ± 2.4	5.5 % ± 0.8
week 4	13.0 % ± 2.8	10.0 % ± 2.2	9.3 % ± 1.6	6.4 % ± 0.8

[1]Mean ± standard deviation: all such values.

Discussion

We showed that the percentage of ^{13}C-labeled folate in the total plasma folate pool was lower when the intake of unlabeled folic acid was higher; the inverse of the percentage of ^{13}C-labeled folate in plasma was highly, linearly correlated with the amount of folic acid that was ingested.

Our study required an LC-MS/MS technique sensitive to low concentrations of ^{13}C-labeled folate. We showed that the method was sensitive enough to detect percentages of labeled folate of 5-15%. In addition, the method allowed us to detect small

differences in percentages of labeled folate between the different folic acid treatment groups.

In a future experiment we can apply this stable isotope method to assess bioavailability of dietary sources of folate relative to folic acid. In that planned experiment, four groups of subjects will daily ingest a capsule with a fixed low dose of ^{13}C-labeled folic acid. In addition, we will provide three of these groups with a diet low in folate and with a supplement of unlabeled folic acid that differs per group. With the data from these three groups we can draw a calibration curve of the percentage of labeled folate in plasma folate versus intake of folic acid. The fourth group of subjects will also daily ingest a capsule with a low dose of labeled folic acid; however, this group will consume a diet high in dietary folates. In this group the absorbed amount of dietary folate determines the percentage of labeled folate in the plasma folate. This percentage will be interpolated on the calibration curve to assess what the absorbed amount of dietary folate is. In addition, we will analyze the amounts of dietary folate in all groups. Subsequently, we can calculate the percentage of dietary folate that was absorbed from the amount of dietary folate that was available in the food, which is the bioavailability of dietary folate.

We did not monitor or control dietary intake of the subjects during the intervention, but assumed that due to the randomization of the subjects the background diets of all groups were comparable. If the intake of dietary folate had been controlled, the variation within treatment groups would probably have been less and the correlation between the percentage of labeled folate and intake of folic acid would have been higher.

The current study was the final step in the validation process of a stable isotope dilution method. We showed that the association between the inverse of the percentage of labeled folate in the plasma folate and the intake of unlabeled folic acid could be approached linearly. In future experiments this method will be used to study the bioavailability of dietary folates relative to synthetic folic acid.

References

1. Scott JM. Folate and vitamin B12. Proc Nutr Soc 1999;58:441-8.
2. Brody T, Shane B. Folic acid. In: Rucker R, Suttie J, McCormick D, eds. Handbook of vitamins. New York: Academic Press Marcel Dekker, 2001:427-463.
3. Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. MRC Vitamin Study Research Group. Lancet 1991;338:131-7.
4. Rogers LM, Pfeiffer CM, Bailey LB, Gregory JF. A dual-label stable-isotopic protocol is suitable for determination of folate bioavailability in humans: evaluation of urinary excretion and plasma folate kinetics of intravenous and oral doses of [13C5] and [2H2]folic acid. J Nutr 1997;127:2321-7.
5. Sauberlich HE, Kretsch MJ, Skala JH, Johnson HL, Taylor PC. Folate requirement and metabolism in nonpregnant women. Am J Clin Nutr 1987;46:1016-28.
6. Brouwer IA, van Dusseldorp M, West CE, et al. Dietary folate from vegetables and citrus fruit decreases plasma homocysteine concentrations in humans in a dietary controlled trial. J Nutr 1999;129:1135-9.
7. Hannon-Fletcher MP, Armstrong NC, Scott JM, et al. Determining bioavailability of food folates in a controlled intervention study. Am J Clin Nutr 2004;80:911-918.
8. Melse-Boonstra A, Verhoef P, West CE, et al. A dual-isotope-labeling method of studying the bioavailability of hexaglutamyl folic acid relative to that of monoglutamyl folic acid in humans by using multiple orally administered low doses. Am J Clin Nutr 2006;84:1128-1133.
9. Melse-Boonstra A, West CE, Katan MB, Kok FJ, Verhoef P. Bioavailability of heptaglutamyl relative to monoglutamyl folic acid in healthy adults. Am J Clin Nutr 2004;79:424-9.
10. Konings EJ. A validated liquid chromatographic method for determining folates in vegetables, milk powder, liver, and flour. J AOAC Int 1999;82:119-27.
11. Bland JM, Altman DG. Statistics Notes: Transforming data. BMJ 1996;312:770-.
12. Melse-Boonstra A, Verhoef P, West C. Quantifying folate bioavailability: a critical appraisal of methods. Curr Opin Clin Nutr Metab Care 2004;7:539-45.
13. Frosst P, Blom HJ, Milos R, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10:111-3.

4

Bioavailability of food folates is 80% of that of folic acid

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Abstract

Background: The bioavailability of natural food folates is lower than synthetic folic acid, but there is no agreement how much lower.

Objective: We determined the aggregate bioavailability of food folates from fruits, vegetables and liver relative to folic acid in a 4-week dietary intervention study.

Design: Seventy-two healthy men and women were randomly divided over 4 treatment groups. Group A [n=29] received a high-folate diet with 369 μg of food folate per day and a placebo capsule, groups B, C and D [n=14 or 15] received a low-folate diet with 73 μg of food folate per day and folic acid capsules. These capsules contained 92 μg of folic acid per day for group B, 191 μg for group C and 289 μg for group D. In addition, all 72 subjects daily ingested a capsule with 58 μg of $[^{13}C_{11}]$-labeled folic acid. We measured the percentage of $[^{13}C_{11}]$-labeled folate in plasma folate at the end of the intervention, and the changes in serum folate concentrations over the four weeks of the intervention.

Results: Bioavailability of food folate relative to folic acid was 78% [95%CI: 48 to 108%] according to $[^{13}C_{11}]$-labeled folate and 85% [52 to 118%] according to changes in serum folate concentrations.

Conclusion: The aggregate bioavailability of folates from fruits, vegetables and liver is about 80% relative to folic acid. The consumption of a diet rich in food folate can improve the folate status of a population more efficiently than is generally assumed.

Introduction

Folate is a generic term for the various biochemical moieties of the B vitamin pteroyl glutamic acid or folic acid. Important sources of folate are fruits, (fortified) breakfast cereals, vegetables, dairy products and liver products (1-3). Folic acid, the fully oxidized and stable form of this vitamin, is used extensively in dietary supplements and for food fortification, but does not occur naturally in significant amounts (4).

An inadequate folate intake will lead to a decrease in serum and erythrocyte folate, to increased blood concentrations of homocysteine and ultimately to macrocytic anemia (5). It might also increase the risk of neurocognitive diseases, cancer and cardiovascular disease (6-9). Increased intake of folic acid by women of childbearing age reduces the risk of neural tube defects in the babies of these women (10).

Folate bioavailability is defined as the proportion of an ingested amount of folate that is absorbed in the gut and becomes available for metabolic processes. In human intervention studies relative bioavailability is usually assessed by comparison with a reference dose of folic acid. Bioavailability of food folate is generally lower than that of folic acid but it is unclear how much lower (11, 12).

Recommended daily allowances for food folate take into account its lower bioavailability. The basis for adapting the US recommendations for folate to the lower bioavailability of food folate was formed by a study of Sauberlich et al. (5, 13). Sauberlich et al. (13) stated that bioavailability of food folate was no more than 50% compared with folic acid. Unfortunately the authors did not indicate how this finding was obtained.

Other long-term dietary intervention studies found bioavailability estimates between 30 and 98% (14, 15). Thus, there is no clear figure for the bioavailability of natural folates compared with folic acid.

We therefore performed a large, long-term dietary intervention study in which we compared bioavailability of food folate with that of different doses of folic acid; we assessed bioavailability simultaneously both from changes in serum folate concentrations and from stable isotope dilution.

Methods

Subjects

The Medical Ethical Committee of Wageningen University approved the study. We recruited subjects from staff and students of Wageningen University and from the Wageningen population. Ninety-three healthy men and women volunteered to take part. During a screening visit, four weeks before the start of the trial, they gave their written informed consent, filled out a questionnaire, and donated a fasting blood sample in which we assessed the concentration of serum vitamin B12, serum folate (Access Immunoassay, Beckman Coulter, Fullerton, CA, USA) serum creatinine (Synchron LX20, Beckman Coulter, Fullerton, CA, USA), plasma total homocysteine (16), and methylenetetrahydrofolate reductase (MTHFR) 677 C→ T genotype (17).

We excluded eighteen subjects based on the following criteria: use of B vitamins within the period three months prior to the study; BMI below 18 kg/m^2 or above 30 kg/m^2; use of drugs that interfere with folate metabolism; serum vitamin B12 concentration below 119 pmol/L; serum creatinine concentration above 125 µmol/L; plasma total homocysteine concentration above 26 µmol/L; presence of cardiovascular disease, cancer, rheumatoid arthritis, epilepsy, or gastro-intestinal disorders.

The remaining 75 subjects were stratified by serum folate, MTHFR 677C→T genotype and gender. Within each stratum, randomized blocks assigned the subjects to one of the four intervention groups.

Design

The trial included four parallel intervention groups, **Figure 4.1**. Group A, the food folate group, consumed a diet high in food folate and a placebo capsule. Groups B, C and D, the folic acid groups, all received the same diet low in food folate plus a daily capsule which for group B contained 92 µg of folic acid, for group C 191 µg and for group D 289 µg. In addition, all 75 subjects received a capsule with 58 µg of [$^{13}C_{11}$]-folic acid every day. The folic acid groups (B through D) served to calibrate our outcome measurements, namely the percentage of [$^{13}C_{11}$]-labeled folate in the total pool of plasma folate and the change in serum folate, in terms of folic acid intake. We

constructed calibration lines of the percentage of $[^{13}C_{11}]$-labeled folate in the total pool of plasma folate and of the change in serum folate as a function of folic acid intake in groups B through D. We then projected the mean percentage of labeled folate in plasma folate in group A and the mean change in serum folate in group A on the corresponding calibration line to assess to which dose of folic acid the food folate in group A was equivalent to. The duration of the study was 31 days; the folate intervention started at day 3 and ended at day 31. Two fasting blood samples were drawn at baseline at day 1 and day 3 and after four weeks of intervention at day 29 and 31.

Diet

Dietitians from the Division of Human Nutrition of Wageningen University estimated the habitual energy intake of the subjects with a validated food frequency questionnaire before the start of the intervention (18). We provided ~90 percent of their total daily
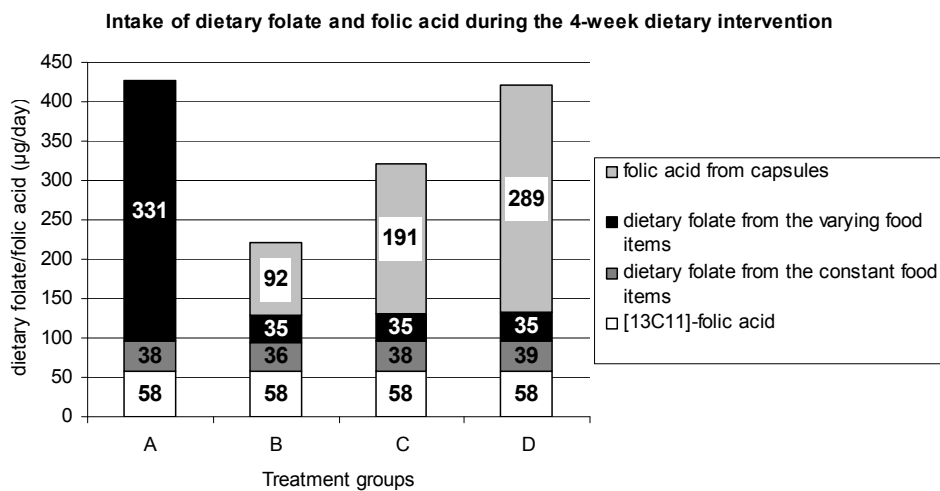
Figure 4.1: mean intake of food folate and folic acid from food and capsules as analyzed by HPLC. Group A consumed a diet high in food folate and a placebo capsule; groups B, C and D consumed a diet low in food folate and folic acid capsules. All groups daily received a capsule with $[^{13}C_{11}]$-labeled folic acid. The constant food items were similar for all groups, while the varying food items of group A differed from groups B through D; for group A the varying food items were products rich in food folate, for groups B through D these products were replaced by similar products low in food folate.

energy requirement to the subjects during the intervention. Subjects daily had a limited free choice from a list of products which provided the remaining 10 percent of energy. These free-choice items were mainly non-alcoholic drinks, alcoholic drinks (no more than one beer each day), candies and sweet sandwich fillings. The items contained a low amount of folate and fat. Subjects kept a diary in which they recorded which free-choice items they consumed, whether they took their capsules, illnesses, use of medication and any deviations from the diet.

The diets we supplied consisted of constant food items and of varying food items. The constant part of the diet consisted of whole wheat bread, margarine, sweet sandwich fillings, cheese and/or ham, cookies, milk, boiled potatoes or rice or pasta and meat or a meat replacement. These constant food items provided about 9 MJ (~2,100 kcal) per day for a typical participant and were similar for all groups. The varying food items provided about 2 MJ (~480 kcal) per day and differed between group A on the one hand and groups B through D on the other hand, **Table 4.1**.

Although the constant food items were similar for all groups, the amounts of food we supplied varied dependent on the energy requirement of the subjects. The food folate in this constant part of the diet was as low as possible, ~100 μg of folate per 9 MJ of constant foods as calculated from food tables (19).

The varying food items for group A were products rich in food folate, ~350 μg per 2 MJ of varying foods as calculated from food tables; for groups B through D these products were replaced by similar products low in food folate, ~30 μg per 2 MJ as calculated from food tables (**Table 4.1**). Vegetables, fruit juices, liver paste and fruits contributed most to the folate intake in the high folate group, **Figure 4.2**. The varying part of the diet was nearly the same for all 29 subjects in group A, independent of the subject's energy requirement; only the amounts of dressing, sauce and dessert were adjusted to individual energy requirements (**Table 4.1**). Likewise, the varying part of the diet for groups B through D was nearly the same for all 43 subjects in these groups. During weekdays at lunch time, we served hot lunches to all subjects; subjects consumed these lunches under our supervision at the Division of Human Nutrition of

Wageningen University. We weighed out all foods and drinks for each subject. After lunch subjects received their package with foods and drinks for their evening meal and for breakfast. On Fridays subjects received a package with foods and drinks for the breakfasts, lunches and hot meals of the weekend plus instructions for the preparation of these foods.

Meals were prepared from conventional foods and drinks. Folic acid fortification is not mandatory in the Netherlands and the foods and drinks that we used did not contain added folic acid.

Measurement of nutrients in food samples

We collected a total of four duplicate diets on each day of the trial: two duplicate diets representative for group A and two for groups B through D; the energy content of these duplicates was 11 MJ (~2,600kcal) based on food tables. During weekdays we daily collected the duplicates at lunch time. We also collected a duplicate of a package with foods and drinks for the weekend. This was prepared for consumption during the actual weekend and then worked up as described below. We collected, pooled and analyzed the items of the constant part of the diet separately from the varying food items. For the food folate analyses hot items were cooled down to ~10 ºC immediately after collection in a blast chiller before they were added to the cold items. We added 250 mL of chilled CHES/HEPES buffer pH 7.85 with 2% ascorbic acid and 0.2 M 2-mercapto-ethanol, per kg of food and homogenized this in a blender under a flow of nitrogen and stored the samples at -80 ºC until analysis.

Food folate was analyzed in a selection of the duplicate diets: we selected one random Monday duplicate out of the four Mondays of the study of group A and analyzed food folate in the constant food items and in the varying food items. The same was done for all other weekdays. We repeated this selection for diets from groups B through D. In this way we avoided the thawing and homogenization that would have been necessary if we had pooled the diets of all four Mondays of the study. We decided not to analyze folate in all samples, since the food folate analysis is time and labor intensive. Food folate was analyzed with an HPLC method (20). In short, duplicate diets were thawed,

extracted with CHES/HEPES buffer in a boiling water bath for 10 min and cooled down in a water bath at 0˚C. Samples were subjected to tri-enzyme treatment, purified on an affinity column (Folate Binding Protein, Scipps, San Diego, CA) and analyzed by HPLC with fluorescence and UV detection. All analyses were performed in duplicate by splitting the sample after it had been thawed.

Duplicate diets for the analyses of energy in the diets were stored at -20 ºC. After the study we thawed these duplicates, pooled the constant parts of each diet per week and pooled the varying food items of each diet per week and homogenized them. Samples were stored at -20 ºC until analysis.

Total fat, protein, dry matter, ash and fiber were analyzed in these duplicates. From these analyses we calculated the amount of fat, protein, carbohydrates, fiber and energy in the diets actually eaten by the subjects.

Capsules

The capsules with folic acid (Merck Eprova, Schaffhausen, Switserland) were especially produced for this study. We ordered capsules containing 100, 200 and 300 µg, respectively. To analyze the actual amount of folic acid, we dissolved capsules in CHES/HEPES buffer pH 7.85 with 2% ascorbic acid and 0.2 M 2-mercapto-ethanol in a boiling water bath and measured folic acid by HPLC with UV detection (20). The amount of folic acid in a random sample of 20 capsules of each dose was about 95% of the expected dose.

$[^{13}C_{11}]$-Folic acid was synthesized for us by ARC (Apeldoorn, the Netherlands). Folic acid was labeled with six ^{13}C atoms in the benzoic acid structure and with five ^{13}C atoms in the glutamate part (21). These capsules contained 58 ± 11 µg of folic acid (mean $\pm$ SD, n=20) according to HPLC analysis (20), which was ~85% of the expected dose.

An independent research assistant from Wageningen University coded the placebo and folic acid capsules: subjects and investigators were blinded to the folic acid treatment.

The assistant did not reveal the code until all data had been gathered and double-checked. Subjects ingested their capsules just before the hot meal.

