Folic acid, folate and homocysteine: Human intervention studies

Ingeborg A Brouwer

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Fifty years after its first discovery and synthesis, folate is coming of age with the recognition that folate is important not only in the most widespread form of vitamin-related anemia in the world, but also in four conditions as dreaded as the horsemen of the apocalypse: cancer, heart disease, stroke, and neural tube defects (Irwin H Rosenberg, Am J Clin Nutr 1997, 65 162-3).

Aan mijn ouders

Abstract

Folic acid, folate and homocysteine: Human intervention studies

PhD Thesis by Ingeborg A Brouwer, Department of Obstetrics and Gynaecology, University Hospital Nijmegen St Radboud, Nijmegen, and Division of Human Nutrition and Epidemiology, Wageningen University, Wageningen, the Netherlands

An elevated plasma total homocysteine concentration is a risk factor for women for having offspring with a neural-tube defect, and for both men and women for cardiovascular diseases. Extra intake of folic acid decreases plasma total homocysteine concentrations and has been proven to contribute to the prevention of neural-tube defects. Studies on the role of folic acid in preventing cardiovascular diseases are underway.

In this thesis we investigated whether low doses of supplemental folic acid (tablets) and folates in foods improve blood folate status and decrease plasma total homocysteine concentrations. We also studied a mechanism by which inadequate supply of folate to the foetus could be a risk factor for spina bifida, a neural-tube defect.

A daily intake of 250 µg of supplemental folic acid for four weeks was sufficient to improve folate status and decrease plasma total homocysteine concentrations in healthy young women. In a dietary controlled experiment, we showed that not only intake of supplemental folic acid, but also intake of folate derived from vegetables and citrus fruit improved the folate status and decreased plasma total homocysteine concentrations.

Folate and plasma total homocysteine concentrations of spina bifida patients were comparable to those of control patients. Four weeks of supplementation with 500 µg of folic acid per day improved the folate status and decreased plasma total homocysteine concentrations to a similar extent in the two groups.

In conclusion, we found no indication that an impaired folate and homocysteine metabolism in spina bifida patients is an important risk factor for having a neural-tube defect.

A high intake of dietary folate from vegetables and citrus fruit improves folate status and decreases plasma total homocysteine concentrations, which may lower the risk of cardiovascular diseases and of having offspring with a neural-tube defect. This provides a new argument for advising the general population to increase their intake of vegetables and fruits. However, women planning to become pregnant **should** still be advised to take supplemental folic acid as well. Folate from foods may prevent neural-tube defects, but folic acid supplements have been proven to do so.

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List of abbreviations

$\mu\text{g}/2\text{d}$ = microgram per 2 days

$\mu\text{g}/\text{d}$ = microgram per day

B12 = vitamin B12

B6 = vitamin B6

BHMT = betaine homocysteine methyltransferase

CS = cystathionine synthase

CV = coefficient of variance

DHFR = dihydrofolate reductase

H_2PteGlu = dihydrofolate

H_4PteGlu = tetrahydrofolate

MS = methionine synthase

MTHFR = methylenetetrahydrofolate reductase

PLP = pyridoxal-5'-phosphate

PteGlu = pteroylglutamate

PteGlu_1 = pteroylmonoglutamate (= folic acid)

PteGlu_n = pteroylglutamate with side chain containing between 1 and 7 glutamates

tHcy = plasma total homocysteine

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Introduction

Rationale and objectives

Folate is the generic name for all derivatives of the B-vitamin with the biological activity of pteroylmonoglutamic acid (FAO, 1988). Folic acid is the fully oxidised monoglutamate form of the vitamin, whereas the other naturally occurring forms are referred to as folates. Folic acid supplementation around conception decreases the risk of having offspring with a neural-tube defect (Czeizel and Dudás, 1992; MRC Vitamin Study Research Group, 1991). Therefore, in 1993 the Food and Nutrition Council in the Netherlands advised all women planning to become pregnant to consume folic acid preparations to prevent neural-tube defects. Women who have not previously given birth to a child with a neural-tube defect are advised to take a tablet containing 400 to 500 µg of folic acid each day from at least four weeks before pregnancy until at least the eighth week of pregnancy (Food and Nutrition Council, 1993). Although lower doses may be effective, no randomised trials have been carried out to test whether this is indeed the case. The underlying mechanism of the preventive effect of folic acid is unclear.

Folate in food occurs in many different forms. It is unclear whether high intakes of folate from food can also prevent neural-tube defects. Furthermore, it is difficult for women to reach a daily intake of 400 µg of dietary folate. The average intake is only about 250 µg per day (de Bree *et al.*, 1997). On the other hand, not all women in the target group do take folic acid tablets around conception (de Jong-van den Berg *et al.*, 1998; Sayers *et al.*, 1997).

Women who previously gave birth to a child with a neural-tube defect were shown to have elevated plasma total homocysteine concentrations (Mills *et al.*, 1995; Steegers Theunissen *et al.*, 1994). Moreover, an elevated plasma total homocysteine concentration is an established risk factor for cardiovascular diseases (Boushey *et al.*, 1995). In a meta-analysis Boushey *et al.* (1995) estimated that a plasma total homocysteine concentration of 15 µmol/L is associated with a 43% higher risk for coronary heart disease in men than a concentration of 10 µmol/L. Intakes of folic acid in doses above 650 µg have been shown to decrease plasma total homocysteine concentrations and may thereby reduce the risk of cardiovascular diseases (Ubbink, 1997). Less is known about the homocysteine-lowering effect of lower doses of folic acid or dietary folate.

Most studies which have investigated the effect of folic acid supplementation on plasma total homocysteine concentration have focused on persons with plasma total homocysteine concentrations above the normal range (Brattström *et al.*, 1988;

Guttormsen *et al* , 1996, Ubbink *et al* , 1993, Ubbink *et al* , 1994) Refsum *et al* (1998) concluded from a review of the literature that the relationship between plasma total homocysteine level and cardiovascular disease is continuous without an apparent threshold. Therefore, it would be worthwhile to investigate the effect of intake of folic acid and dietary folate on total plasma homocysteine concentrations in subjects with plasma total homocysteine concentrations in the normal range.

Thus, this thesis has the following objectives

- 1 To investigate whether supplementation with low doses of folic acid improves the folate status and decreases plasma total homocysteine concentrations in healthy subjects
- 2 To study the underlying mechanism by which additional folic acid around the time of conception prevents neural-tube defects. More specifically, whether folate and homocysteine metabolism of patients with spina bifida differs from that of control subjects and whether folic acid supplementation in spina bifida patients improves their folate status and decreases their plasma total homocysteine concentrations
- 3 To investigate whether intake of folate from vegetables and citrus fruit improves the folate status and decreases plasma total homocysteine concentrations in healthy subjects and to relate such effects to those obtained with the ingestion of folic acid

This introduction will provide background information on several aspects of synthetic folic acid and naturally occurring folates. Then, the metabolism of homocysteine will be described. This is followed by a description of the consequences of a low folate intake for neural-tube defects and cardiovascular diseases. Subsequently, the strengths and weaknesses of study designs generally used in human intervention studies are discussed. Finally, the introduction ends with the outline of the thesis.

Folate and its metabolism

Nomenclature

Folate is the generic term for all compounds of the B-vitamin that exhibit vitamin activity similar to pteroylmonoglutamic acid (folic acid) (Figure 1.1). In this thesis folic acid refers to the fully oxidised pteroylmonoglutamic acid. Folic acid is not a natural compound and it is much more stable than most reduced forms (O'Broin *et al* , 1975). The synthetic form of this vitamin is used in tablets and fortified foods.

In nature the pteridine ring of folate is reduced to either dihydrofolate ($H_2PteGlu$) or tetrahydrofolate ($H_4PteGlu$). The tetrahydrofolates are present unsubstituted or with various one-carbon groups (Figure 1.2). Natural folates often exist as polyglutamates ($PteGlu_n$), with a side chain of up to seven glutamate residues in γ -peptide linkage. In the human body folate is transported across membranes as monoglutamates ($PteGlu_1$). Only monoglutamates are found in plasma and urine. In tissues folates are synthesised to polyglutamates. Polyglutamates are in general better substrates for enzymes than monoglutamates (Wagner, 1995). In food folates mainly occur as polyglutamates.

Figure 1.1 Structure of tetrahydrofolate. From Shane (1995)

Folate one-carbon pool

The main function of folate is to carry one-carbon moieties, such as methyl and formyl groups. These one-carbon groups are used in the synthesis of DNA precursors. Figure 1.2 depicts the reactions and enzymes involved in one-carbon metabolism. The folates in this figure are shown as monoglutamates, though, as mentioned before, most folates in human tissues are present as polyglutamates. For most conversions polyglutamates will serve as substrates. Folate has to be fully reduced to tetrahydrofolate before it can carry a one-carbon unit (Wagner, 1995). The enzyme dihydrofolate reductase reduces folic acid via dihydrofolate to tetrahydrofolate.

Figure 1.2 Schematic overview of the main conversions of the folate one-carbon pool. Modified from Wagner 1995. Folates in this figure are shown as monoglutamates although most folates in human tissues are polyglutamates. The boxes refer to the different folate coenzymes, the italic terms refer to enzymes.

Tetrahydrofolate can via 5,10-methylene-tetrahydrofolate be converted to 5-methyl-tetrahydrofolate. The latter form is a methyl donor for the remethylation of homocysteine to methionine.

Intestinal absorption of folate

Dietary polyglutamyl folates must be deconjugated by the enzyme pteroylpolyglutamate hydrolase (folate conjugase) to a monoglutamyl form before absorption. After hydrolysis two different transport systems are present. In the first transport system, folates are bound to membrane-associated folate-binding proteins and transported across the brush border membrane by a carrier-mediated mechanism. However, at high intraluminal concentration of folate ($>10 \mu\text{M}$) a nonsaturable diffusion-mediated transport system plays a major role in folate absorption (Mason, 1990).

Before folic acid enters the portal circulation it is reduced to dihydrofolate and subsequently to tetrahydrofolate, which is either methylated or formylated in the mucosal cells (Perry and Chanarin, 1973, Selhub *et al* , 1973, Selhub *et al* , 1983, Strum, 1979). However, at amounts above 266 μg of folic acid unmetabolised folic acid has been shown to appear in serum (Kelly *et al* , 1997).

Bioavailability and bioefficacy of folate

Folate bioavailability can be defined as the fraction of folate ingested which may have the potential to meet functional demands in target tissues. Homocysteine is a functional parameter of the folate status. Therefore, the term bioefficacy can be used if effects on plasma total homocysteine are used as the determinant of bioavailability (Strain and West, 1999).

Homocysteine metabolism

Methionine is an essential amino acid that is converted to S-adenosylmethionine by methionine adenosyltransferase. S-adenosylmethionine is the main methyl donor. S-adenosylhomocysteine is formed after donation of the methyl group. S-adenosylhomocysteine is hydrolysed to homocysteine. Homocysteine can either be remethylated to methionine, or transsulphurated to cysteine via cystathionine. Figure 1.3 shows the main conversions in the homocysteine metabolism.

The enzyme methyltetrahydrofolate-homocysteine methyltransferase (methionine synthase) uses folate, in the 5-methyl-tetrahydrofolate form, as a methyl group donor for the remethylation of homocysteine to methionine. Vitamin B12 (methylcobalamin) is involved in this remethylation reaction as a co-factor.

Figure 1.3 Schematic view of the main conversions of the homocysteine metabolism.

Betaine-homocysteine methyltransferase is an enzyme involved in an alternative remethylation pathway. Betaine is converted to dimethylglycine and serves as a methyl donor for the remethylation from homocysteine to methionine by betaine-homocysteine methyltransferase. The latter enzyme is mainly found in the liver and only minor activity has been shown in kidneys and adrenal glands (Finkelstein, 1990).

Vitamin B6 is a co-factor in the transsulphuration of homocysteine to cysteine. Homocysteine is first condensed with serine to form cystathionine by the B6-dependent enzyme cystathionine β -synthase. This reaction is irreversible. Cystathionine is then further metabolised to cysteine by the B6-dependent enzyme γ -cystathionase (Ueland *et al*, 1993).

Consequences of low folate intake

Neural-tube defects

Neural-tube defects are one of the most common severe congenital malformations of the central nervous system in man. The mean birth prevalence rate of neural-tube defects in the Netherlands is around 1.4 per 1,000 live births. This implies that each year approximately 250 children in the Netherlands are born with a neural-tube defect (EUROCAT Working Group, 1998). Neural-tube closure takes place in the third and fourth week after conception. Failure of the neural-tube to close results in a neural-tube defect. The most common forms of neural-tube defects are spina bifida and anencephaly. Spina bifida develops when the neuroporus posterior fails to close. The severity of the spina bifida depends on the size of the part of the spinal cord that remains open. Meningomyeloschisis is an open defect of the spinal cord, whereas spina bifida occulta is a defect by which the skin is closed. Anencephaly is a lethal malformation which occurs when the neuroporus anterior remains open and the underdeveloped forebrains remain in contact with the amniotic fluid and subsequently degenerates. Meningocele and meningo-encephalocele are closed brain defects (Rubin and Farber, 1995).

In 1965, Hibbard and Smithells were the first to suggest that the B-vitamin folic acid plays a role in the pathogenesis of neural-tube defects (Hibbard and Smithells, 1965). In 1983, Smithells' group showed a 70% lower neural-tube defect recurrence rate in women who received supplements containing folic acid (Smithells *et al*, 1983). However, the study was not randomised and therefore selection bias could not be excluded. Two later randomised trials confirmed that supplementation of women with folic acid in the period around conception decreases the occurrence and

recurrence risk of having offspring with a neural-tube defect (Czeizel and Dudás, 1992; MRC Vitamin Study Research Group, 1991). Governments of several countries responded to the results of these trials by launching advice for women planning to become pregnant (Centers for Disease Control, 1992; Expert Advisory Group, 1992; Food and Nutrition Council, 1992; Food and Nutrition Council, 1993). In the Netherlands, all women planning to become pregnant are advised to take each day a folic acid tablet, containing 400 to 500 µg of folic acid in addition to their habitual diet, from at least four weeks before conception up to the eighth week of pregnancy. Women with a history of a neural-tube defect affected pregnancy are advised to take 4-5 mg supplemental folic acid daily (Food and Nutrition Council, 1993). Since January 1998, all fortified flour in the US contains 140 µg folic acid per 100 g by law. This action was taken by the US government to increase the folic acid intake of women in the reproductive age category. These reproductive women are recommended to consume 400 µg of synthetic folic acid from fortified foods and/or supplements in addition to intake of folate from a varied diet (Yates *et al.*, 1998). Fortification of the food partly overcomes the problem that women become pregnant without taking extra folic acid in spite of the advice (de Jong-van den Berg *et al.*, 1998).

The underlying mechanism of the preventive effect of folic acid is unclear. Steegers Theunissen *et al.* noticed a derangement in the homocysteine metabolism in some women with neural-tube defect affected offspring (Steegers Theunissen *et al.*, 1991; Steegers Theunissen *et al.*, 1994). This finding was confirmed by Mills *et al.* (1995). Folic acid supplementation can decrease plasma total homocysteine concentrations (Chapters 2 and 4 of this thesis; Homocysteine Lowering Trialists' Collaboration). Therefore, homocysteine might play a role in the aetiology of neural-tube defects.

Cardiovascular disease

Cardiovascular diseases are a major health problem in the Western world. In the Netherlands, they are responsible for approximately 40% of total mortality (Dutch Heart Foundation, 1997). Several studies have shown that patients with cardiovascular diseases have higher plasma total homocysteine concentrations than healthy control subjects (Boushey *et al.*, 1995; Clarke *et al.*, 1991; den Heijer *et al.*, 1996; Falcon *et al.*, 1994; Pancharuniti *et al.*, 1994; Selhub *et al.*, 1995; Stampfer *et al.*, 1992). Boushey *et al.* (1995) estimated that a total of 10% of the population's risk for coronary artery disease is attributable to elevated plasma total homocysteine concentrations. Furthermore, the relationship between the plasma total homocysteine concentration and cardiovascular disease seems to be continuous

with no apparent threshold (Refsum *et al.*, 1998). The European Concerted Action Project showed that an increased plasma total homocysteine concentration conferred not only an independent risk of cardiovascular disease, but the risk increased strongly in combination with smoking and hypertension (Graham *et al.*, 1997). Moreover, a low folate intake has also been shown to be a risk factor for cardiovascular disease (Giles *et al.*, 1995; Morrison *et al.*, 1996; Pancharuniti *et al.*, 1994; Selhub *et al.*, 1995). Intakes of doses of folic acid above 650 µg per day have been shown to decrease plasma total homocysteine concentrations which may decrease the risk of cardiovascular disease (Ubbink, 1997). Intervention studies are currently running to investigate whether homocysteine-lowering therapies indeed lead to a decrease in cardiovascular disease.

Study designs

Several strategies can be used to investigate the relationship between folate and folic acid intake and folate and homocysteine concentrations in the blood. Different strategies can be distinguished by differences in study design. Firstly, in observational studies subjects are studied without intervening in their dietary habits or lifestyle. This implies that the burden on the subjects and the costs of these studies are relatively low. On the other hand, selection bias and possible confounding are more difficult to control than in intervention studies. De Bree *et al.* summarised studies investigating habitual folate intake of adult populations in Europe (de Bree *et al.*, 1997). In this thesis we studied the relationship between intake and status using intervention studies.

Intervention studies actively interfere in lifestyle and usual customs of subjects. The ideal design of an intervention study is a cross-over, placebo-controlled randomised trial. In a cross-over study all subjects receive both placebo and intervention treatment in random order. If necessary, a wash-out period should be taken in between two treatments. Studies investigating a longer period of intervention with folic acid or folate have to take into account that the washout period after treatment with folic acid should be at least four months (see Chapter 2). This implies that cross-over studies are time-consuming, which increases the chance that subjects withdraw from the study. Therefore, parallel designs have been used in most studies investigating the effects of supplementation with folic acid or folate on folate and homocysteine status. In a parallel design, groups of subjects receive different treatments at the same time. All studies described in this thesis have a parallel design.

To ensure that the observed effects are caused by the treatment and not by other factors, a placebo group should be included. The placebo group needs to be treated in the same way as the intervention groups, but has to receive placebo treatment instead of the chosen intervention treatment. Therefore, changes irrespective of treatment, such as random drifts with time, will also occur in the placebo group. Studies described in Chapters 2, 3 and 6 include a placebo group. An alternative for a placebo group, in case the number of subjects is limited, is a sequential design with a placebo period followed by an intervention period (see Chapter 5). In such a design subjects receive first placebo treatment followed by intervention treatment. The change in the treatment period can then be corrected for the change in the placebo period. The main disadvantage of this design is that period effects cannot be excluded. A placebo group can be omitted for efficiency reasons if the effect of one of the intervention strategies is already established (see Chapter 4). However, inclusion of a placebo group is preferred.

Subjects should always be randomly divided into the groups to prevent selection bias. This implies that neither subjects nor researchers can choose who will be assigned to which group. To prevent differences in baseline values among intervention groups, researchers can choose to stratify for these values and randomise after stratification (see Chapter 6).

Studies should be of a blind nature to prevent subjects influencing the response to their treatment. In intervention studies with folic acid, tablets or fortified products can be manufactured in such a manner that the treatments are indistinguishable. However, in intervention studies using natural food folate it is not possible to mask the differences between dietary treatments (Cuskelly *et al.* 1996; Chapter 6).

Another important factor in intervention studies is the measure of control. The least controlled situation is a study in which subjects are merely advised to change their normal habits, for example to take tablets or to eat different products. This strategy can be used to test whether an advice given by a government can be effective or not (see Cuskelly *et al.* 1996). To enlarge the chance that subjects will indeed change their habits, supplements can be given to them. This is more convenient for the subjects and therefore compliance will be better. Folic acid and placebo tablets were therefore provided in the studies described in Chapters 2, 3, 4 and 5, as in many other studies (Homocysteine Lowering Trialists' Collaboration, 1998). Some studies provided foods fortified with folic acid (Malinow *et al.*, 1998; Schorah *et al.*, 1998). Cuskelly *et al.* (1996) provided products naturally rich in folate to their dietary folate group. The advantage of advising subjects or supplying

supplemental items is that it can easily be implemented in the free-living situation. However, when no effect is observed it is not clear whether this is caused by lack of compliance of the subjects or because the intervention treatment is not effective. To diminish the risk of non-compliance, intake should be checked. Compliance can be checked by counting the remaining tablets, examining tablet calendars or diaries (Chapters 2, 3, 4 and 5), assessment of concentrations in blood or tissues, and assessment of dietary intake by a validated method (see Cuskelly *et al.* 1996; Chapters 2 and 3).

To increase compliance even further, investigators may choose to perform a completely controlled study, in which the investigators supply the full diet and supervise the subjects. In the most controlled setting, subjects are living in a metabolic ward, under supervision, 24 hours per day (Jacob *et al.*, 1995; Jacob *et al.*, 1994; Sauberlich *et al.*, 1987). The investigators know exactly what the subjects consumed during the study. However, the burden on the subjects is high and such studies usually involve a limited number of subjects. Supplying a complete diet to subjects, but not trying to control their daily activities (Chapter 6) can reduce these disadvantages. Deviations from the protocol occur more frequently in a setting in which only the diet is controlled compared to a completely controlled trial, but are still considerably less in studies where only supplements to the diets are supplied. By supervising one or more meals per day subjects have even less chance to deviate from the protocol (Chapter 6). All strictly controlled trials have the disadvantage that they do not investigate whether these strategies can be implemented in normal life.

The choice of study design should mainly depend on the research question, although practical limitations play a role as well.

Outline of the thesis

Supplementation with high doses of folic acid is known to decrease plasma total homocysteine concentrations and to improve the folate status in healthy men and women. Investigating the effects of natural food folate on the folate and homocysteine metabolism is a complicated task. Therefore, it is important to first know whether low doses of supplemental folic acid can decrease plasma total homocysteine concentrations and improve the folate status in healthy subjects (objective 1). Chapter 2 answers the question whether supplementation with low doses of folic acid (250 - 500 µg per day) decreases plasma total homocysteine and improves the folate status in healthy volunteers. This chapter also describes whether eight weeks of washout is sufficient for the subjects to return to baseline concentrations of blood folate and plasma total homocysteine.

A decrease of plasma total homocysteine concentrations by folic acid supplementation would theoretically lead to an increase in methionine concentrations because folate is a methyl donor in the remethylation reaction of homocysteine to methionine. Chapter 3 reports whether folic acid supplementation indeed leads to increased plasma methionine concentrations.

One group described in Chapters 2 and 3 received a tablet containing 500 µg of folic acid and a placebo tablet on alternate days. Therefore, Chapter 4 describes a study investigating whether providing subjects with 500 µg of folic acid every other day leads to effects on folate status and plasma total homocysteine concentrations that differ from those of providing subjects with 250 µg of folic acid every day.

Chapter 5 describes a case-control study in patients with spina bifida and disabled control subjects. We investigated their homocysteine and folate status at baseline. Furthermore, we studied the impact of folic acid supplementation on the folate status and homocysteine concentrations of the spina bifida patients and the control subjects (objective 2).

The effects of a folate rich diet on folate status and homocysteine concentrations are largely unknown. Therefore, Chapter 6 reports results of a dietary controlled intervention study. The study investigated whether natural food folate from vegetables and citrus fruit would improve the folate status and decrease plasma total homocysteine concentrations (objective 3).

Chapter 7 reviews the literature investigating the many factors that may influence folate bioavailability and bioefficacy.

Chapter 8 discusses the main conclusions and implications of the studies described in this thesis.

Low-dose folic acid supplementation decreases plasma homocysteine: a randomized trial

Ingeborg A Brouwer, Marijke van Dusseldorp, Chris MG Thomas, Marinus Duran, Joseph GAJ Hautvast, Tom KAB Eskes, Régine PM Steegers Theunissen.
The American Journal of Clinical Nutrition 1999; 69: 99-104.

Abstract

Background: An elevated plasma total homocysteine concentration is a risk factor for cardiovascular disease and neural-tube defects. A high daily intake of supplemental folic acid is known to decrease total homocysteine concentrations.

Objective: We studied the effect of low-dose folic acid administration (250 or 500 µg/d) for four weeks on plasma total homocysteine concentrations and folate status. We also investigated whether total homocysteine concentrations and blood folate concentrations returned to baseline after an eight-week washout period.

Design: In this placebo-controlled study 144 healthy women aged 18-40 years received 500 µg folic acid/d, 500 µg folic acid every second day (250 µg/d), or a placebo tablet with their habitual diet (mean dietary folate intake 280 µg/d).

Results: Administration of 250 and 500 µg folic acid/d for 4 weeks significantly increased folate concentrations in plasma ($P < 0.001$) and red cells ($P < 0.01$). Total homocysteine concentrations decreased significantly ($P < 0.001$) in women ($n=50$) who took 250 µg folic acid/d [mean ($\pm$ SEM) deviation from baseline $-11.4 \pm 1.98\%$] and in women ($n=45$) who took 500 µg folic acid/d ($-21.8 \pm 1.49\%$). Eight weeks after the end of the intervention period (week 12), plasma total homocysteine concentrations in the folic acid-supplemented groups had not returned to baseline (week 0).

Conclusions: Doses of folic acid as low as 250 µg/d, on average, in addition to the usual dietary intakes, significantly decreased plasma total homocysteine concentrations in healthy, young women. An eight-week washout period was not sufficient for blood folate and plasma total homocysteine concentrations to return to baseline concentrations.

Introduction

Folic acid has received a great deal of attention because of its role in the prevention of neural-tube defects. Sufficient evidence is now available to advise women to increase their folic acid intake if they are planning to become pregnant (Czeizel and Dudás, 1992; MRC Vitamin Study Research Group, 1991). Doses of supplemental folic acid between 360 and 4000 µg/d in addition to usual dietary intakes of folate have been proven to prevent neural-tube defects (Czeizel and Dudás, 1992; Kirke *et al.*, 1992; Laurence *et al.*, 1981; MRC Vitamin Study Research Group, 1991; Smithells *et al.*, 1983).