Measurements in blood

For the measurement of serum folate, blood samples were drawn into coagulation tubes, stored in the dark for 30 to 120 minutes at room temperature and centrifuged at 2600g for 10 minutes at 4 ˚C. Serum was pipetted off and stored at -80 ˚C. We measured the serum folate concentration with a chemiluminescent immunoassay (Access Immunoassay system, Beckman Coulter, Fullerton, CA, USA) in the samples of days 1, 3, 29 and 31. Baseline values represent the mean of days 1 and 3 and week 4 values the mean of days 29 and 31. Intra- and interassay CV of this immunoassay were <15%.

For the measurement of labeled folate, blood samples were drawn into EDTA-containing tubes, immediately placed on ice and centrifuged within 30 minutes at 2600g for 10 minutes at 4 ˚C. Plasma was pipetted off and stored at -80 ˚C. We measured the percentage of labeled folate in the total pool of folate in plasma with liquid chromatography tandem mass spectrometry (LC-MS/MS), see formula 1(21).

1) Percentage of labeled folate =

$$100 * \frac{[^{13}C_{11}]\text{-}5\text{-}methyltetrahydrofolate}{\{[^{13}C_{11}]\text{-}5\text{-}methyltetrahydrofolate + [^{13}C_{0}]\text{-}5\text{-}methyltetrahydrofolate\}}$$

The numerator is the area under the curve of the $[^{13}C_{11}]$-5-methyltetrahydrofolate LC-MS/MS peak and the denominator is the sum of the areas under the curve of the $[^{13}C_{11}]$-5-methyltetrahydrofolate and the $[^{13}C_{0}]$-5-methyltetrahydrofolate LC-MS/MS peaks.

Labeled folate was analyzed only in the samples of day 1 and 29. We chose to analyze labeled folate only in one baseline sample and in one follow-up sample, since we expected that the estimate for bioavailability derived from labeled folate data would be

more precise than the estimate derived from serum folate data even if analyzed only in one sample. The labeled compound does not occur naturally and was not detected in baseline samples. We restricted our measurements to the 5-methyltetrahydrofolate fraction of the plasma, since this is the most abundant folate vitamer in plasma (22).

Calculation of bioavailability

We plotted the individual percentages of labeled folate in the plasma folate of subjects in groups B, C and D against the intake of supplemental folic acid. We fitted a linear regression line through these points to construct a calibration line of percentage of labeled folate against intake of supplemental folic acid. The mean percentage of labeled folate in group A was then projected on this calibration line to assess by interpolation to which dose of folic acid the additional amount of food folate in group A (folate$_{additional}$) was equivalent, **Figure 4.3**; this dose was called X_a. Relative bioavailability of food folate was derived from X_a and folate$_{additional}$ according to formula 2.

2) $\quad Bioavailability_{relative} = 100\% * \dfrac{X_a}{folate_{additional}}$

Where folate$_{additional}$ was calculated with formula 3:

3) $\quad folate_{additional} = food\ folate\ in\ group\ A - food\ folate\ in\ groups\ B\ through\ D\ (in$
$\qquad \mu g\ per\ day)$

The 95% CI associated with this bioavailability estimate was calculated with formula 4:

4) [lower limit, upper limit]

$$= \left[100\% \frac{X_a - t_{0.975;n-2} * s_{X_a}}{folate_{additional}}, 100\% * \frac{X_a + t_{0.975;n-2} * s_{X_a}}{folate_{additional}} \right]$$

Where the standard error of prediction for X_a (s_{Xa}) was calculated with formula 5 (23):

5) $$s_{X_a} = \sqrt{\frac{MSE}{b_1^2}\left[\frac{1}{m} + \frac{1}{n} + \frac{(X_a - \overline{X})^2}{\sum(X_i - \overline{X})^2}\right]}$$

MSE = mean square error

b_1 = slope of the regression line in percentage change in labeled folate per μg of folic acid

m = number of subjects in group A

n = number of subjects in groups B, C and D together

$\overline{X}$ = mean intake of folic acid in groups B, C and D in μg

X_i = individual intake of folic acid of subjects in groups B, C and D in μg

We also calculated relative bioavailability from changes in serum folate. Similar to the approach for the labeled folate data, we plotted the individual change in serum folate of subjects in groups B through D against the intake of supplemental folic acid and fitted a linear regression line through the data points.
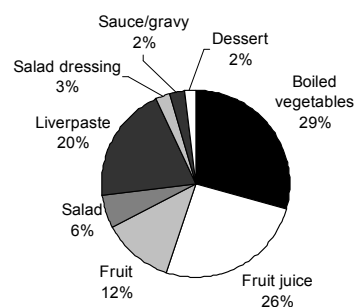

Figure 4.2: contribution of the various food items to the additional food folate in the diet of group A compared with groups B through D.

Table 4.1: varying food items of the diets in a study of the bioavailability of food folate versus folic acid. The food folate group (A) was fed foods and drinks high in food folate, and the folic acid groups (B through D) were fed equivalent foods and drinks low in food folate plus various doses of folic acid.

	Group	Per day	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Boiled vegetables	A	*200 g*	Spinach	Brussels sprouts & red pepper	Broad beans & corn	Broccoli	Spinach, corn & red pepper	Sugar peas	Broccoli & cauliflower
	B-D	*100 g*	Green beans	Peas & carrots	Mushrooms, leek & carrots	Red cabbage	Corn, carrots, green pepper & mushrooms	Carrots	Cabbage, green pepper & mushrooms
Salad	A	*50 g*	Cabbage, green pepper, tangerines & cashew nuts	Endive, red pepper, tangerine & hazelnut	Lettuce, chick peas, pineapple & walnuts	Pakchoi, red pepper, peach & cashew nuts	Iceberg lettuce, chick peas, dates & hazelnut	Broccoli, corn, peach & walnuts	Kohlrabi, corn, dates & hazelnuts
	B-D	*50 g*	Blanched celery, tomato & dates	Cucumber, onion, tomato & pineapple	Chicory, apple & raisins	Fennel, cucumber, tomato & mixed fruit	Cabbage, tomato, pickle & peach	Radish, cucumber, onion & pineapple	Carrot, cucumber & raisins
Fruit	A	*2 pieces*	1 orange and 1 banana or kiwi	1 orange and 1 banana or kiwi	1 orange and 1 banana or kiwi	1 orange and 1 banana or kiwi	1 orange and 1 banana or kiwi	1 orange and 1 banana or kiwi	1 orange and 1 banana or kiwi
	B-D	*1 piece*	Apple	100 g of grapes	Apple	100 g of melon	Pear	Apple	Pear
Fruit juice	A	*400 mL*	Orange juice	Orange juice	Orange juice	Orange juice	Orange juice	Orange juice	Orange juice
	B-D	*400 mL*	Apple- or grape juice	Apple- or grape juice	Apple- or grape juice	Apple- or grape juice	Apple- or grape juice	Apple- or grape juice	Apple- or grape juice
Liver paste	A	*25 g[1]*	Liver paste	Liver paste	Liver paste	Liver paste	Liver paste	Liver paste	Liver paste
	B-D	-	-	-	-	-	-	-	-

Table 4.1: continued

	Group	Per day	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Salad dressing[2]	A	*15 g [10-18][3]*	Dressing	Dressing	Dressing	Dressing	Dressing	Dressing	Dressing
	B-D	*17 g [14-20][3]*	Dressing	Dressing	Dressing	Dressing	Dressing	Dressing	Dressing
Sauce/ gravy[2]	A	*53 g [41-68][3]*	Sauce/Gravy	Sauce/Gravy	Sauce/Gravy	Sauce/Gravy	Sauce/Gravy	Sauce/Gravy	Sauce/Gravy
	B-D	*69 g [60-75][3]*	Sauce/Gravy	Sauce/Gravy	Sauce/Gravy	Sauce/Gravy	Sauce/Gravy	Sauce/Gravy	Sauce/Gravy
Dessert	A	*140 g [115-155][3]*	Flavored yoghurt	Flavored yoghurt	Flavored yoghurt	Flavored yoghurt	Flavored yoghurt	Flavored yoghurt	Flavored yoghurt
	B-D	*110 g [83-135][3]*	Flavored custard	Flavored custard	Flavored custard	Flavored custard	Flavored custard	Flavored custard	Flavored custard

[1]Vegetarians received a yeast-based vegetarian spread (Tartex®, Freiburg, Germany) 12.5 g per day

[2]Salad dressing and sauce/gravy were prepared with egg yolk for group A and with egg white for groups B through D

[3]The amount of dressing, sauce and dessert differed per subject, based on the energy requirement of that subject. Values are given as medians [25percentile -75percentile].

Bioavailability

Table 4.2: characteristics of subjects, assessed four weeks before the start of the 4-week experimental period. Data are means with standard deviations in parentheses, unless stated otherwise[1].

	Food folate group	Folic acid groups		
	A	**B**	**C**	**D**
	n=29	**n=15**	**n=14**	**n=14**
Female (%)	76	73	64	71
Age (y), median [range]	22	22	23	24
	[19-41]	[19-48]	[20-49]	[19-49]
BMI (kg/m^2)	23	22	22	22
	(3)	(4)	(2)	(3)
Serum folate (nmol/L)	11.3	11.6	11.5	11.9
	(3.7)	(2.9)	(4.1)	(3.5)
Plasma total homocysteine (μmol/L)	10.1	9.7	10.3	10.3
	(2.6)	(2.3)	(3.4)	(3.2)
Serum vitamin B12 (pmol/L)	219	230	228	191
	(69)	(68)	(68)	(55)
MTHFR-genotype, numbers of 677 CC/CT/TT	15/13/1	8/7/0	7/7/0	8/6/0
Vegetarians (%)	17	7	29	14

[1]There were no statistically significant group differences by ANOVA or chi-square test.

Results

One person in group A became ill before the start of the study and one each in group C and D dropped out for personal reasons within the first two weeks. Analyses are based on the 72 subjects that completed the study.

The diaries of the subjects did not show any deviations from the provided diets that might have affected the results. Capsule intake was verified by counting the returned capsules and by checking the subjects' diaries: mean capsule intake was 99%, the lowest value was 90%. Characteristics measured at screening were not significantly different between groups (**Table 4.2**). The mean intakes of energy protein, fat, carbohydrates and fiber during the trial were not significantly different between groups (**Table 4.3**). According to analysis of duplicate diets, the food folate intake in group A was 369 μg per day while the food folate intake in groups B through D was 73 μg per day (**Figure 4.1**); thus group A had 296 μg per day more food folate than did groups B-

D. Homocysteine concentrations decreased slightly with increasing intakes of folic acid (**Table 4.4**).

As expected, the percentage of labeled folate in the plasma folate decreased with increased intakes of folic acid in groups B through D, **Figure 4.3**. The equation of the calibration line was:

% of labeled folate in plasma folate = - 0.01616 *(µg of supplemental folic acid/day) + 13.47

The mean percentage of labeled folate in plasma of group A was 9.7% (**Table 4.4**). This value was entered into the equation of the calibration line (**Figure 4.3**), which showed that the extra 296 µg of food folate ingested by group A was equivalent to ingestion of 232 µg of folic acid. Therefore relative bioavailability of food folate calculated from labeled folate data (ie 232/296*100) was 78%, [95%CI: 48, 108%].

Serum folate concentrations increased linearly with increasing intakes of folic acid in groups B through D (**Figure 4.4**). The equation of the calibration line was:

change in serum folate (nmol/L) = 0.02369 *(µg of supplemental folic acid/day) - 0.083

The mean increase in serum folate in group A was 5.9 nmol/L (**Table 4.4**). This value was entered into the equation of the calibration line (**Figure 4.4**). This showed that the extra 296 µg of food folate ingested in group A was equivalent to ingestion of 252 µg of folic acid. Therefore relative bioavailability of food folate calculated from serum folate data (ie 252/296*100) was 85%, [52%, 118%].
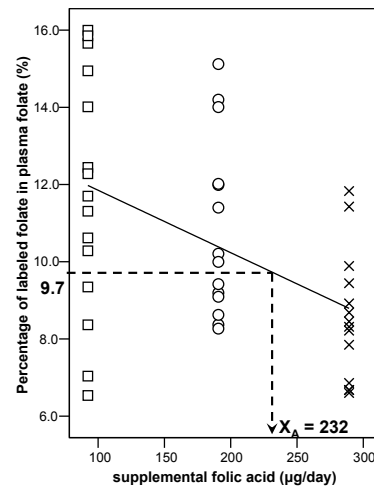
Figure 4.3: percentage of labeled folate in the plasma folate after 4 weeks of intervention in folic acid groups (B through D). Each symbol represents 1 subject; □ = group B, ○ = group C, × = group D. The solid line is the linear regression line through the individual data points of groups B, C and D. The mean percentage of labeled folate in group A was 9.7%. This was projected on the regression line and corresponded to an estimated intake of folic acid of 232 μg (broken lines). The R^2 of the regression line was 0.23, while the slope was - 0.01616, 95%CI [-0.02550, -0.00683]..

Figure 4.4: change in serum folate from baseline to week 4 in the folic acid groups (B through D). Each symbol represents 1 subject; □ = group B, ○ = group C, × = group D. The solid line is the linear regression line through the individual data points of groups B, C and D. The mean change in serum folate in group A was 5.9 nmol/L. This was projected on the regression line and corresponded to an estimated intake of folic acid of 252 μg (broken lines).The R^2 of the regression line was 0.22, while the slope was 0.02369, 95%CI [0.00948, 0.03789].
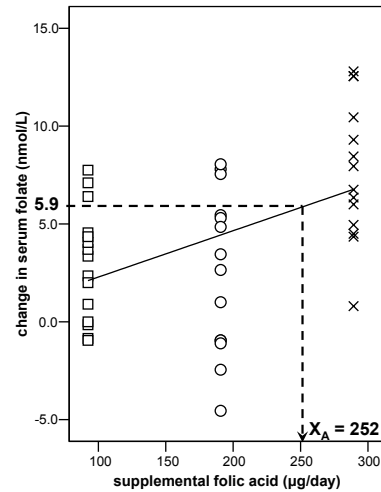
Table 4.3: intake of total energy and natural folate from the diets consumed by the 4 different treatment groups (A-D) plus the amount of folic acid measured in the capsules taken by groups B through D. Values are means with standard deviations in parentheses, unless stated otherwise.

		Food folate group	Folic acid groups		
		A	B	C	D
		n=29	n=15	n=14	n=14
Energy intake[1], in MJ/day	Constant food items [2]	7.6 (2.1)	8.5 (1.8)	9.3 (2.6)	9.2 (1.9)
	Varying food items [2]	2.2 (0.1)	1.4 (0.1)	1.5 (0.1)	1.5 (0.1)
	TOTAL DIET	**9.8 (2.2)**	**9.9 (1.9)**	**10.8 (2.7)**	**10.7 (2.0)**
Food folate intake, in µg/day [range][3]	Constant food items [2]	38 [32-47]	36 [28-49]	38 [30-54]	39 [30-54]
	Varying food items [2]	331 [259-426]	35 [14-51]	35 [14-51]	35 [14-51]
	TOTAL DIET	**369 [295-463]**	**71 [50-101]**	**73 [52-105]**	**74 [52-105]**
Folic acid[4], in µg/day	capsules	n.d.[5]	92 (6)	191 (11)	289 (24)

[1] The mean energy intake per group was calculated from food tables (19) and subsequently adjusted for the difference between calculated and analyzed amount of energy in a diet that provided 11MJ/day (~2,600 kcal). Figures for the constant part of the diet include the amount of energy in the free-choice items which was calculated from food tables (19). Mean intakes of macronutrients based on analyses were: protein 13 energy%, fat 32-33 energy%, carbohydrates 52-53 energy%. Mean fiber content of the diet based on analyses was 3.7 g/MJ (37 g/day) for group A and 3.4 g/MJ (36 g/day) for groups B through D. There were no statistically significant differences in energy intake between groups by ANOVA.

[2] The constant part of the diet was similar for all groups, but amounts varied with the energy requirement of each individual. The varying food items were high in folate for group A and low in folate for groups B through D, amounts were largely the same for all individuals.

[3] Food folate was analyzed in duplicate diets. The amount of folate was analyzed in the constant food items and in the varying food items once for each day of the week in group A and once for each day of the week for groups B through D. Thus, each value is the mean of seven analyses; Monday through Sunday. The amount of food folate varied per day due to the daily variation in menus; the range indicates the highest and lowest amount of daily food folate over the seven days of the week. All analyses were performed in duplicate. All food folate analyses are performed in duplicate diets providing 11MJ (~2,600kcal) per day. The differences between the amounts of folate in the constant part of diets B, C and D arose because amounts of folate were recalculated to the actual energetic intakes, which differed slightly between groups B, C and D.

[4] Folic acid was analyzed in a random sample of 20 capsules per dose.

[5] n.d. not detectable in the placebo capsules

Table 4.4: effect of a high-folate diet (group A) and of low-folate diets with increasing amounts of folic acid (groups B through D) on the percentage of labeled folate in plasma folate, concentration of serum folate and concentration of plasma total homocysteine. Values are means with standard deviations in parentheses.

		Food folate group	Folic acid groups		
		A	**B**	**C**	**D**
		n=29	**n=15**	**n=14**	**n=14**
Labeled folate, as % of total folate	**Week 4[1]**	9.7 (2.0)	**11.8 (3.1)**	**10.9 (2.3)**	8.6 (1.7)
Serum folate. in nmol/L	Baseline	12.1 (4.0)	12.0 (3.5)	12.8 (4.5)	12.3 (4.9)
	Week 4	18.0 (4.1)	15.0 (3.4)	15.3 (3.9)	20.0 (6.5)
	Change [2]	**5.9** **(3.9)**	**3.0** **(2.8)**	**2.6 (4.1)**	**7.7** **(3.6)**
Plasma total homocysteine, in μmol/L	**Change [3]**	**-0.8** **(1.4)**	**-0.6** **(1.2)**	**-0.7 (1.2)**	**-1.2** **(1.2)**

[1]The labeled compound does not occur naturally and was not detected in baseline samples.

[2]There were statistically significant differences between groups in changes in serum folate (ANOVA with post hoc Bonferroni tests). Group A differed from group C (p=0.043), group B differed from group D (p=0.006) and group C differed from group D (p=0.003).

[3]There were no statistically significant differences between groups in changes in homocysteine by ANOVA.

Discussion

In a four-week, dietary controlled study, we found that bioavailability of food folate relative to folic acid was 78% based on an isotope method and 85% based on changes in serum folate. The fact that both methods yielded similar estimates strengthens the confidence in our finding. As we have no reason to believe that one method is superior to the other, we consider their average of 82% our best estimate for the bioavailability of natural folates.

In our trial we carefully controlled the intake of food folate and folic acid. A problem in studies that aim to assess relative bioavailability of food folate versus folic acid has been the fact that the actual intake of food folate may differ from the targeted dose, since the stability of food folates during cooking is poor and since the compliance of subjects to the intervention diet can be low (12) and these factors will lead to underestimation of the bioavailability of folate from foods. In contrast to food folate, folic acid is very stable and compliance with taking a pill is likely to be higher than

compliance with a prescribed diet. We strictly controlled the intake of both food folate and folic acid in our subjects: the subjects came to our laboratory daily during weekdays to consume a hot meal plus supplements, and thus a large part of the daily intake of folate and folic acid was supervised. Furthermore, we provided ~90% of all the foods and drinks for consumption off-site and asked subjects to note in a diary if they deviated from these supplies. In addition, we based our estimates of bioavailability on the analyzed amounts of folic acid and folate in the capsules and in the duplicates of the diets as actually eaten.

We measured two markers for folate bioavailability: the percentage of labeled folate in the plasma folate and changes in serum folate. We expected that the most precise estimate for bioavailability would result from the labeled folate data. Since the labeled compound does not occur naturally, the percentage of labeled folate in plasma only had to be evaluated after the intervention: this eliminated the extra error in subtracting two measurements from each other. However, the precision was similar for labeled folate and serum folate: the confidence intervals surrounding both estimates for bioavailability had a similar width. For serum folate the estimate for bioavailability and the width of the confidence interval did not change when we based our calculations only on the measurements in samples from day 1 and day 29 (data not shown). Therefore, in this experimental set-up the expensive stable isotope method yielded no advantage over serum folate measurements. However, the fact that both methods yielded similar numbers emphasizes the internal consistency of the study and strengthens the confidence in our outcome. We used folic acid labeled with 11 ^{13}C-atoms in our intervention, because we had it in stock from earlier experiments. Using cheaper forms of folic acid would not have made the intervention much cheaper because the LC-MS/MS analysis is much more expensive than is the use of the labeled compound itself.

The participants ingested the folic acid capsules just before their hot meal, which might have affected the serum folate responses in groups B through D, and hence the estimate for relative bioavailability. Pfeiffer et al. found that the serum folate response to a folic acid supplement taken together with a light meal was 15% lower than the

folate response to a folic acid supplement taken on an empty stomach (24). The difference was not statistically significant, but if it were valid, then the calibration line as plotted in **figure 4.4** would have been steeper and the bioavailability estimate for food folates would have been lower if subjects had ingested the capsules on an empty stomach. We plotted a linear calibration line in **figure 4.2**. However, a lack of fit test showed that the linear model was not the best model to describe our data: addition of a 2nd order term to the model improved the R^2 and the model's fit to the data (data not shown). Estimation of bioavailability from this 2nd order regression line produced a slightly higher value for bioavailability of food folate than the linear model: 89% compared to 85% in the linear model. This reinforces our conclusion that the bioavailability of folate is higher than previously reported. We decided to use a linear regression model to fit our data, since other larger studies found that the relationship between intake of folic acid and change in serum folate is linear over the range of intakes used by us (25, 26).

HPLC analysis of folate in food generally results in lower values than microbiological analysis (1, 27) and therefore bioavailability figures of food folate generally will be higher when based on HPLC analysis. Previous bioavailability studies (13-15) analyzed food folate with microbiological assays. Therefore, we also analyzed our food samples microbiologically (28). This yielded a food folate intake in group A of 474 µg per day, and 136 µg per day in groups B through D; the additional food folate in group A compared with groups B through D was therefore 338 µg per day. This resulted in a bioavailability of (232/338)*100 = 68% [42 to 95%] based on labeled folate data or (252/338)*100 = 75% [45 to 103%] based on changes in serum folate. Thus, the estimate for bioavailability was ~10% lower with microbiologically derived figures than when based on HPLC analysis.

Our findings disagree with a statement in a paper from Sauberlich et al (13). The authors stated that "when compared with synthetic PGA (pteroyl glutamic acid), dietary folates appeared to be no more than 50% available". Unfortunately, the authors do not indicate how this statement was derived. In our opinion, the less than suitable design and small size of this study, with 3 to 4 subjects per treatment group, and the

absence of a folic acid group, make it impossible to estimate the bioavailability of natural folates compared with folic acid from these data. Our results also differ from those of Hannon-Fletcher et al (15). In that study subjects consumed a folate-depleted carrier meal to which food folates extracted from spinach or from yeast were added or they consumed the carrier meal together with folic acid in a tablet; meals were provided daily for 30 days. Based on changes in serum folate, spinach folate was 36% bioavailable, while yeast folate was 62% bioavailable compared with folic acid. However, addition of these two sources of folate to meals is not representative for folates from whole diets: in whole diets folates originate from various sources and matrices of foods. Furthermore food intake was not controlled. These factors limit the usefulness of the bioavailability findings in this study. Our findings are in excellent agreement with the findings of Brouwer et al (14). They conducted a four week highly-controlled intervention study and found that bioavailability of food folate from fruit and vegetables relative to folic acid was 78% based on changes in plasma folate.

We composed a diet high in folate by selecting fruits and vegetables rich in folate, by providing liver paste as sandwich filling and by adding egg-yolk to sauces and salad dressings. Fruits and vegetables were the main source and provided 73% of the additional folate in group A (**Figure 4.2**). Besides fruits and vegetables, unfortified bread and cereals are important sources of folate in general populations (1, 2). However, it was not feasible to include these foods in the varying food items of group A, as we could not replace them with similar products low in folate for groups B through D. Bioavailability of folates from food is influenced by a number of food-related factors, e.g. the species of folate in the food, the number of glutamate residues attached to the folate molecule and the food matrix (29). Since cereals and fruits and vegetables are all plant foods, there are likely to be similarities in factors that affect bioavailability for these food groups. Therefore we speculate that our findings will also be applicable for diets in which cereals are an important folate source, but this requires confirmation.

Our findings and those of Brouwer et al. (14) indicate that bioavailability of food folates is higher than generally assumed, namely about 80% for folate as measured by

HPLC, relative to folic acid from capsules taken with a meal. Subjects in group A consumed a total of 369 μg of folate from foods per day, equivalent to 295 μg of folic acid. Their diet did include 25 g of liver paste and 400 mL of orange juice per day, which may not be to everyone's liking. Nevertheless we think it is quite feasible for a healthy varied diet to provide the equivalent of 300 μg of folic acid per day. The value of 50% bioavailability for folates from food, as used in the construction of recommended daily allowances (5) underestimates the bioavailability of food folates. The use of this low number could lead to skepticism about the importance of a healthy diet as a source of folate: our data suggest that such skepticism may be unwarranted. Our data show that consumption of a diet rich in folate can improve the folate status of a population more efficiently than is generally assumed.

Acknowledgements:

References:

1. Konings EJ, Roomans HH, Dorant E, Goldbohm RA, Saris WH, van den Brandt PA. Folate intake of the Dutch population according to newly established liquid chromatography data for foods. Am J Clin Nutr 2001;73:765-76.
2. National Food Survey Committee. National Food Survey 2000 annual report on food expenditure, consumption and nutrient intakes. London United Kingdom, Ministry of Agriculture, Fisheries and Food, 2000:161.
3. Tucker KL, Selhub J, Rosenberg IH, Wilson PWF. Dietary intake pattern relates to plasma folate and homocysteine concentrations in the Framingham Heart Study. J Nutr 1996;126:3025-31.
4. Brody T, Shane B. Folic acid. In: Rucker R, Suttie J, McCormick D, eds. Handbook of vitamins. New York: Academic Press Marcel Dekker, 2001:427-463.
5. Institute of Medicine. Folate. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. Washington: National Academy Press, 2000:196-305.
6. Clarke R. Homocysteine-lowering trials for prevention of heart disease and stroke. Semin Vasc Med 2005;5:215-22.

7. Lucock M. Is folic acid the ultimate functional food component for disease prevention? BMJ 2004;328:211-4.
8. Selhub J, Bagley LC, Miller J, Rosenberg IH. B vitamins, homocysteine, and neurocognitive function in the elderly. Am J Clin Nutr 2000;71:614S-620S.
9. Seshadri S, Beiser A, Selhub J, et al. Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. N Engl J Med 2002;346:476-83.
10. Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. MRC Vitamin Study Research Group. Lancet 1991;338:131-7.
11. Gregory III JF. The bioavailability of folate. In: Bailey LB, ed. Folate in health and disease. I ed. Gainesville, Florida: Marcel Dekker, 1995: 195-236.
12. McNulty H, Pentieva K. Folate bioavailability. Proc Nutr Soc 2004;63:529-36.
13. Sauberlich HE, Kretsch MJ, Skala JH, Johnson HL, Taylor PC. Folate requirement and metabolism in nonpregnant women. Am J Clin Nutr 1987;46:1016-28.
14. Brouwer IA, van Dusseldorp M, West CE, et al. Dietary folate from vegetables and citrus fruit decreases plasma homocysteine concentrations in humans in a dietary controlled trial. J Nutr 1999;129:1135-9.
15. Hannon-Fletcher MP, Armstrong NC, Scott JM, et al. Determining bioavailability of food folates in a controlled intervention study. Am J Clin Nutr 2004;80:911-918.
16. Ubbink JB, Hayward Vermaak WJ, Bissbort S. Rapid high-performance liquid chromatographic assay for total homocysteine levels in human serum. J Chromatogr 1991;565:441-6.
17. Frosst P, Blom HJ, Milos R, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10:111-3.
18. Hiemstra G, de Roos N, de Vries J, et al. Anamnesemethode: snel en doelmatig? (In Dutch. English translation: Method of anamnesis: quick and efficient?). Nederlands tijdschrift voor dietisten 2005; 60: 88-96
19. Stichting-NEVO. Dutch Food Composition Table 2001. The Hague: Voedingscentrum, 2001.
20. Konings EJ. A validated liquid chromatographic method for determining folates in vegetables, milk powder, liver, and flour. J AOAC Int 1999;82:119-27.
21. Melse-Boonstra A, Verhoef P, West C, et al. A dual isotopic labeling method for studying the bioavailability of hexaglutamyl folic acid relative to monoglutamyl folic acid in humans using orally administered multiple low doses. Am J Clin Nutr 2006;(in press).
22. Herbert V, Larrabee A, Buchanan J. Studies on the identification of a folate compound of human serum. J Clin Invest 1962;41:1134-1138.
23. Neter J, Kutner MH, Nachtsheim CJ, Wasserman W. Simultaneous inferences and other topics in regression analysis. In: Applied linear statistical models
4th ed. ed: McGraw-Hill, 1996:152-175.
24. Pfeiffer CM, Rogers LM, Bailey LB, Gregory JF, 3rd. Absorption of folate from fortified cereal-grain products and of supplemental folate consumed with or without food determined by using a dual-label stable-isotope protocol. Am J Clin Nutr 1997;66:1388-97.
25. van Oort FV, Melse-Boonstra A, Brouwer IA, et al. Folic acid and reduction of plasma homocysteine concentrations in older adults: a dose-response study. Am J Clin Nutr 2003;77:1318-23.
26. Wald DS, Bishop L, Wald NJ, et al. Randomized trial of folic acid supplementation and serum homocysteine levels. Arch Intern Med 2001;161:695-700.
27. Arcot J, Shrestha A. Folate: Methods of analysis. Trends in Food Science and Technology 2005;16:253-266.
28. DeVries JW, Rader JI, Keagy PM, et al. Microbiological assay-trienzyme procedure for total folates in cereals and cereal foods: Collaborative study. J AOAC Int 2005;88:5.
29. Brouwer IA, Dusseldorp Mv, West CE, Steegers-Theunissen RPM. Bioavailability and bioefficacy of folate and folic acid in man. Nutr Res Rev 2001;14:267-294.

5

Bread co-fortified with folic acid and vitamin B12 improves folate and vitamin B12 status of healthy older people: a randomized controlled trial

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Abstract

Introduction: Mandatory fortification of flour with folic acid has reduced the number of neural tube defects in North America. Concerns that high intakes of folic acid might mask vitamin B12 deficiency in older people have delayed the introduction of fortification in many European countries. Co-fortification of flour with folic acid and vitamin B12 could simultaneously improve folate and vitamin B12 status.

Aim: To estimate the effect of consumption of bread fortified with modest amounts of folic acid and vitamin B12 on folate and vitamin B12 status in healthy older people living in the Netherlands, where folic acid fortification is not taking place.

Design: A double blind randomised placebo-controlled trial of bread consumption for 12 weeks in men and women aged 50 to 75y. One group (n=72) ate bread fortified with 138 μg of folic acid and 9.6 μg vitamin B12 per day while the other group (n=70) ate unfortified bread.

Results: Fortified bread increased serum folate concentrations by 45% (mean 6.3 nmol/L; 95%CI 4.5-8.1nmol/L) and serum vitamin B12 concentrations by 49% (mean: 102 pmol/L; 95%CI 82-122 pmol/L) relative to the placebo group. Fortified bread increased erythrocyte folate concentrations by 22% and holotranscobalamin concentrations by 35%; it decreased homocysteine concentrations by 13% and methylmalonic acid concentrations by 10%. Consumption of fortified bread decreased the proportion of individuals with marginal serum vitamin B12 concentration (<133pmol/L) from 8% at enrolment to 0% after 12 weeks.

Conclusion: Bread fortified with modest amounts of folic acid and vitamin B12 will improve folate and vitamin B12 status and a considerable proportion of vitamin B12 deficiency in older people.

ClinicalTrials.gov Identifier: NCT00353353

Introduction

Periconceptional intake of folic acid by women of childbearing age reduces their risk of having a baby with a neural tube defect (1-5). Several European countries are considering folic acid fortification to ensure sufficient intake of folic acid for women of childbearing age (6, 7), because many women who plan their pregnancies do not take folic acid supplements in the advised period (8) and many other pregnancies are unplanned (9).

Excessive intake of folic acid may cure ("mask") vitamin B12-related anemia and thereby delay the diagnosis of a vitamin B12 deficiency (10, 11). About one fifth of older people have marginal vitamin B12 status (12-14) and these people have a high risk of developing a vitamin B12 deficiency. Therefore, folic acid fortification could potentially harm a large proportion of the population. Co-fortification of grain products with modest amounts of both vitamin B12 and folic acid (15, 16) could simultaneously increase folate status and minimize hazards for vitamin B12 deficient people, but the effects of co-fortification on folate and vitamin B12 status in the general population have not been previously examined. Previous interventions examined the effects of very high doses of vitamin B12 supplements (17-20), were performed only in elderly individuals with marginal B-vitamin status (17-22), or used only a limited assessment of vitamin B12 status (23).

We examined to what extent consumption of bread fortified with modest amounts of folic acid and vitamin B12 can improve the folate and vitamin B12 status of older people living in the Netherlands, where fortification is not taking place. The intervention aimed to increase the intake of folic acid by at least 100 μg/day, comparable with the current fortification level in the USA (24) and to increase the intake of vitamin B12 by at least 6 μg/day, which is ~2 times the reference nutrient intake in the US (25). We chose to fortify bread with 6 μg of vitamin B12 per day, since a recent study reported that a dietary intake of at least that dose optimized all vitamin B12 related markers in 98 post-menopausal women (26).

Methods

Participants

We recruited participants from a database of persons who had previously expressed interest in participation in trials from the division of Human Nutrition of Wageningen University, and through advertisements in local newspapers. Participants had to be between 50 and 75 years old and had to consume at least three slices of bread/day, see **Figure 5.1**. Exclusion criteria were use of B-vitamin supplements in the 3 months prior to the study, treatment with B12-injections in the last 5 years, or illness or use of medication that interfered with folate or vitamin B12 metabolism (eg pernicious anemia or use of antacids). We invited 154 individuals, who met these criteria, to provide a fasting blood sample at the study centre at Wageningen University. We excluded 11 applicants with a serum vitamin B12 concentration below 118 pmol/L and/or serum folate below 6.8 nmol/L (Access Immunoassay, Beckman Coulter, cut-off values are laboratory reference limits for deficiency). It was considered unethical in the Netherlands to withhold vitamins from individuals with vitamin concentrations below these cut-offs. We stratified the remaining 143 participants for serum vitamin B12, serum folate concentration, and age. An independent research assistant randomized participants within each stratum to one of the two intervention groups using the random number generator from Excel (MS Office Excel 2003). The 27 participants, who shared a household with another participant, were allocated to the same treatment to avoid a mix up of treatments. All participants gave written informed consent. Both trial staff and participants remained blinded to the treatment until all data had been gathered and double-checked. The Medical Ethical Committee of Wageningen University approved the study. Recruitment started in June 2006; the trial ran from September through December 2006.