Folate is an important substrate in the remethylation of homocysteine to methionine. Steegers Theunissen *et al.* showed that a subgroup of women who had previously experienced an neural-tube defect affected pregnancy had higher plasma total homocysteine concentrations than healthy control women (Steegers Theunissen *et al.*, 1994). A mildly elevated plasma total homocysteine concentration is suggested to be a marker for a defect in folate metabolism or for folate deficiency and is thus a risk factor for having a child with a neural-tube defect (Mills *et al.*, 1995; Steegers Theunissen *et al.*, 1994).

High plasma total homocysteine concentrations are also considered a risk factor for cardiovascular disease. The prevalence of elevated plasma total homocysteine concentrations is higher in patients with coronary artery disease (Pancharuniti *et al.*, 1994; Stampfer *et al.*, 1992), cerebrovascular (Clarke *et al.*, 1991; Selhub *et al.*, 1995) and peripheral vascular diseases and thrombosis (den Heijer *et al.*, 1996; Falcon *et al.*, 1994) than in healthy control subjects. Evidence is accumulating that folate is involved in the prevention of these diseases. Low folate concentrations in blood are associated with cardiovascular disease (Giles *et al.*, 1995; Morrison *et al.*, 1996; Pancharuniti *et al.*, 1994; Selhub *et al.*, 1995) and folate intake is related to carotid artery stenosis (Selhub *et al.*, 1995).

Intakes of supplemental folic acid ≥ 650 µg/d are known to decrease plasma total homocysteine concentrations (Ubbink, 1997). Some studies have investigated the effect of low doses of supplemental folic acid on plasma total homocysteine concentrations (den Heijer *et al.*, 1998; Guttormsen *et al.*, 1996a; Jacob *et al.*, 1994; O'Keefe *et al.*, 1995; Ward *et al.*, 1997). However, this is the first placebo-controlled, in-depth study of the effects of supplemental folic acid (as low as 500 µg every other day) on plasma total homocysteine concentrations in healthy subjects with normal plasma total homocysteine concentrations. If low doses of supplemental folic acid also decrease plasma total homocysteine concentrations, then intervention with foods rich in folate could also be an option to reduce plasma total homocysteine.

Therefore, the aim of the present study was to investigate whether an average daily intake of 250 or 500 µg of supplemental folic acid decreases plasma total

homocysteine concentrations in healthy women. In addition, we investigated whether plasma total homocysteine concentrations returned to baseline (week 0) within eight weeks after the intervention ended (week 12) and how these treatments affected plasma and red blood cell folate concentrations.

Subjects and Methods

Subjects

Healthy, nonpregnant women aged 18-40 years were recruited by advertisements in local newspapers and by the distribution of leaflets. Women were excluded if they were smokers, had a gastrointestinal disorder, or had used any of the following preparations during the two months preceding the trial: vitamins, minerals, yeast or seaweed, malaria prophylaxis, or anticonvulsants. On the basis of these criteria 158 women were eligible for participation. After recruitment, seven women withdrew, five were excluded because they could not attend the first blood collection session, and two because they were already participating in another nutritional study. The final study group consisted of 144 women.

The study design was approved by the Medical Ethical Committee of the Wageningen Agricultural University. All subjects gave written informed consent. The fieldwork of the study was performed at the Division of Human Nutrition and Epidemiology of the Wageningen Agricultural University.

Methods

After stratification by the use and type (brand) of oral contraceptives used, the women were divided at random (by alternation) into three intervention groups: 500 µg folic acid/d (500-µg group), 500 µg folic acid every other day (on average 250 µg/d; 250-µg group), or a placebo (placebo group) for four weeks. To maintain the blind nature of the study, all subjects received two containers of tablets, one marked with a red sticker and one with a yellow sticker. The tablets were indistinguishable from each other in appearance and taste.

Subjects received a diary and a calendar in which the days were alternately marked red and yellow. Subjects were asked to maintain their regular diet, but to refrain from consuming liver and marmite, a yeast extract. All subjects kept a diary in which they reported the intake of tablets, illnesses experienced, the days on which they menstruated, the days on which they did not take oral contraceptives, any extraordinary foods consumed, and excessive physical activity. To assess compliance, we counted any remaining tablets and checked the reported intake of tablets in the diaries. On the days blood was collected, subjects took their tablets after blood sampling. Tablets were always taken before breakfast. A 24-hour recall was obtained from each subject once during the intervention period to check their intake of macronutrients and folate.

Venous blood samples were collected after subjects had fasted overnight

beginning at the start of the four-weeks intervention period (week 0), after 1, 2 and 4 weeks of intervention (weeks 1, 2 and 4) and 4 and 8 weeks after the intervention period ended (weeks 8 and 12) Total plasma homocysteine and plasma folate concentrations were determined in all blood samples Red blood cell folate concentrations were assessed at baseline (week 0) and at the end of both the intervention (week 4) and washout periods (week 12) We determined plasma vitamin B12 concentrations at the start of the intervention period

Blood samples were drawn into EDTA-containing evacuated tubes (Venoject II, Terumo, Madrid, Spain) For the determination of plasma total homocysteine, plasma folate and plasma vitamin B12, samples were immediately placed on ice and centrifuged within 60 min at 3000xg for 10 min at 4°C Plasma was separated and stored at -35°C for folate and vitamin B12 and at -80°C for plasma total homocysteine determination For the determination of folate concentrations in red blood cells, haematocrit samples were analysed and a 1:4 dilution of whole blood was stored in sodium ascorbate (10g/L) at -35°C The hemolysates were further diluted with IMx Folate RBC Lysis Reagent (Abbott Diagnostics, Maidenhead, United Kingdom) before measurement Total homocysteine concentrations were measured by HPLC technique and fluorimetric detection (intra- and inter-assay CVs <8%) (Araki and Sako, 1987) All samples from each subject were analysed in the same run Folate concentrations in plasma and red blood cells and vitamin B12 in plasma were determined with the IMx automated immunoassay system (Abbott Laboratories, North Chicago), the vitamin B12 assays is based on microparticle technology (microparticle intrinsic factor assay) and the plasma and red blood cell folate assays are based on ion-capture technology The intra-assay CV of the folate assay varied between 3% and 6%, whereas the inter-assay CV varied between 6% and 10%, depending on the folate concentration For vitamin B12, both the intra- and inter-assay CVs were <5%

Statistical analyses

The response to the various treatments was calculated for each subject as the change in plasma total homocysteine, plasma folate, or red blood cell folate, between the start (week 0) and the end (week 4) of the intervention period Estimation of group sizes was based on a 10% decrease in plasma total homocysteine concentrations after four weeks of supplementation with 400 µg folic acid in young women in Germany (Dierkes, 1995) On the basis of these data, 50 women per group would be sufficient to detect a change in plasma total homocysteine of 1 µmol/L with a power of 90% and an α of 0.05 Furthermore, changes in plasma total homocysteine and plasma and red blood cell folate were calculated per subject between the start of the intervention period and the end of the washout period (week 0 - week 12) The changes in folate and plasma total homocysteine concentrations were normally distributed One-way analysis of variance was used to analyse differences in responses of and in baseline concentrations of plasma total homocysteine, folate and vitamin B12 among the

three groups. When this analysis indicated a significant difference ($P < 0.05$) multiple comparisons were made by Student's t tests, using a significance level of $P < 0.05/3 = 0.017$ (to maintain an overall significance level of $P < 0.05$).

Results

There were no significant differences among the three groups with respect to age, body mass index, use of oral contraceptives, or baseline concentrations of vitamin B12, plasma and red blood cell folate (Table 2.1). Although the subjects had been randomly assigned into the three groups, the baseline concentrations of plasma total homocysteine differed significantly among the groups. The mean dietary intakes of folate and macronutrients during the intervention period did not differ significantly among the three intervention groups.

Subject compliance was determined to be good on the basis of tablet counts and diaries. Twenty-three subjects forgot to take one tablet during the whole study period and only five subjects forgot to take two tablets.

After four weeks of intervention, plasma and red blood cell folate concentrations had increased significantly in the two treatment groups compared to the placebo group (Table 2.2). The increases in plasma folate were significantly higher in the 500- μg group than in the 250- μg group ($P < 0.001$). During the eight weeks after the end of the intervention period, the so-called washout period, plasma folate concentrations decreased significantly in the two treatment groups compared to the placebo group. However, the plasma folate concentration between weeks 0 and 12 was still significantly higher in the two treatment groups than in the placebo group ($P < 0.01$). Red blood cell folate concentrations remained stable during the washout period.

Total homocysteine concentrations decreased during the four-weeks intervention period in the two treatment groups, whereas they remained stable in the placebo group (Figure 2.1). Four weeks of supplementation with 500 μg folic acid every other day resulted in a decrease in the plasma total homocysteine concentrations of 11.4% compared to 0.8% in the placebo group (Figure 2.1). In the 500- μg group, plasma total homocysteine decreased 21.8%. No significant changes occurred in plasma total homocysteine concentrations between weeks 4 and 12 (during the washout period). At the end of the washout period, plasma total homocysteine concentrations in the two treatment groups were still significantly lower than those in the placebo group.

The quintiles of subjects with the highest baseline plasma total homocysteine concentrations showed the most pronounced decrease in plasma total homocysteine (Figure 2.2).

Table 2.1 Characteristics of the study population of 144 healthy women (18-40 years)

	500 µg/d group (n=45)	250 µg/d group (n=50)	placebo group (n=49)
Age (years)	23.6 ± 4.1	21.7 ± 2.0	22.3 ± 3.1
BMI (kg/m ²)	22.3 ± 2.5	22.8 ± 2.8	22.1 ± 2.3
Use of OC (%)	71	66	65
Homocysteine (µmol/L) ²	11.1 ± 2.6 ¹	9.7 ± 2.2	10.4 ± 2.5
Plasma folate (nmol/L)	11.3 ± 4.3	11.4 ± 3.4	12.0 ± 4.2
RBC folate (nmol/L)	400 ± 107	386 ± 85	422 ± 181
Vitamin B12 (pmol/L)	265 ± 117	290 ± 88	286 ± 110
Folate intake (µg/d) ³	290 ± 100	175 ± 99	277 ± 111
Energy intake (MJ/d)	8.9 ± 2.0	9.5 ± 2.5	9.7 ± 3.2
Protein intake (En%)	16.0 ± 3.6	14.7 ± 3.0	15.3 ± 3.2
Fat intake (En%)	33.2 ± 7.1	33.4 ± 8.6	34.0 ± 6.4
CHO intake (En%)	51.0 ± 6.6	51.2 ± 8.7	49.8 ± 7.4

BMI, body mass index OC, oral contraceptives RBC, red blood cell CHO, carbohydrate En%, percentage energy

Values are expressed as means ± SD

¹ Significant difference from 250 µg group (t-test) $P < 0.01$ ² Significant difference between three groups (ANOVA) $P < 0.05$ ³ 24-hour recalls were obtained once from each subject during the intervention period

Table 2.2 Plasma folate and red blood cell folate concentrations during and after four weeks of folic acid supplementation, and at 4 (week 8) and 8 weeks (week 12) after the end of the intervention period.¹

	500 µg/d group (n=45)	250 µg/d group (n=50)	placebo group (n=49)
Plasma folate (nmol/L)			
Week 0	11 ± 4.0	11 ± 3.4	12 ± 4.2
Week 1	21 ± 7.0	19 ± 6.2	16 ± 6.0
Week 2	21 ± 6.5	18 ± 5.4	14 ± 4.8
Week 4	27 ± 5.1	23 ± 5.3	17 ± 6.1
Week 4 - 0	16.1 (14.8, 17.4) ^{a,b}	11.3 (9.8, 12.8) ^a	5.2 (3.8, 6.6)
Week 8	22 ± 5.2	20 ± 4.7	16 ± 5.3
Week 12	21 ± 4.6	21 ± 5.1	18 ± 5.0
Week 12 - 0	9.9 (8.6, 11.2) ^a	9.1 (7.6, 10.5) ^c	6.4 (5.1, 7.7)
Red blood cell folate (nmol/L)			
Week 0	400 ± 107	386 ± 85	422 ± 181
Week 4	508 ± 176	432 ± 126	401 ± 182
Week 4 - 0	93 (65, 121) ^a	47 (11, 83) ^c	-17.3 (-37.5, 2.9)
Week 12	500 ± 152	433 ± 99	402 ± 152
Week 12 - 0	91 (65, 118) ^a	45 (17, 74) ^c	-14.0 (-35.3, 7.2)

¹ Absolute values are presented as means ± SD. Changes from week 0 to 4 and from week 0 to 12 are given as means (95% CI). Changes were normally distributed, absolute values not.

^a Significant difference with placebo group, $P < 0.001$.

^b Significant difference with 250 µg group, $P < 0.001$.

^c Significant difference with placebo group, $P < 0.01$.

Figure 2.1 Mean plasma total homocysteine (tHcy) levels and extent of deviation from baseline (percent change $\pm$ SEM) during 4 weeks of folic acid supplementation and 4 and 8 weeks after the end of the intervention period in 144 healthy, non-pregnant women (18-40 years)¹.

Footnote by Figure 2.1:

¹ Differences only tested at weeks 4 and 12. The extent of deviation from week 0 to 4 and from week 0 to 12 was significantly different between the three groups (ANOVA): $P < 0.001$.

* Significant difference between intervention group and placebo group ($P < 0.01$).

** Significant difference between intervention group and placebo group ($P < 0.001$).

† Significant difference between 500 µg/d group and 250 µg/d group ($P < 0.001$).

Figure 2.2 The mean change (lower 95% CI limit) in plasma total homocysteine (tHcy) concentrations ($\mu\text{mol/L}$) after four weeks of folic acid supplementation by quintiles of baseline tHcy concentrations for each intervention group in 144 healthy, non-pregnant women (18-40 yrs).

Footnote by Figure 2.2:

Mean ($\pm$ SD) baseline tHcy concentrations ($\mu\text{mol/L}$) per quintile:

Quintile 1: $7.5 \pm 0.7 \mu\text{mol/L}$

(placebo $n=8$, 250 $\mu\text{g/day}$ $n=12$, 500 $\mu\text{g/day}$ $n=7$)

Quintile 2: $8.9 \pm 0.3 \mu\text{mol/L}$

(placebo $n=8$, 250 $\mu\text{g/day}$ $n=14$, 500 $\mu\text{g/day}$ $n=8$)

Quintile 3: $9.9 \pm 0.4 \mu\text{mol/L}$

(placebo $n=15$, 250 $\mu\text{g/day}$ $n=6$, 500 $\mu\text{g/day}$ $n=8$)

Quintile 4: $11.5 \pm 0.5 \mu\text{mol/L}$

(placebo $n=10$, 250 $\mu\text{g/day}$ $n=11$, 500 $\mu\text{g/day}$ $n=9$)

Quintile 5: $14.3 \pm 1.9 \mu\text{mol/L}$

(placebo $n=8$, 250 $\mu\text{g/day}$ $n=5$, 500 $\mu\text{g/day}$ $n=13$)

Discussion

Intervention period

The present study clearly showed that four weeks of folic acid supplementation with a daily dose of 500 µg or 500 µg every second day rapidly and significantly increased plasma and red blood cell folate concentrations and significantly decreased plasma total homocysteine concentrations. Supplementation with 500 µg folic acid/d caused the most distinct decrease in plasma total homocysteine concentrations in the first two weeks of intervention (Figure 2.1). Assuming that plasma total homocysteine is a valid functional index of folate status (Stites *et al.*, 1997; Ubbink *et al.*, 1994), these results showed that the recommended period of folic acid use of four weeks before conception is sufficient to improve folate status. Neural-tube closure takes place during the third and fourth weeks of pregnancy. Our results suggest that even if women did not take folic acid before pregnancy, for example in the case of an unplanned pregnancy, it might still be worthwhile to start taking extra folic acid immediately after missing the first menstrual period because plasma total homocysteine and folate status improved after as little as one week of supplementation.

The extent of the decrease in plasma total homocysteine was influenced by the initial plasma total homocysteine concentrations of the study subjects (Figure 2.2). Subjects in the fifth quintile of initial plasma total homocysteine concentrations showed a much greater decrease in plasma total homocysteine concentration after intervention with folic acid than did subjects with low initial plasma total homocysteine concentrations. This could not have been due to regression towards the mean because the placebo group did not show this phenomenon. These findings agree with the results of Ward *et al.* (1997). This also explains the discrepancy with the results of Ubbink *et al.* who showed a 42% decrease in plasma total homocysteine concentrations after six weeks of supplementation with 650 µg folic acid/d (Ubbink *et al.*, 1994). Their study population was men with hyperhomocysteinemia (mean $\pm$ SD: 28.8 ± 14.5 µmol/L), whereas our subjects had normal plasma total homocysteine concentrations, with a mean baseline concentration of 10.5 µmol/L (Ubbink *et al.*, 1994).

Despite random allocation of the treatments, the mean baseline homocysteine concentration in the 250-µg group was significantly lower than in the 500-µg group. These differences may have occurred by chance. They cannot be explained by differences in folate and vitamin B12 concentrations because they were comparable in all three groups (Table 2.1).

During the four weeks of intervention, plasma total homocysteine concentrations gradually decreased in women in the 250-µg group (Figure 2.1). In contrast, during the first two weeks of intervention the decrease in the 500-µg group was more or less linear, whereas in the subsequent two weeks the decrease was more gradual. An explanation may be that the plasma total homocysteine

concentrations of many subjects in the 500- μ g group had already plateaued between the second and fourth week of supplementation, whereas four weeks of intervention with 250 μ g/d was not sufficient to have this effect. Several studies suggest that a high dose of folic acid will have no additional plasma total homocysteine-lowering effect after a plateau has been reached. However, the lowest effective folate intake and the optimal duration of supplementation are not clear. The results depicted in Figure 2.2 suggest that, at least over a four-weeks intervention period, 500 μ g/d has a more pronounced effect on plasma total homocysteine concentrations than does 250 μ g/d.

Washout period

Eight weeks after the end of the supplementation period, plasma total homocysteine concentrations had not returned to baseline. Although plasma folate concentrations in the 250- and 500- μ g groups decreased after supplementation had ceased, concentrations remained higher than those in the placebo group throughout the study period. This was not unexpected because the whole-body folate pool has a slow turnover of < 1%/d (Stites *et al.*, 1997). Therefore, plasma total homocysteine was expected to remain lower and blood folate concentrations were expected to remain higher than at the start of the study for more than three months.

Red blood cell folate concentrations did not change between the end of the intervention period and the end of the study (Table 2.2). This finding agrees with the suggestion that red blood cells incorporate folate at erythropoiesis (Shane, 1995, Zettner *et al.*, 1981) and retain it during their whole life-span (Shane, 1995).

Data suggest that lowering plasma total homocysteine may have important implications for the prevention of cardiovascular diseases in the general population. Boushey *et al.* (1995) estimated that an increment in plasma total homocysteine of 5 μ mol/L would result in an increase in the relative risk to 1.4. However, studies in which plasma total homocysteine-lowering therapy, for example, by folic acid supplementation, resulted in a reduction in the incidence of cardiovascular disease have not yet been reported.

Our results indicate that 4 weeks of folic acid supplementation with an average daily dose of 250 or 500 μ g decreases plasma total homocysteine by 11% and 22% (1.3 and 2.6 μ mol/L), respectively. In this study we supplied the 250- μ g group with 500 μ g every other day. We think that this might have underestimated the effect in the 250- μ g group, because Kelly *et al.* (1997) showed that intake of >266 μ g folic acid/d in addition to the normal diet leads to unmetabolised folic acid in the blood. This suggests that not all folic acid supplied is available for the remethylation of homocysteine to methionine. It is not clear whether this has negative effects or not, but possible negative effects of the use of supplemental folic acid should not be ignored (Campbell, 1996, Savage and Lindenbaum, 1995, Tamura, 1995). Therefore, the search for the lowest effective dose still remains important.

Although the question of whether a 250- μ g/d dose long-term is as effective as

a 500- μ g dose still needs to be answered, this study showed that even a dose as low as 250 μ g folic acid/d effectively lowered plasma total homocysteine concentrations and increases the folate status of young healthy women. The fact that such low doses are effective suggests that intervention with natural food folate may also be feasible. Thus, the bioavailability of natural folates in foods should receive close attention (Wei *et al.*, 1996). The results of this study indicate that food fortification with low doses of folic acid is a good option.

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3

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

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Abstract

An elevated plasma total homocysteine concentration is a risk factor for cardiovascular disease and for having offspring with a neural-tube defect. Homocysteine is a transmethylation product of methionine and can be remethylated to methionine. Folate is a methyl donor in this remethylation reaction. Folic acid supplementation can decrease plasma total homocysteine concentrations. However, the effect of folic acid supplementation on plasma methionine concentrations is still unclear. There is also concern that folic acid supplementation would negatively affect the vitamin B12 status.

We studied the effects of low-dose folic acid supplementation on plasma methionine and on plasma vitamin B12 concentrations. We also investigated whether baseline plasma methionine and plasma total homocysteine concentrations correlated with the baseline folate and vitamin B12 status.

The experiment had a parallel design. During four weeks, 144 young women received either 500 µg of folic acid each day, or 500 µg of folic acid and placebo tablets on alternate days, or a placebo tablet each day. Plasma methionine, plasma total homocysteine and plasma vitamin B12 concentrations were measured at the start and at the end of the intervention period. Folic acid supplementation had no effect on plasma methionine or plasma vitamin B12 concentrations although it significantly decreased plasma total homocysteine concentrations. Plasma methionine concentrations showed no correlation with either plasma total homocysteine concentrations (Spearman $r_s = -0.01$; $P = 0.89$), or any of the blood vitamin parameters at baseline. Baseline plasma total homocysteine concentrations showed a slight inverse correlation with baseline concentrations of plasma vitamin B12 ($r_s = -0.25$, $P < 0.001$), plasma folate ($r_s = -0.24$, $P < 0.01$) and red blood cell folate ($r_s = -0.19$, $P < 0.05$).

In conclusion, low-dose folic acid supplementation did not influence plasma methionine or plasma vitamin B12 concentrations. Furthermore, no correlation between plasma methionine concentrations and the blood folate and vitamin B12 status was shown.

Introduction

Homocysteine has received a lot of attention because hyperhomocysteinemia has been shown to be a risk factor for cardiovascular disease and for having offspring with a neural-tube defect (Boushey *et al.*, 1995; Mills *et al.*, 1995; Steegers Theunissen *et al.*, 1994). Homocysteine is the transmethylation product of the essential amino acid methionine. Methionine can be transmethylated via S-adenosylmethionine and S-adenosylhomocysteine to homocysteine, which in turn can be remethylated to methionine (Fig.1.3 Chapter 1). Thus, in theory an increase in plasma methionine is expected after a decrease in plasma homocysteine. N-5-methyltetrahydrofolate, the predominant form of the B-vitamin folic acid in the blood, serves as a methyl donor for this remethylation reaction and supplementation with folic acid decreases homocysteine concentrations (Brouwer *et al.*, 1999; Ward *et al.*, 1997). Vitamin B12 (methylcobalamin) is a co-factor in this remethylation reaction. Guttormsen *et al.* (1996b) showed that in folate or vitamin B12 deplete subjects supplementation with folic acid or vitamin B12 increased plasma methionine concentrations.

Already since 1947 there is concern that folic acid supplementation may have deleterious effects on cobalamin deficiency (Heinle and Welch, 1947). We therefore checked in this study whether low-dose folic acid supplementation influenced plasma vitamin B12 concentrations.

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

Subjects and Methods

The methods of this experiment have previously been described in more detail in Chapter 2 (Brouwer *et al.*, 1999). In short, these are as follows.

Subjects

We recruited healthy, non-pregnant women (18-40 years). Exclusion criteria were: smoking, gastrointestinal disorder, use of: vitamins, minerals, yeast or seaweed,

malaria prophylaxes and anti-convulsants. The final study group comprised 144 women.

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

Methods

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

Venous blood samples were collected after overnight fasting at the start (week 0) and at the end of the four weeks intervention period (week 4). We determined plasma methionine concentrations and plasma total homocysteine, plasma folate, red blood cell folate and plasma vitamin B12 concentrations in all samples.

Measurements

Samples for determinations in plasma were immediately placed on ice and centrifuged within 60 min at 3,000 x g for 10 min. Plasma was separated and stored at -35°C for folate and vitamin B12, at -80°C for plasma total homocysteine and at -20°C for methionine. For determination of folate concentrations in red blood cells, whole blood was diluted 5-fold with aqueous sodium ascorbate (10 g/L) and stored at -35°C. The haemolysates were further diluted with IMx Folate RBC Lysis Reagent (Abbott Laboratories, North Chicago, IL, USA). To be able to express the folate concentration in red blood cells, haematocrits were also measured.

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

Plasma total homocysteine was measured by high-performance liquid chromatography (HPLC) technique and fluorimetric detection (intra- and inter-assay coefficients of variation <8%) (Araki and Sako, 1987). Folate concentrations in plasma and red blood cells and vitamin B12 in plasma were determined with the Abbott IMx vitamin B12 and Folic Acid assays (Abbott Laboratories, North Chicago, IL, USA). The intra-assay CV of the folate assay was <6%, while the inter-assay CV was <10%. For vitamin B12, both the intra- and inter-assay CVs were lower than 5%.

Statistics

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

Results

Four weeks of low-dose folic acid supplementation had no effect on plasma methionine concentrations, whereas folate concentrations in plasma and red blood cell increased and plasma total homocysteine concentrations decreased (Table 3.1; Brouwer *et al.*, 1999). Intervention with folic acid had no effect on plasma vitamin B12 concentrations (Table 3.1).

The change (mean, 95% CI) in plasma methionine in the 500- μ g group corrected for the change in the placebo group was 0.37 (-1.6, 2.3) μ mol/L; the change in plasma methionine in the 250- μ g group corrected for the change in the placebo group was -1.2 (-3.2, 0.7) μ mol/L. Figure 3.1 shows the individual values of plasma methionine and plasma total homocysteine before and after intervention.