182 persons aged 50-75y who habitually consumed ≥3 slices of bread per day volunteered for the study

Excluded n=28:
   -use of B-vitamin-supplements for 3 months prior to the study (n=3)
   -illness or use of medication which interfered with folate or vitamin B12 metabolism (n=17)
   -practical reasons (eg absent during the planned trial period) (n=8)

154 subjects were eligible and provided a blood sample

Excluded: n=11
- serum vitamin B12 < 118 pmol/l (n=5)
- serum folate < 6.8 nmol/L (n=5)
- both folate and vitamin B12 too low (n=1)

143 subjects randomized

n= 73
Fortified bread

n=70
Placebo bread

2 dropped out after 7 weeks due to medical reasons not related to the trial, 1 participant still provided a blood sample, the other was lost to follow-up

n= 72
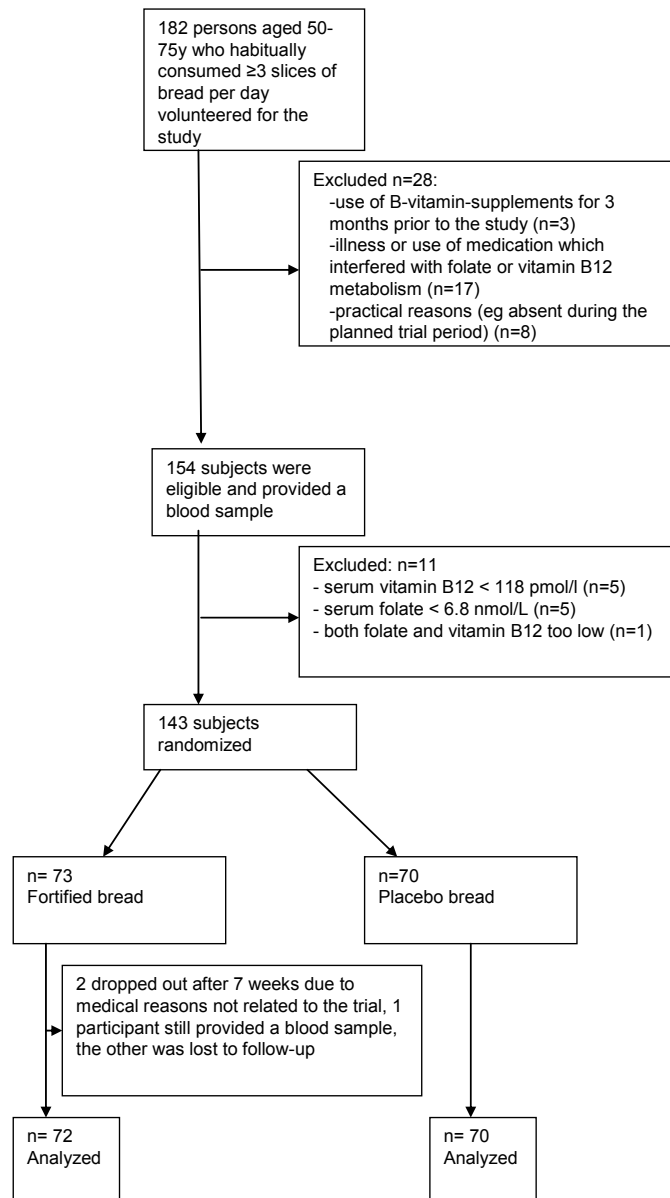Analyzed

n= 70
Analyzed

FIGURE 5.1: Recruitment and flow of participants during the study

Design

The fortified bread group ate bread fortified with folic acid and vitamin B12 and the placebo group ate unfortified bread. Participants donated a fasting blood sample in the week before the treatment started (baseline). Then they consumed the bread for 12 weeks and donated a fasting blood sample on the last day of treatment (week 12). We asked the participants to substitute the bread that they usually ate with the bread provided by us, to consume at least three slices of this bread per day, and to record the number of slices eaten in a food diary. Furthermore, participants returned all unused slices of bread at the end of each week when they collected a fresh batch of bread for the next week. We also asked participants to consume their habitual diet and avoid the use of vitamin supplements.

Sample size

We calculated that 55 participants would be required in each group to detect an absolute difference in serum vitamin B12 response of 50 pmol/L between the groups (18, 22, 23) with a power of 90% and $\alpha = 0.025$ (one-sided testing) assuming a standard deviation of the response of 80 pmol/L (17, 18, 22). We added another 20 persons per group, because bread intake varies between persons, which increases variability. The trial had >99% power to detect a 6 nmol/L difference in response in serum folate (27), assuming a standard deviation of 5 nmol/L (27).

Bread

We wanted to increase the intake of folic acid by 100 µg/day and the intake of vitamin B12 by 6 µg/day. Individuals in this age group in the Netherlands typically consume 3-5 slices of bread per day (28); thus our aim was to add 33 µg of folic acid and 2 µg of vitamin B12 per slice of bread. We used whole wheat bread as this type of bread is consumed most widely in the Netherlands (29). One loaf of bread weighed about 800 g and contained 24 slices.

Pilot studies of bread fortification: We first tested procedures to make fortified bread. We prepared a solution containing 800 mg of folic acid (folic acid from Merck Eprova,

Schaffhausen, Switzerland) and 10 g of baking powder (mixture of sodium carbonate and ammonium carbonate) per litre of water (baking powder improved solubility of folic acid) and a solution containing 480 mg of vitamin B12 (cyanocobalamin from DSM Nutritional Products, Basel, Switzerland) per litre of water. We mixed 5mL of folic acid solution with 0.5mL of vitamin B12 solution. A commercial baker added this mixture to the water used to make dough and baked 5 loaves on each of three separate days using a standard recipe. Two samples of 100 gram of each bread were freeze-dried, ground, and stored at -20ºC. Samples were weighted and internal standard ($^{13}C_5$-folic acid) was added. Samples were extracted in an autoclave at 119ºC for 15 min in buffer of pH 7.8 and the extract was filtrated and adjusted to pH 4.5 and folic acid was analyzed with a reversed phase HPLC method with MS/MS detection (TNO Quality of Life, Zeist, the Netherlands, SOP VIT/071). To analyse vitamin B12, samples were extracted in an autoclave (119ºC for 15 min) using a 0.1M acetate buffer pH 4.6 with 50mg/L potassium cyanide. Vitamin B12 was analysed in the filtrated extract with a competitive binding radioassay with ^{57}Co-cyanocobalamin (TNO Quality of Life, Zeist, the Netherlands, SOP VIT/065). The analyzed amount of folic acid was 760 mg/L in the folic acid solution and 408 µg/loaf of bread: recovery of folic acid during bread making was therefore 54%. We performed a second pilot study in a research bakery (DSM Food Innovation Centre, Delft, the Netherlands), recovery of folic acid during those experiments was 73%. Average recovery was therefore 64%, consistent with values previously resported (30, 31). Analyzed amounts of vitamin B12 were 482 mg/L in the vitamin B12 solution and 37 µg/loaf of bread: thus, recovery of vitamin B12 was 77%. The coefficient of variation (CV) of vitamin concentrations within and between loaves of bread was no more than 5% for both folic acid and vitamin B12.

Trial: One of the authors (RMW) prepared vitamin and placebo solutions. The vitamin solutions were now prepared with 1288 mg of folic acid and 640 mg of vitamin B12 respectively, to correct for the incomplete recovery. Since the vitamin solution was red, the placebo solution was coloured with tartrazine E102 and carmoisine E122; an assistant coded all solutions, stored them at -20ºC and delivered them to the baker. The baker weekly baked and sliced bread for our trial; he baked fortified and placebo bread

on separate days. We analyzed samples of each batch of bread and found that the recovery of folic acid was 64% and of vitamin B12 was 81%, comparable to our pilot experiments. The fortified bread contained 30 µg of folic acid per slice and 2 µg of vitamin B12 per slice. The CV of vitamin concentrations between loaves of bread was no more than 7%.

We (RMW, IAB) could not detect differences between fortified and non-fortified bread in taste, smell, or appearance. Participants collected fresh bread once a week on the day of production; they stored bread in the freezer or at room temperature.

Blood analysis

Blood samples were collected into coagulation tubes, stored in the dark for 30-120 minutes at room temperature, and centrifuged at 3000g for 10 min at 4°C. Serum was pipetted off and stored at -80°C. Serum folate and vitamin B12 were analyzed with a chemiluminescence immunoassay (Access Immunoassay, Beckman Coulter; CV for folate 5% and for vitamin B12 7%) and serum holotranscobalamin with a Microparticle Enzyme Immunoassay (AxSYM-HoloTC, Axis-shield, CV 9%). To obtain plasma, blood samples were collected into EDTA-containing tubes. These tubes were placed on ice immediately after venipuncture and centrifuged within 30 min at 3000g for 10min at 4°C. Plasma was pipetted off and stored at -80°C . Plasma methylmalonic acid concentration was analyzed by HPLC with MS/MS detection (32) (CV 4%) and plasma total homocysteine concentration by HPLC with fluorescence detection (33) (CV 7%). We collected whole blood into an EDTA-containing tube, haemolysed the blood and analyzed folate with a chemiluminescence immunoassay (Access Immunoassay, Beckman Coulter, CV 8%).

Approximately 50 individuals provided additional blood samples for construction of a quality control pool. We coded these pools as simulated participants and randomly submitted at least 20 samples of this pool to the laboratory in between participant samples: we calculated the CV for each assay from these measurements.

Data analysis

Main outcomes of the trial were the differences of the changes from baseline to week 12 between the fortified bread and the placebo group in serum vitamin B12 and serum folate. Secondary outcomes were the differences in the changes in methylmalonic acid, holotranscobalamin, homocysteine and erythrocyte folate between the fortified bread and placebo group.

In addition, we assessed if the response in markers of vitamin B12 status depended on baseline vitamin B12 status. The serum vitamin B12 assay (Beckman Coulter) defined 133 pmol/L as the cut-off value for marginal or indeterminate vitamin B12 status. We assessed the number of participants with serum vitamin B12 concentrations below this cut-off at baseline and at the end of the intervention. We also defined our own cut-offs for low vitamin B-12 status based on the lowest quintiles for serum vitamin B12 or serum holotranscobalamin concentration, and the upper quintile for plasma methylmalonic acid concentration before the intervention. Furthermore, we divided the participants in quintile categories based on baseline serum vitamin B12, baseline holotranscobalamin and baseline methylmalonic acid. We assessed the changes in each biomarker within the various quintile categories of the biomarkers.

To test differences between groups, we used unpaired Students t-tests or ANOVA with post-hoc Bonferroni for normally distributed variables and Mann-Whitney U tests for variables that were not normally distributed. All statistical analyses were carried out on an intention-to-treat basis with SPSS 12.0.1.

Results

Table 5.1 shows that the characteristics of the participants assessed during the recruitment were similar for both the fortified and the placebo groups. Serum folate and vitamin B12 concentrations were within the normal range for this age group in the Netherlands (34) but lower than levels typically found in the US, probably due to the use of fortified breakfast cereals, spreads and supplements in the US, **Table 5.1**.

Compliance was assessed from the food diaries; 139 of the participants consumed at least three slices of bread during at least 90% of the trial period. The fortified bread

Table 5.1: Characteristics of the participants assessed during the recruitment phase, four weeks before the start of the trial

	Fortified bread group n=73	Placebo group n=70
Age, in y	60 ± 6^1	61 ± 6
Gender, number of men/women	34/39	22/48
Body mass index, in kg/m² [2]	26.5 ± 5.1	26.4 ± 4.3
Creatinine, in μmol/L[3]	84 ± 12	82 ± 12
Serum folate, in nmol/L	15.5 ± 7.4	14.4 ± 5.5
Serum vitamin B12, in pmol/L	219 ± 68	204 ± 57

[1] mean ± SD

[2] 39% of all subjects had a BMI ≤25, 17% of all subjects had a BMI ≥30

[3] one participant had a creatinine level >125 μmol/L

group on average consumed 4.6 slices of bread per day and the placebo group 4.3 slices, **Table 5.2**. The mean additional intake of folic acid from fortified bread was 138 ± 47 (SD) μg and of vitamin B12 9.6 ± 3.2 μg per day. **Table 5.2** shows that fortified bread increased serum folate concentrations by 45% or 6.3 nmol/L (95%CI: 4.5-8.1nmol/L) and serum vitamin B12 concentrations by 49% or 102 pmol/L (95%CI: 82-122 pmol/L) relative to unfortified bread. Fortified bread increased erythrocyte folate concentrations by 22% and holotranscobalamin concentrations by 35%. Fortified bread decreased homocysteine concentrations by 13% and methylmalonic acid concentrations by 10% relative to unfortified bread, **Table 5.2**. Exclusion of the 27 participants that shared a household with another participant did not substantially alter the findings of this study (data not shown).

Figure 5.2 shows that the proportion of participants with marginal or indeterminate vitamin B12 status (serum vitamin B12 <133 pmol/L) decreased from 8% at baseline to 0% at the end of the intervention in the fortified bread group and the proportion increased from 4% at baseline to 9% after the intervention in the placebo group. In addition, the proportion of participants with marginal vitamin B12 status according to the other markers of vitamin B12 status decreased substantially due to consumption of fortified bread, **Figure 5.2**.

Figure 5.3 shows that the response of vitamin B12 and holotranscobalamin was similar within the different quintile categories of baseline vitamin B12 and holotranscobalamin status, but that participants in the highest quintile category of

baseline methylmalonic acid status showed a statistically significantly larger response to the fortified bread than participants in the first two quintile categories.

DISCUSSION

In this population of healthy older people, bread fortified with modest amounts of folic acid and vitamin B12 eliminated the presence of low serum vitamin B12 concentrations. It improved serum folate concentrations by 45% and serum concentrations of vitamin B12 by 49% compared with unfortified bread. Consequently, co-fortified bread appears promising as a means of simultaneously improving the folate and vitamin B12 status of older people.
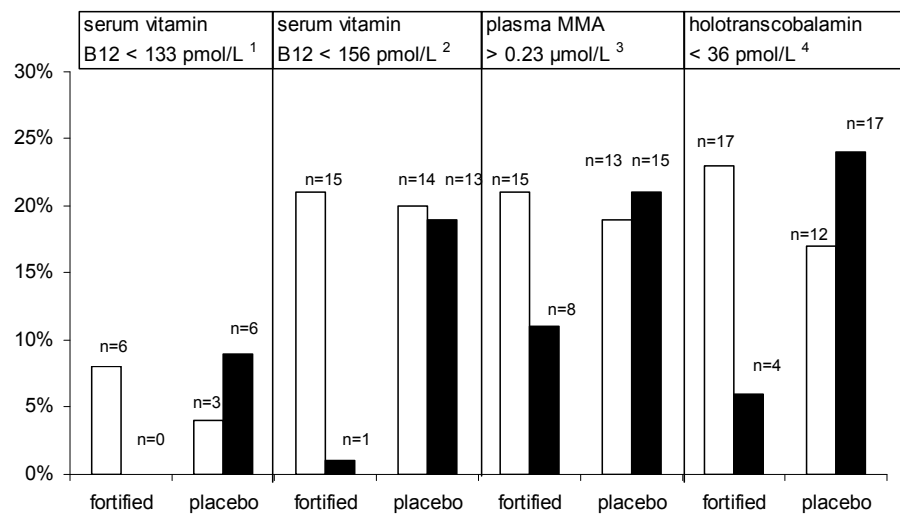

FIGURE 5.2: Percentage of subjects with blood values below or above selected cut-off values, before (white bars) and at the end of the intervention (black bars).

[1] cut-off level for the serum vitamin B12 concentration that indicates marginal or indeterminate vitamin B12 status according to the description in the assay (Access Immunoassay, Beckman Coulter)

[2] the lowest quintile of serum vitamin B12 before the intervention was 156 pmol/L

[3] the highest quintile of methylmalonic acid before the intervention was 0.23 μmol/L

[4] the lowest quintile of holotranscobalamin before the intervention was 36 pmol/L

Table 5.2: Consumption of bread and concentrations and changes in blood variables during the intervention with fortified or placebo bread in healthy elderly participants

		Fortified bread group n= 72	Placebo group n=70	p-value[1]
Slices of bread consumed per day[2]		4.6 ± 1.5[3]	4.3 ± 1.3	
Serum folate (nmol/L)	Baseline	15.7 ± 6.0	14.9 ± 5.6	
	Week 12	20.2 ± 6.6	13.3 ± 5.6	
	Absolute change	**4.7 ± 6.1**	**-1.5 ± 4.6**	**<0.001**
	95%CI for absolute change	**3.3, 6.2**	**-2.6, -0.5**	
	Relative change	**38% ± 42**	**-7% ± 28**	
Serum vitamin B12 (pmol/L)	Baseline	223 ± 74	207 ± 53	
	Week 12	331 ± 113	211 ± 65	
	Absolute change	**107 ± 76**	**4 ± 38**	**<0.001**
	95%CI for absolute change	**89, 124**	**-5, 13**	
	Relative change	**51% ± 32**	**2% ± 17**	
Erythrocyte folate (pmol/L)	Baseline	683 ± 223	618 ± 208	
	Week 12	790 ± 208	595 ± 199	
	Absolute change	**107 ± 141**	**-23 ± 122**	**<0.001**
	95%CI for absolute change	**74, 141**	**-52, 6**	
	Relative change	**20% ± 23**	**-2% ± 19**	
Total homocysteine (µmol/L)	Baseline	10.5 ± 2.3	10.5 ± 2.2	
	Week 12	8.9 ± 2.1	10.3 ± 2.2	
	Absolute change	**-1.6 ± 1.7**	**-0.2 ± 1.6**	**<0.001**
	95%CI for absolute change	**-2.0, -1.2**	**-0.6, 0.1**	
	Relative change	**-14% ± 14**	**-1% ± 15**	
Methylmalonic acid (µmol/L)	Baseline[4]	0.17 [0.13, 0.21]	0.17 [0.14, 0.21]	
	Week 12[4]	0.15 [0.13, 0.19]	0.17 [0.14, 0.20]	
	Absolute change[4]	**-0.01 [-0.03, 0.01]**	**0.00 [-0.01, 0.02]**	**0.001**
	Relative change[4]	**-9% [-16, 6%]**	**1% [-8, 10%]**	
Holotranscobalamin (pmol/L)	Baseline[4]	46.7 [36.9, 59.7]	46.5 [39.5, 55.5]	
	Week 12[4]	63.5 [54.8, 86.1]	44.1 [37.2, 55.2]	
	Absolute change[4]	**19.2 [9.3, 29.8]**	**-2.4 [-7.1, 2.3]**	**<0.001**
	Relative change[4]	**40% [22, 61%]**	**-5% [-15,4%]**	

[1]p-value for unpaired t-test or the Mann-Whitney U test (methylmalonic acid and holotranscobalamin) comparing the change in the fortified bread group to the change in the placebo group

[2]mean consumption of bread was calculated from the food diaries of the participants