Table 3.1 Plasma folate, red blood cell folate, plasma vitamin B12, total plasma homocysteine and plasma methionine concentrations before and after four weeks of folic acid supplementation ¹

	500 µg/d group (n=45)	250 µg/d group (n=50)	placebo group (n=49)
Initial plasma folate (nmol/L)	11 ± 4 0	11 ± 3 4	12 ± 4 2
Final plasma folate (nmol/L)	27 ± 5 1	23 ± 5 3	17 ± 6 1
Initial red blood cell folate (nmol/L)	400 ± 107	386 ± 85	422 ± 181
Final red blood cell folate (nmol/L)	508 ± 176	432 ± 126	401 ± 182
Initial plasma vitamin B12 (nmol/L)	265 ± 117	290 ± 88	286 ± 110
Final plasma vitamin B12 (pmol/L)	265 ± 110	300 ± 98	287 ± 121
Initial total plasma homocysteine (µmol/L)	11 1 ± 2 6	9 7 ± 2 2	10 4 ± 2 5
Final total plasma homocysteine (nmol/L)	8 5 ± 1 7	8 5 ± 1 7	10 3 ± 2 5
Initial plasma methionine (µmol/L)	22 6 ± 4 8	23 4 ± 4 3	23 4 ± 4 1
Final plasma methionine (µmol/L)	23 7 ± 5 7	22 9 ± 4 3	24 1 ± 4 7

¹ Values are presented as means ± SD

Figure 3.1 Individual baseline concentrations versus concentrations after four weeks of low-dose folic acid supplementation in healthy young women (n=144).

Footnote by Figure 3.1:

Left panel: Plasma methionine concentrations. Right panel: Plasma total homocysteine concentrations. Values on the diagonal line had the same value before and after intervention.

Baseline plasma total homocysteine and methionine concentrations of the subjects were not correlated (Spearman $r_s = -0.01$; $P = 0.89$). Baseline plasma total homocysteine concentrations showed a slight, but significant correlation with baseline concentrations of plasma vitamin B12 ($r_s = -0.25$, $P = 0.003$), plasma folate ($r_s = -0.24$, $P = 0.004$) and red blood cell folate ($r_s = -0.19$, $P = 0.03$). However, none of these blood vitamin concentrations correlated with plasma methionine concentrations.

Users of oral contraceptives ($n = 97$) had significantly lower baseline plasma vitamin B12 concentrations (252 ± 84 pmol/L) than non-users ($n = 47$; 346 ± 118 pmol/L). However, no differences were observed for plasma total homocysteine and folate concentrations between users and non-users of oral contraceptives.

Discussion

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

In contrast to our study, Guttormsen *et al.* (1996b) showed significantly increased fasting plasma methionine concentrations after intervention with folic acid (5 mg per day for 10 days) and/or intramuscular injections with cobalamin (3-5 mg in 2-3 wk) (Guttormsen *et al.*, 1996b). However, they supplied much higher doses of folic acid and all subjects in that study had a deficient or marginal folate and vitamin B12 status and elevated plasma homocysteine concentrations (Guttormsen *et al.*, 1996b). Our results are in accordance with Jacob *et al.* (1995) who reported no effect of folate depletion and repletion on plasma methionine concentrations in healthy young men, although plasma total homocysteine concentrations increased after depletion and decreased after repletion (Jacob *et al.*, 1994). All our subjects had plasma methionine concentrations in the normal range (Armstrong and Stave, 1973; Scriver *et al.*, 1985), but they were not in optimal folate status because folic acid supplementation decreased their plasma total homocysteine concentrations (Brouwer *et al.*, 1999). Effects of folic acid supplementation might be different in subjects with either high or low plasma methionine and plasma total homocysteine

concentrations. Repetition of the analysis with exclusion of those subjects with plasma total homocysteine concentrations above 15 $\mu\text{mol/L}$ did not change the results of the current study.

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

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

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

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

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

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4

Homocysteine-lowering effect of 500 µg of folic acid every other day versus 250 µg per day

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Abstract

An elevated plasma total homocysteine concentration is considered a risk factor for neural-tube defects and for cardiovascular disease. Several studies showed that supplementation with low doses of folic acid decreases plasma total homocysteine concentrations. We investigated whether supplementation with 500 µg of folic acid every other day is as effective in lowering plasma total homocysteine concentrations as supplementation with 250 µg of folic acid each day.

In a four-week intervention study, 22 healthy young women (18-40 yrs) took either 500 µg of folic acid every other day (500-µg/2d group) or 250 µg of folic acid each day (250-µg/d group). Fasting blood was collected at the start and the end of the intervention period.

Plasma folate concentrations increased by 11.4 nmol/L (6.8 to 15.9) in the 250-µg/d group and by 9.1 nmol/L (95% CI: 1.9 to 16.3) in the 500-µg/2d group. These increases were not significantly different from each other. Plasma total homocysteine concentrations decreased by 1.52 µmol/L (95% CI: -2.09 to -0.95; $P < 0.001$) in the 250-µg/d group and by 0.88 µmol/L (-1.53 to -0.23; $P < 0.05$) in the 500-µg/2d group. The difference in decrease of 0.64 µmol/L between the 250-µg/d group and the 500-µg/2d group was not significant ($P = 0.11$). Although not conclusively, this study suggests that supplying subjects with folic acid each day decreases plasma total homocysteine more effectively than a double dose of folic acid every other day. The frequency of supplementation with folic acid might influence plasma total homocysteine concentrations.

Introduction

Several studies have shown that low-dose folic acid supplementation decreases plasma total homocysteine concentrations (Ward *et al.*, 1997; Homocysteine Lowering Trialists' Collaboration, 1998; Brouwer *et al.*, 1999). This is of importance because elevated homocysteine concentrations are considered a risk factor for cardiovascular disease and neural-tube defects (Steegers Theunissen *et al.*, 1994; Mills *et al.*, 1995; Boushey *et al.*, 1995; Graham *et al.*, 1997). Moreover, plasma total homocysteine is regarded as a functional marker of the folate status (Jacob *et al.*, 1994).

In the study of Brouwer *et al.* (1999) we supplemented healthy young women for a period of four weeks with tablets containing either 500 µg of folic acid each day, or every other day with placebo tablets on alternate days. Although not to the same extent, both intervention strategies decreased plasma total homocysteine concentrations. The intervention with 500 µg each day decreased plasma homocysteine concentrations with on average 22%, while 500 µg of folic acid every other day decreased the concentrations by 11%. Kelly *et al.* (1997) showed that unaltered folic acid appears in the serum of subjects receiving more than 266 µg of folic acid in addition to the usual diet. This unaltered folic acid is fully oxidised. Folate in the reduced tetrahydrofolate form can bind to one-carbon units and serve as a methyl group donor in the reaction of homocysteine to methionine (Wagner, 1995). Therefore, folic acid needs to be reduced before it can lower homocysteine. Besides, storage of folic acid is likely to be less effective than of other folate forms (Shane, 1995; Wagner, 1995). Thus, a dose of folic acid of 500 µg per 2 days might be less effective than 250 µg each day.

In the current study we investigated whether supplementation of women with 500 µg of folic acid every other day is as effective in lowering plasma total homocysteine concentrations as supplementation with 250 µg of folic acid each day. We hypothesised that the two intervention strategies would result in a similar increase in plasma folate concentrations. However, 250 µg of folic acid each day was expected to decrease plasma total homocysteine concentrations more effectively than 500 µg of folic acid every other day.

Subjects and Methods

Subjects

Healthy, non-pregnant women (18-40 years) were recruited within the University Hospital St Radboud in Nijmegen, the Netherlands. Women were excluded if they were smokers, had a gastrointestinal disorder, or had used any of the following preparations during the three months preceding the trial: vitamins, minerals, yeast or seaweed, malaria prophylaxis and anti-convulsants. Based on these criteria we included 22 women in the study.

The Medical Ethical Committee of the University Hospital Nijmegen St Radboud approved the study design. All women gave written informed consent.

Methods

Subjects were divided into two intervention groups. During four weeks, they either received one tablet of 500 µg of folic acid every other day and placebo tablets on the alternate days (500 µg/2d group), or 250 µg of folic acid each day (250 µg/d group) (Pharmachemie B.V., Haarlem, the Netherlands). Subjects in the group receiving 500 µg of folic acid and placebo tablet on alternate days received two containers of tablets, one marked with a red and one with a yellow sticker. These subjects received a calendar in which the days were alternately marked red and yellow. Subjects in the group receiving 250 µg of folic acid each day also received a tablet calendar. Subjects were asked to follow their regular diet, except for refraining from the consumption of liver and marmite, a yeast extract. All subjects reported intake of tablets on their tablet calendar. To study compliance, we counted any remaining tablets and checked the reported intake of tablets on the calendar. The subjects always took their tablets before breakfast and on blood collection days after fasting blood sampling.

Venous blood samples were collected after overnight fasting at the start (week 0) and the end (week 4) of the four-week intervention period. Plasma total homocysteine and plasma folate concentrations were determined in all samples.

Blood samples were drawn in EDTA vacutainer tubes (Venoject II, Terumo, Madrid, Spain). For the determination of plasma total homocysteine and plasma folate samples were immediately placed on ice and centrifuged within 60 minutes at 3,000 x g for 10 minutes. Plasma was separated and stored at -35°C for folate and at -80°C for plasma total homocysteine determination until required for analysis. Folate concentrations in plasma were determined with the Abbott IMx Folic Acid assay, which is based on ion-capture technology for the IMx automated immunoassay

system (Abbott Laboratories, North Chicago, IL, USA). The intra-assay coefficients of variance (CV) of the folate assay varied between 3% and 6%, while the inter-assay CV varied between 6% and 10%, depending on the folate concentration. Homocysteine was measured by high performance liquid chromatography (HPLC) essentially according to the method of Fiskerstrand *et al.* (Fiskerstrand *et al.*, 1993) with some modifications (te Poele-Pothoff *et al.*, 1995). The intra- and inter-assay CV were <6.5%. All samples from each subject were analysed in the same run.

Statistical analyses

The responses to the various treatments were calculated for each subject as the change in plasma total homocysteine and plasma folate, between the start (week 0) and the end (week 4) of the intervention period. The changes in folate and plasma total homocysteine concentrations were normally distributed. Student t-tests were used to analyse differences in response of plasma total homocysteine and plasma folate between the two groups ($P < 0.05$). Paired t-tests were used for comparisons within the groups ($P < 0.05$).

Results

Four weeks of supplementation with 250 µg of folic acid each day or 500 µg of folic acid and placebo treatment on alternate days increased plasma folate concentrations in both groups (Figure 4.1). The mean increase of 11.4 nmol/L (6.8 to 15.9) in the 250 µg/d group was not significantly different from the 9.1 nmol/L (95% CI: 1.9 to 16.3) increase in the 500 µg/2 d group.

Plasma total homocysteine concentrations significantly decreased in both intervention groups (Figure 4.2). Plasma total homocysteine concentrations decreased in the 250 µg/d group by 1.52 µmol/L (95% CI: -2.09 to -0.95; $P < 0.001$) and in the 500 µg/2d group by 0.88 µmol/L (-1.53 to -0.23; $P < 0.05$). Although plasma total homocysteine concentrations in the 250 µg/d group decreased 0.64 µmol/L more than in the 500 µg/2d group, this difference was not significant ($P = 0.11$).

Although not significantly different, the mean baseline concentration of plasma total homocysteine was slightly higher in the 250 µg/d group (mean 11.3 µmol/L; 95% CI: 9.9 to 12.7) than in the 500 µg/2 d group (10.3 µmol/L; 9.2 to 11.4). The decrease in plasma total homocysteine by intervention with folic acid is known to be dependent on the initial plasma total homocysteine concentration. Therefore, we

corrected for this by repeating the analysis with exclusion of the subjects with the two highest and the two lowest baseline concentrations of plasma total homocysteine. The mean baseline concentration of plasma total homocysteine of the 18 remaining subjects ($n=9$ per group) was $10.5 \mu\text{mol/L} \pm 1.4$ (SD) in the $250 \mu\text{g/d}$ group and $10.8 \mu\text{mol/L} \pm 1.7$ in the $500 \mu\text{g/d}$ group. The mean change over the intervention period was now -1.48 ($-2.19, -0.76$) $\mu\text{mol/L}$ in the $250 \mu\text{g/d}$ group and -0.99 ($-1.79, -0.19$) $\mu\text{mol/L}$ in the $500 \mu\text{g/d}$ group. The difference between the two intervention groups was not significantly different, but the decrease in the $250 \mu\text{g/d}$ group was still 150% of the decrease in the $500 \mu\text{g/d}$ group.

Figure 4.1 Individual baseline plasma folate concentrations versus concentrations after four weeks of low-dose folic acid supplementation in healthy young women ($n=22$). Values on the diagonal line had the same value before and after intervention.

Figure 4.2 Individual baseline plasma total homocysteine concentrations versus concentrations after four weeks of low-dose folic acid supplementation in healthy young women (n=22). Values on the diagonal line had the same value before and after intervention.

Discussion

Although this study cannot give conclusive evidence, it suggests that supplying subjects with folic acid each day decreases plasma total homocysteine more effectively than a double dose of folic acid every other day. Thus, the frequency of supplementation with folic acid might influence plasma total homocysteine concentrations.

Folic acid supplementation in doses as low as 200 to 500 μg daily has been shown to be effective in lowering plasma total homocysteine concentrations (Ward *et*

al , 1997, Homocysteine Lowering Trialists' Collaboration, 1998, Brouwer *et al* , 1999) Kelly *et al* (1997) showed that unaltered folic acid appears in the serum two hours after intake of a single dose of folic acid above 266 µg. This unaltered folic acid has no homocysteine lowering potential and needs to be reduced before it becomes metabolically active.

We compared the strategy of supplying subjects with 500 µg of folic acid every other day with supplying subjects with 250 µg each day. The reduction in plasma total homocysteine tended to be more pronounced in the 250 µg/d group than in the 500 µg/2d group. However, we could not show any significant differences in responses between the two intervention strategies. Eleven subjects per group participated in this study. With this sample size we could have shown a significant difference of 1.1 µmol/L ($\alpha=0.05$, $\beta=0.20$). Therefore, the lack of significance is likely to be caused by the small sample size.

We hypothesised that 250 µg of folic acid daily would increase plasma folate to the same extent as 500 µg every other day. The folate increasing effect was comparable for the 250 µg/d group (mean 11.4, 95% CI 6.8 to 15.9 µmol/L) and the 500 µg/2d group (9.1, 1.9 to 16.3 µmol/L). Based on the study of Kelly *et al* (1997) it is likely that a dose of folic acid of 500 µg results in the appearance of unaltered folic acid in the blood. However, the assay we used to determine plasma folate measures unaltered folic acid as well as 5-methyl-tetrahydrofolate. Therefore, we expected to find no difference in plasma folate response. On the other hand, it is known that only reduced folate can bind one-carbon units (Wagner, 1995). Moreover, monoglutamates can be elongated to polyglutamates by adding glutamate units with the enzyme folylpoly- γ -glutamate synthetase. This is of importance because tissues can only retain folylpolyglutamates and not monoglutamates. In contrast to dihydrofolate and tetrahydrofolate is folic acid a very poor substrate for folylpoly- γ -glutamate synthetase (Shane, 1995). Therefore, less folic acid will be converted to pteroylpolyglutamates compared to the reduced forms of folate and more unaltered folic acid than other folate forms may be excreted. Furthermore, polyglutamates of tetrahydrofolate forms are better substrates for enzymes of folate metabolism than monoglutamates (Shane, 1995).

Previous studies investigating the effect of folic acid on plasma total homocysteine have mainly used either one tablet daily, or a single dose of fortified food (Ward *et al* , 1997, Homocysteine Lowering Trialists' Collaboration, 1998, Brouwer *et al* , 1999, Malinow *et al* , 1998, Schorah *et al* , 1998). With doses above 266 µg per day it might be expected that several small doses spread out over the day have more homocysteine-lowering effect than one single high dose. Food

fortification programs as implemented in the US may therefore be more effective than the use of folic acid tablets. Our study lasted only four weeks, therefore it is possible that over a longer period a high dose supplied every other day has a similar homocysteine lowering potential as half the dose supplied each day. However, supplementation with a dose above 266 µg implies that unaltered folic acid is present in the blood. Whether this has negative consequences or not is unclear (Kelly *et al.*, 1997).

In conclusion, our study suggests that the frequency of supplementation might influence plasma total homocysteine concentrations. Future studies could investigate the effect of several doses spread out over the day versus a single dose with the same total folic acid content.

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Homocysteine metabolism and effects of folic acid supplementation in patients affected with spina bifida

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Abstract

Folic acid supplementation around conception decreases the risk of having offspring with a neural-tube defect. In most cases however the aetiology behind it is still unknown. This study investigated whether patients with spina bifida have lower concentrations of blood folate and higher concentrations of fasting and post-methionine-load plasma total homocysteine than control patients. Moreover, effects of supplementation with 500 µg folic acid/d on the folate status and plasma total homocysteine concentrations in spina bifida and control patients were determined.

The study population consisted of two groups: spina bifida patients (n=12) and control patients (n=15) living in the same institute. Subjects in both groups received four weeks of placebo treatment followed by four weeks of intervention with 500 µg folic acid/d. Blood was collected at day 0, after 4 and after 8 weeks. A methionine-loading test was performed at the start and the end of the study.

At baseline, blood folate and plasma total homocysteine concentrations were not significantly different between the spina bifida and the control group. Intervention with folic acid significantly increased plasma and red blood cell folate concentrations in both groups. Folic acid decreased fasting plasma total homocysteine concentrations in the control group by 1.6 ± 0.5 µmol/L ($P < 0.01$) and in the spina bifida patients by 2.2 ± 1.3 µmol/L ($P = 0.10$).

This study does not show a derangement in homocysteine metabolism in spina bifida compared to control patients. Moreover, folic acid supplementation seems at least as effective in spina bifida patients as in controls.

Introduction

Folic acid supplementation around conception decreases the risk of having offspring with a neural-tube defect by more than 50% (Czeizel and Dudás, 1992; MRC Vitamin Study Research Group, 1991). Women with no history of a neural-tube defect pregnancy are advised to take 400-500 µg extra folic acid from at least four weeks before conception up until the eighth week of pregnancy (Centers for Disease Control, 1992; Expert Advisory Group, 1992). The US government has mandated food producers to fortify flour with 140 µg of folic acid per 100 g to increase the intake of folic acid in women of reproductive age (US Department of Health and Human Services. Food and Drug Administration, 1996).

Although folic acid supplementation clearly decreases the risk of having a child with a neural-tube defect, in most cases the aetiology behind it is still unknown. Steegers Theunissen *et al.* (1994) showed that a sub-group of women who previously had a pregnancy affected by a neural-tube defect had elevated concentrations of total homocysteine in plasma. Homocysteine is a demethylation product of the essential amino acid methionine. Folate donates a methyl group to homocysteine to form methionine in this remethylation reaction. Intake of low doses of folic acid (200-500 µg/d) decreases plasma total homocysteine concentrations in healthy volunteers (Brouwer *et al.*, 1999; den Heijer *et al.*, 1998; Dierkes *et al.*, 1998; Ward *et al.*, 1997).

The C677T mutation in the methylenetetrahydrofolate reductase gene is one of the causes of an elevated plasma total homocysteine concentration. This polymorphism has been shown to be more frequent in patients with a neural-tube defect than in controls (van der Put *et al.*, 1995; Whitehead *et al.*, 1995). However, it explains only approximately 8% of all cases (van der Put *et al.*, 1997a).

Van der Put *et al.* (1997b) studied the folate and plasma total homocysteine status of patients with spina bifida, the most common form of a neural-tube defect. They showed that plasma folate concentrations were lower and plasma total homocysteine and red blood cell folate concentrations were elevated in spina bifida patients compared to control subjects. However, studies investigating folate concentrations in serum and red blood cells of fetuses affected with a neural-tube defect found no differences with control fetuses (Economides *et al.*, 1992; Thorand *et al.*, 1996).

This study investigated whether patients with a spina bifida have lower baseline concentrations of blood folate and higher baseline concentrations of plasma

total homocysteine than control patients. We also studied the effects of methionine-loading on plasma total homocysteine concentrations in both groups. Moreover, this is the first study investigating the effects of folic acid supplementation on the folate status and plasma total homocysteine concentrations of spina bifida and control patients.

Subjects and Methods

Subjects

Subjects, aged 14 to 55 years (13 men and 15 women) were recruited from two institutes for disabled people in the Netherlands (Werkenrode, Groesbeek and Het Dorp, Arnhem). Both spina bifida patients and control subjects were living in similar circumstances in the same institutes. Exclusion criteria were metabolic diseases, epilepsy, use of drugs interfering with folate metabolism, and use of vitamin or mineral supplements in the three months preceding the experiment. None of the women were pregnant or planning to become pregnant during the study. Only patients with spina bifida aperta (n=12) were included in the spina bifida group. Subjects in the control group (n=15) were suffering from transverse lesion (n=4), history of polio myelitis (n=2), contusio cerebri (n=2), femur fibula ulna (FFU) syndrome (n=1), spasms (n=1), hernia diafragmatica (n=1), attention deficit hyperkinetic disorder (ADHD) (n=1), achondroplasia (n=1), or status after removal of a tumor of the cerebellum (n=1), status after several medical problems in the past (n=1).

The study was approved by the Medical Ethical Committee of the Catholic University Nijmegen, and all participants gave their written informed consent. Parents or caretakers of all subjects under the age of 18 gave written permission for the adolescents to participate.

Design

The trial had a parallel design with a run-in period. All subjects received a placebo tablet daily for the first four weeks and a 500 µg folic acid tablet for the following four weeks. Subjects kept their regular diet during the intervention period.

Tablets

Placebo tablets and folic acid tablets were indistinguishable from each other. The subjects were blind to whether they received placebo or folic acid tablets.

Methionine-loading test

An oral methionine-loading test was performed at the beginning of the experiment (i.e., at the start of the run-in period) and eight weeks later, at the end of the experiment (i.e., at the end of the folic acid intervention period). After an overnight fast, venous blood was collected for measurement of total homocysteine and vitamin concentrations. Immediately after the fasting blood collection, the subjects took 0.1 g L-methionine/kg body weight mixed in 200 mL of orange juice. All participants consumed a standardised breakfast and luncheon. For practical reasons, a second blood sample for measuring the (post-load) total homocysteine concentration was drawn from each subject five hours after the fasting sample. Both blood samples were taken at the same time for each subject for the first and the second methionine-loading test.

Blood sampling and analysis

Venous blood samples were collected after overnight fasting at the start of the placebo period, at the end of the placebo period (start of the folic acid intervention period), and at the end of the folic acid intervention period. Plasma total homocysteine, plasma folate, red blood cell folate and whole blood pyridoxal-5'-phosphate (PLP) concentrations were determined in all samples. Plasma vitamin B12 concentrations were assessed at the start of the experiment.

Blood samples for plasma total homocysteine, folate and vitamin B12 were drawn into EDTA vacutainer tubes. Blood was collected into heparinised vacutainer tubes for determination of PLP in whole blood. For the determination of plasma total homocysteine and plasma folate concentrations, samples were immediately placed on ice and centrifuged within 2 hours at 3,000 x g for 10 min. Plasma was separated and stored at -35°C for folate determination and at -80°C for total homocysteine determination. For determination of folate concentrations in red blood cells, whole blood was diluted 5-fold with aqueous sodium ascorbate (1g/100 mL) and stored at -35°C. Before measurement, the haemolysates were further diluted with IMx Folate RBC Lysis Reagent (Abbott Laboratories, North Chicago, IL, USA). To be able to express the folate concentration in red blood cells, haematocrits were also measured. Correction was made for the concentration of folate in plasma. Homocysteine was measured by high performance liquid chromatography (HPLC) essentially according to the method of Fiskerstrand *et al.* (Fiskerstrand *et al.*, 1993) with some modifications (te Poele-Pothoff *et al.*, 1995). The intra- and interassay CVs were <6.5%. Folate concentrations in the haemolysates were determined with the Abbott IMx Folic acid assay, which is based on ion-capture technology for the IMx automated immunoassay system (Abbott Laboratories, North Chicago, IL, USA).

The intra-assay CV was <6%, while the interassay CV was <10%. All samples from each subject were analysed in the same run. Determination of vitamin B6 as PLP in whole blood was performed by HPLC technique (Schrijver *et al.*, 1981). The intra- and interassay CV for the method was 1.6% and 5.5% respectively.

The C677T mutation was determined by polymerase chain reaction of a genomic DNA fragment followed by restriction enzyme analysis with *HinfI* (Frosst *et al.*, 1995).

Statistical analyses

The frequency distribution curves of the absolute values were skewed. Therefore, these values are reported as medians with 25 and 75 percentile values. For the statistical analysis of the absolute values the data were log-transformed to obtain normality. In cases where normality could not be obtained by log-transformation (age) a Wilcoxon non-parametric test was used.

The response to treatment was calculated for each subject as the change in fasting plasma total homocysteine, plasma or red blood cell folate, or whole blood vitamin B6 during the intervention period minus the change during the placebo period. Post-load homocysteine was only measured at the start and the end of the study. Therefore, change in post-load homocysteine was calculated as concentration after the intervention period minus concentration at the start of the study. Changes in folate and homocysteine concentrations were normally distributed as checked by visual inspection of the normal probability plots (univariate procedure; SAS Institute Inc, Cary, NC, USA). To analyse differences between the groups in response to treatment Student t-tests were used and considered significant at $P < 0.05$. Paired t-tests were used to analyse changes within groups.

Results

Start of the study

At the start of the study, no significant differences were apparent between the spina bifida and the control group for any of the measured variables (Table 5.1). The mean age of the spina bifida patients was 25.3 ± 12.6 years and the mean age of the control subjects was 29.3 ± 16.2 years. This difference was also not significant.

Two subjects from the control group withdrew from the study after the first blood collection for personal reasons.

Effect of intervention with folic acid

We excluded one subject in the spina bifida group because of non-compliance to the folic acid therapy.

Table 5.1 Baseline biochemical indices in blood of spina bifida patients and disabled control subjects.