[3]unless otherwise indicated table values are mean ± SD
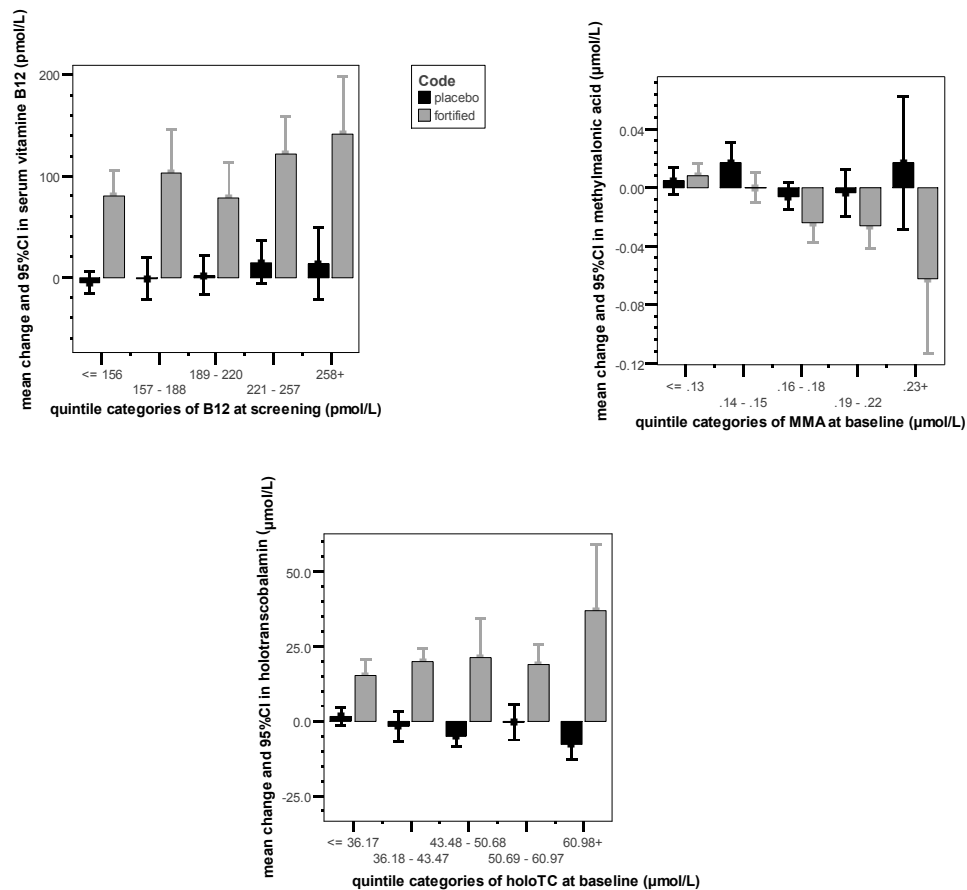
[4]median [interquartile range]

FIGURE 5.3: Mean change in serum vitamin B12, methylmalonic acid and holotranscobalamin within different quintile categories of baseline status. ANOVA-tests showed that there were no statistically significant differences between the different quintile categories of the fortified group in change in serum vitamin B12 and in change in holotranscobalamin. The change in methylmalonic acid in participants in the highest quintile category of the fortified group was larger than the change of the participants in the first and second quintile categories; the changes of methylmalonic acid in the other quintile categories of the fortified group did not differ statistically significantly

The sensitivity and specificity of serum vitamin B12 concentrations to diagnose deficiency is reportedly low (35) and methylmalonic acid and holotranscobalamin concentrations have been suggested as better indicators (35-37). As there is no consensus about the best biochemical test to diagnose vitamin B12 deficiency, we

assessed the effect of vitamin B12 fortification on all available biochemical markers of vitamin B12 status. Moreover, as there is no agreement on the absolute values for definition of vitamin B12 deficiency (38), we used two different cut-off points for serum vitamin B12 and demonstrated that consumption of fortified bread markedly improved vitamin B12 status irrespective of which biomarker or cut-off point was used to define vitamin B12 deficiency.

We fortified bread with folic acid at a level comparable with that applied in flour fortification in the USA (24). The average actual additional intake of folic acid due to fortification in the USA was estimated to be about 200 μg/day (39, 40) due to overage. Neural tube defects decreased by 25% since the start of the fortification program in the USA (41) and by 50% in Canada (42). Folic acid fortification at the level used in the current study might prevent about 20% of neural tube defect affected pregnancies in the UK (5). In addition to the established effect of folic acid on birth defects, folic acid has been suggested to reduce the risk of cardiovascular disease, certain types of cancer, and the rate of cognitive decline in elderly people (43-45). Vitamin B12 fortification may reduce both the rate of cognitive decline (46) and the risk of neural tube defects (47).

We chose to fortify bread with 6 μg of vitamin B12 per day, rather than the current RDA of about 3 μg per day. Actual intake of vitamin B12 was somewhat higher because participants ate more than the minimum three slices per day. We chose this 6 μg for several reasons. First, the current RDA was established based on the absence of anemia and neurologic symptoms, whereas it is unknown whether it will also prevent other adverse health consequences (26). Second, a recent study reported that a dietary intake of at least 6 μg/day optimized all vitamin B12 related markers in 98 post-menopausal women (26). Last, studies with vitamin B12 deficient individuals showed that 2.5 to 10 μg/day will improve serum vitamin B12 concentrations by 20-60 pmol/L (18, 21, 22) and will decrease methylmalonic acid concentrations by about 50% of the effect observed at very high doses (18).

A limitation of our study is that it was too short to assess the effect of fortification on other aspects of health status, such as cardiovascular disease (48) and cognitive

impairment (46, 49). Indeed, assessment of the effects of co-fortification on other markers of health would have required a much larger number of participants and would require that the trial be continued for a much longer duration. Furthermore, since we lacked data on the causes of low vitamin B12 status, we were unable to specifically evaluate the ability of co-fortified bread to improve the vitamin B12 status of older people with atrophic gastritis, the most common cause of low vitamin B12 status in older people (50).

There are other concerns about folic acid fortification, besides concerns about masking of vitamin B12 deficiency that have delayed the introduction of folic acid fortification in many countries. Specifically, although high folate intake has been associated with a decreased risk of cancer in epidemiological studies, animal studies suggest that high doses of folic acid could promote the growth of existing tumours (51). Studies conducted after the introduction of fortification in the USA in 1998, reported a possible increase in colorectal cancer rates (52) and a higher prevalence of anemia and cognitive impairment in elderly people with a high serum folate and a low vitamin B12 status compared with elderly with normal status (53). Furthermore, folic acid may interfere with anti-folate chemotherapy and with anticonvulsant therapy. However, the evidence underpinning possible negative effects of folic acid fortification is far from conclusive (5). Countries need to consider all possible benefits and harms before they decide to start fortification (5, 54, 55).

Will the low doses of vitamin B12 administered in our trial be beneficial for individuals with marginal or deficient status? Individuals with pernicious anemia, i.e. lack of intrinsic factor, can only passively absorb about 1% of crystalline vitamin B12 (50); such individuals are unlikely to benefit from fortification and will still require parenteral vitamin B12 therapy by intramuscular injections. However, the main cause of low vitamin B12 status in older people is the reduced ability to extract vitamin B12 from food protein, due to age-related gastric atrophy (36, 46, 56, 57). Absorption of crystalline vitamin B12 should be unimpaired in such cases and hence most older people with low vitamin B12 status will probably be responsive to fortified food. On the other hand, a study from Eussen et al suggested that daily oral doses of 650 to 1000

μg per day were needed to maximally decreased methylmalonic acid concentrations in individuals with marginal status (18). This finding appears consistent with the persistence of elevated methylmalonic acid concentrations in 11% of the treated participants in our trial despite normalization of other biomarkers of vitamin B12 status. However, it is important to emphasize that our cut-off point for an elevated methylmalonic acid concentration was 0.23 nmol/L, which is lower than the 0.32 nmol/L in the study from Eussen (18).

In conclusion, bread fortified with modest amounts of folic acid and vitamin B12 will improve folate and vitamin B12 status and may prevent a considerable proportion of vitamin B12 deficiency in older people. Several large-scale trials of folic acid and vitamin B12 are currently assessing the effects of long-term dietary supplementation with folic acid and vitamin B12 on vascular and non-vascular outcomes (48). However, further large-scale trials of vitamin B12 supplementation are needed to assess the effects on non-vascular outcomes of vitamin B12 supplementation in elderly populations in the absence of anemia or cognitive impairment.

Acknowledgements

References

1. Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. MRC Vitamin Study Research Group. Lancet 1991;338:131-7.
2. Czeizel AE, Dudas I. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. N Engl J Med 1992;327:1832-5.
3. Voedingsraad. Advice concerning folic acid supply in relation to neural tube defects [In Dutch: Advies inzake foliumzuurvoorziening in relatie tot neuralebuisdefecten]. Den Haag: Voorlichtingsbureau voor de Voeding, 1992.
4. Voedingsraad/Gezondheidsraad. Follow-up advice concerning folic acid supply in relation to neural tube defects [In Dutch: Vervolgadvies inzake foliumzuurvoorziening in relatie tot neuralebuisdefecten]. Den Haag: Voorlichtingsbureau voor de voeding, 1993.
5. Scientific Advisory Committee on Nutrition. Folate and disease prevention. Norwich, UK: The Stationery Office, 2006:1-211.
6. Abramsky L, Dolk H. Should Europe fortify a staple food with folic acid? Lancet 2007;369:641-642.

7. Czernichow S, Noisette N, Blacher J, et al. Case for folic acid and vitamin B12 fortification in Europe. Semin Vasc Med 2005;5:156-62.
8. De Walle HEK, De Jong-Van Den Berg LTW. Growing gap in folic acid intake with respect to level of education in the Netherlands. Community Genetics 2007;10:93-96.
9. Eurocat folic acid working group. Special Report: Prevention of neural tube defects by periconceptional folic acid supplementation in Europe (december 2005). 2005:Available at: http://www.eurocat.ulster.ac.uk/pubdata/Folic-Acid.html, accessed May 09, 2007.
10. Health Council of the Netherlands: Risks of folic acid fortification. The Hague: Health Council of the Netherlands, 2000:48.
11. Committee on Medical Aspects of Food and Nutrition Policy (COMA). Folic acid and the prevention of disease. Report on health and social subjects. Department of Health, UK, 2000.
12. van Asselt DZ, de Groot LC, van Staveren WA, et al. Role of cobalamin intake and atrophic gastritis in mild cobalamin deficiency in older Dutch subjects. Am J Clin Nutr 1998;68:328-34.
13. Clarke R, Grimley Evans J, Schneede J, et al. Vitamin B12 and folate deficiency in later life. Age Ageing 2004;33:34-41.
14. Clarke R, Refsum H, Birks J, et al. Screening for vitamin B-12 and folate deficiency in older persons. Am J Clin Nutr 2003;77:1241-7.
15. Recommended levels of folic acid and vitamin B12 fortification: conclusions. Nutr Rev 2004;62:S62-6.
16. Oakley GP, Jr. Let's increase folic acid fortification and include vitamin B-12. Am J Clin Nutr 1997;65:1889-90.
17. Dhonukshe-Rutten RA, van Zutphen M, de Groot LC, Eussen SJ, Blom HJ, van Staveren WA. Effect of supplementation with cobalamin carried either by a milk product or a capsule in mildly cobalamin-deficient elderly Dutch persons. Am J Clin Nutr 2005;82:568-74.
18. Eussen SJ, de Groot LC, Clarke R, et al. Oral cyanocobalamin supplementation in older people with vitamin B12 deficiency: a dose-finding trial. Arch Intern Med 2005;165:1167-72.
19. Ubbink JB, Vermaak WJH, van der Merwe A, Becker PJ, Delport R, Potgieter HC. Vitamin Requirements for the Treatment of Hyperhomocysteinemia in Humans. J. Nutr. 1994;124:1927-1933.
20. Woodside JV, Yarnell JW, McMaster D, et al. Effect of B-group vitamins and antioxidant vitamins on hyperhomocysteinemia: a double-blind, randomized, factorial-design, controlled trial. Am J Clin Nutr 1998;67:858-66.
21. Blacher J, Czernichow S, Raphael M, et al. Very Low Oral Doses of Vitamin B-12 Increase Serum Concentrations in Elderly Subjects with Food-Bound Vitamin B-12 Malabsorption. J Nutr 2007;137:373-378.
22. Seal EC, Metz J, Flicker L, Melny J. A Randomized, Double-Blind, Placebo-Controlled Study of Oral Vitamin B12 Supplementation in Older Patients with Subnormal or Borderline Serum Vitamin B12 Concentrations. Journal of the American Geriatrics Society 2002;50:146-151.
23. Tucker KL, Olson B, Bakun P, Dallal GE, Selhub J, Rosenberg IH. Breakfast cereal fortified with folic acid, vitamin B-6, and vitamin B-12 increases vitamin concentrations and reduces homocysteine concentrations: a randomized trial. Am J Clin Nutr 2004;79:805-811.
24. Stover PJ. Physiology of folate and vitamin B12 in health and disease. Nutr Rev 2004;62:S3-12; discussion S13.
25. Institute of Medicine. Vitamin B12. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. Washington: National Academy Press, 2000:306-356 and 527-530.
26. Bor MV, Lydeking-Olsen E, Moller J, Nexo E. A daily intake of approximately 6 {micro}g vitamin B-12 appears to saturate all the vitamin B-12-related variables in Danish postmenopausal women. Am J Clin Nutr 2006;83:52-58.
27. van Oort FV, Melse-Boonstra A, Brouwer IA, et al. Folic acid and reduction of plasma homocysteine concentrations in older adults: a dose-response study. Am J Clin Nutr 2003;77:1318-23.
28. Voedingscentrum. Results of the Dutch Nutrition Survey 1998 [In Dutch: Zo eet Nederland 1998. Resultaten van de Voedselconsumptiepeiling 1998]. Delft: Voedingscentrum, 1998.
29. Kistemaker C, Bouman M, Hulshof KFAM. The consumption of seperate products by Dutch population groups - Dutch Nutrition Survey 1997-1998. [In Dutch: De consumptie van

afzonderlijke producten door Nederlandse bevolkingsgroepen - Voedselconsumptiepeiling 1997-1998]. TNO-report V98.812. Zeist: Stiching Voedingscentrum Nederland, 1998.

30. Johansson M, Witthoft CM, Bruce A, Jagerstad M. Study of wheat breakfast rolls fortified with folic acid. The effect on folate status in women during a 3-month intervention. Eur J Nutr 2002;41:279-86.

31. Pfeiffer CM, Rogers LM, Bailey LB, Gregory JF, 3rd. Absorption of folate from fortified cereal-grain products and of supplemental folate consumed with or without food determined by using a dual-label stable-isotope protocol. Am J Clin Nutr 1997;66:1388-97.

32. Blom HJ, Van Rooij A, Hogeveen M. A simple high-throughput method for the determination of plasma methylmalonic acid by liquid chromatography-tandem mass spectrometry. Clin Chem Lab Med 2007;45:645-50.

33. Ubbink JB, Hayward Vermaak WJ, Bissbort S. Rapid high-performance liquid chromatographic assay for total homocysteine levels in human serum. J Chromatogr 1991;565:441-6.

34. Brussaard JH, Lowik MR, van den Berg H, Brants HA, Goldbohm RA. Folate intake and status among adults in the Netherlands. Eur J Clin Nutr 1997;51 Suppl 3:S46-50.

35. Clarke R, Sherliker P, Hin H, et al. Detection of vitamin B12 deficiency in older people by measuring vitamin B12 or the active fraction of vitamin B12, holotranscobalamin. Clin Chem 2007;53:963-70.

36. Hvas AM, Nexo E. Diagnosis and treatment of vitamin B12 deficiency--an update. Haematologica 2006;91:1506-12.

37. Refsum H, Johnston C, Guttormsen AB, Nexo E. Holotranscobalamin and Total Transcobalamin in Human Plasma: Determination, Determinants, and Reference Values in Healthy Adults. Clin Chem 2006;52:129-137.

38. Thorpe SJ, Heath A, Blackmore S, et al. International Standard for serum vitamin B(12) and serum folate: international collaborative study to evaluate a batch of lyophilised serum for B(12) and folate content. Clin Chem Lab Med 2007;45:380-6.

39. Choumenkovitch SF, Selhub J, Wilson PW, Rader JI, Rosenberg IH, Jacques PF. Folic acid intake from fortification in United States exceeds predictions. J Nutr 2002;132:2792-8.

40. Quinlivan EP, Gregory Iii JF. Effect of food fortification on folic acid intake in the United States. Am J Clin Nutr 2003;77:221.

41. Centers for Disease Control and Prevention. Spina bifida and anencephaly before and after folic acid mandate-United States, 1995-1996 nad 1999-2000. MMWR Morb Mortal Wkly Rep 2004;53:362-365.

42. Mills JL, Signore C. Neural tube defect rates before and after food fortification with folic acid. Birth Defects Res A Clin Mol Teratol 2004;70:844-5.

43. Lucock M. Is folic acid the ultimate functional food component for disease prevention? BMJ 2004;328:211-4.

44. Molloy AM, Scott JM. Folates and prevention of disease. Public Health Nutr 2001;4:601-9.

45. Durga J, van Boxtel MPJ, 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;369:208-216.

46. Hin H, Clarke R, Sherliker P, et al. Clinical relevance of low serum vitamin B12 concentrations in older people: the Banbury B12 study. Age Ageing 2006;35:416-422.

47. Ray JG, Wyatt PR, Thompson MD, et al. Vitamin B12 and the risk of neural tube defects in a folic-acid-fortified population. Epidemiology 2007;18:362-366.

48. Clarke RJ. Homocysteine-lowering trials for prevention of cardiovascular events: A review of the design and power of the large randomized trials. American Heart Journal 2006;151:282-287.

49. Eussen SJ, de Groot LC, Joosten LW, et al. Effect of oral vitamin B-12 with or without folic acid on cognitive function in older people with mild vitamin B-12 deficiency: a randomized, placebo-controlled trial. Am J Clin Nutr 2006;84:361-70.

50. Baik HW, Russell RM. Vitamin B12 deficiency in the elderly. Annual Review of Nutrition 1999;19:357-377.

51. Kim YI. Will mandatory folic acid fortification prevent or promote cancer? Am J Clin Nutr 2004;80:1123-8.

52. 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.