Variable	Spina Bifida		Controls	
	Median (25-75%)	n	Median (25-75%)	n
Hemoglobin (mmol/L)	8.3 (7.8 to 8.5)	12	8.8 (8.3 to 9.5)	15
Hematocrit (L/L)	0.40 (0.39 to 0.42)	12	0.42 (0.40 to 0.45)	15
ASAT (IU/l)	13 (10 to 19)	12	15 (13 to 21)	15
ALAT (IU/L)	12 (10 to 20)	12	14 (10 to 32)	15
Alkaline Phosphatase (IU/L)	67 (52 to 72)	12	71 (61 to 116)	15
Creatinine (μmol/L)	62 (57 to 76)	12	75 (58 to 85)	15
Plasma folate (nmol/L)	11 (10 to 13)	12	14 (11 to 16)	15
Red blood cell folate (nmol/L)	370 (340 to 400)	12	330 (290 to 430)	15
Plasma vitamin B12 (pmol/L)	250 (165 to 370)	12	290 (200 to 360)	15
Whole blood vitamin B6 (nmol/L)	38 (33 to 45)	12	36 (26 to 47)	15
Fasting total plasma homocysteine (μmol/L)	11.1 (10.1 to 13.9)	12	10.2 (8.8 to 12.4)	15
Post-load total plasma homocysteine (μmol/L)	30.1 (28.7 to 43.4)	11	28.7 (26.7 to 37.3)	14

Table 5.2 Change in plasma folate, red blood cell folate, whole blood vitamin B6, and tHcy concentrations (pre- and postload) during four weeks of intervention with 500 µg of folic acid corrected for the change during four weeks of placebo treatment in patients with a spina bifida and controls.

	Spina bifida mean ± SE	Controls mean ± SE
Plasma folate (nmol/L)	15.4 ± 1.4*** (n=11)	14.8 ± 3.1*** (n=12)
Red blood cell folate (nmol/L)	151 ± 15*** (n=11)	102 ± 33* (n=12)
Whole blood vitamin B6 (nmol/L)	2.6 ± 1.9 (n=11)	2.0 ± 2.7 (n=12)
Fasting total plasma homocysteine (µmol/L)	-2.2 ± 1.3 (n=11)	-1.6 ± 0.5** (n=11)
Total plasma homocysteine after methionine-loading test (µmol/L)	-4.3 ± 1.1** (n=11)	-1.8 ± 1.7 (n=12)

Significant effect of intervention with 500 µg folic acid: *** $P < 0.001$,

** $P < 0.01$, * $P < 0.05$.

Figure 5.1 Plasma total homocysteine concentrations before (start) and after intervention with 500 µg of folic acid every day for four weeks in spina bifida and control patients.

Footnote: Upper panel: Fasting plasma total homocysteine concentrations. Lower panel: Plasma total homocysteine concentrations after methionine-loading test.

*patients homozygous for the C677T mutation in the gene encoding for methylenetetrahydrofolate reductase.

Plasma and red blood cell folate concentrations increased significantly during four weeks of intervention with 500 µg folic acid per day compared to the placebo period in both the spina bifida and control group (Table 5.2). There were no significant differences in any of the blood parameters between the spina bifida and the control patients. Intervention with folic acid decreased fasting plasma total homocysteine concentrations in the control group ($P < 0.01$) and in the spina bifida patients ($P = 0.10$) (Table 5.2; Figure 5.1). The same intervention with folic acid significantly decreased post-load plasma total homocysteine concentrations in the spina bifida patients ($P < 0.01$), but not in the control group ($P = 0.32$) (Table 5.2). In those subjects in whom folic acid supplementation did not decrease the high post-load plasma total homocysteine concentrations, it did increase plasma and red blood cell folate concentrations and decreased fasting concentrations of plasma total homocysteine.

One subject in the spina bifida group and one subject in the control group were homozygous for the C677T mutation, which is known to decrease enzyme activity of methylenetetrahydrofolate reductase.

Discussion

This study aimed at investigating whether spina bifida patients have a derangement in their folate dependent homocysteine metabolism. We could not show any significant difference in the folate status or in plasma total homocysteine concentrations between spina bifida and control patients at baseline. Furthermore, the effects of treatment with 500 µg folic acid/d for four weeks on folate status and total homocysteine concentrations were not significantly different between the groups. Four weeks of folic acid supplementation significantly increased plasma folate and red blood cell folate concentrations in spina bifida patients as well as in control subjects. It also decreased plasma total homocysteine concentrations in spina bifida patients and controls. Although not significantly different, the effects of folic acid treatment on both fasting and post-load plasma total homocysteine concentrations were slightly stronger in the spina bifida than the control group (Table 5.2; Figure 5.1). In the presence of a disorder in the homocysteine metabolism, it could be expected that more folic acid would be needed to establish a similar decrease in plasma total homocysteine concentration in spina bifida patients compared to control subjects. Thus, our study gives no indication for such a disorder in spina bifida patients.

In contrast to our baseline results, van der Put *et al.* (1997b) and Bjørke-Monsen *et al.* (1997) showed higher fasting plasma total homocysteine concentrations in spina bifida patients than in control subjects. Several investigators studied the folate and vitamin B12 status of fetuses affected with a neural-tube defect. Concordant with our study, none of these studies found significant differences in the folate status between fetuses affected with a neural-tube defect and controls (Bunduki *et al.*, 1995; Economides *et al.*, 1992; Thorand *et al.*, 1996).

Plasma total homocysteine concentrations in the amniotic fluid were shown to be higher in mothers pregnant with a fetus with a neural-tube defect than in control mothers pregnant with a healthy child (Steegers Theunissen *et al.*, 1995). Moreover, vitamin B12 levels in amniotic fluid of pregnancies affected by a neural-tube defect were lower than in healthy pregnancies (Gardiki-Kouidou and Seller, 1997; Weekes *et al.*, 1992). Both the elevated plasma total homocysteine concentrations and the lower vitamin B12 concentrations suggest either a defect in the homocysteine metabolism of mother or child, a transport problem, or leakage of homocysteine from the neural-tube defect into the amniotic fluid. Our study suggests that either a metabolic derangement in the mother, or an abnormality in the transport from mother to child are more important causes for spina bifida than an abnormality in the child itself. Leakage of homocysteine from the neural-tube defect cannot be excluded, but does not explain the more than 50% of neural-tube defects that can be prevented by intake of folic acid by the mother (Czeizel and Dudás, 1992; MRC Vitamin Study Research Group, 1991).

Van der Put *et al.* (1995) and Whitehead *et al.* (1995) showed that the C677T polymorphism in the methylenetetrahydrofolate reductase gene could explain part of the more than 50% of neural-tube defect cases that can be prevented by the intake of extra folic acid around conception (Czeizel and Dudás, 1992; MRC Vitamin Study Research Group, 1991). This polymorphism was more frequent in patients with a neural-tube defect than in the control population (van der Put *et al.*, 1995; Whitehead *et al.*, 1995). In contrast, in 137 spina bifida patients and 153 control subjects there was no evidence for an association between the frequency of the C677T mutation and the occurrence of spina bifida (Koch *et al.*, 1998). The C677T mutation increases plasma total homocysteine concentrations in patients and control subjects (Bjørke-Monsen *et al.*, 1997; van der Put *et al.*, 1995). Bjørke-Monsen *et al.* (1997) showed that the difference in elevated fasting plasma total homocysteine was confined to those subjects with one or two mutated C→T alleles. Only two subjects in our study, one spina bifida patient and one control subject were homozygous for the C677T mutation. We repeated the statistical analyses with exclusion of these

subjects. This did not change the results of our study. Our study gives no indications for other differences in the homocysteine metabolism between spina bifida patients and control subjects. However, our small sample size encounters us from drawing firm conclusions.

In conclusion, this study does not show that a derangement in homocysteine metabolism is more frequent in spina bifida patients than in control patients. Moreover, folic acid supplementation seems at least as effective in spina bifida patients as in controls. Therefore, a metabolic derangement in the mother or a defect in the transport of folate from mother to child seem more obvious causes for spina bifida than an abnormality in the homocysteine metabolism of the child itself.

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6

Dietary folate from vegetables and citrus fruit decreases plasma homocysteine concentrations: a dietary controlled trial

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Abstract

Elevated plasma total homocysteine concentrations are considered a risk factor for neural-tube defects and cardiovascular disease. Supplementation with folic acid decreases the risk of women having children with neural-tube defects. In both sexes it decreases plasma total homocysteine levels. The present study investigated the efficacy of natural dietary folate in improving folate and homocysteine status. We performed a four-week dietary controlled, parallel design intervention trial with sixty-six healthy subjects (18-45 years) divided into three treatment groups. We supplied daily to each of the groups: the dietary folate group, a diet high in vegetables and citrus fruit (total folate content circa 560 μg) plus a placebo tablet; the folic acid group, a diet naturally low in folate (circa 210 μg) plus folic acid (500 μg folic acid and placebo tablet on alternate days, i.e., 250 μg folic acid/d); the placebo group, the same low folate diet plus a placebo tablet. After four weeks of intervention, folate status improved and plasma total homocysteine concentrations decreased in both the dietary folate and the folic acid group. From the amount of additional folate (350 $\mu\text{g}/\text{d}$) and folic acid (250 $\mu\text{g}/\text{d}$) consumed, the relative bioavailability of dietary folate compared to folic acid was, for this experiment, calculated to be 60 to 98%, depending on the endpoint used. In conclusion, increasing the consumption of vegetables and citrus fruit, both good sources of folate, will improve the folate status and decrease plasma total homocysteine concentrations. This may contribute to the prevention of cardiovascular disease and neural-tube defects in the general population.

Introduction

Increased intake of folate can have a high impact on public health. Folic acid supplementation around conception decreases the risk of women having offspring with neural-tube defects (Czeizel and Dudás, 1992; MRC Vitamin Study Research Group, 1991). In both men and women folic acid supplementation improves the folate status and decreases elevated plasma total homocysteine concentrations, which are considered to be a risk factor for cardiovascular disease (Boushey *et al.*, 1995) and neural-tube defects (Mills *et al.*, 1995; Steegers Theunissen *et al.*, 1994).

In several countries, women planning to become pregnant are advised to take 400-500 µg of folate per day (Expert Advisory Group, 1992; US Public Health Service, 1992). It is generally thought that this intake can only be reached by using folic acid, the synthetic form of folate. Daily doses of 200-300 µg of synthetic folic acid improve not only the folate status, but also decrease plasma total homocysteine concentrations (Brouwer *et al.*, 1999; Jacob *et al.*, 1994; O'Keefe *et al.*, 1995; Ward *et al.*, 1997). The effectiveness of these low doses of folic acid suggests that a nutritional intervention with foods rich in folate could also be feasible and successful. We therefore tested the hypothesis that increased intake of dietary folate from vegetables and citrus fruit improves the folate status and decreases plasma total homocysteine concentrations.

Subjects and Methods

Subjects

Healthy men and women aged 18-45 years were recruited. A total number of 66 women and 28 men applied for enrolment in the study. Exclusion criteria were: smoking; known gastrointestinal disorders; screening plasma total homocysteine concentrations of > 20 µmol/L; or use of vitamins, minerals, yeast or seaweed, malaria prophylactics or anti-convulsants in the four months prior to the experiment. Food products fortified with folic acid are not available in the Netherlands. Therefore, the habitual diet of the subjects contained no folic acid. None of the women were pregnant or planning to become pregnant within the first six months after the study. Based on the selection criteria, 62 women and 25 men were eligible for participation. We included 52 of the women and 25 men into the trial. Ten of these subjects received treatment as part of a study on carotene bioavailability that will be published separately. Because only 77 subjects were required, all 25 men and 52 of the women

entered the trial: two women decided not to participate for personal reasons and eight were chosen at random for exclusion. Thus, the present study included 67 subjects. The study was approved by the Medical Ethical Committee of the Division of Human Nutrition and Epidemiology of Wageningen Agricultural University, and all participants gave their written informed consent.

Design

The trial had a parallel design with three treatments. Group sizes were aimed at showing an 11% decrease in plasma total homocysteine concentrations after four weeks of intervention with 500 µg of folic acid every other day with a power of 80% (Brouwer *et al.* 1999). The treatments comprised: firstly, a diet high in natural folate plus a placebo tablet (dietary folate group; n=23); secondly, a diet low in folate plus supplemental folic acid (folic acid group; n=22); and thirdly, the same low folate diet plus a placebo tablet (placebo group; n=22).

All subjects were stratified over the groups for screening concentration of plasma total homocysteine, energy intake, vegetarianism and gender.

Diets

Before the trial started, trained dieticians estimated the habitual energy intake of the subjects using a validated food frequency questionnaire (Feunekes *et al.*, 1993). This method has been validated for estimating fat and energy intake, however it is not suitable for estimating folate intake. Therefore, we did not estimate habitual folate intake. The energy content of the diets was calculated using the Dutch Nutrient Data Base (Brants and Hulshof, 1995; Stichting NEVO, 1993).

Each day, all subjects received a basal diet with a similar nutrient composition for all groups. Table 6.1 describes the diets, Table 6.2 the nutrient composition. The quantity of the basal diet was based on the habitual energy intake for each subject. Body weights without shoes, jackets, and heavy clothing were recorded twice weekly. The level of energy intake was adjusted when necessary to compensate for any weight change. If subjects complained about having either too much or not enough food we weighed them more often. In exceptional cases, for example when the subjects were hungry or underwent more than usual physical activity, we provided 'energy buns' which had a similar macronutrient composition as the rest of the diet and a known folate content. The meals contained conventional foods and drinks. Six different menus were provided over the four-week intervention period.

Table 6.1 Six-day cycle menus

	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6
Basal diet	whole wheat bread margarine low-fat margarine (35%) cheese cheese spread ham strawberry jam	whole wheat bread margarine low-fat margarine cheese cheese spread corned beef honey	whole wheat bread margarine low-fat margarine cheese cheese spread ham sprinkles no chocolate	whole wheat bread margarine low fat margarine cheese cheese spread minced meat loaf fried blue berry jam	whole wheat bread margarine low-fat margarine cheese cheese spread ham apple syrup	whole wheat bread margarine low fat margarine cheese cheese spread porc fricandeau cherry jam
Basal diet	milk (egg)* treacle waffle apple free-choice items	milk (egg)* spicy biscuit pear free-choice items	milk (egg)* biscuit grapes free-choice items	milk (egg)* biscuit apple free-choice items	milk (egg)* shortbread biscuit melon free-choice items	milk (egg)* cracknel pear free-choice items
Basal diet (hot meal)	potatoes green beans gravy beef sausage or vegetarian (soja product) curry dressing caramel custard	bulgur vegetable mix (broccoli, carrot, cauliflower, corn) tomato sauce pork chop or cheese honey dressing chocolate custard	pasta spinach sour cream Kromeski meat filled or brown beans tomato dressing pear/ melon custard	potatoes peas sweet-sour sauce hamburger or vegetarian (soja burger) orange dressing rhubarb custard	rice broccoli curry sauce beef or egg chives dressing vanilla custard	rice Brussel sprouts cheese sauce ham or tahoe mustard dressing apple/cinnamon custard
Supplement Dietary folate group	green beans salad broccoli/ corn/ Brussels sprouts soup spinach orange juice clementines	vegetable mix salad broccoli/ carrot ragout peas/ corn/ carrot orange juice orange	spinach salad green beans/ onion/ tomato soup broccoli orange juice clementines	peas salad peas/ corn/ pepper ragout vegetable mix orange juice orange	broccoli salad broccoli/ corn/ peas soup vegetable mix orange juice clementines	Brussel sprouts salad green beans/ cucumber/ radish ragout green beans/ pepper/ onion orange juice orange
Supplement Folic acid group and Placebo group	salad rice/cucumber cup-a-soup pear grape juice	salad bulgur/ celery/ pepper cup-a-soup apple apple juice	salad pasta/ cucumber/ pickled gerkins/ pepper curry soup pear grape juice	salad rice/ chicory/ apple/ raisins cup-a-soup apple apple juice	salad rice/ celery/ pepper mushroom ragout pear grape juice	salad pasta/ chicory/ apple/ raisins onion soup apple apple juice

*Eggs were provided once per week. Subjects consumed eggs on random days during the week (no more than one per day)

Table 6.2 Mean daily intake of nutrients and energy during dietary intervention period¹

Energy/Nutrient	Intervention		
	Dietary folate group	Folic acid group ³	Placebo group
Folate ($\mu\text{g/day}$) ²			
calculated	594 $\pm$ 27	226 $\pm$ 9	226 $\pm$ 9
analysed	560 $\pm$ 184	210 $\pm$ 49	210 $\pm$ 49
Folic acid ($\mu\text{g/day}$)	0	250 ³	0
Protein (energy%)	14.1	13.7	13.6
Fat (energy%)	31.7	30.7	30.5
Carbohydrates (energy%)	53.0	55.6	55.9
Alcohol (energy%)	1.3	1.5	0.5
Dietary fibre (g/MJ)	4.8	4.2	4.1
Energy (MJ/day)	9.89 $\pm$ 2.53	9.61 $\pm$ 2.57	9.85 $\pm$ 2.47
Energy (kcal/day)	2364 $\pm$ 605	2297 $\pm$ 614	2354 $\pm$ 590

¹Values are based on the analysis of six complete duplicate diets (one for each day of the menu cycle) plus its calculated contribution from the free choice items (see methods section)

²The folate content represents the daily amount for a subject receiving 11 MJ/day. Differences in folate content were similar for all energy levels.

³The folic acid group received one 500 μg folic acid tablet and one placebo tablet on alternate days.

In addition to the basal diet (containing ca 200 µg of folate per day by calculation), we supplied subjects in the placebo and folic acid group with foods low in folate (containing ca 25 µg/d by calculation), while the dietary folate group received foods rich in folate (containing ca 395 µg/d by calculation) (Table 6 2). The main sources of dietary folate were vegetables (spinach, green peas, broccoli, Brussels sprouts, green beans or a mixed vegetable dish, ca 320 µg/d) and citrus fruit (1 orange or 2 clementines and orange juice, ca 75 µg/d) Soup or ragout with less vegetables, rice or pasta salad, and non-citrus fruit (apples/pears) instead of citrus fruit were provided to the folic acid and placebo groups The energy content of the foods supplied in addition to the basal diet was similar for all subjects in all three intervention groups (1380 kJ/d)

All foodstuffs were weighed out for each subject On weekdays at noon, hot meals were prepared and served under supervision at the Division of Human Nutrition and Epidemiology If subjects could not finish their whole meal, dessert was packed and taken home At lunch-time, food for the rest of the day was provided and taken home On Friday food for the weekend was taken home Subjects checked whether their own package of food was complete before leaving the Division If the participant later discovered that something was missing it was delivered to their house If subjects could not come to the division to eat their hot meal, food was either collected or delivered to their house In addition to the food supplied, subjects were allowed a limited number of free-choice items low in folate and fat chosen from a list The free-choice items were mainly non-alcoholic soft-drinks, alcoholic drinks (subjects were allowed to have no more than one beer each day), candy and sweet bread fillings. All participants kept a diary in which they recorded illness, medication used, consumption of free-choice items, and any deviations from their diet At the end of the trial, subjects were asked to complete an anonymous questionnaire regarding problems and non-compliance during the study

Duplicate portions of the low and high folate diet were collected on each of the 29 trial days for a fictitious participant with a daily energy intake of 11 MJ The folate content was analysed in a sub-sample (one from each menu, in total 12 samples) by microbiological assay with *Lactobacillus casei* as described by Horne & Patterson (Horne and Patterson, 1988) and modified by Tamura, Freeberg & Cornwell (Tamura *et al* , 1990) The assay had an intra-assay coefficient of variance (CV) of <10%. Macronutrients were analysed in pooled samples The energy and nutrient content of the free choice items was estimated from data in food consumption tables (Stichting NEVO, 1993)

Tablets

In addition to the food, the folic acid group received a tablet containing 500 µg folic acid every other day and a placebo tablet on alternate days, while the other two groups received one placebo tablet daily. Subjects were blind to whether they received folic acid or placebo tablets. Because of the varying amounts of vegetables, participants were aware of their diet category. All subjects noted on a 'tablet calendar' whether they took the tablet or not. Supplementation compliance was monitored by counting the remaining tablets and inspection of the tablet calendars.

Blood sampling and analysis

Venous blood samples were collected after an overnight fast at the start (days 0 and 1), after two weeks (day 15) and at the end of the intervention period (days 29 and 30). Plasma total homocysteine and plasma folate concentrations were assessed in all samples. Red blood cell folate concentrations were determined on days 1 and 30.

Blood samples were drawn into EDTA vacutainer tubes (Venoject II, Terumo, Madrid, Spain). For the determination of plasma total homocysteine and plasma folate concentration, samples were immediately placed on ice and centrifuged within 60 min at 3,000 x g for 10 min. Plasma was separated and stored at -35°C for folate determination and at -80°C for total homocysteine determination. For the determination of folate concentrations in red blood cells, whole blood was diluted four-fold with aqueous sodium ascorbate (10 g/L) and stored at -35°C. Before measurement, the hemolysates were further diluted with IMx Folate RBC Lysis Reagent (Abbott Laboratories, North Chicago, IL, USA). To be able to express the folate concentration in red blood cells, haematocrits were also measured. Correction was made for the concentration of folate in plasma. Plasma total homocysteine was measured by high performance liquid chromatography with fluorimetric detection (Araki and Sako, 1987). The intra- and inter-assay CV were <8%. Folate concentrations in blood were determined with the Abbott IMx Folic Acid assay, which is based on ion-capture technology for the IMx automated immunoassay system (Abbott Laboratories, North Chicago, IL, USA). The intra-assay CV was <6%, and the inter-assay CV was <10%. All samples from each subject were analysed in the same run.

Calculations of bioavailability of folic acid

We calculated the bioavailability of dietary folate from vegetables and citrus fruits relative to the bioavailability of folic acid. For each endpoint (change in concentration of plasma folate, red blood cell folate and plasma total homocysteine), it was calculated as follows:

$$\text{Relative bioavailability (\%)} = \frac{\begin{array}{l} \text{Change in endpoint in} \\ \text{dietary folate group} \\ \text{during intervention} \\ \text{minus change in} \\ \text{placebo group} \end{array}}{\begin{array}{l} \text{Change in endpoint in} \\ \text{folic acid group} \\ \text{during intervention} \\ \text{minus change in} \\ \text{placebo group} \end{array}} \times \frac{\begin{array}{l} \text{Additional} \\ \text{folic acid} \\ \text{provided} \\ (250 \mu\text{g}) \\ \text{Additional} \\ \text{dietary} \\ \text{folate} \\ \text{provided} \\ (\mu\text{g}) \end{array}}{\text{Additional}} \times 100$$

For each subject, average values for plasma total homocysteine and plasma folate concentration were taken for day 0 and day 1 (week 0), and for day 29 and day 30 (week 4). Response to the various treatments was calculated for each subject as the change in each endpoint, between the start (week 0) and the end (week 4) of the intervention period.

Statistics

Changes in folate and total homocysteine concentrations were normally distributed as checked by visual inspection of the normal probability plots (univariate procedure; SAS Institute Inc, Cary, NC). To analyse differences in endpoint response between the intervention groups and the placebo group, Student t-tests were used with a significance level of $P < 0.025$ to maintain an overall significance level of $P < 0.05$. One-way ANOVA was used to analyse differences in baseline levels of plasma total homocysteine, plasma folate and red blood cell folate concentrations among the three groups.

Results

Diets and adherence

The nutrient and energy intakes of the subjects are shown in Table 6.1. The energy intake from the free-choice items was fixed for each level of daily energy intake and was on average $11.2\% \pm 1.3$ (SD) of total energy.

Neither the questionnaires, nor the diaries revealed any deviations from the protocol that might have affected the results. Two subjects missed three supervised hot meals, six subjects two supervised meals, and 16 subjects missed one supervised meal. In all these cases the meals were eaten outside the division. The remaining 42

subjects attended all supervised hot meals. One man withdrew from the study on the second day because he could not adhere to the diet.

Counting of the remaining tablets and checking the tablet calendars revealed that seven subjects in the folic acid group failed to take one tablet during the intervention period. None of the subjects failed to take more than one folic acid tablet during the study.

Stratification was successful because plasma total homocysteine, energy intake, vegetarianism and gender were not different among the groups. The mean age $\pm$ SD was 23.0 ± 7.5 years and their body mass index was 22.4 ± 2.0 kg/m². Over the 29 days of the trial, body weights decreased by 0.6 ± 0.9 kg (mean $\pm$ SD).

Folate status

Baseline plasma folate and red blood cell folate concentrations were not significantly different among the three groups. After four weeks of intervention, the plasma folate concentrations increased in both the dietary folate and the folic acid group (Table 6.3). The most distinct increase in plasma folate concentrations occurred during the first two weeks. Red blood cell folate concentrations also increased in both the dietary folate group and the folic acid group (Table 6.3).

Homocysteine

At baseline, the median (25%, 75%) of the plasma total homocysteine concentration was 10.1 (8.0, 11.6) μ mol/L. Baseline plasma total homocysteine concentrations were not significantly different among the groups. Four weeks of intervention decreased the plasma total homocysteine concentrations in both the dietary folate and the folic acid group (Table 6.3). After correction for changes in the placebo group, the mean decrease was 2.0 μ mol/L (95% CI, 1.0 to 3.0) in the dietary folate group and 2.4 μ mol/L (1.4 to 3.4) in the folic acid group. Plasma total homocysteine concentrations decreased gradually during the entire four-week intervention period (Table 6.3).

Bioavailability

The bioavailability of folate from vegetables and citrus fruits relative to folic acid was for the different endpoints: 60% based on plasma total homocysteine concentration; 78% based on plasma folate concentration; and 98% based on red blood cell folate concentration.

Table 6.3 Effect of dietary folate and folic acid on plasma folate, red blood cell folate and total plasma homocysteine concentrations.

	Dietary folate group (additional folate: 350 µg/d) (n=23) ¹	Folic acid group (additional folic acid: 500 µg/2d) (n=22)	Placebo group (additional folate / folic acid: 0 µg/d) (n=22)
Plasma folate (nmol/L)			
Week 0 ²	12.8 (11.8 to 16.2)	13.5 (11.0 to 16.4)	12.7 (10.2 to 16.1)
Week 2 ²	19.8 (17.7 to 23.3)	17.2 (16.3 to 21.7)	11.7 (10.0 to 15.9)
Week 4 ²	19.4 (18.3 to 23.0)	19.8 (17.8 to 22.5)	12.4 (10.4 to 14.6)
Change from baseline ³	7.0 (5.4 to 8.6)**	6.4 (4.7 to 8.0)**	
Red blood cell folate (nmol/L)			
Week 0 ²	336 (268 to 386)	328 (285 to 370)	353 (283 to 403)
Week 4 ²	367 (320 to 447)	379 (338 to 418)	334 (299 to 411)
Change from baseline ³	60.5 (31.1 to 89.9)**	44.1 (14.7 to 73.4)*	

	Dietary folate group (additional folate 350 µg/d) (n=23) ¹	Folic acid group (additional folic acid 500 µg/2d) (n=22)	Placebo group (additional folate / folic acid 0 µg/d) (n=22)
Plasma homocysteine (µmol/L)			
Week 0 ²	9.6 (8.4 to 11.4)	10.0 (8.3 to 12.1)	10.4 (7.9 to 11.7)
Week 2 ²	9.0 (8.0 to 10.0)	9.1 (7.4 to 10.3)	9.9 (7.6 to 11.5)
Week 4 ²	8.4 (7.9 to 9.8)	8.8 (7.4 to 10.0)	10.2 (8.5 to 12.1)
Change from baseline ³	-2.0 (-3.0 to -1.0)**	-2.4 (-3.4 to -1.4)**	

¹ One of the subjects withdrew from the study after two days of intervention

² Median (25%,75%)

³ Mean (95% confidence interval) change in intervention group (n=22) over 4-week period corrected for change in placebo group (n=22). The data are presented as medians for absolute values and means for changes because changes, but not absolute values are normally distributed. Student t-test * $P < 0.01$, ** $P < 0.001$

Discussion

This controlled dietary intervention study demonstrates that a diet rich in vegetables and citrus fruit favourably affects plasma folate, red blood cell folate and plasma total homocysteine concentrations in young healthy volunteers. Many studies have shown that folic acid, the synthetic monoglutamate, decreases plasma total homocysteine concentrations and improves the folate status (Brouwer *et al.*, 1999; Cuskelly *et al.*, 1996; Jacob *et al.*, 1994; O'Keefe *et al.*, 1995; Ward *et al.*, 1997). However, studies investigating the effects of natural food folate on plasma total homocysteine and folate status are scarce. A cross-sectional study showed that intake of dietary folate was inversely correlated with plasma total homocysteine concentrations and positively correlated with the plasma folate concentration (Tucker *et al.*, 1996). Cuskelly *et al.* (1996) found a non-significant increase of 11% (95% CI: -6, 29) in red blood cell folate concentration in young healthy women after three months of intervention with natural food folate. Our study shows a significant 17% (CI: 8 to 26) increase in red blood cell folate concentration after four weeks of intervention (Table 6.3). This discrepancy can be explained by differences in the study hypotheses and designs. Cuskelly *et al.* (1996) investigated whether fortified foods, supplements and consumption of natural food folate would have equal effects on folate status in a free living situation, in which the subjects selected their own foods. They included 10 women in each group and the average intake of additional dietary folate was calculated to be 200 µg per day (Cuskelly *et al.*, 1996). In contrast, our study was a controlled dietary intervention study with 22 subjects in each group. We supplied the subjects in the dietary folate group with vegetables and fruits containing 350 µg of additional dietary folate per day, a relatively high amount. Nevertheless, because their study lasted three times longer than ours a higher response would have been expected in the study of Cuskelly *et al.* (1996).

The 350 µg of additional dietary folate consumed daily in the dietary folate group was 1.4 times the 250 µg of folic acid provided to subjects in the folic acid group. Depending on the endpoint chosen, the bioavailability of dietary folate, from vegetables and citrus fruit, relative to folic acid was calculated to be between 60% and 98%. This estimate is higher than the 50% or less suggested by the study of Sauberlich *et al.* (1987) who determined bioavailability of folate from a mixed diet by using responses in plasma folate. Based on the response in red blood cell folate concentration our estimate of relative folate bioavailability of 98% is high compared to the 39% estimated from the study of Cuskelly *et al.* (1996). The differences in bioavailability between the

studies might be explained by the fact that we used only vegetables and citrus fruit as a source of folate, while the other two studies fed a wider range of food products (Cuskelly *et al* , 1996, Sauberlich *et al* , 1987) However, the difference might also be attributable to the higher degree of compliance in fruit and vegetables consumption in our study

In our study the relative bioavailability is considerably lower when calculated from the change in plasma total homocysteine (60%) than from the change in folate concentrations in plasma (78%) and red blood cells (98%) These differences cannot be explained by differences in intestinal absorption between synthetic folic acid and folate from foods, but would appear to be related to the chemical form of the vitamin ingested Tablets contain the fully oxidised form of pteroylglutamic acid while in food, the vitamin exists with two or four additional hydrogen atoms and is conjugated to glutamic acid Food folate appears to be less effective in reducing plasma total homocysteine concentrations than folic acid On the other hand, it tends to accumulate more in red blood cells than in plasma

For practical reasons we provided 500 µg of folic acid on alternate days instead of 250 µg each day It cannot be ruled out that the estimate of bioavailability based on changes in plasma total homocysteine (60%) would have been slightly lower if we had provided 250 µg of folic acid each day, because Kelly *et al* (1997) have shown that an intake of folic acid in addition to that in the diet of more than 266 µg/d results in significant amounts of unmetabolised folic acid in the blood (Kelly *et al* , 1997) This suggests that not all folic acid supplied is available for the remethylation of homocysteine to methionine

Folate is the nutrient most likely to be responsible for the reduction in plasma total homocysteine concentrations because it was present in large quantities in the diet of the dietary folate group and because folate concentrations in plasma and red blood cells increased significantly during the intervention period In addition to folate, vitamin B12 is also involved as a co-factor in the remethylation process However, vitamin B12 is only present in animal products and therefore could not have caused the observed reduction in plasma total homocysteine concentrations in our study Apart from the remethylation to methionine, homocysteine can also be converted to cysteine by transsulfuration by the vitamin B6-dependent enzyme cystathionine synthase Even though vitamin B6 is present in vegetables and fruit and even though an intake of vitamin B6 above the recommended daily allowances might protect women against coronary heart disease (Rimm *et al* , 1998), it is not likely to have caused the observed effect on fasting plasma total homocysteine Vitamin B6 administration in tablet form has been shown to have a marked effect on plasma total homocysteine concentrations

after methionine loading, while the effect of vitamin B6 administration on fasting plasma total homocysteine concentrations is regarded as negligible (Ubbink *et al.*, 1994).

This study was designed to determine the efficacy of dietary folate from vegetables and citrus fruit to improve the folate status and to decrease plasma total homocysteine concentrations. Therefore, we chose the strategy of supplying the subjects with a high amount of folate in a controlled setting rather than to examine the effects that can be observed under field conditions. As a consequence, the intake of 350 g of vegetables and one piece of citrus fruit and 200 mL of citrus fruit juice given in our study in addition to the basal diet (total folate content in the dietary folate group: 560 µg/d) is higher than what can be expected to be eaten by the general population.

In summary, we have shown that the intake of folate-dense vegetables and citrus fruit significantly enhances the folate status and decreases plasma total homocysteine concentrations in healthy volunteers.

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7

Bioavailability of folate and folic acid: Review of the literature

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Introduction

Folate is a B-vitamin that serves as a methyl group donor in one-carbon metabolism. The term folate refers to all derivatives with the biological activity of pteroylmonoglutamic acid (folic acid). Folic acid is the synthetic fully oxidised monoglutamate of the vitamin that is not present in significant quantities in nature. In nature, various reduced forms of folate with one or more glutamate groups occur (see Figure 1 2).

During the last decade, folic acid has received much attention because new functions apart from those related to the classical treatment of megaloblastic anaemia have been discovered. Folic acid supplementation around the time of conception has been proven to make a contribution to the prevention of neural-tube defects (Czeizel and Dudás, 1992, MRC Vitamin Study Research Group, 1991). The Federal Government of the USA responded to these findings by mandating food producers to fortify grain products with folic acid. This stresses the importance of understanding the factors which affect bioavailability of folic acid added to foods.

Furthermore, dietary folate and folic acid both effectively decrease total homocysteine levels in plasma (Brouwer *et al.*, 1999; Homocysteine Lowering Trialists' Collaboration, 1998, Chapter 6). This is important because an elevated plasma homocysteine concentration has been identified as an independent risk factor for cardiovascular disease (Boushey *et al.*, 1995; Graham *et al.*, 1997). Plasma homocysteine concentration can be regarded as a functional indicator of the folate status (Jacob *et al.*, 1995).

This review will discuss the factors influencing bioavailability of natural food folate and of folic acid from fortified food products. It also includes discussion of the efficacy of folate and folic acid in decreasing plasma homocysteine levels.

Intestinal absorption

Dietary folates are a mixture of various pteroylpolyglutamates (PteGlu_n). These pteroylpolyglutamates have between 2 and 7 glutamate residues in their side chain. Before absorption, which takes place in the jejunum, dietary polyglutamyl folates must first be deconjugated by the enzyme pteroylpolyglutamate hydrolase (folate conjugase) to a monoglutamyl form (PteGlu₁).

Before the fully oxidised monoglutamyl form (PteGlu₁) of the vitamin, folic acid, enters the portal circulation it is reduced to tetrahydrofolate (H₄PteGlu₁). This form is either methylated or formylated in the mucosal cells of the jejunum (Perry and

Chanarin, 1973; Selhub *et al.*, 1973; Selhub *et al.*, 1983; Strum, 1979). However, when single doses of more than 266 µg of folic acid have been fed, unmetabolised folic acid has been shown to be present in serum (Kelly *et al.*, 1997).

Definition of bioavailability

In pharmacokinetics bioavailability is described as the ratio of area under the curve derived from an oral dose to the area under the curve derived from an intravenous reference dose (Rowland and Tozer, 1989). However, this definition is not valid in folate bioavailability because it requires that the total clearance is independent of the route of administration. This is not the case for folates (Gregory *et al.*, 1992). Rogers *et al.* showed that for that reason administration of folate via the same route is preferred in folate bioavailability studies (Rogers *et al.*, 1997). Most studies in the field of folate determined relative instead of absolute bioavailability. In those studies bioavailability of the compound is assessed relative to the bioavailability of folic acid, the fully oxidised monoglutamate (PteGlu₁).

At the Bioavailability meeting held in Wageningen in May 1997, bioavailability was defined as the fraction of an ingested nutrient which has the potential to meet functional demands in target tissues. This definition implies that bioavailability refers to post-absorptive processes, namely bioefficacy and biodelivery, as well as absorption (Strain and West, 1999). Plasma total homocysteine is a functional parameter of folate status. Therefore, changes in plasma total homocysteine can be used as a measurement of bioefficacy according to this definition. Changes in plasma folate or red blood cell folate can be regarded as measurements of biodelivery. In this review, bioavailability covers measurements of absorption, biodelivery and bioefficacy.

Factors influencing folate and folic acid bioavailability: SLAMENGHI

In 1996 de Pee & West published a review on dietary carotenoids and their role in combating vitamin A deficiency. They introduced the mnemonic 'SLAMANGHI' to order the factors influencing the bioavailability of carotenoids (de Pee and West, 1996). During the Wageningen Bioavailability meeting the word was modified to SLAMENGHI. The SLAMENGHI factors are not specific for carotenoid bioavailability, but can also be applied to the bioavailability of other nutrients. In this review we will discuss the factors influencing bioavailability of folate with reference to SLAMENGHI: **S**pecies of folate; **L**inkage at molecular level; **A**mount of folate/folic acid consumed; **M**atrix; **E**ffect modifiers; **N**utrient status; **G**enetic factors; **H**ost-related factors; **M**athematical **I**nteractions between the various factors.

Species of folate

In this section, the effects of different species of folate on bioavailability will be discussed. Folate occurs in many different forms. Folic acid (PteGlu.), which only exists in small amounts in nature, is the fully oxidised monoglutamate of the vitamin and does not have moieties which can be transferred as one-carbon units. The more reduced forms of folate, dihydrofolate and tetrahydrofolate, which do exist in nature, can be substituted with such moieties (Wagner, 1995). These reduced forms, for example 5-methyltetrahydrofolate and formyltetrahydrofolates are much more common in nature (Figure 1.2).

The bioavailability of oxidised and reduced folates with or without various one-carbon units has been investigated in a series of human intervention studies (Table 7.1). Findings from these studies are not consistent. Perry & Chanarin found a greater increase in serum folate levels after ingestion of reduced folates than after ingestion of folic acid. However, urinary excretion of folic acid was higher than that of the other monoglutamyl folate forms (Perry and Chanarin, 1970). Brown *et al* (1973) found that the bioavailability of other monoglutamate forms was greater than that of folic acid except that the bioavailability of 5-formyltetrahydrofolate was similar and of tetrahydrofolate was less (Brown *et al*, 1973). On the basis of a study using the ratio of urinary excretion of orally administered folate labelled with [^3H] and intravenously administered folate labelled with [^3H], Gregory *et al* (1992) concluded that folic acid was more bioavailable than the reduced forms of the vitamin. Other studies have found no differences in bioavailability between folic acid and the reduced forms (Bhandari and Gregory, 1992, Pietrzik and Remer, 1989, Tamura and Stokstad, 1973).

One problem with most studies investigating folate bioavailability is that the variation in response between subjects can be quite substantial. Another problem is that it is not possible to determine whether these differences are due to differences in absorption or in postabsorption processes. In all studies, except that of Pietrzik and Remer (1989), subjects received one or more doses of folic acid for periods up to seven days in order to saturate the tissues with folic acid.

To our knowledge, there are no studies published investigating the effect of different species of folate on plasma total homocysteine concentrations.

Table 7.1 Effect of Species of folate on folate bioavailability (human intervention studies)

<i>Authors</i>	<i>Design</i>	<i>Results</i>	<i>Conclusions and comments</i>
Perry & Chanarin, 1970	<p>Subjects: 69 medical students (5 groups, n=11-16 per group).</p> <p>Design: Parallel.</p> <p>Prior loading: 20 mg folic acid (PteGlu₁) for three days before experiment.</p> <p>Treatment: Single dose of 10 µg/kg (20 µg/kg for H₂PteGlu₁).</p> <p>Folate species: PteGlu₁, H₂PteGlu₁, H₄PteGlu₁, 5-formyl-H₄PteGlu₁, 5-methyl-H₄PteGlu₁.</p> <p>Blood collection at 0, 1, 2 and 3 hours + 6 hours urine collection.</p> <p>Measurement: plasma folate and urinary excretion.</p>	<p>Bioavailability relative to folic acid was for both measurements highest for 5-methyl-H₄PteGlu₁, followed by 5-formyl-H₄PteGlu₁, and lowest for H₄PteGlu₁.</p> <p>However, bioavailability of all reduced forms was higher compared to folic acid when measured by 2 hour change in serum folate, whereas it was lower when measured by urinary excretion.</p>	<p>This study suggests that differences exist between the several monoglutamyl forms of folate. However, the short duration of the measurement period (6 hours urine collection and 3 hours blood collection) might not be long enough to measure true differences in study.</p>
Brown et al., 1973	<p>Subjects: 21 medical students and hospital staff volunteers (fasting).</p> <p>Design: Cross-over, with one week between tests (parallel for pteroate, 5-formimino-H₄PteGlu₁ and 5,10-methylene-H₄PteGlu₁, n=7 per group).</p> <p>Prior loading: 10 mg PteGlu₁ dose orally one week prior to the first test. Thereafter, 5 mg loading doses after each test.</p> <p>Treatment: Oral administration of 0.68 µmol (300 µg).</p> <p>Folate species: Pteroate, 5-formimino-H₄PteGlu₁, 5,10-methylene-H₄PteGlu₁, H₄PteGlu₁, PteGlu₁, 5-formyl-H₄PteGlu₁, 5-methyl-H₄PteGlu₁, 5,10-methenyl-H₄PteGlu₁, H₂PteGlu₁, 10-formyl-H₄PteGlu₁.</p> <p>Blood collection after 1 and 2 hours.</p> <p>Measurement: Increase in serum folate.</p>	<p>Pteroate, H₄PteGlu₁ and 5,10-methylene-H₄PteGlu₁ increased by <3 ng/mL.</p> <p>PteGlu₁, 5-formyl-H₄PteGlu₁, 5-formimino-H₄PteGlu₁ and 5-methyl-H₄PteGlu₁ increased 6 to 9 ng/mL.</p> <p>5,10-methenyl-H₄PteGlu₁, H₂PteGlu₁ and 10-formyl-H₄PteGlu₁ increased by >10 ng/mL.</p>	<p>This study suggests that the various monoglutamyl forms of folate have different bioavailability. However, the short duration of the serum collection might also be due to differences in absorption time.</p>

<i>Authors</i>	<i>Design</i>	<i>Results</i>	<i>Conclusions and comments</i>
Tamura & Stokstad, 1973	Subjects 6 healthy males (fasting) Design Within person comparison Prior loading with PteGlu ₁ 2 x 10 mg on first day, 5 mg on second day, 2 mg on third day Then 2 mg every other day Treatment Oral dose of 0.5 mg (equivalent to PteGlu ₁) of H ₄ PteGlu ₁ and 5-methyl-H ₄ PteGlu ₁ , a dose of 0.75 mg for 5-formyl-H ₄ PteGlu ₁ Folate species H ₄ PteGlu ₁ , 5-methyl-H ₄ PteGlu ₁ , 5-formyl-H ₄ PteGlu ₁ Measurement 24 hours urinary excretion of folate	Folate bioavailability compared to PteGlu ₁ H ₄ PteGlu ₁ 104.7% (range 40-136) (n=6) 5-methyl-H ₄ PteGlu ₁ 120.8% (82-152) (n=6) 5-formyl-H ₄ PteGlu ₁ 70.0% (64-76) (n=2)	No significant difference in relative bioavailability between H ₄ PteGlu ₁ , 5-formyl-H ₄ PteGlu ₁ and 5-methyl-H ₄ PteGlu ₁ compared to PteGlu ₁ . There was a wide variation in response between subjects
Pietrzik & Remer, 1989	Subjects 12 healthy adults Design Cross-over with one week between tests No prior loading Treatment Single dose of 1 mg 5-formyl-H ₄ PteGlu or PteGlu Folate species 5-formyl-H ₄ PteGlu versus PteGlu Measurement Area under the curve (AUC) for serum folate (3 days)	The ratio for AUC for PteGlu ₁ versus 5-formyl-H ₄ PteGlu ₁ was 1.02	This study suggests a similar bioavailability for PteGlu ₁ and 5-formyl-H ₄ PteGlu ₁ in aqueous solution
Gregory III et al, 1992	Subjects 7 adult males (fasting) Design Within person comparisons (3 weeks intervals) Prior saturation with 2 mg folic acid/day for 7 days Treatment The different species are given orally D ₂ -labelled [² H ₂]PteGlu ₁ (50% [² H ₂], 51% [² H ₁], and 9% [² H ₀]), D ₄ -labelled [² H ₄] PteGlu ₁ (88% [² H ₄], 12% [² H ₂], and 0% [² H ₀]) is given intravenously as control Folate species PteGlu ₁ , 10-formyl-H ₄ PteGlu ₁ , 5-methyl-H ₄ PteGlu ₁ , 5-formyl-H ₄ PteGlu ₁ , H ₄ PteGlu ₁ Measurement Urinary excretion of D ₂ /D ₄ for 48 hours	Urinary D ₂ /D ₄ excretion ratios PteGlu ₁ 1.53 10-formyl-H ₄ PteGlu ₁ 1.02 5-methyl-H ₄ PteGlu ₁ 0.99 5-formyl-H ₄ PteGlu ₁ 1.13 H ₄ PteGlu ₁ 0.71	This study indicates that differences in bioavailability of monoglutamyl folates exist. Bioavailability of folic acid seems better than of the reduced forms

Linkage at molecular level

Folate not only occurs as different species as discussed above but also with more than one glutamate residue. In this section, bioavailability of folate with different numbers of glutamate residues in its side chain (PteGlu_n, n=1-7) in human volunteers will be discussed (Table 7.2).

As discussed above, pteroylpolyglutamates which are the major forms of folate in foods, have first to be hydrolysed to monoglutamates before absorption in the small intestine can take place. A conjugase present in the jejunum is responsible for removing glutamate residues from pteroylpolyglutamates (Reisenauer *et al*, 1977). Under normal circumstances, the activity of this folate conjugase enzyme is not rate limiting in the absorption process (Reisenauer and Halsted, 1987). This finding is in line with that from earlier studies using ³H-labelled PteGlu. PteGlu₇ is absorbed nearly as well as PteGlu₁ (Godwin and Rosenberg, 1975, Rosenberg and Godwin, 1971). Two studies using 24 hour urinary excretion of folate and area under the curve of serum folate also found no significant differences in bioavailability of PteGlu₁, PteGlu₃ and PteGlu₇ (Bailey *et al*, 1988, Tamura and Stokstad, 1973). However, a well designed study using labelled folates suggested that PteGlu₆ is less bioavailable than PteGlu₁ (Gregory *et al*, 1991). Earlier studies also suggested that less PteGlu₁ disappeared from the jejunum than PteGlu₇ (Halsted *et al*, 1975, Halsted *et al*, 1978). Although the results of the studies are not equivocal, absorption of PteGlu_n is often found to be less than that of PteGlu₁. This may imply that bioavailability of polyglutamates is less than that of monoglutamates. However, it cannot be excluded that uptake of polyglutamates takes longer, but that the net effect in the long-term is similar to monoglutamates.

Table 7.2 Effect of Linkage at molecular level, pteroylglutamate chain length, on folate bioavailability (human intervention studies)

<i>Authors</i>	<i>Design</i>	<i>Results</i>	<i>Conclusions and comments</i>
Tamura & Stokstad, 1973	Subjects 6 healthy males (fasting) Design Within person comparison Prior loading with PteGlu ₃ 2 x 10 mg on first day, 5 mg on second day, 2 mg on third day Then 2 mg every other day for the duration of the study Treatment Oral dose of 0.75 - 2.0 mg equivalent to PteGlu ₃ Chain length PteGlu ₃ , PteGlu ₇ versus PteGlu ₁ Measurement 24 hours urinary excretion of folate	Folate availability compared to PteGlu ₁ PteGlu ₃ 85.2 % (range 27-144) (n=6) PteGlu ₇ 90.4 % (13-140) (n=14)	No significant differences in relative bioavailability between PteGlu ₃ , PteGlu ₇ and PteGlu ₁ There was a wide variation in response between the subjects
Godwin & Rosenberg, 1975	Subjects 11 healthy fasting volunteers (4 men, 7 women) Design Cross-over with 3 or 4 days interval No prior loading Treatment oral dose of 300 mL water with either 0.6 µmol [³ H]PteGlu ₁ or 0.6 µmol [³ H]PteGlu ₇ After 4 hours a flushing dose of 15 mg unlabelled folic acid was given Chain length PteGlu ₁ versus PteGlu ₇ Measurement urinary excretion of folate over 48 hours	Folate urinary excretion PteGlu ₁ 70.8 ± 13.0 (mean ± SD) PteGlu ₇ 56.1 ± 11.2	This study shows that physiological doses of both monoglutamyl and heptaglutamyl folic acid are absorbed PteGlu ₁ seems to be slightly better absorbed than PteGlu ₇
Halsted, Baugh & Butterworth, 1975	Subjects 5 healthy volunteers Design Jejunal perfusion of [³ H]PteGlu ₁ and [¹⁴ C]PteGlu ₇ No prior loading Treatment Equimolar solutions of [³ H]PteGlu ₁ and [¹⁴ C]PteGlu ₇ were provided Chain length PteGlu ₇ versus PteGlu ₁ Measurement luminal disappearance	Percentage luminal disappearance PteGlu ₁ 74.7% PteGlu ₇ 52.6%	PteGlu ₁ and PteGlu ₇ are both taken up by the jejunum The uptake of PteGlu ₁ seems higher than the uptake of PteGlu ₇

<i>Authors</i>	<i>Design</i>	<i>Results</i>	<i>Conclusions and comments</i>
Halsted et al 1978	Subjects 6 healthy adults, 4 patients with coeliac sprue Design Single 48-hours experiment No prior loading Treatment Jejunal perfusion with solution containing 2 $\mu\text{mol/L}$ ^3H -PteGlu ₇ and 2 $\mu\text{mol/L}$ ^{14}C PteGlu ₁ Chain length PteGlu ₇ versus PteGlu ₁ Measurement urinary isotopic recovery after jejunal folate perfusion	The urinary recovery of PteGlu ₇ was lower than that of PteGlu ₁	The bioavailability of the monoglutamate seems to be greater than the bioavailability of the polyglutamate
Bailey et al 1984	Subjects 13 healthy male subjects in the age categories 20-29 yrs and 65-83 yrs Design Single 48-hours experiment Treatment Jejunal perfusion with solution containing 3 $\mu\text{mol/L}$ ^3H -PteGlu ₁ and 3 $\mu\text{mol/L}$ ^{14}C -PteGlu ₇ Chain length PteGlu ₇ versus PteGlu ₁ Measurement urinary isotopic recovery after jejunal folate perfusion and luminal disappearance	Both urinary recovery and luminal disappearance were higher for PteGlu ₁ than for PteGlu ₇ in subjects of both age categories	This study suggests higher bioavailability of folic acid than of pteroylheptaglutamate
Bailey et al , 1988	Subjects 9 healthy males (fasting) Design Cross-over with two weeks interval No prior loading Treatment Single dose of 750 μg PteGlu ₇ or equivalent amount of PteGlu ₁ given in solution or with bran or spinach Chain length PteGlu ₇ versus PteGlu ₁ Measurement Area under the curve (AUC) of serum folate for 8 hours	AUC's of PteGlu ₇ and PteGlu ₁ were not different when ingested as solution or with spinach AUC for PteGlu ₇ ingested with bran was lower than PteGlu ₁ ingested with bran	This study suggests no effect of chain length when ingested as solution, but an inhibiting effect of dietary fibre on bioavailability of PteGlu ₇ compared to PteGlu ₁

<i>Authors</i>	<i>Design</i>	<i>Results</i>	<i>Conclusions and comments</i>
Keagy, Shane & Oace, 1988	Subjects 7 healthy young men Design All subjects received all treatments Constant loading with 500 µg of folic acid per day Treatment Folate absorption tests were conducted during the last four days of a 9 d period in which subjects received PteGlu ₁ and PteGlu ₇ in a formula on alternate days Chain length PteGlu ₁ and PteGlu ₇ Measurement Serum folate after 1 and 2 hours and urine excretion (24 hrs)	PteGlu ₇ produced a lower rise in serum folate and in urinary excretion PteGlu ₇ excretion was 63% of PteGlu ₁ excretion	This study suggests that bioavailability of PteGlu ₇ is either lower or takes longer than bioavailability of PteGlu ₁
Gregory et al 1991	Subjects 7 adult males (fasting) Design Within person comparison (3 weeks interval) Prior loading 2mg PteGlu ₁ for 7 days Treatment Oral dose of 677 nmol d2-folate followed by intravenous dose of 502 nmol d4-PteGlu ₁ Chain length PteGlu ₆ versus PteGlu ₁ Urine was collected for 48 hours Measurement Excretion ratio of urinary folates (% d2-folate dose/ % d4-PteGlu dose)	Urinary excretion ratios PteGlu ₁ 1.45 ± 0.10 (mean ± SEM) PteGlu ₆ 0.67 ± 0.04	This study suggests that PteGlu ₆ is less bioavailable than PteGlu ₁
Wei et al 1996	Subjects 7 adult males (fasting) Design Cross-over with 3 weeks intervals Prior loading 10 mg PteGlu ₁ for 1 week starting 3 weeks before experiment, followed by 2 mg/d until the end of the experiment Treatment Oral dose of 677 nmol d2-PteGlu ₆ and 677 nmol d4-PteGlu ₁ given in combination with water, orange juice, tomato homogenate, lima bean homogenate, or with citric acid Chain length PteGlu ₆ versus PteGlu ₁ Measurement Excretion ratio of urinary D ₂ -PteGlu ₆ /D ₄ -PteGlu ₁ (48 hour urine collection)	The D ₂ /D ₄ excretion ratio for tomato, lima beans and citrate buffer was similar to the ratio of the control (1.00 ± 0.17, mean ± SD) The D ₂ /D ₄ ratio for orange juice was significantly lower (~66% relative to that in the control trials)	These findings indicate that orange juice affects the absorption of orally taken polyglutamate This would imply that the effect occurs at the level of the intestinal deconjugation process by pteroyl polyglutamate hydrolyse

Amount of folate / folic acid

Bioavailability of folate or folic acid is likely to be influenced by the amount ingested. For absorption, there are two different transport systems. In the first transport system, folates are bound to membrane-associated folate-binding proteins and transported across the brush border membrane by a carrier-mediated mechanism. However, at high intraluminal concentration of folate ($>10 \mu\text{mol/L}$) a second nonsaturable diffusion-mediated transport system plays a major role in folate absorption (Mason, 1990). The amount ingested is most likely to be of significance if the saturable transport system is saturated. At physiological concentrations ($<5 \mu\text{mol/L}$) of folate in the lumen transport occurs mainly via the saturable transport system (Mason, 1990). The amount of natural food folate normally ingested is not likely to reach an intake level that causes saturation of this transport system. However, with intake of synthetic folic acid this level can easily be reached.