53. Morris MS, Jacques PF, Rosenberg IH, Selhub J. Folate and vitamin B-12 status in relation to anemia, macrocytosis, and cognitive impairment in older Americans in the age of folic acid fortification. Am J Clin Nutr 2007;85:193-200.
54. Rosenberg IH. Science-based micronutrient fortification: which nutrients, how much, and how to know? Am J Clin Nutr 2005;82:279-280.
55. Sweeney MR, McPartlin J, Scott JM. Folic acid fortification and public health: Report on threshold doses above which unmetabolised folic acid appear in serum. BMC Public Health 2007;7.
56. Carmel R. Malabsorption of food cobalamin. Bailliere's Clinical Haematology 1995;8:639-655.
57. Stabler SP, Allen RH. Vitamin B12 deficiency as a worldwide problem. Annu Rev Nutr 2004;24:299-326.

6

General
Discussion

General discussion
Outline

In this chapter, I will apply the findings from our research to answer the thesis' central question whether Dutch diets provide enough folate to meet the folate requirements, and if not how to deal with fortification of food with folic acid. The outline of this discussion is as follows:

First, I will discuss the bioavailability findings of chapter 4, and assess how bioavailability and gender affect the folate requirements. I will then answer the first part of the central question of whether Dutch diets provide enough folate to meet the folate requirements. In the last part of the discussion, I will focus on folic acid fortification and answer the last part of the central question of how to deal with fortification of food with folic acid. I will end with some concluding remarks and suggestions for future research.

Bioavailability

In chapter 4, we showed that bioavailability of dietary folate relative to folic acid was 78% (95%CI 48-108%) as assessed with a stable isotope dilution method, and 85% (95%CI 52-118%) as assessed from plasma folate levels. Several methodological aspects should be considered in the interpretation of these findings.

Methodological aspect: use of stable labelled folates

In chapter 3, we developed a method to study the bioavailability of folate from food that was based on dilution of ^{13}C-labeled folate concentrations in blood. We showed that the relation between the percentage of ^{13}C-labelled folate that was measured in plasma and the dose of unlabeled folic acid that was supplemented was hyperbolic; an inverse transformation of the percentage of labelled folate resulted in a stronger correlation with the dose of unlabelled folic acid. We calculated that if we would have applied the same inverse transformation to the stable isotope data of chapter 4, the estimate for bioavailability would have been 74% (95%CI 42-105%); this is comparable to the untransformed estimate of 78% (95%CI 48-108%) that we now reported in that

chapter. Since transformation hardly influenced the estimate of bioavailability, we decided to keep the data presentation of chapter 4 as simple as possible and did not apply the transformation.

In our study, we had hypothesized that the precision of the estimate for bioavailability derived from the stable isotope dilution method would be higher than the precision of the estimate derived from plasma folate levels. The percentage of labeled folate in plasma had to be evaluated only after the intervention, because the labelled compound does not occur naturally, while plasma folate levels had to be measured before and after the intervention. Thus, the estimate from plasma folate levels contained extra error, since 2 measurements had to be subtracted from each other. However, we found a similar precision, indicated by a similar width of the confidence intervals surrounding the estimates for bioavailability. Previous interventions that studied aspects of folate bioavailability did find more precise estimates with stable isotope methods (1).

Methodological aspect: method of analysis of food folate

The estimate for bioavailability depended on the method of analysis for the amount of folate in food. HPLC-analysis of the amount of folate in food resulted in lower folate values than a microbiological method; consequently, the bioavailability estimate was higher for the HPLC-method (82%) than for the microbiological method (72%). Other studies have also reported lower food folate values for HPLC as compared with microbiological methods (2, 3).

We compared the result of the HPLC-method (4) and the microbiological method (5) for various food items (**Figure 6.1)**. The 2 laboratories that used these methods analysed the folate content of four different certified reference materials and of folic acid containing capsules. The homogeneity and stability of certified reference materials is clearly documented; the certified value for the amount of food folate in these items is the average result of the analyses of about 15 laboratories that used a microbiological method to analyse food folate (6).

Figure 6.1 illustrates that, in general, HPLC-analysis resulted in lower folate values than the microbiological analysis, although the results only differed statistically significantly for the fortified milk powder and the mixed vegetables (t-test, $p < 0.05$). Non-folate compounds in the sample could stimulate or inhibit the growth of the micro-organism of the microbiological assay, which may have biased the results of the microbiological analysis (7). HPLC-analysis of food folate is not hampered by this problem, since all folate derivatives are separately detected and quantified (4). In the HPLC-method of food folate, folates are extracted and separated from the food mixture with a column of folate-binding-protein, the mixture of food folates is separated into its various folate derivatives using an HPLC-column, and each folate derivative is separately detected and quantified.

Figure 6.1 also suggests that the results vary the least between methods for the folic acid containing items (fortified milk powder and capsules), which other researchers have also shown (7). The main form of folate in these samples – folic acid - is used in microbiological methods to make a standard curve of the growth of the micro-organism. However, the growth of the micro-organism on other folate derivatives may differ from the growth on folic acid (7, 8), which is another significant disadvantage of this method. For HPLC-analysis, vigorous extraction and purification of the food samples is necessary to remove compounds with identical chemical and chromatographical properties. This intensive sample clean-up could lead to a loss of the sensitive folate compounds, or to interconversion of folate derivatives. Moreover, every folate derivative has its own detection limit. These factors could lead to a slight underestimate of the folate content of foods by HPLC-methods. We chose to report HPLC-values in chapter 4 because the HPLC-method has less important disadvantages than the microbiological assay. We also decided to focus on the HPLC-values since the current food table in the Netherlands mainly contains HPLC-based values for food folate (9). Nevertheless, we added the microbiological results to chapter 4, since the food tables of many other countries contain and since many other studies reported microbiological food folate data.

In summary, the analysis of food folate is complicated. The results of different methods vary greatly (10, 11), but there is currently no 'golden standard'. However, a combination of HPLC-separation of folate derivatives with microbiological detection of the folate derivatives (3, 12) may help to explain some of the discrepancies between these methods. Because food folate analysis varies so much, it is important always to consider the method of analysis when interpreting results of studies with food folate.
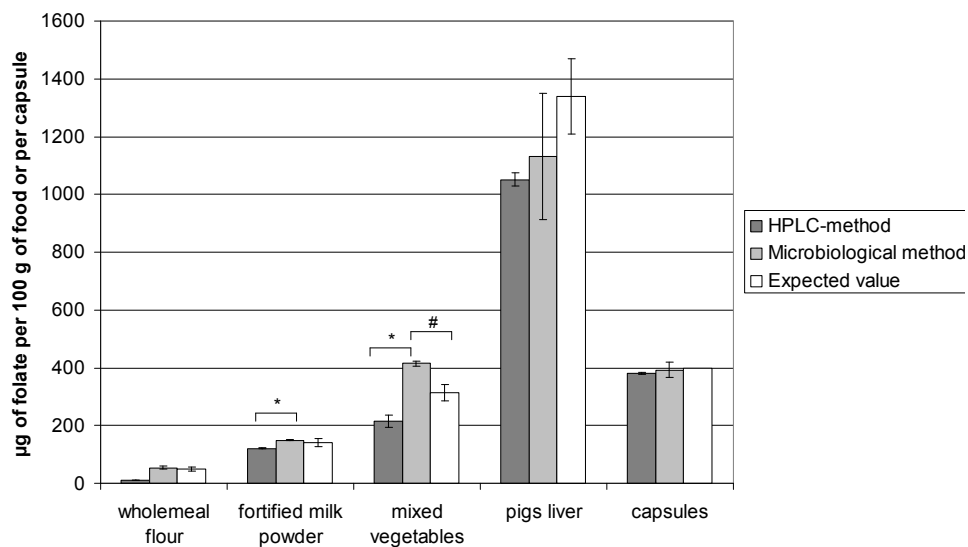

Figure 6.1: Food folate content of various food items and folic acid content of capsules as assessed with HPLC and with a microbiological method. Food items were certified reference materials, the expected value of these food items is the certified value for microbiological methods that is reported by the Community Bureau of Reference. Error bars represent the SD as reported by the labs; for the expected values of the food items, error bars represent the 95% confidence interval, for the capsules we did not have a measure of dispersion for the expected value.

* Statistically significantly different between methods (t-test, α=0.05) .

\# The microbiological result differed statistically significantly from the expected value (t-test, α=0.05). We did not compare the results of the HPLC-method with the expected value, since this expected value only applies to microbiological methods.

Influence of bioavailability and gender on dietary folate equivalents and folate requirements

Dietary folate equivalents

Folate requirements are expressed as folate from food or as dietary folate equivalents (**Table 6.1**) (13, 14). In the calculation of dietary folate equivalents bioavailability is taken into account. However, the assumptions about bioavailability in the calculation of these equivalents are challengeable. The first assumption is that bioavailability of food folate relative to folic acid is 50%. This 50% was somewhat arbitrarily chosen and it underestimated the bioavailability of food folate to folic acid (chapter 4). The second assumption is that bioavailability of folic acid ingested with food is 15% lower than folic acid ingested on an empty stomach. This was based on a study that showed that the ingestion of supplemental folic acid together with a light meal lowered bioavailability of folic acid by ~15% (p=0.088, n= 14)(15). More research should elucidate if ingestion of folic acid together with food indeed lowers bioavailability. The last assumption is that folic acid taken with food is (85/50) = 1.7 times more available than food folate. This assumption is incorrect because it implicitly assumes that the 50% bioavailability estimate adapted from the Sauberlich study (5) is an estimate of the bioavailability of food folate relative to folic acid taken on an empty stomach. The authors from the Sauberlich paper clearly state that folic acid in that study was taken with a meal. Thus, the 50% estimate is relative to folic acid taken with a meal.

In conclusion, the underlying assumptions in the calculation of dietary folate equivalents are not well-founded. Next, I will discuss how the research from this thesis affects the calculation of dietary folate equivalents and folate requirements.

Bioavailability and gender

Table 6.1 and 6.2 show how our findings of bioavailability and gender influence the calculation of dietary folate equivalents and the folate requirements. Our bioavailability findings suggest that 1 µg of dietary folate is equivalent to 0.82 µg of folic acid taken with food for food folate analyzed with an HPLC-method. For food

folate analyzed with a microbiological method 1 µg of dietary folate is equivalent to 0.72 µg of folic acid taken with food (**Table 6.1**).

Our findings in chapter 2 suggested that men needed 45 µg of folic acid more than women to achieve a similar response in erythrocyte folate. Thus, folate requirements probably need to be 45 µg of folic acid higher for men than for women (**Table 6.2**).

Table 6.1: calculation of dietary folate equivalents

Origin of data:	The Dutch Health Council (13) and the US Institute of Medicine (14)		
Bioavailability of	**Relative to**		**Dietary folate equivalents (DFE)**
Food folate	Folic acid taken on empty stomach	50%	1 µg of DFE = 0.5 µg of folic acid on empty stomach
Folic acid taken with food	Folic acid taken on empty stomach	85%	
Food folate	Folic acid taken with food	50/85 = 0.6	1 µg of DFE = 0.6 µg of folic acid taken with food
Folic acid taken with food	Food folate	85/50 =1.7	1 µg of folic acid taken with food = 1.7 µg of DFE
The amount of DFE in a mixture of food folate and folic acid is calculated as follows:			µg of DFE = µg of food folate + 1.7 * µg of folic acid
Origin of data:	**This thesis**		
Bioavailability of	**Relative to**		**Dietary folate equivalents (DFE)**
Food folate[1]	Folic acid taken with food	82%	1 µg of DFE = 0.82 µg folic acid taken with food
Folic acid with food	Food folate[1]	1/0.82 = 1.2	1 µg of folic acid taken with food = 1.2 µg of DFE
The amount of DFE in a mixture of food folate and folic acid is calculated as follows[1]:			µg of DFE = µg of food folate + 1.2 * µg of folic acid
Food folate[2]	Folic acid taken with food	72%	1 µg of DFE = 0.72 µg folic acid taken with food
Folic acid with food	Food folate[2]	1/0.72 = 1.4	1 µg of folic acid taken with food = 1.4 µg of DFE
The amount of DFE in a mixture of food folate and folic acid is calculated as follows[2]:			µg of DFE = µg of food folate + 1.4 * µg of folic acid

[1]For food folate analyzed with an HPLC-method

[2]For food folate analyzed with a microbiological method

Table 6.2: adapting the EAR for folate in the Netherlands according to the results of the studies described in this thesis

Current situation		EAR	
	μg of dietary folate equivalents[1]	200	
	μg of folic acid on empty stomach[2]	100	
	μg of folic acid with meal[3]	118	
New situation (based on the research in this thesis)		**EAR**	**EAR**
Method of analysis of food folate		HPLC	Microbiological
Bioavailability of dietary folate relative to folic acid [4]		82%	72%
	μg of folic acid[6]	118 for women	118 for women
		163 for men	163 for men
	μg of dietary folate equivalents	140 for women[6]	165 for women[7]
		200 for men[6]	2 for men[7]

[1] in the report of the Dutch Health Council the EAR is expressed as μg of folate from food and not with the term dietary folate equivalents. Nevertheless, the amount of folate from food is calculated in exactly the same way as the amount of dietary folate equivalents (13, 14)

[2] the Dutch Health Council assumed that bioavailability of food folate is 50% relative to folic acid taken on an empty stomach

[3] the Dutch Health Council assumed that bioavailability of folic acid taken with a meal is 85% relative to folic acid taken on an empty stomach

[4] see chapter 4

[5] chapter 2 suggested that the EAR for folate should be 45 μg higher for men than for women

[6] calculated by dividing the new EAR in μg of folic acid by 0.82

[7] calculated by dividing the new EAR in μg of folic acid by 0.72

Folate requirements

The current estimated average requirement for folate in the Netherlands is 200 μg of folate from food per day for adult men and women (13). Considering the findings of this thesis, the estimated average requirement for folate should be 140 μg of dietary folate equivalents for women and 200 μg for men for food folate assessed with an HPLC-method (see **Table 6.2** for calculations). With microbiological analysis of food folate the estimated average requirement should be 165 μg of dietary folate equivalents for women and 225 μg for men.

Do Dutch diets provide enough folate to meet the folate requirements?

Folate intake in the Netherlands

I compared the usual intake of folate in the Netherlands with our estimated average requirement derived for HPLC-analysis of food folate.

Since 2001, the Dutch food table has mainly contained food folate values resulting from HPLC-analysis (9). Median folate intake reported in the 1997/1998 Dutch Nutrition Survey analyzed with the 2001 Dutch food table (16) was 167 μg (10th-90th percentile 124-227 μg) in adult women and 208 μg (159-280 μg) in adult men, **Table 6.3**. I assessed the proportion of individuals with a usual intake below the estimated average requirement with the cut-point method (17, 18). The cut-point method showed that 25% of women and 50% of men had an intake below our newly calculated estimated average requirement.

Mean dietary folate intake reported in the 1996/1997 Dutch MORGEN cohort was 192 μg (SD 54 μg) in adult women and 239 μg (73 μg) in adult men (19). This study did not report percentiles, and thus we could not apply the cut-point method to evaluate the intake.

Mean dietary folate intake in young adults (age 19-35) in the 2003 Dutch Nutrition Survey was 155 μg (SD 36 μg) in women and 233 μg (60 μg) in men (20), which is comparable to the other surveys. This survey also did not report percentiles. Nevertheless, the figures of the MORGEN cohort and of the 2003 Dutch Nutrition Survey are similar to the 1997/1998 Dutch Nutrition Survey, and thus prevalence of insufficient intake of folate will probably not substantially deviate from that survey.

In summary, folate intake does not meet the folate requirements for 25% of Dutch adult women and for 50% of Dutch adult men. Nevertheless, folate intake is better than previously reported (16), where about 50% of men and about 75% of women did not reach the current requirement of 200 μg per day (**Table 6.3**).

Although the intake of dietary folate is low, this does not mean that a large part of the population has macrocytic anemia. There are no indications that the prevalence of macrocytosis in the general adult population in the Netherlands is high, but

representative data are lacking (20). In the trial participants of chapter 2, the prevalence of deficient erythrocyte folate status (<305 nmol/L) was no more than 2%. Information about the prevalence of macrocytosis in the general Dutch population is needed to evaluate if the folate status should be improved.

Table 6.3: folate intake and prevalence of inadequate intake in the Netherlands[1]

		Folate intake in µg	Prevalence of inadequate intake according to:	
			Current EAR (200 µg)	New EAR (men 200 µg, women 140 µg)
Dutch Nutrition Survey '97/'98 (age 19-50)	men n=1437	208 (159-280)[2]	50%	50%
	women n=1655	167 (124-227)[2]	75%	25%
MORGEN cohort '93/'96 (age 20-65)	men n=1821	239 (73)[3]	-	-
	women n=2045	192 (54)[3]	-	-
Dutch Nutrition Survey 2003 (age 19-35)	men n=352	233 (60)[3]	-	-
	Women n=398	155 (36)[3]	-	-

[1] Intake for dietary sources only

[2] Median (10th -90th percentile)

[3] Mean (sd)

Folic acid fortification

In the next part of the discussion, I focus on some of the aspects that should be considered in the debate about folic acid fortification. Currently, less than half of all Dutch women use folic acid supplements in the advised period, and this number is even lower among non-native and lower educated Dutch women (20, 21).

Number of neural tube defects that could be prevented due to fortification in the Netherlands

Sufficient intake of folic acid can maximally prevent about 70% of all cases of neural tube defects (22). In Canada and the US the prevalence of neural tube defects

decreased by 25-50% after folic acid fortification started in 1996-1998 (23, 24). The prevalence of neural tube defects in the Netherlands is about 8 cases per 10,000 births, which means that each year about 130 children will be born with a neural tube defect (20, 25). The Health Council of the Netherlands calculated that folic acid fortification at a level of about 100 μg per 100 kilocalories of food could probably prevent about 15 cases of neural tube defects in the Netherlands (~12% reduction) each year (20).

A higher level of fortification would probably mean that more neural tube defects would be prevented, but we currently have only indirect data (26) about the dose-response relation between folic acid intake and risk reduction. The level of fortification of 100 μg of folic acid in the flour fortification program in the US was chosen to prevent a substantial part of the population from having a higher intake level than the tolerable upper intake level of folic acid of 1 mg/day (14), (27). This upper intake level is primarily based on preventing the masking of a vitamin B12 deficiency.