Many studies investigated effects of amount of synthetic folic acid (PteGlu,) on bioavailability (Table 7.3). Heseke & Schmitt showed that plasma folate reached a steady state after four weeks of supplementation with 1 mg of folic acid daily. Levels in red blood cells increased over the total intervention period of 17 weeks (Heseke and Schmitt, 1987). This synchronises with the lifetime of the red blood cell, which is known to incorporate folate only during erythropoiesis (Shane, 1995). Truswell and Kounnavong (1997) gave in several experiments folic acid supplements, containing either 100 μg , or 500 μg , or 1000 μg of folic acid per day, for 3 weeks in addition to the regular diet. The relative biggest increase in plasma folate was established by the 100 μg of folic acid, but the absolute biggest increase was established by intake of the highest dose of folic acid. The study does not make clear whether on the long term the same level of serum folate can be reached (Truswell and Kounnavong, 1997). Malinow *et al.* (1998) also showed in a cross-over study that a dose of 127 μg of folic acid daily for 5 weeks was relatively more effective in raising plasma folate (30.8%) than doses of 499 μg (64.8%) and 665 μg (105.7%) daily. This suggests that lower doses of folic acid increase plasma folate concentrations most effectively (Malinow *et al.*, 1998). However, the plasma folate raising effect might be slightly underestimated in the groups receiving the higher doses because the washout period between the intervention periods was only five weeks (Malinow *et al.*, 1998). This period is probably too short to avoid a carry-over effect (Brouwer *et al.*, 1999).

Plasma total homocysteine concentration is a functional parameter of folate status. Therefore, the effect of folic acid on plasma total homocysteine concentrations can be described as bioefficacy. Many authors have investigated the effects of different amounts of folic acid on plasma total homocysteine concentrations. Clarke compared in a meta-analysis (Homocysteine Lowering Trialists' Collaboration, 1998) most of these studies. This meta-analysis showed similar homocysteine-lowering effects for doses between 0.5 and 5 mg of folic acid. Thus, it seemed as if doses above 500 µg of folic acid had no additional homocysteine-lowering effect. Besides the articles mentioned in the meta-analysis, a few other authors studied effects of lower doses of folic acid on plasma total homocysteine concentrations. Ward *et al.* (1997) showed that 200 µg of folic acid had a similar effect as 400 µg of folic acid per day. However, six weeks of supplementation with 100 µg of folic acid was not sufficient to reach a similar level of plasma total homocysteine. The latter study does not exclude the possibility that giving the 100 µg of folic acid for a longer period would have resulted in lower plasma total homocysteine concentrations. Malinow *et al.* (1998) found in their study no significant decrease in plasma total homocysteine concentrations after five weeks of supplementation with 127 µg of folic acid per day. However, the design of the study makes it difficult to interpret their results.

Non-metabolised folic acid

Kelly *et al.* (1997) found unmetabolised fully oxidised folic acid (PteGlu₁) in serum of subjects receiving more than 266 µg of folic acid per day. They suggest that the excess of folic acid might not be available for lowering plasma total homocysteine. This is in line with other studies that show no additional homocysteine lowering effect of doses of folic acid above 500 µg, or even 200 µg of folic acid (Bonnette *et al.*, 1998; Homocysteine Lowering Trialists' Collaboration, 1998; Schorah *et al.*, 1998; Ward *et al.*, 1997).

Table 7.3 Effect of Amount of folic acid consumed on folic acid bioavailability (human intervention studies)

<i>Authors</i>	<i>Design</i>	<i>Results</i>	<i>Conclusions and comments</i>
Heseker & Smith, 1987	Subjects: 6 healthy subjects (4 men, 2 women). Design: parallel. No prior loading. Treatment: 500 µg folic acid was administered 2 times daily for 17 weeks in addition to their regular diet. Measurement: Plasma and red blood cell folate.	The mean folate concentration in plasma reached a steady state after $\pm$ 4 weeks. This level was maintained by continuous doses of folic acid. Levels in red blood cell increased continuously during 17 weeks.	A daily dose of 1 mg was sufficient to reach a steady state for plasma folate after four weeks. Red blood cells only incorporate folate during erythropoiesis. Life time of the red blood cell is approximately 120 days. Therefore, it will take around 120 days to reach steady state in red blood cells.
Ward et al. 1997	Subjects: 30 healthy males. Design: Sequential design. No prior loading. Treatment: folic acid was administered daily at doses increasing from 100 µg (6 weeks), to 200 µg (6 weeks), to 400 µg (14 weeks) in addition to the regular diet. Measurement: Fasting total plasma homocysteine, serum folate and red blood cell folate (measured at start and end of study).	<ul style="list-style-type: none">- Both 100 and 200 µg of folic acid significantly decreased total plasma homocysteine, 400 µg had no further decreasing effect.- Serum folate increased gradually with all three doses.- Red blood cell folate increased over the study period.	A dose of 200 µg of folic acid daily seems as effective as 400 µg daily in lowering total plasma homocysteine. It is not clear whether 100 µg daily in the long term could be as effective as 200 µg.

<i>Authors</i>	<i>Design</i>	<i>Results</i>	<i>Conclusions and comments</i>
Truswell & Kounnavong, 1997	<p>Subjects exp 1 n=13, exp 2 n=16, exp 3 n=6 (n=6 completed all 3 experiments)</p> <p>Design Parallel, consecutive exp (28 days between exp)</p> <p>No prior loading</p> <p>Treatment Subjects received folic acid supplements (in aqueous solution) in addition to their regular diet</p> <p>Exp 1 100 µg for 3 weeks, then 1000 µg for 3 weeks</p> <p>Exp 2 500 µg for 3 weeks, then 1500 µg for 3 weeks</p> <p>Exp 3 1000 µg for 3 weeks, then 2000 µg for 3 weeks</p> <p>Measurement response in serum folate after 2 and 3 weeks</p>	<p>The relative biggest increase was for the first 100 µg of folic acid</p> <p>Serum folates appeared to take longer to reach the highest possible level after small doses of folic acid than after doses of 1000 µg/d or more</p>	<p>The wash out period between the experiments was only four weeks Therefore the starting value of serum folate increased from experiment 1 to 3</p>
Kelly et al 1997	<p>Subjects Exp 1 n=14, Exp 2 n=6 (+5), Exp 3 n=30 elderly, Exp 4 n=16 elderly</p> <p>Design parallel</p> <p>Treatment</p> <p>Exp 1 Subjects received folic acid fortified foods for 5 days (amount of folic acid ranging from 90 µg/d to 1200 µg/d)</p> <p>Exp 2 Each subjects received 400, 300 and 200 µg of folic acid (in isotonic saline), separated by 2-weeks intervals</p> <p>Exp 3 After pretreatment with 400 µg of folic acid for 18 d, each subject (geriatric patients) was given a constant dose of 150 µg to 600 µg in bread for 3 consecutive days</p> <p>Exp 4 Elderly patients routinely received fortified milk and cereals (172-190 µg/d) Each subject was given low-fat milk (200 mL) fortified with folic acid (200 µg/d)</p> <p>Measurements Fasting and post-prandial (2 25 hrs after meal) blood samples were taken Total folate and folic acid were determined in serum</p>	<p>Unaltered folic acid was found in subjects consuming more than 266 µg of folic acid per day</p>	<p>It is not clear what the implications are of having free folic acid in the serum</p>

<i>Authors</i>	<i>Design</i>	<i>Results</i>	<i>Conclusions and comments</i>
Caudill et al 1997 + 1998	Subjects 12 pregnant and 12 nonpregnant women No prior loading Design parallel Treatment Women received daily 120 µg folate/day and either 330 µg or 730 µg folic acid/day for 12 weeks Measurement (1997) Serum folate, red blood cell folate and urinary 5-methyl-PteGlu excretion Measurement (1998) Excretion of folate catabolites, para-aminobenzoylglutamate (pABG) and acetamidobenzoylglutamate (apABG)	Less excretion of folate catabolites in the group receiving 450 µg of folate than in the group receiving 850 µg of folate	The amount of folate of 450 µg seems to be used more efficiently than the amount of 850 µg
Homocysteine Lowering Trialists' Collaboration, 1998	Meta-analysis of randomised trials Subjects Individual data on 1114 people included in 12 trials Design meta-analysis of effects of folic acid based supplements on total plasma homocysteine concentrations Folic acid was provided in various doses (0.4 to 5 mg) in addition to the diet Measurement Total plasma homocysteine	There was no evidence for differences in homocysteine lowering effects between daily doses of < 1 mg (mean dose 0.5 mg), of 1-3 mg, or of > 3 mg folic acid	Doses of 0.5 mg of folic acid seemed as effective in lowering total plasma homocysteine as doses above that level. There were no studies in the meta-analysis investigating doses lower than 0.4 mg folic acid.
Malinow et al 1998	Subjects 75 subjects with coronary artery disease (n=25 per treatment) No prior loading Design Cross-over between treatment and placebo Treatment Subjects received 30 g cereal supplemented with either 127 µg of folic acid, 499 µg of folic acid or 665 µg of folic acid for five weeks. Wash-out period was five weeks Measurement Total plasma homocysteine and plasma folate	All doses significantly increased plasma folate levels. A dose of 499 µg and 665 µg of folic acid decreased total plasma homocysteine significantly by respectively 11 and 14 percent. A dose of 127 µg of folic acid resulted in a non-significant decrease of 3.7 percent.	A wash-out period of 5 weeks is too short for total plasma homocysteine concentrations to return to baseline. It is not clear whether subjects were already taking folic acid fortified foods before the trial started and even during the trial.

<i>Authors</i>	<i>Design</i>	<i>Results</i>	<i>Conclusions and comments</i>
Schorah et al 1998	<p>Subjects 94 healthy volunteers</p> <p>No prior loading</p> <p>Design Parallel</p> <p>Treatment Subjects received either unfortified cereals, or cereals fortified with 200 µg of folic acid, or cereals fortified with 200 µg of folic acid and other vitamins</p> <p>Measurement Total homocysteine, cysteine and vitamin B12 in plasma, serum folate and red blood cell folate at weeks 0, 4, 8 and 24</p>	<p>Folic acid supplementation significantly increased serum folate (66%) and red blood cell folate (24%) and decreased homocysteine (10%) There was no additional effect of the other vitamins in the fortified cereals.</p>	<p>A dose of 200 µg of folic acid in addition to the regular diet was sufficient to significantly decrease total plasma homocysteine concentrations and increase serum and red cell folate</p>
Bonnette et al 1998	<p>Dietary controlled trial</p> <p>Subjects 12 pregnant women (weeks 14 to 26) and 12 nonpregnant women</p> <p>Design Two-by-two factorial</p> <p>No prior loading</p> <p>Treatment All subjects received daily 120 µg food folate and either 330 µg or 730 µg supplemental folic acid for 12 weeks</p> <p>Measurements Total plasma homocysteine weekly</p>	<p>Total plasma homocysteine concentrations in pregnant and nonpregnant women remain constant with both doses within four weeks</p> <p>No significant difference between the doses was shown</p>	<p>No significant differences between the doses were shown</p> <p>However, the power of the study was such that only differences among the groups of more than 3 µmol tHcy could be detected</p>

Matrix

Matrix effects on bioavailability involve effects of encapsulation and binding. Natural food folate can be encapsulated in plant cells or subcellular components. Generally for folic acid added to food binding would be the main problem although in the preparation encapsulation may occur.

Comparison of folate bioavailability among different foods involves besides matrix effects of molecular linkage, species and effect modifiers. However, studies comparing folate bioavailability among foods cannot distinguish between these factors. Matrix is probably the main factor, therefore we have decided to discuss human studies in which folate bioavailability of different foods is compared in this section (Table 7.4).

Retief was one of the first researchers to study effects of different foods on folate bioavailability (Retief, 1969). However, this study is not included in Table 7.4 because he studied only one subject. Studies investigating the effect of food matrix of single foods on the bioavailability of dietary folate showed that folate was absorbed to some extent (Babu and Srikantia, 1976; Keagy *et al.*, 1988; Retief, 1969; Sauberlich *et al.*, 1987; Tamura and Stokstad, 1973; Chapter 6). However, the bioavailability of food folate relative to folic acid differed enormously between products (Babu and Srikantia, 1976; Keagy *et al.*, 1988; Retief, 1969; Sauberlich *et al.*, 1987; Tamura and Stokstad, 1973).

Few studies investigated bioavailability of folate from mixed diets. Sauberlich *et al.* (1987) estimated from a strictly controlled trial that the bioavailability of a mixed diet would be no higher than 50%. Our group (Chapter 6) found a bioavailability of folate from vegetables and citrus fruit of 60 to 98% relative to folic acid, depending on the endpoint chosen. The fact that the folic acid tablets were taken every other day may have overestimated the effect of food folate slightly (Chapter 4). Cuskelly *et al.* (1996) provided dietary folate to women in the free-living situation and found no significant improvement in red blood cell folate concentrations. The small number of participants per group in that study might have affected the results (Cuskelly *et al.*, 1996). Thus, folate from a mixed diet might be absorbed for more than 50%. However, it is difficult to predict how much folate from a mixed diet is absorbed because folate occurs in many different food products. Bioavailability of a mixed diet might also be expected to depend on other factors besides the matrix.

The US Federal Government has introduced mandating fortification with folic acid of flour products as from January 1, 1998. This was accomplished to increase

the folic acid intake of women in the fertile age group because a higher intake lowers the risk of having offspring with a neural-tube defect (US Department of Health and Human Services. Food and Drug Administration, 1996). Therefore, it is important to know the effect of flour and of other food which may be eaten at the same time and to know the bioavailability of folic acid added as fortificant.

Several studies from a research group in Johannesburg (Colman, 1982; Colman *et al.*, 1975; Margo *et al.*, 1975) investigated the effects of maize meal, rice and bread on the bioavailability of folic acid (Table 7.4). They found that the bioavailability of folic acid consumed with bread was 58% (range, 42 - 84%) of that when it was consumed with water (Colman *et al.*, 1975). In contrast, wheat bran was found to stimulate rather than inhibit the serum folate response to feeding folic acid (Keagy *et al.*, 1988). The enhancing effect of bran might be due to endogenous folate in wheat bran, but interaction of bran with folate inhibitors cannot be excluded. Bailey *et al.* (1988) also showed no inhibiting effect of bran on the absorption of folic acid, although bran decreased the absorption of PteGlu7. Pfeiffer *et al.* (1997) used a dual-label stable isotope protocol to determine absorption of folic acid fortified cereal-grain products. No significant differences were shown between white bread, whole wheat bread, rice, pasta or solution. The fact that the between subject variation was high in this study might have considerably affected the results (Pfeiffer *et al.*, 1997).

Table 7.4 Effect of food Matrix of single foods and mixed diets on bioavailability of folate and folic acid

	<i>Design</i>	<i>Results</i>	<i>Conclusions and comments</i>
Tamura & Stokstad, 1973	<p>Human intervention study</p> <p>Subjects n=6 healthy males</p> <p>Design Cross-over</p> <p>Prior loading with folic acid 2 x 10 mg on first day, 5 mg on second day, 2 mg on third day Then 2 mg every other day</p> <p>Treatment Each subject received different foods orange juice, romaine lettuce, romaine lettuce extract, egg yolk, banana, lima beans (dry and frozen), liver, brewer's yeast, brewer's yeast extract, cabbage (cooked and raw), defatted soybean meal, wheat germ</p> <p>Measurement Urinary folate excretion (24 hours)</p>	<p>Bioavailability of food relative to folic acid</p> <p>Orange juice 31% (17-40)</p> <p>Romaine lettuce 25% (12-37)</p> <p>Romaine extract 48% (38-52)</p> <p>Egg yolk 59% (23-129)</p> <p>Banana 82% (0-148)</p> <p>Lima beans (dry, cooked) 70% (0-138)</p> <p>Lima beans (frozen, cooked) 96% (48-181)</p> <p>Liver (cooked) 50% (22-103)</p> <p>Brewer's yeast 60% (55-67)</p> <p>Brewer's yeast extract 63% (59-69)</p> <p>Cabbage (cooked) 47% (0-127)</p> <p>Cabbage (raw) 47% (0-93)</p> <p>Defatted soybean meal 46% (6-83)</p> <p>Wheat germ 30% (0-64)</p>	<p>The large amounts of food, e.g. 500-700 g of cabbage and lettuce may have adversely affected the bioavailability</p> <p>The bioavailability of folate varies considerably between products. For all products is the mean relative bioavailability lower than for folic acid</p>

	<i>Design</i>	<i>Results</i>	<i>Conclusions and comments</i>
Colman et al 1975	<p>Human intervention study</p> <p>Design parallel (intervention period on average 30 days)</p> <p>Subjects women in late stage pregnancy</p> <p>No prior loading</p> <p>Treatment groups provided with a single dose daily</p> <p>control subjects receiving 0 µg of folic acid (n=18)</p> <p>subjects receiving 1000 µg of folic acid in maize meal (n=20)</p> <p>subjects receiving 500 µg of folic acid in maize meal (n=27)</p> <p>Subjects receiving 300 µg of folic acid in maize meal (n=23)</p> <p>Subjects receiving 300 µg of folic acid in tablet (n=34)</p> <p>Measurement Red blood cell folate and serum folate at delivery</p>	<p>Red blood cell folate</p> <p>The slope of the group receiving 500 µg of folic acid in maize meal was similar to that of the group receiving 300 µg of folic acid in a tablet</p> <p>Serum folate</p> <p>The slope of the regression lines in all four intervention groups was significantly greater than in the control group The slopes seemed similar for 300 µg of folic acid in maize, 300 µg of folic acid in tablet and for 500 µg of folic acid in maize</p>	<p>This study suggests that 500 µg of folic acid in maize meal was equally efficient as 300 µg of folic acid in a tablet</p>
Colman, Green & Metz, 1975	<p>Human intervention study</p> <p>Subjects n=7 healthy adults</p> <p>Design Cross-over</p> <p>Prior loading with 15 mg of folic acid daily for 3 days</p> <p>Treatment Subjects received 1 mg of folic acid in either an aqueous solution, or in maize, in bread, or rice for 4 consecutive days</p> <p>Measurement Sum of rise in serum folate 1 and 2 hours after ingesting the folic acid on day 4</p>	<p>Relative bioavailability of fortified products compared to folic acid in aqueous solution</p> <p>Maize 53% (range 32-77%)</p> <p>Rice 58% (42-84%)</p> <p>Bread 38% (18-68%)</p>	<p>This study suggests that folic acid provided in combination with maize, rice or bread takes either a longer time to be absorbed or is less bioavailable than folic acid in a solution</p> <p>The folic acid is supplied to the products before preparation Preparation might have destroyed some of the folic acid</p>

	<i>Design</i>	<i>Results</i>	<i>Conclusions and comments</i>
Margo et al. 1975	Human intervention study. Design. One group compared with Colman et al 1975. Subjects: women in late stage pregnancy (n=15). No prior loading Treatment. Subjects received daily 900 µg of folic acid in bread Measurement Red cell folate concentrations.	The increase in red blood cell folate concentrations was similar to the increase observed in the groups receiving 300 µg of folic acid in a tablet or 500 µg of folic acid in maize meal (Colman et al. 1975).	It is not clear whether the amount of folic acid in the bread was measured before of after processing of the bread.
Babu & Srikantia, 1976	Human intervention study. Subjects. n=10 healthy males. Design. cross-over Prior loading with 5 mg for 6 days, followed by 2 mg every other day + 400 µg of folic acid was provided with each food. Treatment: A single meal of the following foods was provided: Bengal gram (282 µg of folate). Green gram (314 µg of folate). Tomato (300 µg of folate). Spinach (310 µg of folate). Banana (192-252 µg of folate). Egg (210-350 µg of folate) Goat liver (315 µg of folate). Brewer's yeast (300 µg of folate). Measurement: 24 hours urinary excretion (dose response curve).	Availability as measured by dose response curves: Bengal gram: 69% Green gram. 55% Tomato: 37% Spinach: 63% Banana: 46% Egg: 72% Liver: 70% Yeast: 10%	There was considerable variation in availability between the subjects.

	<i>Design</i>	<i>Results</i>	<i>Conclusions and comments</i>
Sauberlich et al 1987	<p>Human intervention study</p> <p>Subjects n=10 healthy nonpregnant women</p> <p>Design parallel/consecutive</p> <p>No prior loading with folic acid</p> <p>Treatment After a depletion period of 28 days subjects received increasing amounts of food folate or synthetic folic acid</p> <p>Measurement plasma and red blood cell folate concentrations, lymphocyte deoxyuridine suppression, neutrophil segmentation and urinary folate excretion</p>	<p>Plasma folate concentrations decreased 60% during the depletion period and continued to decrease until subjects received 200 µg of dietary folate per day</p> <p>An amount of 300 µg of dietary folate was sufficient to increase plasma folate slightly</p> <p>Red blood cell folate concentrations still continued to decrease Dietary folates seemed to be no more than 50% bioavailable compared to synthetic folic acid</p>	<p>This strictly controlled study revealed that dietary folate from a mixed diet was no more than 50% bioavailable compared to folic acid</p>
Keagy, Shane & Oace, 1988	<p>Human intervention study</p> <p>Subjects n=7 healthy young men</p> <p>Design All subjects received all treatments</p> <p>Constant loading with 500 µg of folic acid per day</p> <p>Treatment Four folate absorption tests were conducted during the last four days of each 9 d period in which subjects received either a formula, or wheat bran, or white beans PteGlu and PteGlu7 were given on alternate days during the absorption tests</p> <p>Measurement Increase in serum folate after 1 and 2 hours and 24 hours urine excretion</p>	<p>Adding wheat bran to the formula diet increased the AUC for PteGlu PteGlu7 was not affected by wheat bran Wheat bran increased the absorption of PteGlu relative to PteGlu7 whereas beans minimised the difference between the AUC</p>	<p>The enhancing effect of wheat bran on PteGlu bioavailability could be due to endogenous folate of the wheat bran or from interactions of bran with inhibitors of folate absorption</p>

	<i>Design</i>	<i>Results</i>	<i>Conclusions and comments</i>
Bailey et al , 1988	Human intervention study Subjects Nine healthy males (fasting) Design. Cross-over with two weeks interval No prior loading Treatment: Single dose of 750 µg PteGlu ₇ and equivalent amount of PteGlu ₁ given in solution or with bran or spinach Measurement Area under the curve (AUC) of serum folate for 8 hours	AUC's of PteGlu ₇ and PteGlu ₁ were not different when ingested as solution or with spinach. AUC for PteGlu ₇ ingested with bran was lower than PteGlu ₁ ingested with bran.	This study suggests no effect of chain length when ingested as solution, but an inhibiting effect of dietary fibre on bioavailability of PteGlu ₇ compared to PteGlu ₁ .
Cuskelly, McNulty & Scott, 1996	Human intervention study Design Parallel (3 months) Subjects: 41 women (5 groups). No prior loading Treatment: Subjects received either folic acid supplements (400 µ/d) or folic acid fortified food (additional 400 µg/d), or dietary folate, or dietary advice or nothing (control) for three months Measurement Red cell folate concentrations	Folic acid supplements and foods fortified with folic acid both effectively increased red blood cell folate concentrations, while food folate had no significant effect on red blood cell folate concentrations	The study is based on small numbers of women in each group and the food and tablet intake was not controlled. However, intake of dietary folate was assessed by a validated dietary assessment method.
Pfeiffer et al. 1997	Human intervention study Design: Cross-over. Subjects: 14 adults. No prior loading. Treatment: 1. Subjects received [¹³ C ₅]folic acid in white bread, whole wheat bread, rice or pasta or in solution concurrently with [² H ₂] folic acid intravenously 2. Subjects received [¹³ C ₅]folic acid with or without a light breakfast. Measurement. Urinary excretion. % [¹³ C ₅]folate dose /% [² H ₂] folate.	The bioavailability of [¹³ C ₅]folic acid in the fortified products seemed lower than folic acid in solution, but these differences were not significantly different (<i>P</i> =0.607) The absorption of folic acid seemed slightly lower when consumed after breakfast than without food. This difference was also not significant (<i>P</i> < 0.085).	The bioavailability of [¹³ C ₅]folic acid in fortified cereal grains was high. The between subject variation in this study was high in comparison to studies using prior loading with folic acid. The sensitivity of this protocol was such that only urinary excretion ratios that were 50% of lower of the control could be detected.

	Design	Results	Conclusions and comments
Brouwer et al 1999	Human intervention study Design Parallel, dietary controlled study (4 weeks) Subjects 66 subjects (22 per group) No prior loading Treatment Subjects received either a diet with a normal folate content plus placebo tablets daily (placebo group), or a diet with the same folate content plus 500 µg folic acid and placebo tablets every other day (folic acid group), or a diet high in folate (extra 350 µg/day, dietary folate group) Measurement Change in plasma folate, red blood cell folate and plasma total homocysteine	Compared to folic acid the relative bioavailability was depending on the endpoint plasma folate 78% red blood cell folate 98% plasma homocysteine 60%	Folate from vegetables and fruit decreased total plasma homocysteine concentrations and improved the folate status. The effects are probably slightly overestimated because folic acid tablets were given every other day instead of each day.

Effect modifiers

Effect modifiers are components in foods which influence the amount of a nutrient, folate, reaching the target organ. The effect of folate antagonists and other drugs will not be discussed in this review.

Since the intestinal brush border conjugase is zinc dependent, zinc can be expected to affect folate absorption. However, no studies investigating effects of zinc supplementation on the availability of dietary folate are available. Daily consumption of 3.5 mg or 14.5 mg of zinc in combination with folic acid supplementation for 25 days had no effect on the concentration of folate in serum, red blood cells and urine. This suggests that absorption of folic acid is not influenced by zinc intake (Kauwell *et al.*, 1995).

Certain components in the food may have the ability to inhibit activity of the folate conjugase enzyme and thereby decrease the bioavailability of PteGlu_n. Tomatoes and orange juice inhibited the pteroylglutamate hydrolase (folate conjugase) activity in the human intestine (Bhandari and Gregory, 1990). Furthermore, citrate and to a lesser extent malate and formate, have been shown to affect intestinal brush border conjugase activity in vitro (Wei and Gregory, 1998). This suggests that organic acids affect the absorption of dietary polyglutamate folate by interfering with the intestinal deconjugation of the glutamate chain.

Alcohol could be another effect modifier. Folate deficiency is prevalent among chronic alcoholic patients which often have an inadequate dietary intake of minerals and vitamins. However, alcohol may also affect folate absorption (Halsted, 1995). In alcoholic pigs hydrolysis of pteroylpolyglutamates seems to be disturbed by intake of ethanol (Naughton *et al.*, 1989; Reisenauer *et al.*, 1989). This has not been confirmed in human studies although ethanol ingestion in five chronic alcoholic patients increased urinary excretion of folic acid (Russell *et al.*, 1983). In combination with a diet deficient in folate, intake of ethanol decreased the uptake of folic acid in alcoholic subjects (Halsted *et al.*, 1971). In normal, non-alcoholic, subjects ingestion of ethanol also decreased plasma folate concentrations (Eichner and Hillman, 1973). Thus, alcohol seems to affect folate bioavailability.

Nutrient status

The folate, vitamin B12 and zinc status of the host might influence folate bioavailability. Only a few studies investigated the effect of folate status on folate bioavailability. Babu & Lakshmaiah (1987) showed no effect of folate deficiency on jejunal conjugase activity in rats. To our knowledge, there are no studies available comparing folate bioavailability in folate deplete and folate replete subjects. Although the study of Bower *et al* (1993) shows that the rise in serum folate after a pteroylpolyglutamate load (4.5 mg PteGlu7) is higher in subjects with higher serum folate compared to subjects with lower baseline serum folate levels. This might be explained by a longer circulation of folate in serum of replete subjects. This would imply that in deplete subjects folate is rapidly removed from the serum to the tissues (Bower *et al*, 1993).

Distribution of folate over the tissues changes during folate deficiency. Livers of folate deficient rats contain more polyglutamates of higher chain length than folate replete rats (Cassady *et al*, 1980, Varela-Moreiras and Selhub, 1992, Ward and Nixon, 1990). Folate concentrations decrease and chain length increases in liver, spleen and kidney in folate deficient rats, but both concentrations and chain length are the same in brain of folate deficient and folate replete rats (Richardson *et al*, 1979). Thus, folate status affects folate distribution over the tissues, but it is not clear whether it also affects folate bioavailability.

Vitamin B12 can influence folate bioefficacy since it is directly interrelated with folate. Methylcobalamin serves as a co-factor for methionine synthase, the enzyme responsible for the remethylation of homocysteine into methionine. In the same reaction is 5-methyl-tetrahydrofolate demethylated into tetrahydrofolate (Savage and Lindenbaum, 1995). In cobalamin deficiency 5-methyltetrahydrofolate cannot be converted to tetrahydrofolate. This lack of formation of tetrahydrofolate is named "the methyl folate trap". The lack of tetrahydrofolate has consequences for the formation of other folate coenzymes (Herbert and Zalusky, 1962). Although this theory has been criticised and many variations of the theory have been suggested (Savage and Lindenbaum, 1995), all these variations suggest that vitamin B12 deficiency influences folate bioefficacy because it changes the distribution of the different folate forms. This influences also the overall folate status because tetrahydrofolate is a much better substrate than 5-methyltetrahydrofolate for the enzyme folate polyglutamate synthetase. This enzyme is needed for formation of polyglutamates (Cichowicz and Shane, 1987a, Cichowicz and Shane, 1987b). Polyglutamyl folates

are better retained in cells and are more effective co-enzymes than monoglutamyl folates (Lowe *et al.*, 1993).

A sufficient supply of zinc is known to be important in folate bioavailability. Tamura *et al.* (1978) showed that zinc depletion reduced the rise in serum folate after supplementation with pteroylheptaglutamate by 53% while absorption of the monoglutamate form seemed unaffected. This suggested that intestinal pteroylpolyglutamate hydrolase is zinc dependent and that zinc depletion inhibits hydrolysis of polyglutamates. Chandler, Wang and Halsted (Chandler *et al.*, 1986) confirmed the zinc dependency of the brush border folate hydrolase. Tamura reviewed in 1995 the literature concerning the nutrient interaction of folate and zinc. He concluded that although pteroylpolyglutamate hydrolase is zinc dependent its clinical significance is not clear.

Genetic factors

Some genetic mutations are known to influence folate metabolism. This section will discuss some common occurring genetic factors influencing folate bioavailability and efficacy.

In mice the RFC-1 gene expression regulates the pH-dependent folate absorption in the small intestine (Chiao *et al.*, 1997). The organisation and structure of the human RFC-1 gene that encodes for a folate transporter is determined as well (Tolner *et al.*, 1998). However, the significance of this gene for folate absorption needs further investigation.

Another gene that is linked to the folate status is the gene encoding for methylenetetrahydrofolate reductase (MTHFR). A variant of MTHFR was found to have lower specific activity and lower sensitivity to heat (Kang *et al.*, 1988). This thermolabile variant is caused by an alanine-to-valine missense mutation (Goyette *et al.*, 1994). Jacques *et al.* (1996) demonstrated that individuals homozygous for this mutation with lower plasma folate concentrations (<15.4 nmol/L) had 24% higher fasting plasma total homocysteine concentrations than individuals with the normal genotype and similar plasma folate concentrations. No difference between genotypes was seen among individuals with plasma folate concentrations ≥ 15.4 nmol/L. They suggested that individuals homozygous for this polymorphism need more folate to regulate their plasma homocysteine concentrations (Jacques *et al.*, 1996). This implies that the bioefficacy of folate is diminished by this polymorphism when the folate status is not optimal.

Methionine synthase is the enzyme involved in the remethylation reaction from homocysteine to methionine. To our knowledge, no polymorphisms in the gene encoding for the methionine synthase enzyme have been shown to influence the folate status or bioefficacy.

Host-related factors

Host-related factors are factors of the host besides the nutrient status and the genetic component that could influence bioavailability or bioefficacy. Examples of such factors are age, pregnancy, illness and malabsorption.

Bailey *et al.* (1984) investigated the absorption of poly- and monopteroylglutamates in different age groups. They found no indication that either the absorption or the activity of the folate conjugase was affected by age (Bailey *et al.*, 1984).

Pregnancy increases the demand for folate. This higher demand can be explained by an accelerated folate breakdown (Kownacki Brown *et al.*, 1993; McPartlin *et al.*, 1993). However, Caudill *et al.* (1998) found in a dietary controlled trial no significant difference in catabolism between pregnant women and nonpregnant controls. Their study suggested that pregnant women made more efficient use of an amount of 450 µg of folic acid than of 850 µg of folic acid (Caudill *et al.*, 1998). The same group (Caudill *et al.*, 1997) found also no differences in serum folate or red blood cell folate response or in excretion of urinary 5-methylTHF after supplementation with 450 µg and 850 µg of folate per day. Thus, it is not clear what causes the higher demand for folate during pregnancy.

Two randomised trials showed that folic acid supplementation in the periconceptional period reduces the risk of having offspring with neural-tube defects (Czeizel and Dudás, 1992; MRC Vitamin Study Research Group, 1991). A decreased capability for the absorption of folate in the mother has been suggested as a cause for the folate-related cases of neural-tube defects. However, Bower *et al.* (1993) showed that intestinal hydrolysis of pteroylpolyglutamates was not impaired in women who previously had a child with a neural-tube defect. Moreover, Davis *et al.* (1995) found no difference in the absorption of folic acid between mothers with and without a history of a neural-tube defect. In contrast, Neuhouser *et al.* (1998) found that women who previously gave birth to a child with a neural-tube defect needed a larger dose of folic acid or folate to elicit a plasma response equivalent to the general

population. Thus, a diminished bioavailability of folate in the mother could be a cause for neural-tube defects in their offspring.

Halsted (1990) summarised studies from his group that investigated the effect of gastrointestinal diseases on the absorption of [^3H]PteGlu and [^{14}C]PteGlu. Absorption of [^3H]PteGlu and [^{14}C]PteGlu was not affected by ulcerative colitis. However, uptake of both PteGlu and PteGlu was diminished in tropical and celiac sprue patients.

The saturable folate transport system in the jejunum is pH dependent. Thus, folate bioavailability is pH dependent. Folate transport has an acidic pH optimum. This subject has been extensively reviewed in the past (Halsted, 1979, Mason, 1990).

Mathematical Interactions

Mathematical interactions account for the difference in effect observed when two or more factors taken together have an effect that is different from the sum of the effect of the separate factors. To our knowledge there are no articles in which this complicated problem has been studied.

Conclusion

Various factors can influence bioavailability of nutrients. Of the factors influencing bioavailability of folate and folic acid two stand out in effect: the effect of the food matrix and the amount of folic acid consumed. Bioavailability of folate from some foods is less than 50% of that of folic acid. The most likely explanation for this would be matrix factors: encapsulation and binding. However, matrix effects can often not be distinguished from other factors. Other factors include the form and chain length of folate in food. Food folate can have various one-carbon groups and these various folate forms can have a chain length of 1-7 glutamates. Although some studies suggest that the form of folate affects bioavailability this seems to be only a minor factor. Chain length might indeed affect bioavailability: studies in this review suggest that polyglutamates have a lower bioavailability than monoglutamates. However, we think that differences in chain length can explain at most half of the difference in bioavailability between food folate and folic acid. Bioavailability might also be influenced by other factors in food, such as organic acids. Indeed, organic acids

inhibited the conjugase responsible for the breakdown of polyglutamates into absorbable monoglutamates *in vitro*. This suggestion that organic acids decrease the bioavailability of folate has to be confirmed by *in vivo* studies. On the basis of the studies in this review we conclude that matrix is the main factor influencing bioavailability.

The amount of folic acid consumed seems to be a very important factor as well. The bioavailability of supplemental folic acid is good. However, the homocysteine-lowering capacity of doses of folic acid above 500 µg is limited and it is not clear whether unmetabolised folic acid poses health risks. This is of importance, because food fortification is implemented in many countries nowadays and folic acid tablets are freely available.

In particular circumstances, host-related factors, such as illness and pH of the jejunum can influence bioavailability. Genetic factors also deserve our attention for future research. Mutations in certain genes might influence folate bioavailability. In this respect, we should not only search for mutations, but also investigate the clinical implication and possible therapies to overcome the negative impact of such mutations.

The present techniques used to measure bioavailability make quantification of the effect of the various factors very difficult. The accurateness of most techniques depends on a steady state situation in the body. To establish this, most studies have used a folic acid preloading scheme to saturate tissues with folic acid. Saturation of the tissues reduces the intra-individual variation in the responses to treatment. However, it is not clear how this affects bioavailability. Therefore, further development of techniques such as the stable isotope techniques is needed.

In conclusion, food matrix and the amount of folic acid consumed are the major factors influencing bioavailability in healthy people. Food manufacturers can play an important role in increasing the bioavailability. Development of new food preparation methods could change the food matrix in a way that folate will be more bioavailable. Many people do not consume sufficient folate (Brussaard *et al.* 1995). Better bioavailability of food folate would make it easier for people to reach a sufficient level of intake. Folate status can also be improved by increasing the intake of folic acid. This could be established by consumption of foods fortified with folic acid and of folic acid tablets. However, intakes above 500 µg per day have no additional improving effect on the functional parameter of folate status, plasma total homocysteine concentrations. Moreover, it is unclear whether such doses pose health risks. Therefore, intakes of high doses of folic acid should be avoided.

8

General Discussion

Folic acid, folate and homocysteine

This thesis describes intervention studies with low doses of supplemental folic acid and dietary folate. The effects of supplementation with low doses of folic acid on folate status and plasma total homocysteine concentrations in healthy volunteers and spina bifida patients are discussed. Bioavailability of food folate is the next issue.

The main conclusions, recommendations and possibilities for future research are presented and the thesis ends with the concluding remarks.

Low-dose folic acid supplementation

Healthy volunteers

Intervention studies with healthy young women with normal baseline levels of total plasma homocysteine (Chapters 2, 3 and 4) show that supplementation with low doses of folic acid improves the folate status and decreases total plasma homocysteine concentrations. Previous research had shown that supplementation with high doses of folic acid, often in combination with other vitamins and minerals, improved folate status and decreased total plasma homocysteine concentrations. Most of the earlier studies focused on subjects with hyperhomocysteinemia (Franken *et al.*, 1994; Naurath *et al.*, 1995; Ubbink, 1997; Ubbink *et al.*, 1994; van den Berg *et al.*, 1994). The studies described in Chapters 2, 3 and 4 and the Homocysteine Lowering Trialists' (1998) showed that supplementation with doses of folic acid between 250 and 500 µg per day can also decrease plasma total homocysteine concentrations in healthy people with homocysteine concentrations in the normal range.

Our study investigating effects of low doses of folic acid (Chapter 2) and the study of Ward *et al.* (1997) showed that the extent of decrease in total plasma homocysteine concentrations depends on the baseline concentration of total plasma homocysteine. Higher baseline concentrations of total plasma homocysteine result in a more dramatic decrease in total plasma homocysteine after folic acid supplementation than lower baseline concentrations (Brouwer *et al.*, 1999; Ward *et al.*, 1997). In our study, the effect could not be due to regression to the mean because it was not shown in the placebo group.

Folic acid supplementation is most likely to stimulate the remethylation from homocysteine into methionine because 5-methyltetrahydrofolate is the methyl donor

in this reaction (Ueland *et al* , 1993) Therefore, an increase in plasma methionine concentrations could have been expected after folic acid supplementation However, our study (Chapter 3) could not show such an increase In contrast, Guttormsen *et al* (1996b) showed an increase in plasma methionine after supplementation with folic acid or injections with vitamin B12 However, the subjects in that study were deficient in either folate or vitamin B12 and used a much higher dose of folic acid (Guttormsen *et al* , 1996b) Thus, the discrepancy in results might be due to the dose of folic acid used in our study (500 µg/d or 500 µg every other day) and the fact that the subjects were in a good vitamin status However, with respect to plasma total homocysteine the status of our subjects seemed not optimal because folic acid did decrease their plasma total homocysteine concentrations

Kelly *et al* (1997) showed that unmetabolised folic acid appears in serum after ingestion of a dose of folic acid of more than 266 µg The study described in Chapter 4 suggests that 250 µg folic acid taken each day decreases plasma total homocysteine more effectively than 500 µg folic acid taken every other day This observation together with the findings of Kelly *et al* (1997) indicates that unaltered folic acid has no homocysteine lowering capacity Therefore, low doses of folic acid should be recommended to be taken each day or divided over the day (food fortification) to lower plasma total homocysteine concentrations

Spina Bifida patients

In Chapter 5 were no apparent differences between spina bifida and disabled control subjects in plasma and red blood cell folate, in plasma vitamin B12 or plasma total homocysteine concentrations at baseline The outcomes of earlier studies were not conclusive with respect to differences in plasma total homocysteine concentrations in patients with a neural-tube defect and control subjects Some studies showed higher fasting plasma total homocysteine concentrations in spina bifida than in control subjects (Bjørke-Monsen *et al* , 1997, van der Put *et al* , 1997b), whereas other studies found no significant differences in the folate and vitamin B12 status of fetuses affected with a neural-tube defect (Bunduki *et al* , 1995, Economides *et al* , 1992, Thorand *et al* , 1996) Bjørke-Monsen *et al* (1997) showed that the differences in total plasma homocysteine concentrations between the spina bifida and the control group were confined to those patients with one or two C677T-mutated alleles in the methylenetetrahydrofolate reductase gene Furthermore, plasma total homocysteine concentrations are only increased in those subjects homozygous for the C677T mutation with a plasma folate concentration lower than 15.4 nmol/L (Jacques *et al* , 1996) Thus, a sufficient intake of folate can counteract

the increased risk of the C677T mutation. Repeating the statistical analysis with exclusion of those subjects with two mutated C677T alleles did not change the outcome of our study (Chapter 5).

Our study (Chapter 5) is the first to investigate effects of four weeks of supplementation with 500 µg of folic acid per day on plasma total homocysteine concentrations in spina bifida patients. This intervention strategy seemed as effective in lowering plasma total homocysteine concentrations and improving the folate status in spina bifida patients as in control subjects. We have no indication that an impaired folate and homocysteine metabolism in spina bifida patients is an important cause for their neural-tube defect. Therefore, our study (Chapter 5) provides no new insights on the mechanism by which inadequate supply of folate causes spina bifida. We suggest that either a higher need for folic acid in the mother, or a defect in intra-uterine folate transport from mother to child are more important causes for neural-tube defects than a defect in the homocysteine metabolism of the affected child itself.

Bioavailability of folate

In Chapter 2 we show that it is possible to decrease plasma total homocysteine concentrations with low doses of folic acid in healthy volunteers. This suggested that there could be possibilities for successful lowering of plasma total homocysteine concentrations with dietary folate. A controlled dietary experiment (Chapter 6) shows that dietary folate can improve the folate status and decrease plasma total homocysteine concentrations in healthy volunteers. In contrast, Cuskelly *et al.* (1996) found no significant improvement of red blood cell folate concentrations after 3 months of intervention with dietary folate. However, our study focused on folate from vegetables and citrus fruit only whereas the study of Cuskelly *et al.* (1996) used a broader range of folate rich products. Besides, Cuskelly *et al.* (1996) studied whether supplying subjects with folate rich foods in the free living situation would improve their folate status, whereas our study is an efficacy study. This implied that the diets were much stricter controlled. In addition, the study in Chapter 6 consisted of 22 subjects per group instead of no more than 10 per group in the study of Cuskelly *et al.* (1996). Sauberlich *et al.* (1987) concluded from changes in plasma folate in healthy volunteers in a very strictly controlled trial that the bioavailability of folate from a mixed diet was no more than 50%. We found in our study, folate from vegetables and citrus fruit to be between 60 and 98% bioavailable compared to the

folic acid treatment. The bioefficacy, using changes in total plasma homocysteine as endpoint, was 60%. The bioavailability was 78% for changes in plasma folate, and 98% for changes in red blood cell folate concentrations. However, in this dietary intervention study we supplied folic acid tablets every other day instead of each day. The study in which tablets were taken every day versus a double dose every other day (Chapter 4) suggests that 250 µg folic acid taken each day more effectively decreases plasma total homocysteine than 500 µg of folic acid taken every other day. Therefore, we might have underestimated the effect of folic acid in our controlled dietary experiment (Chapter 6). By doing so we overestimated the relative bioavailability of dietary folate from vegetables and citrus fruit. Even though, the results of Chapter 6 show that dietary folate from vegetables and citrus fruit improved the folate status and decreased plasma total homocysteine concentrations in healthy volunteers.

Chapter 6 and other studies suggest that food folate is less bioavailable than folic acid (Cuskelly *et al.*, 1996; Sauberlich *et al.*, 1987). Many studies have investigated factors affecting the bioavailability of folate. These studies are reviewed in Chapter 7. Two factors stand out in effect on folate bioavailability: food matrix and amount of folic acid consumed. The length of the glutamate chain of the folate molecule is likely to play a role as well. Polyglutamates seem to be less bioavailable than monoglutamates.

The present techniques used to measure bioavailability make quantification of the effects of the various factors difficult. Therefore, further development of techniques, such as stable isotope techniques will be very useful for future research.

Main conclusions

The main conclusions from the intervention studies are the following:

- Low-dose folic acid supplementation (250 or 500 µg/day) improves folate status and decreases total plasma homocysteine concentrations in healthy subjects.
- The homocysteine and folate metabolism of spina bifida patients does not appear to differ drastically from control patients. Although the C677T mutation in the methylenetetrahydrofolate reductase gene may contribute to the occurrence of neural-tube defects. Furthermore, supplementation with low-doses of folic acid seems as effective in spina bifida patients as in control subjects.
- Dietary folate from vegetables and citrus fruit also has the potential to improve the folate status and to lower total plasma homocysteine concentrations. The relative effect on plasma total homocysteine (bioefficacy) of dietary folate from vegetables and citrus fruit was 60%. The relative bioavailability was 78% for changes in plasma folate and 98% for changes in red blood cell folate.

Implications

Folic acid/ folate for the prevention of neural-tube defects

Earlier studies have clearly shown that additional folic acid taken in the period around the conception lowers the risk of having offspring with a neural-tube defect (Czeizel and Dudas, 1992, MRC Vitamin Study Research Group, 1991) Other studies suggest that elevated homocysteine concentrations are a risk factor for having offspring with a neural-tube defect (Mills *et al* , 1995, Steegers Theunissen *et al* , 1994) We showed that additional dietary folate from vegetables and citrus fruit, as well as low-doses of folic acid decrease total plasma homocysteine concentrations in healthy volunteers In spite of this finding, women planning to become pregnant should still be advised to take 400 to 500 µg of synthetic folic acid in addition to their habitual diet from at least four weeks before conception until the eighth week of pregnancy (Food and Nutrition Council, 1993)

There are a few reasons why this advice to take supplemental folic acid is still valid Firstly, synthetic folic acid has in randomised trials been proven to prevent neural-tube defects (Czeizel and Dudas, 1992, MRC Vitamin Study Research Group, 1991) Dietary folates may prevent neural-tube defects, but have not been proven to do so Some investigators even report a diminished absorption of dietary folate in women with prior pregnancies with a neural-tube defect (Neuhouser *et al* , 1998) It is also no longer possible to test whether a high intake of dietary folate prevents neural-tube defects because it would be unethical to withhold folic acid from women in the critical period around the conception For the same ethical reasons it also no longer possible to investigate the minimal daily dose of folic acid that prevents neural-tube defects Secondly, the Netherlands Food and Nutrition Council advises women planning to become pregnant to take 400 to 500 µg of folic acid daily in addition to the dietary intake (Food and Nutrition Council, 1993) Therefore, even if a higher intake of dietary folate would also be effective in preventing neural-tube defects, it would be very hard for women to take an equivalent amount of folate with their diet Thirdly, intake of folic acid in the right period is expected to prevent more than 50% of the neural-tube defect cases (MRC Vitamin Study Research Group, 1991) The underlying mechanism of these folic acid preventable neural-tube defects is not clear The homocysteine-lowering potential of folic acid might play a role in some cases, but other factors may play a role as well Therefore, although we show that dietary folate decreases plasma total homocysteine concentrations, it does not prove that neural-tube defects can be prevented in this manner

On the other hand, although women are advised to take additional folic acid in the period around conception this advice is not always followed (de Jong-van den Berg *et al.*, 1998; Sayers *et al.*, 1997). For women who do not take extra folic acid it may be worthwhile to take as much dietary folate as possible. To increase the chance that women who become pregnant receive sufficient folic acid to prevent neural-tube defects, the US government has decided to fortify flour with folic acid (US Department of Health and Human Services, Food and Drug Administration, 1996).

Vegetables and fruit are not only good sources of folate, they also have other health promoting components. Therefore, women planning to become pregnant should be advised to take 400 to 500 µg of folic acid and to follow the dietary guidelines as suggested by the Netherlands Health and Nutrition Council (Food and Nutrition Council, 1993; Voedingsraad, 1992). These guidelines include an advice to eat at least 200 grams of vegetables and 2 servings of fruit (Voedingsraad, 1992).