Masking of a vitamin B12 deficiency: experiences in the US and Canada

In 1996, the Dutch Health Council advised against folic acid fortification, mainly because of the possibility of masking of a vitamin B12 deficiency (28). The US and Canada decided otherwise and started folic acid fortification around 1996-1998 (29, 30). Several groups have researched if folic acid fortification has led to an increase in the number of subjects with a masking of a vitamin B12 deficiency in the US and Canada, but the data are not equivocal. A study in Canadian women aged ≥65 years suggested that fortification had led to a six-fold increase in the number of women with a supraphysiological concentration of folate in serum (above 45 nmol/L) in combination with a deficiency of serum vitamin B12 (31), but the prevalence of macrocytosis was not reported. Another Canadian study showed no changes in mean hemoglobin concentrations, in mean corpuscular volume of erythrocytes, or in the proportion of seniors with an abnormally high mean corpuscular volume in the period from pre- to post-fortification (32). A study in the US in people aged 19 years and older indicated that the proportion of subjects with low serum vitamin B12 without macrocytosis was significantly higher after fortification was implemented compared

with the period before fortification was implemented (33), which could suggest an increased prevalence of masking. However, a second US study carried out from 1992 to 2000 did not show an increase in the proportion of hospital patients with a low vitamin B12 concentration without anemia (34). The conclusion is that some but not all epidemiological studies suggest that after the implementation of fortification the prevalence of masking of a vitamin B12 deficiency increased.

Several studies suggested that low vitamin B12 status is prevalent in about 20% of elderly individuals and that this is associated with cognitive impairment (35-38). If future research shows that vitamin B12 supplementation improves cognition (37, 39), this could form an additional argument to implement vitamin B12 fortification.

Possible positive and negative effects of folic acid fortification
In addition to a reduction in the number of neural tube defects, folic acid fortification may have additional benefits. In the 1990s, several epidemiological papers suggested that folic acid could lower the risk of cardiovascular disease (40, 41) and several large intervention trials were designed to study this hypothesis (42). So far, the trials have failed to show that folic acid can substantially lower cardiovascular disease risk (43, 44), although there are some indications that folic acid lowers the risk for stroke (45, 46). We have to await a meta-analysis of finished and ongoing intervention trials to see if folic acid affects vascular disease risk and other age-related diseases (42, 47).

While we are unsure of folic acid's effects on age-related diseases, folic acid fortification will partly solve a health inequality: all women of childbearing age, higher and lower educated, will profit from folic acid fortification and lower their risk of neural tube defect-affected pregnancies. Studies in the Netherlands and the US showed that women with a higher level of education are more likely to take folic acid supplements than lower educated women (21, 48, 49), and the latter will concurrently have a higher risk of a neural tube defect-affected pregnancies.

While folic acid fortification helps women of childbearing age, it is unclear how folic acid fortification will interfere with the efficacy of anti-folate drugs. These drugs can be used to combat diseases such as rheumatoid arthritis and childhood leukemia (50-52).

When folic acid fortification is implemented, especially children might exceed the safe upper intake level of folic acid (20, 53). This safe upper limit of intake is derived from adult data and is not based on conclusive data. Nevertheless, children are in a rapid stage of development, and no-one has yet researched the effects of folic acid on child development (50).

Folate is a critical factor in cell division and growth. It is involved in nucleotide synthesis and DNA methylation. Because of this, folate could play a role in cancer etiology and/or progression, but this role may be two-sided: folate may protect against the initiation of cancer, but may also facilitate the growth of existing lesions (54-57). Cohort studies suggested that high intake of dietary folate is associated with a 25% lower risk of colorectal cancer (58). A time-trend study of colorectal cancer incidence in the US and Canada (59) reported a sudden reversal in the decline in colorectal cancer when folic acid fortification was implemented. A folic acid supplementation trial (1mg/day for 3-5y) found that folic acid does not affect the risk of adenomas in patients with a history of adenomas (60) - adenomas are considered a precursor lesion of colorectal cancer (61). A subgroup analysis suggested that folic acid might increase the risk of advanced lesions and of multiple adenomas (60). Yet, this may have been a chance finding (62). Another folic acid trial (0.5mg/day for 3y) did not show an effect of folic acid on adenoma recurrence (63). Additional trials should further explain the role of folic acid in colorectal carcinogenesis, although it will be difficult to do so, since it would not be ethical to study if folic acid supplementation can enhance the growth of colorectal tumors in a human trial. The ongoing homocysteine lowering trials for prevention of vascular disease could possibly shed some light on the potential role of folic acid in cancer (42). Additionally, validated biomarkers of colorectal cancer risk are needed to study the role of folic acid in cancer etiology (57).

Folic acid fortification in the Netherlands?

How should we deal with folic acid fortification in the Netherlands? Folic acid fortification of bread and other staple foods will reduce the number of neural tube defects and simultaneous fortification with vitamin B12 could reduce the risk of folic acid fortification for elderly individuals who have a vitamin B12 deficiency. However, folic acid fortification should not be implemented in the Netherlands because of the concern that folic acid might facilitate the growth of existing lesions in colorectal cancer. The fact that this concern has been raised should halt folic acid fortification, although it is far from established that fortification will indeed have a detrimental effect on colorectal carcinogenesis. There are other options to improve folic acid intake of women of childbearing age, without having to expose the whole population chronically to folic acid. Moreover, folic acid fortification could lead to ethical problems concerning the free choice of people; if folic acid fortification of bread products is implemented it will be very hard to choose products that do not contain additional folic acid.

Other options to stimulate folic acid intake

What other options can improve folic acid intake of women who could become pregnant? The government should adopt a combination of strategies to stimulate folic acid and dietary folate intake of Dutch women.

Our studies showed that the bioavailability of dietary folate is ~80%. This suggests that public health advice to improve dietary folate intake may help to improve folate status (64-68). However, women would have to eat an additional 600 g of spinach, 100 g of liver or drink an additional 2.5 liters of orange juice to reach an amount of folate equivalent to the recommended 400 µg of folic acid per day. This is hardly feasible, and not advisable due to the high amount of sugar in orange juice and vitamin A in liver. Thus, use of folic acid supplements is advised.

To educate women on the benefits of folic acid intake, folic acid use before pregnancy should receive more attention during biology lessons at secondary schools, and in other youth care programs.

Preconceptional care programs should be initiated and/or improved (20, 69). In such programs, couples who wish to conceive can receive information about many topics that are important to consider before conception, such as nutrition (use of folic acid) alcohol and drugs, medication, illness, and work-related issues. Studies have shown that preconception counseling can improve intake of folic acid (70).

Pharmacies can also play an important role in increasing knowledge about the use of folic acid. Studies have shown that pharmacy campaigns can be effective (71, 72). In those campaigns, boxes with oral contraceptives were labeled with a text sticker: "Are you planning to have a baby? Ask for information about folic acid in your pharmacy". Additionally, women received a leaflet with information about folic acid use. Approximately 70% of Dutch women take oral contraceptives at some time before their first pregnancy, and thus a large part of the women can be reached in pharmacies. Also, mass media campaigns to stimulate folic acid use should be held, and repeated regularly (73). In all campaigns, special emphasis and focus should be on reaching lower educated and non-native Dutch women (21, 48, 49). Information about folic acid in popular soap operas might also be an interesting opportunity to reach these women (74).

Concluding remarks and suggestions for future research

This thesis showed that bioavailability of dietary folate is 80%, which is higher than the 50% that has been generally assumed. Consequently, the current dietary reference intakes for folate are too high. If we adapt the dietary reference intakes to the findings of this thesis, dietary intake of folate in 25% of women and 50% of men in the Netherlands is lower than the requirement. However, it is not clear if this is causing any clinical problems. Not all women of childbearing age know that they should take folic acid supplements in the periconceptional period or use folic acid. Folic acid fortification could be an option to improve folate intake of these women. However,

folic acid fortification should not be implemented country-wide as long as there is uncertainty about the role of folic acid in colorectal carcinogenesis. Therefore, it is important to invest in mass media campaigns, education, and other campaigns to promote folic acid supplements for women of childbearing age.

This thesis also showed that many interesting topics in the field of dietary folate and folate requirements still need to be researched:

- It would be interesting to study the bioavailability of folic acid taken with food compared with the bioavailability of folic acid from supplements. Does the concurrent ingestion of food lead to a decrease in the bioavailability?

- Dietary reference intakes for folate need to be reconsidered; taking into account that bioavailability is higher than generally assumed. Additionally, future studies should examine if the folate requirement of men and women indeed differs, and if so, should the dietary reference intakes be higher for men than for women.

- The dietary intake of folate in the adult population in the Netherlands is not optimal. However, no data clearly indicates if low intake leads to clinical problems (20). Thus, intake data as it relates to folate status needs to be reconsidered to see if public health measures are necessary to improve the folate status of the Dutch population.

- The prevalence of macrocytosis, of neural tube defects and of age-related diseases that could be affected by folic acid need to be monitored carefully in the Netherlands, so that any public health initiative to improve folate status can be evaluated.

- A validated standard method of analysis for food folate would make it easier to compare folate data between countries and would be a step forward in research on food folate bioavailability. Currently, different countries use several methods of analysis of food folate to construct their food tables. These differences make it difficult to compare the intake of folate between countries.

- Differences between HPLC and microbiological methods for food folate analysis should be further examined. It is not completely clear why HPLC-methods of food folate generally result in lower folate values than microbiological values. Analyses that use HPLC-methods to separate the folate derivatives in a food sample combined with the microbiological detection of the various derivatives might help to explain why methods differ.

- It is unclear if folic acid fortification will change the incidence rate of colorectal cancer. Validated biomarkers of colorectal cancer risk are needed to study the role of folic acid in cancer etiology (57).

References

1. Melse-Boonstra A, Verhoef P, West C. Quantifying folate bioavailability: a critical appraisal of methods. Curr Opin Clin Nutr Metab Care 2004;7:539-45.
2. Ginting E, Arcot J. High-Performance Liquid Chromatographic Determination of Naturally Occurring Folates during Tempe Preparation. J. Agric. Food Chem. 2004;52:7752-7758.
3. Kariluoto S, Vahteristo LT, Finglas PM, De Meer K, Nau H, Kehlenbach U. Population estimates of folate intake from food analyses. Am J Clin Nutr 2002;76:689-90; author reply 690-1.
4. Konings EJ. A validated liquid chromatographic method for determining folates in vegetables, milk powder, liver, and flour. J AOAC Int 1999;82:119-27.
5. DeVries JW, Rader JI, Keagy PM, et al. Microbiological assay-trienzyme procedure for total folates in cereals and cereal foods: Collaborative study. J AOAC Int 2005;88:5.
6. Commission of the European Communities. Community Bureau of Reference. The certification of the mass fractions of vitamins in four reference materials: wholemeal flour (CRM 121), milk powder (CRM 421), lyophilized mixed vegetables (CRM 485) and lyophilized pigs liver (CRM 487). Report EUR 18320 EN 1998.
7. Koontz JL, Phillips KM, Wunderlich KM, et al. Comparison of total folate concentrations in foods determined by microbiological assay at several experienced U.S. commercial laboratories. J AOAC Int 2005;88:805-13.
8. Newman EM, Tsai JF. Microbiological analysis of 5-formyltetrahydrofolic acid and other folates using an automatic 96-well plate reader. Anal Biochem 1986;154:509-15.
9. Stichting-NEVO. Dutch Food Composition Table 2001. The Hague: Voedingscentrum, 2001.
10. Puwastien P, Pinprapai N, Judprasong K, Tamura T. International inter-laboratory analyses of food folate. Journal of Food Composition and Analysis 2005;18:387.
11. Arcot J, Shrestha A. Folate: Methods of analysis. Trends in Food Science and Technology 2005;16:253.
12. Kehlenbach U. [Optimisation and application of an analytical method for the selective and sensitive determination of folate patterns in clinical samples and foodstuff] In German: Optimierung und Anwendung einer Analysemethode zur selektiven und sensitiven Bestimmung von Folatmustern in klinischen Proben und Lebensmitteln, . Zentrum fur Lebensmittelwissenschaften, Institut fur Lebensmitteltoxikologie und Chemische Analytik der Stiftung Tierarztliche Hochschule Hannover, PhD thesis,
, 2004.
13. Health Council of the Netherlands: Dietary reference intakes: Vitamin B6, folic acid and vitamin B12. The Hague: Health Council of the Netherlands, 2003:142.
14. Institute of Medicine. Folate. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. Washington: National Academy Press, 2000:196-305.

15. Pfeiffer CM, Rogers LM, Bailey LB, Gregory JF, 3rd. Absorption of folate from fortified cereal-grain products and of supplemental folate consumed with or without food determined by using a dual-label stable-isotope protocol. Am J Clin Nutr 1997;66:1388-97.

16. Waijers P, Slob W, Ocke MC, Feskens EJ. Methode voor schatting van de prevalentie van inadequate innemingen van micronutrienten. Toepassing: foliumzuur. RIVM Bilthoven, , 2004:RIVM report no 350010001/2004.

17. Barr SI, Murphy SP, Poos MI. Interpreting and using the Dietary References Intakes in dietary assessment of individuals and groups. J Am Diet Assoc 2002;102:780-788.

18. Carriquiry AL. Assessing the prevalence of nutrient inadequacy. Public Health Nutrition 1999;2:23-33.

19. De Bree A, Verschuren WMM, Blom HJ, Kromhout D. Association between B vitamin intake and plasma homocysteine concentration in the general Dutch population aged 20-65 y. Am J Clin Nutr 2001;73:1027-1033.

20. Health Council of the Netherlands. Naar een optimaal gebruik van foliumzuur. [Towards an optimal use of folic acid]. Publication number 2008/02. Den Haag, 2008.

21. De Walle HEK, De Jong-Van Den Berg LTW. Growing gap in folic acid intake with respect to level of education in the Netherlands. Community Genetics 2007;10:93-96.

22. Mitchell LE, Adzick NS, Melchionne J, Pasquariello PS, Sutton LN, Whitehead AS. Spina bifida. Lancet 2004;364:1885-95.

23. Centers for Disease Control and Prevention. Spina bifida and anencephaly before and after folic acid mandate-United States, 1995-1996 nad 1999-2000. MMWR Morb Mortal Wkly Rep 2004;53:362-365.

24. Mills JL, Signore C. Neural tube defect rates before and after food fortification with folic acid. Birth Defects Res A Clin Mol Teratol 2004;70:844-5.

25. Eurocat folic acid working group. Special Report: Prevention of neural tube defects by periconceptional folic acid supplementation in Europe (december 2005). 2005:Available at: http://www.eurocat.ulster.ac.uk/pubdata/Folic-Acid.html, accessed May 09, 2007.

26. Daly S, Mills JL, Molloy AM, et al. Minimum effective dose of folic acid for food fortification to prevent neural-tube defects. Lancet 1997;350:1666-9.

27. Stover PJ. Physiology of folate and vitamin B12 in health and disease. Nutr Rev 2004;62:S3-12; discussion S13.

28. Health Council of the Netherlands: Risks of folic acid fortification. The Hague: Health Council of the Netherlands, 2000:48.

29. Herbert V, Bigaouette J. Call for endorsement of a petition to the Food and Drug Administration to always add vitamin B-12 to any folate fortification or supplement. Am J Clin Nutr 1997;65:572-573.

30. Oakley GP, Jr. Let's increase folic acid fortification and include vitamin B-12. Am J Clin Nutr 1997;65:1889-90.

31. Ray JG, Vermeulen MJ, Langman LJ, Boss SC, Cole DE. Persistence of vitamin B12 insufficiency among elderly women after folic acid food fortification. Clin Biochem 2003;36:387-91.

32. Liu S, West R, Randell E, et al. A comprehensive evaluation of food fortification with folic acid for the primary prevention of neural tube defects. BMC Pregnancy Childbirth 2004;4:20.

33. Wyckoff KF, Ganji V. Proportion of individuals with low serum vitamin B-12 concentrations without macrocytosis is higher in the post folic acid fortification period than in the pre folic acid fortification period. Am J Clin Nutr 2007;86:1187-1192.

34. Mills JL, Von Kohorn I, Conley MR, et al. Low vitamin B-12 concentrations in patients without anemia: the effect of folic acid fortification of grain. Am J Clin Nutr 2003;77:1474-7.

35. Clarke R. Vitamin B12, folic acid, and the prevention of dementia. N Engl J Med 2006;354:2817-9.

36. Clarke R, Birks J, Nexo E, et al. Low vitamin B-12 status and risk of cognitive decline in older adults. Am J Clin Nutr 2007;86:1384-1391.

37. Eussen SJ, de Groot LC, Joosten LW, et al. Effect of oral vitamin B-12 with or without folic acid on cognitive function in older people with mild vitamin B-12 deficiency: a randomized, placebo-controlled trial. Am J Clin Nutr 2006;84:361-70.

38. Stabler SP, Allen RH. Vitamin B12 deficiency as a worldwide problem. Annu Rev Nutr 2004;24:299-326.

39. Malouf M, Grimley EJ, Areosa SA. Folic acid with or without vitamin B12 for cognition and dementia. Cochrane Database Syst Rev 2003:CD004514.
40. Klerk M, Verhoef P, Clarke R, Blom HJ, Kok FJ, Schouten EG. MTHFR 677C?T polymorphism and risk of coronary heart disease: A meta-analysis. Journal of the American Medical Association 2002;288:2023-2031.
41. Lewis SJ, Ebrahim S, Davey Smith G. Meta-analysis of MTHFR 677C->T polymorphism and coronary heart disease: does totality of evidence support causal role for homocysteine and preventive potential of folate? BMJ 2005;331:1053.
42. Clarke R, Armitage J, Lewington S, Collins R, Clarke R. Homocysteine-lowering trials for prevention of vascular disease: Protocol for a collaborative meta-analysis. Clinical Chemistry and Laboratory Medicine 2007;45:1575-1581.
43. Davey Smith G, Ebrahim S. Folate supplementation and cardiovascular disease. Lancet 2005;366:1679.
44. Bazzano LA, Reynolds K, Holder KN, He J. Effect of Folic Acid Supplementation on Risk of Cardiovascular Diseases: A Meta-analysis of Randomized Controlled Trials. Jama 2006;296:2720-2726.
45. Wang X, Qin X, Demirtas H, et al. Efficacy of folic acid supplementation in stroke prevention: a meta-analysis. Lancet 2007;369:1876-1882.
46. Carlsson C. Lowering homocysteine for stroke prevention. Lancet 2007;369:1841-1842.
47. Clarke R. Homocysteine-lowering trials for prevention of heart disease and stroke. Semin Vasc Med 2005;5:215-22.
48. de Walle HEK, Cornel MC, de Jong-van den Berg LTW. Three Years after the Dutch Folic Acid Campaign: Growing Socioeconomic Differences. Preventive Medicine 2002;35:65-69.
49. Use of supplements containing folic acid among women of childbearing age--United States, 2007. MMWR Morb Mortal Wkly Rep 2008;57:5-8.
50. Smith AD, Kim Y-I, Refsum H. Is folic acid good for everyone? Am J Clin Nutr 2008;87:517-533.
51. Arabelovic S, Sam G, Dallal GE, et al. Preliminary Evidence Shows That Folic Acid Fortification of the Food Supply Is Associated with Higher Methotrexate Dosing in Patients with Rheumatoid Arthritis. J Am Coll Nutr 2007;26:453-455.
52. Dinesh Khanna, Grace S. Park, Harold E. Paulus, et al. Reduction of the efficacy of methotrexate by the use of folic acid: Post hoc analysis from two randomized controlled studies. Arthritis & Rheumatism 2005;52:3030-3038.
53. Pfeiffer CM, Caudill SP, Gunter EW, Osterloh J, Sampson EJ. Biochemical indicators of B vitamin status in the US population after folic acid fortification: results from the National Health and Nutrition Examination Survey 1999-2000. Am J Clin Nutr 2005;82:442-450.
54. Kim YI. Will mandatory folic acid fortification prevent or promote cancer? Am J Clin Nutr 2004;80:1123-8.
55. Ulrich CM. Folate and cancer prevention: a closer look at a complex picture. Am J Clin Nutr 2007;86:271-273.
56. Ulrich CM, Potter JD. Folate and Cancer--Timing Is Everything. Jama 2007;297:2408-2409.
57. Sanderson P, Stone E, Kim YI, et al. Folate and colo-rectal cancer risk. Br J Nutr 2007;98:1299-304.
58. Sanjoaquin MA, Allen N, Couto E, Roddam AW, Key TJ. Folate intake and colorectal cancer risk: A meta-analytical approach. International Journal of Cancer 2005;113:825-828.
59. 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.
60. Cole BF, Baron JA, Sandler RS, et al. Folic Acid for the Prevention of Colorectal Adenomas: A Randomized Clinical Trial. Jama 2007;297:2351-2359.
61. Leslie A, Carey FA, Pratt NR, Steele RJC. The colorectal adenoma-carcinoma sequence. British Journal of Surgery 2002;89:845-860.
62. Bayston R, Russell A, Wald NJ, Hoffbrand AV. Folic acid fortification and cancer risk. Lancet 2007;370:2004.
63. Logan RFA, Grainge MJ, Shepherd VC, Armitage NC, Muir KR. Aspirin and Folic Acid for the Prevention of Recurrent Colorectal Adenomas. Gastroenterology 2008;134:29-38.

64. Broekmans WM, Klopping-Ketelaars IA, Schuurman CR, et al. Fruits and vegetables increase plasma carotenoids and vitamins and decrease homocysteine in humans. J Nutr 2000;130:1578-83.

65. Brouwer IA, van Dusseldorp M, West CE, et al. Dietary folate from vegetables and citrus fruit decreases plasma homocysteine concentrations in humans in a dietary controlled trial. J Nutr 1999;129:1135-9.

66. Cuskelly GJ, McNulty H, Scott JM. Effect of increasing dietary folate on red-cell folate: implications for prevention of neural tube defects. Lancet 1996;347:657-9.

67. Vahteristo L, Kariluoto S, Barlund S, et al. Functionality of endogenous folates from rye and orange juice using human in vivo model. Eur J Nutr 2002;41:271-8.

68. Venn BJ, Mann JI, Williams SM, et al. Dietary counseling to increase natural folate intake: a randomized, placebo-controlled trial in free-living subjects to assess effects on serum folate and plasma total homocysteine. Am J Clin Nutr 2002;76:758-65.

69. Health Council of the Netherlands. Preconception care: a good beginning [Preconceptiezorg: voor een goed begin]. The Hague, the Netherlands. 2007; publication no. 2007/19.

70. de Weerd S, Thomas CMG, Cikot RJLM, Steegers-Theunissen RPM, de Boo TM, Steegers EAP. Preconception counseling improves folate status of women planning pregnancy. Obstetrics & Gynecology 2002;99:45-50.

71. Meijer WM, De Smit DJ, Jurgens RA, de Jong-van den Berg LT. Pharmacists' role in improving awareness about folic acid: a pilot study on the process of introducing an intervention in pharmacy practice. Int J Pharmacy Practice 2004;12:1-7.

72. Meijer WM, de Smit DJ, Jurgens RA, de Jong-van den Berg LT. Improved periconceptional use of folic acid after patient education in pharmacies: promising results of a pilot study in the Netherlands. Int J Pharmacy Practice 2005;13:47-51.

73. Bekkers RL, Eskes TK. Periconceptional folic acid intake in Nijmegen, Netherlands. Lancet 1999;353:292.

74. Cassidy J. The soap opera that saves lives. BMJ 2008;336:1102-1103.

Summary in Dutch (Samenvatting)

Samenvatting

Voldoende inname van foliumzuur, één van de B-vitamines, voorkomt bloedarmoede. Brood, groente, fruit, aardappels en melkproducten zijn belangrijke bronnen van foliumzuur. Vrouwen die zwanger kunnen worden, wordt aangeraden om extra foliumzuur te slikken. Zij hebben daardoor minder kans op een baby met een neuralebuisdefect: een afwijking aan de ruggengraat die ernstige handicaps kan veroorzaken.

In dit proefschrift hebben we onderzocht of de Nederlandse voeding voldoende foliumzuur bevat om aan de aanbevelingen te voldoen, en, als er niet voldoende in de voeding zit, of we bijvoorbeeld brood zouden moeten verrijken met foliumzuur. We hebben eerst de aanbevelingen voor foliumzuur onder de loep gelegd: hoe moet hierin rekening worden gehouden met geslacht en biobeschikbaarheid?

Aanbevelingen voor foliumzuur

De aanbevolen dagelijkse hoeveelheid voor foliumzuur is 300 µg per dag: als alle volwassen Nederlanders dat dagelijks binnen zouden krijgen via de voeding, dan zou er vrijwel geen bloedarmoede als gevolg van een foliumzuur-tekort zijn.

Aanbevelingen & geslacht

De huidige aanbeveling is hetzelfde voor mannen en vrouwen. Wij hebben gegevens van twee eerder gedane foliumzuur-onderzoeken bekeken om te onderzoeken of de aanbeveling voor foliumzuur voor mannen en vrouwen inderdaad hetzelfde zou moeten zijn. In het eerste onderzoek kregen 294 mannen en 112 vrouwen gedurende 3 jaar dagelijks een pilletje met 800 µg foliumzuur. In het tweede onderzoek kregen 187 mannen en 129 vrouwen gedurende 12 weken een pilletje met een dosis foliumzuur tussen de 50 en 800 µg per dag. Het foliumzuur gehalte in het bloed van de vrouwen bleek gedurende het onderzoek 5 tot 10% meer gestegen te zijn dan dat in de mannen. We konden dit voor een groot gedeelte verklaren uit het feit dat mannen een groter lichaam hadden, oftewel een groter volume waarin het foliumzuur zich kon verdunnen. De conclusie van dit onderzoek luidde: mannen hebben meer foliumzuur

nodig om een bepaalde foliumzuur concentratie in het bloed te bereiken. De aanbevolen dagelijkse hoeveelheid voor foliumzuur voor mannen zou hoger moeten zijn dan voor vrouwen.

Aanbevelingen & biobeschikbaarheid

Biobeschikbaarheid is de mate waarin foliumzuur vanuit de darmen in het bloed wordt opgenomen en gebruikt kan worden door het lichaam. Foliumzuur in voeding heeft een iets andere chemische structuur dan foliumzuur uit een pilletje. Bovendien moet voedingsfoliumzuur in de darmen eerst uit die voeding worden losgemaakt, voordat het via de darmwand in het bloed kan worden opgenomen. Mogelijk is de biobeschikbaarheid van foliumzuur uit de voeding daarom maar 50%, ten opzichte van foliumzuur uit een pilletje. De aanbevelingen voor foliumzuur worden uitgedrukt als hoeveelheid in voeding, en die moeten dus twee keer zo hoog zijn als wanneer foliumzuur via een pilletje wordt ingenomen, uitgaande van de hiervoor genoemde 50%.

Wij hebben onderzocht of de biobeschikbaarheid van voedingsfoliumzuur werkelijk zoveel lager is, dan die van foliumzuur uit een pilletje. Hiervoor hebben 75 mensen 4 weken lang aan een gecontroleerde voedingsproef mee gedaan. In een dergelijk onderzoek eten de deelnemers vrijwel alleen wat wij, de onderzoekers, hen voorschotelen. Van al het eten dat de deelnemers kregen, stopten we direct een extra portie in de diepvries. In die extra porties hebben we de hoeveelheid foliumzuur gemeten, zodat we precies wisten hoeveel de deelnemers binnen kregen.

De 75 deelnemers werden verdeeld over 4 groepen. Eén groep kreeg voedsel met daarin veel foliumzuur: o.a. citrusfruit, spinazie, leverpastei, spruitjes en sinaasappelsap. De andere drie groepen kregen voedsel waar nauwelijks foliumzuur in zat: o.a. appels, meloen, worteltjes, witlof en champignons. Ter aanvulling kregen deze drie groepen een pilletje met 100 μg, 200 μg of 300 μg foliumzuur per dag. Bij alle deelnemers hebben we voor en na de proef bepaalt wat de concentratie foliumzuur was in het bloed.

De biobeschikbaarheid van foliumzuur uit voeding bleek 82% te zijn ten opzichte van foliumzuur uit een pilletje. De schatting van 50% die in de literatuur vaak wordt aangehouden, is dus een onderschatting.

Bevat de Nederlandse voeding voldoende foliumzuur?

Volgens onze bevindingen moet de aanbevolen dagelijkse hoeveelheid voor foliumzuur hoger zijn voor mannen dan voor vrouwen. Verder kunnen de aanbevelingen naar beneden worden bijgesteld omdat veel meer van het foliumzuur uit de voeding in het bloed wordt opgenomen dan we oorspronkelijk dachten: de biobeschikbaarheid van foliumzuur uit de voeding is geen 50% is, maar 82%. Als we met deze bevindingen rekening houden bevat de voeding van 25% van de Nederlandse vrouwen en van 50% van de Nederlandse mannen te weinig foliumzuur om aan de aanbevelingen te voldoen. Volgens de oorspronkelijke aanbevelingen bevat de voeding van 75% van de Nederlandse vrouwen en van 50% van de Nederlandse mannen te weinig foliumzuur.

Het is niet duidelijk of de lage inname aan foliumzuur ook tot gevolg heeft dat er veel mensen zijn met bloedarmoede in Nederland, of dat het nodig is om de foliumzuurvoorziening van de Nederlandse bevolking te verbeteren.

Foliumzuur in brood

Nog geen 50% van de Nederlandse vrouwen die zwanger kunnen worden slikt foliumzuur. Verrijken van bijvoorbeeld brood met extra foliumzuur kan een manier zijn om te zorgen dat vrouwen extra foliumzuur binnen krijgen. Veel foliumzuur kan echter mogelijk schadelijk zijn voor ouderen met een tekort aan vitamine B12. Zo'n tekort veroorzaakt, net als foliumzuur, bloedarmoede. Een vitamine B12 tekort kan daarnaast op den duur schade aanrichten aan de zenuwbanen. Voldoende foliumzuur verhelpt wel de bloedarmoede maar het beschermt de zenuwbanen niet: het kan een vitamine B12 tekort mogelijk maskeren.

Door brood te verrijken met zowel foliumzuur als vitamine B12 kan dit probleem mogelijk voorkomen worden. In dit proefschrift, onderzochten we het effect van deze dubbele verrijking op zowel de foliumzuur- als de vitamine B12 status van ouderen.

150 mannen en vrouwen in de leeftijd van 50-75 jaar werden verdeeld over twee groepen: één groep at dagelijks gedurende 12 weken brood verrijkt met 138 µg foliumzuur en 9,6 µg vitamine B12, de andere groep at brood dat niet was verrijkt. De hoeveelheid foliumzuur en vitamine B12 in het bloedplasma van de verrijkte groep steeg met 45-49% ten opzichte van de niet-verrijkte groep. Ook andere biomarkers die informatief zijn voor de foliumzuur- en vitamine B12 status (erythrociet folaat, methylmalonzuur, holotranscobalamine en homocysteine) verbeterden aanzienlijk door eten van het verrijkte brood. De conclusie van dit onderzoek was: brood verrijkt met foliumzuur en vitamine B12 kan een manier zijn om vitamine B12 status te verbeteren en om een belangrijk deel van vitamine B12 deficiëntie bij ouderen te voorkomen.

Foliumzuurverrijking in Nederland?

Moet Nederland starten met het toevoegen van foliumzuur aan brood, eventueel in combinatie met vitamine B12? Nee, voorlopig is dit geen goed idee. Foliumzuurverrijking heeft namelijk niet alleen een daling in het aantal neuralebuisdefecten tot gevolg. Het veroorzaakt mogelijk ook een aantal bijwerkingen. Belangrijk punt van zorg is, dat niet duidelijk is wat het effect van foliumzuur is op dikke darm kanker. Onderzoeken laten zien dat het binnen krijgen van voldoende foliumzuur mogelijk beschermt tegen dikke darm kanker, daar staat echter tegenover andere onderzoeken suggereren dat foliumzuur bestaande tumoren sneller zou kunnen laten groeien. Zolang hier geen duidelijkheid over is, is foliumzuurverrijking in Nederland geen optie.

Dit betekent dus dat er andere manieren moeten worden gezocht om vrouwen te stimuleren foliumzuur te slikken. In de biologie-lessen op de middelbare school moet aandacht zijn voor dit onderwerp, via apotheken moet informatie verstrekt worden, er moet geïnvesteerd worden in pre-conceptiezorg, en massa-media campagnes moeten worden opgezet en herhaald.

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Bedankt!
Renate

About the author

Curriculum Vitae

Renate Winkels was born on June 11, 1978 in Nijverdal, the Netherlands. She started the study Food Technology at Wageningen University in 1996. She switched to Human Nutrition at the end of the first year, because she realized that she was more interested in health and health aspects of food than in the technological aspects. During her study, she carried out three trainee projects. The first was a practical period at Asklepion, Rotterdam. Here, she wrote texts about nutrition and health for an educative and entertaining exhibition about the human body. After that, she carried out her first MSc thesis at the department of Human and Animal Physiology of Wageningen University. Here she studied how hormonal processes are timed to the day-night rhythm in animals. For her last MSc thesis she went to Georgia State University, Atlanta, USA, where she studied how neurotransmitter systems of diurnal animals differ from those of nocturnal animals. In 2001 she graduated and started as junior researcher at the Condition and Disease Specific Research department at Numico Research, Wageningen. In 2003 she switched jobs and started her PhD research within the project Diet, Homocysteine and Cardiovascular Disease; a project of the Top Institute Food and Nutrition led by Petra Verhoef. Renate attended several courses, conferences and discussion groups during her PhD program and was a member of the organizing committee of the 2005 PhD excursion to England, Ireland and Scotland. Currently, she works at the BOSK, the association of physically disabled persons and their parents, in Utrecht. Here she leads a projects about the quality of respite care for persons with profound multiple disabilities.

Publications

Winkels RM, Brouwer IA, Clarke R, Katan MB, Verhoef P
Bread cofortified with folic acid and vitamin B-12 improves the folate and vitamin
B-12 status of healthy older people: a randomized controlled trial
Am J Clin Nutr 2008; 88: 348-355

Winkels RM, Brouwer IA, Verhoef P, van Oort FVA, Durga J, Katan MB
Gender and body size affect the response of erythrocyte folate to folic acid treatment
J Nutr 2008; 138: 1456-1461

Winkels RM, Brouwer IA, Siebelink E, Katan MB, Verhoef P
Bioavailability of food folates is 80% of that of folic acid
Am J Clin Nutr 2007; 85: 465–73

Winkels RM
Ambiance in verpleeghuizen doet eten (*article on request about the PhD-studies of Dr Kristel Nijs*)
Voeding Nu 2006; 7/8: 15-17

Winkels RM, Melse-Boonstra A, West CE, Katan MB, Verhoef P
Development of a new method to study bioavailability of folate from total diets in healthy humans, using daily low doses of ^{13}C-labeled folic acid
FASEB-conference proceedings, 2004

Training and educational activities

Courses

Tracer Methodology in Metabolism, Universiteit Maastricht, 2004

Pharmacokinetcs, Leiden/Amsterdam Centre for Drug Research, 2004

Presentation skills, TIFN Wageningen, 2004

Clinical trials and Regression Analysis, NIHES, Rotterdam, 2005

Ecophysiology of the gastro-intestinal tract, VLAG, 2005

Science Journalism, Communicatiebureau de Lynx, Wageningen, 2006

Debating skills for PhD-students, TIFN, 2006

Philosophy and ethics of food science and technology, VLAG, Wageningen, 2006

Scientific writing, Wageningen University, 2006

Career Orientation, Wageningen Graduate Schools, 2006

Masterclass Diet and Cancer, NZO and VLAG, Wageningen, 2007

Design of questionnaires, Wageningen Business School, 2008

Meetings

Eurofoodfolate: Folates – analysis, bioavailability and health, Warsaw, Poland, 2003

Folic acid, vitamin B12 and one-carbon metabolism, FASEB, Snowmass, USA, 2004

Homocysteine metabolism, Milan, Italy, 2005

NWO-nutrition, NWO Papendal, 2004-2006

General

PhD tour England, Ireland, Scotland, 2005

Literature study and discussion, Wageningen University 2003-2007

TIFN seminars, 2003-2008