Folic acid /folate for the prevention of cardiovascular disease

Patients with cardiovascular diseases have been shown to have higher total plasma homocysteine concentrations than healthy control subjects (Clarke *et al.*, 1991; den Heijer *et al.*, 1996; Falcon *et al.*, 1994; Pancharuniti *et al.*, 1994; Selhub *et al.*, 1995; Stampfer *et al.*, 1992). Furthermore, the relationship between plasma total homocysteine and cardiovascular disease seems to be graded with no apparent threshold (Refsum *et al.*, 1998). Moreover, an elevated plasma total homocysteine concentration is besides a strong independent risk factor an even stronger risk factor in combination with the established risk factors smoking and hypertension (Graham *et al.*, 1997). Intervention studies have shown that supplementation with folic acid lowers plasma total homocysteine concentrations (Chapter 2 and 4 of this thesis, (Homocysteine Lowering Trialists' Collaboration, 1998; Ward *et al.*, 1997). Therefore, an elevated plasma total homocysteine concentration seems an important risk factor that can easily be treated with low doses of B-vitamins. However, studies investigating whether homocysteine lowering therapies decrease the risk of cardiovascular disease have not been published, but are currently underway. Therefore, it is too early to advise the whole population to take extra folic acid daily. Of course, the whole population should still be advised to eat according to the dietary guidelines of the Food and Nutrition Council (Voedingsraad, 1992). This implies a sufficient intake of fruits and vegetables. Fruits and vegetables are good sources of folate which help to decrease plasma total homocysteine concentrations in healthy volunteers (Chapter 6). Lowering of plasma total homocysteine concentrations might

decrease the risk of cardiovascular disease. Besides, fruits and vegetables contain other health promoting factors.

Future studies

Neural-tube defects

Folic acid supplementation in the period around conception prevents neural-tube defects. However, the question, how and why folic acid prevents the neural-tube defects is very hard to answer, especially because placebo controlled trials cannot be executed anymore for ethical reasons. Therefore future research on the issue of folate and neural-tube defects should focus on:

- The development of a good animal model for the aetiology of the neural-tube defect. Such a model would be an enormous step forward. However, a main drawback of such a model is that the results cannot always be transferred to human beings (Barber *et al.*, 1998). Of course, good in vitro models can also provide important new insights.
- The search for other genetic mutations, besides the MTHFR C677T mutation, that might have a relationship with neural-tube defects. In this search it is important to not only detect the mutation, but also to investigate the clinical implications of the mutation and whether therapy can overcome the negative impact of the mutation.

Cardiovascular disease

The relationship between cardiovascular disease, folate and homocysteine is the next issue to be solved. Do homocysteine-lowering intervention therapies indeed decrease the risk of cardiovascular disease? Trials investigating this issue are currently underway. Unfortunately, it will take at least 4 or 5 more years before results of these trials will be available. Many of these trials are performed in countries where folic acid fortified food products are available. This implies that subjects in the placebo group will receive folic acid from these foods. Chapter 7 (**A**mount of folate) suggests that the first 100 or 200 µg of folic acid are expected to lower homocysteine concentrations most effectively. Therefore, trials executed in countries where fortified foods are available are at risk of finding a null effect. Thus, it is important that trials will also be performed in countries, such as the Netherlands, where folic acid fortified foods are not on the market.

Further research in the field of folate, homocysteine and cardiovascular disease should focus on:

- The dose response relationship between the vitamins involved in the homocysteine metabolism and plasma total homocysteine concentrations. It is important to find the lowest dose that effectively lowers plasma total homocysteine especially because the effects of unmetabolised folic acid in the blood are unknown (Kelly *et al.*, 1997)
- The investigation of other homocysteine-lowering therapies than supplementation with folic acid. Some studies have investigated effects of vitamin B6 and vitamin B12 mainly in combination with folic acid (Ubbink, 1997). However, very few studies have investigated effects of riboflavin, which donates a prosthetic group to methylenetetrahydrofolate reductase, or betaine, which is involved in the alternate remethylation pathway (homocysteine into methionine).
- The aetiology of the relationship between homocysteine and cardiovascular disease. Even if intervention with folic acid decreases the risk of cardiovascular disease, the question remains whether homocysteine is the causal factor or just an intermediary factor. However, if other homocysteine-lowering therapies would also decrease the risk of cardiovascular disease this would indicate a causal relationship.

Concluding remarks

Intake of low doses of folic acid in addition to the habitual diet and a high intake of dietary folate from vegetables and citrus fruits improves the folate status and decreases plasma total homocysteine concentrations in healthy people. This may lower the risk of having offspring with a neural-tube defect and cardiovascular diseases. The fact that this can be established by intake of folate from vegetables and citrus fruits provides another argument for advising the general population to increase their intake of vegetables and fruits. However, women planning to become pregnant should still take supplemental folic acid as well. Folate from foods may prevent neural-tube defects, but folic acid supplements have been proven to prevent these defects.

We found no indication that an impaired folate and homocysteine metabolism in spina bifida patients is an important cause for their neural-tube defect. We suggest that either a higher need for folic acid in the mother, or a defect in intra-uterine folate transport from mother to child are more important causes for neural-tube defects than a defect in the homocysteine metabolism of the affected child itself.

References

- Andersson, A , Brattstrom, L , Israelsson, B , Isaksson, A , and Hultberg, B (1990) The effect of excess daily methionine intake on plasma homocysteine after a methionine loading test in humans *Clin Chim Acta* **192**, 69-76
- Araki, A and Sako, Y (1987) Determination of free and total homocysteine in human plasma by high performance liquid chromatography with fluorescence detection *J Chromatogr* **422**, 43-52
- Armstrong, M D and Stave, U (1973) A study of plasma free amino acid levels II Normal values for children and adults *Metabolism* **22**, 561-569
- Babu, S and Lakshmaiah, N (1987) Availability of food folate by liver folate repletion in rats *Nutr Reports Int* **35**, 831-836
- Babu, S and Srikantha, S G (1976) Availability of folates from some foods *Am J Clin Nutr* **29**, 376-379
- Bailey, L B , Barton, L E , Hillier, S E , and Cerda, J J (1988) Bioavailability of mono and polyglutamyl folate in human subjects *Nutr Reports Int* **38**, 509-518
- Bailey, L B , Cerda, J J , Bloch, B S , Busby, M J , Vargas, L , Chandler, C J , and Halsted, C H (1984) Effect of age on poly- and monoglutamyl folacin absorption in human subjects *J Nutr* **114**, 1770-1776
- Barber, R C , Shaw, G M , Lammer, E J , Greer, K A , Biela, T A , Lacey, S W , Wasserman, C R , and Finnell, R H (1998) Lack of association between mutation in the folate receptor-alpha gene and spina bifida *Am J Med Genet* **76**, 310-317
- Bhandari, S D and Gregory, J F (1990) Inhibition by selected food components of human and porcine intestinal pteroylpolyglutamate hydrolase activity *Am J Clin Nutr* **51**, 87-94
- Bhandari, S D and Gregory, J F (1992) Folic acid, 5-methyl-tetrahydrofolate and 5-formyl-tetrahydrofolate exhibit equivalent intestinal absorption, metabolism and in vivo kinetics in rats *J Nutr* **122**, 1847-1854
- Björke-Monsen, A L , Ueland, P M , Schneede, J , Vollset, S E , and Refsum, H (1997) Elevated plasma total homocysteine and C677T mutation of the methylenetetrahydrofolate reductase gene in patients with spina bifida *QJM* **90**, 593-596
- Bonnette, R E , Caudill, M A , Boddie, A M , Hutson, A D , Kauwell, G P A , and Bailey, L B (1998) Plasma homocyst(e)ine concentrations in pregnant and nonpregnant women with controlled folate intake *Obstet Gynecol* **92**, 167-170
- Boushey, C J , Beresford, S A , Omenn, G S , and Motulsky, A G (1995) A quantitative assessment of plasma homocysteine as a risk factor for vascular disease Probable benefits of increasing folic acid intakes *JAMA* **274**, 1049-1057
- Bower, C , Stanley, F J , Croft, M , de Klerk, N , Davis, R E , and Nicol, D J (1993) Absorption of pteroylpolyglutamates in mothers of infants with neural tube defects *Br J Nutr* **69**, 827-834

- Brants, H A M and Hulshof, K F A M (1995) *De ontwikkeling van een voedingsmiddelentabel met foliumzuurgehaltes (development of a food table with folic acid values)*, Zeist, the Netherlands, TNO-Nutrition
- Brattstrom, L E , Israelsson, B , Jeppsson, J O , and Hultberg, B L (1988) Folic acid--an innocuous means to reduce plasma homocysteine *Scand J Clin Lab Invest* **48**, 215-221
- Brouwer, I A , van Dusseldorp, M , Thomas, C M G , Duran M, Hautvast, J G A J , Eskes, T K A B , and Steegers Theunissen, R P M (1999) Low-dose folic acid supplementation decreases plasma homocysteine a randomized trial *Am J Clin Nutr* **69**, 99-104
- Brown, J P , Scott, J M , Foster, F G , and Weir, D G (1973) Ingestion and absorption of naturally occurring pteroylmonoglutamates (folates) in man *Gastroenterology* **64**, 223-232
- Brussaard J H , van den Berg H , Brants H A M , van Loon C J A M , and Lowik, M R H *Folate intake and status among adults in the Netherlands (Dutch Nutrition Surveillance System) Descriptive Statistics*, Zeist, the Netherlands, TNO-Nutrition
- Bunduki, V , Dommergues, M , Zittoun, J , Marquet, J , Muller, F , and Dumez, Y (1995) Maternal-fetal folate status and neural tube defects a case control study *Biol Neonate* **67**, 154-159
- Campbell, N R C (1996) How safe are folic acid supplements? *Arch Intern Med* **156**, 1683-1644
- Cassady, I A , Budge, M M , Healy, M J , and Nixon, P F (1980) An inverse relationship of rat liver folate polyglutamate chain length to nutritional folate sufficiency *Biochim Biophys Acta* **633**, 258-268
- Caudill, M A , Cruz, A C , Gregory, J F , Hutson, A D , and Bailey, L B (1997) Folate status response to controlled folate intake in pregnant women *J Nutr* **127**, 2363-2370
- Caudill, M A , Gregory, J F , Hutson, A D , and Bailey, L B (1998) Folate catabolism in pregnant and nonpregnant women with controlled folate intakes *J Nutr* **128**, 204-208
- Centers for Disease Control (1992) Recommendations for the use of folic acid to reduce the number of cases of spina bifida and other neural tube defects *Morbidity and Mortality Weekly Report* **41**, 1-7
- Chandler, C J , Wang X L , and Halsted, C H (1986) Pteroylpolyglutamate hydrolase from human jejunal brush borders, purification and characterization *J Biol Chem* **261**, 928-933
- Chiao, J H , Roy, K , Tolner, B , Yang, C -H , and Sirotnak, F M (1997) RFC-1 gene expression regulates folate absorption in mouse small intestine *J Biol Chem* **272**, 11165-11170
- Cichowicz D J and Shane, B (1987a) Mammalian folypoly-gamma-glutamate synthetase 1 Purification and general properties of the hog liver enzyme *Biochemistry* **26**, 504-512
- Cichowicz, D J and Shane, B (1987b) Mammalian folypoly-gamma-glutamate synthetase 2 Substrate specificity and kinetic properties *Biochemistry* **26**, 513-521
- Clarke, R , Daly, L E , Robinson, K , Naughten, E , Cahalane, S , Fowler, B , and Graham, I (1991) Hyperhomocysteinemia An independent risk factor for vascular disease *N Engl J Med* **324**, 1149-1155

- Colman, N (1982) Addition of folic acid to staple foods as a selective nutrition intervention strategy *Nutr Rev* **40**, 225-233
- Colman, N , Green, R , and Metz, J (1975) Prevention of folate deficiency by food fortification II. Absorption of folic acid from fortified staple foods *Am J Clin Nutr* **28**, 459-464
- Cuskelly, G J , McNulty, H , and Scott, J M (1996) Effect of increasing dietary folate on red-cell folate implications for prevention of neural tube defects *Lancet* **347**, 657-659
- Czeizel, A E and Dudás, I (1992) Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation *N Engl J Med* **327**, 1832-1835
- Davis, B A , Bailey, L B , Gregory, J F , Toth, J P , Dean, J , and Stevenson, R E (1995) Folic acid absorption in women with a history of pregnancy with neural tube defect *Am J Clin Nutr* **62**, 782-784
- de Bree, A , van Dusseldorp, M , Brouwer, I A , van het Hof, K H , and Steegers Theunissen, R P M (1997) Folate intake in Europe recommended, actual and desired intake *Eur J Clin Nutr* **51**, 643-660
- de Jong-van den Berg, L T , de Walle, H E , van der Pal-de Bruin, K M , Buitendijk, S E , and Cornel, M C (1998) Increasing awareness of and behaviour towards periconceptional folic acid consumption in The Netherlands from 1994 to 1995 *Eur J Clin Pharmacol* **54**, 329-331
- de Pee, S and West, C E (1996) Dietary carotenoids and their role in combating vitamin A deficiency a review of the literature *Eur J Clin Nutr* **50 Suppl 3**, S38-S53
- den Heijer, M , Brouwer, I A , Bos, G J , Blom, H J , van der Put, N M , Spaans, A P , Rosendaal, F R , Thomas, C G , Haak, H L , Wijermans, P W , and Gerrits, W J (1998) Vitamin supplementation reduces blood homocysteine levels - A controlled trial in patients with venous thrombosis and healthy volunteers *Arterioscler Thromb Vasc Biol* **18**, 356-361.
- den Heijer, M , Koster, T , Blom, H J , Bos, G M , Briet, E , Reitsma, P H , Vandenbroucke, J P , and Rosendaal, F R (1996) Hyperhomocysteinemia as a risk factor for deep-vein thrombosis *N Engl J Med* **334**, 759-762
- Dierkes, J (1995) *Vitamin requirements for the reduction of homocysteine in healthy young women (thesis)*, Giessen, Germany, Wissenschaftlichen Fachverlag Dr Fleck
- Dierkes, J , Kroesen, M , and Pietrzik, K (1998) Folic acid and vitamin B6 supplementation and plasma homocysteine concentrations in healthy young women *Internat J Vit Nutr Res* **82**, 98-103
- Dutch Heart Foundation (1997) *Cardiovascular diseases in the Netherlands Morbidity and mortality statistics*, The Hague, Dutch Heart Foundation
- Economides, D L , Ferguson, J , Mackenzie, I Z , Darley, J , Ware, I I , and Holmes-Siedle, M (1992) Folate and vitamin B12 concentrations in maternal and fetal blood, and amniotic fluid in second trimester pregnancies complicated by neural tube defects *Br J Obstet Gynaecol* **99**, 23-25
- Eichner, E R and Hillman, R S (1973) Effect of alcohol on serum folate level *J Clin Invest* **52**, 584-591

- EUROCAT Working Group (1998) *EUROCAT registration of congenital anomalies. Northern Netherlands and Southwestern Netherlands. Tables 1981-1996.*, Groningen, Department of Medical Genetics, University of Groningen.
- Expert Advisory Group (1992) *Folic acid and the prevention of neural tube defects*, London, Department of Health.
- Falcon, C.R., Cattaneo, M., Panzeri, D., Martinelli, I., and Mannucci, P.M. (1994) High prevalence of hyperhomocyst(e)inemia in patients with juvenile venous thrombosis. *Arterioscler Thromb* **14**, 1080-1083
- FAO (1988) Requirements of vitamin A, iron, folate and vitamin B12. Report of a joint FOA/WHO Expert Consultation. In. *FAO Food and Nutrition Series*, Rome,
- Feunekes, G.I.J., van Staveren, W.A., de Vries, J.H.M., Burema, J., and Hautvast, J.G.A.J. (1993) Relative and biomarker-based validity of a food-frequency questionnaire estimating intake of fats and cholesterol *Am J Clin Nutr* **58**, 489-496.
- Finkelstein, J.D. (1990) Methionine metabolism in mammals. *J Nutr Biochem* **1**, 228-237.
- Fiskerstrand, T., Refsum, H., Kvalheim, G., and Ueland, P.M. (1993) Homocysteine and other thiols in plasma and urine. automated determination and sample stability. *Clin Chem* **39**, 263-271.
- Food and Nutrition Council (1992) *Report on the relationship between folic acid and neural tube defects*, The Hague, Food and Nutrition Council.
- Food and Nutrition Council (1993) *Follow-up report on the relationship between folic acid intake and neural tube defects*, The Hague, Food and Nutrition Council
- Franken, D.G., Boers, G.H.J., Blom, H.J., Trijbels, F.J., and Kloppenborg, P.W. (1994) Treatment of mild hyperhomocysteinemia in vascular disease patients *Arterioscler Thromb* **14**, 465-470
- Frosst, P., Blom, H.J., Milos, R., Goyette, P., Sheppard, C.A., Matthews, R.G., Boers, G.J., den Heijer, M., Kluijtmans, L.A., van den Heuvel, L.P., and Rozen, R. (1995) A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase *Nat Genet* **10**, 111-113.
- Gardiki-Kouidou, P. and Seller, M.J. (1997) Amniotic fluid folate, vitamin B12 and transcobalamin in neural tube defects *Clin Genet* **33**, 441-448.
- Giles, W.H., Kittner, S.J., Anda, R.F., Croft, J.B., and Casper, M.L. (1995) Serum folate and risk for ischemic stroke. First National Health and Nutrition Examination Survey epidemiologic follow-up study. *Stroke* **26**, 1166-1170.
- Godwin, H.A. and Rosenberg, I.H. (1975) Comparative studies of the intestinal absorption of [3H]pteroylmonoglutamate and [3H]pteroylheptaglutamate in man. *Gastroenterology* **69**, 364-373.
- Goyette, P., Sumner, J.S., Milos, R., Duncan, A.M., Rosenblatt, D.S., Matthews, R.G., and Rozen, R. (1994) Human methylenetetrahydrofolate reductase: isolation of cDNA mapping and mutation identification *Nat Genet* **7**, 551-55s.

- Graham, I M , Daly, L E , Refsum, H M , Robinson, K , Brattstrom, L E , Ueland, P M , Palma-Reis, R J , Boers, G H J , Sheahan, R G , Israelsson, B , Uiterwaal, C S , Meleady, R , McMaster, D , Verhoef, P , Witteman, J , Rubba, P , Bellet, H , Wautrecht, J C , de Valk, H W , Sales Luis, A C , Parrot-Roulaud, F M , Soon Tan, K , Higgins, I , Garcon, D , Medrano, M J , Candito, M , Evans, A E , and Andria, G (1997) Plasma homocysteine as a risk factor for cardiovascular disease *JAMA* **277**, 1775-1781
- Green, T J , Houghton, L A , Donovan, U M , Gibson, R S , and O'Connor, D L (1998) Oral contraceptives did not affect biochemical folate indexes and homocysteine concentrations in adolescent females *J Am Diet Assoc* **98**, 49-55
- Gregory, J F , Bhandari, S D , Bailey, L B , Toth, J P , Baumgartner, T G , and Cerda, J J (1991) Relative bioavailability of deuterium-labeled monoglutamyl and hexaglutamyl folates in human subjects *Am J Clin Nutr* **53**, 736-740
- Gregory, J F , Bhandari, S D , Bailey, L B , Toth, J P , Baumgartner, T G , and Cerda, J J (1992) Relative bioavailability of deuterium-labeled monoglutamyl tetrahydrofolates and folic acid in human subjects *Am J Clin Nutr* **55**, 1147-1153
- Guttormsen, A B , Schneede, J , Ueland, P M , and Refsum, H (1996b) Kinetics of total plasma homocysteine in subjects with hyperhomocysteinemia due to folate or cobalamin deficiency *Am J Clin Nutr* **63**, 194-202
- Guttormsen, A B , Ueland, P M , Nesthus, I , Nygard, O , Schneede, J , Vollset, S E , and Refsum, H (1996a) Determinants and vitamin responsiveness of intermediate hyperhomocysteinemia ($\leq 40 \mu\text{mol/liter}$) - The Hordaland homocysteine study *J Clin Invest* **98**, 2174-2183
- Halsted, C H (1979) The intestinal absorption of folates *Am J Clin Nutr* **32**, 846-855
- Halsted, C H (1990) Intestinal absorption of dietary folates In *Folic acid metabolism in health and disease*, 23-45 Edited by Picciano, M F , Stokstad E L R , and Gregory, J F , New York, Wiley-Liss
- Halsted, C H (1995) Alcohol and folate interactions Clinical implications In *Folate in health and disease*, 313-327 Edited by Bailey, L B , New York, Marcel Dekker Inc
- Halsted, C H , Baugh, C M , and Butterworth-Jr, C E (1975) Jejunal perfusion of simple and conjugated folates in man *Gastroenterology* **68**, 261-269
- Halsted, C H , Reisenauer A M , Shane, B , and Tamura, T (1978) Availability of monoglutamyl and polyglutamyl folates in normal subjects and in patients with coeliac sprue *Gut* **19**, 886-891
- Halsted, C H , Robles, E A , and Mezey, E (1971) Decreased jejunal uptake of labeled folic acid (3H-PGA) in alcoholic patients roles of alcohol and malnutrition *N Engl J Med* **285**, 701-706
- Heinle, R W and Welch, A D (1947) Folic acid in pernicious anemia Failure to prevent neurologic relapse *JAMA* **133**, 739-741
- Herbert, V and Zalusky, R (1962) Interrelations of vitamin B12 and folic acid metabolism folic acid clearance studies *J Clin Invest* **41**, 1263-1276

- Heseker, H and Schmitt, G (1987) Effect of long-term supplementation of folate on folate status in plasma and erythrocytes *J Nutr Sci Vitaminol* **33**, 163-168
- Hibbard, E D and Smithells, R W (1965) Folic acid metabolism and human embryopathy *Lancet* **I**, 1254-1254
- Homocysteine Lowering Trialists' Collaboration (1998) Lowering blood homocysteine with folic acid based supplements meta-analysis of randomised trials *Br Med J* **316**, 894-898
- Horne, D W and Patterson, D (1988) Lactobacillus casei microbiological assay of folic acid derivatives in 96-well microtiter plates *Clin Chem* **34**, 2357-2359
- Jacob, R A , Pianalto, F S , Henning, S M , Zhang, J Z , and Swendseid, M E (1995) In vivo methylation capacity is not impaired in healthy men during short-term dietary folate and methyl group restriction *J Nutr* **125**, 1495-1502
- Jacob, R A , Wu, M M , Henning, S M , and Swendseid, M E (1994) Homocysteine increases as folate decreases in plasma of healthy men during short-term dietary folate and methyl group restriction *J Nutr* **124**, 1072-1080
- Jacques, P F , Bostom, A G , Williams, R R , Ellison, R C , Eckfeldt, J H , Rosenberg, I H , Selhub, J , and Rozen, R (1996) Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations *Circulation* **93**, 7-9
- Kang S -S , Zhou, J , Wong, P W K , Kowalysin, J , and Strokosch, G (1988) Intermediate homocysteinemia a thermolabile variant of methylenetetrahydrofolate reductase *Am J Hum Genet* **43**, 414-421
- Kauwell, G P A , Bailey, L B , Gregory, J F , Bowling, D W , and Cousins, R J (1995) Zinc status is not adversely affected by folic acid supplementation and zinc does not impair folate utilization in human subjects *J Nutr* **125**, 66-72
- Keagy, P M , Shane, B , and Oace, S M (1988) Folate bioavailability in humans effects of wheat bran and beans *Am J Clin Nutr* **47**, 80-88
- Kelly, P , McPartlin, J M , Goggins, M , Weir, D G , and Scott, J M (1997) Unmetabolized folic acid in serum acute studies in subjects consuming fortified food and supplements *Am J Clin Nutr* **65**, 1790-1795
- Kirke, P N , Daly, L E , and Elwood, J H (1992) A randomized trial of low dose folic acid to prevent neural tube defects *Arch Dis Child* **67**, 1442-1446
- Koch, M C , Stegmann, K , Ziegler, A , Schroter, B , and Ermert, A (1998) Evaluation of the MTHFR C677T allele and the MTHFR gene locus in a German spina bifida population *Eur J Pediatr* **157**, 487-492
- Kownacki Brown, P A , Wang, C , Bailey, L B , Toth, J P , and Gregory, J F (1993) Urinary excretion of deuterium-labeled folate and the metabolite p-aminobenzoylglutamate in humans *J Nutr* **123**, 1101-1108
- Laurence, K M , James, N , Miller, M H , Tennant, G B , and Campbell, H (1981) Double-blind randomised controlled trial of folate treatment before conception to prevent recurrence of neural-tube defects *Br Med J* **282**, 1509-1511

- Lowe, K E , Osborne, C B , Lin, B F , Kim, J S , Hsu, J C , and Shane, B (1993) Regulation of folate and one-carbon metabolism in mammalian cells II Effect of folylpoly-gamma-glutamate synthetase substrate specificity and level on folate metabolism and folylpoly-gamma-glutamate specificity of metabolic cycles of one-carbon metabolism *J Biol Chem* **268**, 21665-21673
- Malinow, M R , Duell, P B , Hess, D L , Anderson, P H , Kruger, W D , Phillipson, B E , Gluckman, R A , Block, P C , and Upson, B M (1998) Reduction of plasma homocyst(e)ine levels by breakfast cereal fortified with folic acid in patients with coronary heart disease *N Engl J Med* **338** 1009-1015
- Margo, G , Barker, M , Fernandez-Costa, F , Colman, N , Green, R , and Metz, J (1975) Prevention of folate deficiency by food fortification VII The use of bread as a vehicle for folate supplementation *Am J Clin Nutr* **28**, 761-763
- Mason, J B (1990) Intestinal transport of monoglutamyl folates in mammalian systems In *Folic acid metabolism in health and disease*, 47-64 Edited by Picciano, M F , Stokstad E.L R , and Gregory, J F , New York, Wiley-Liss
- McPartlin, J M , Halligan, A , Scott, J M , Darling, M D , and Weir, D G (1993) Accelerated folate breakdown in pregnancy *Lancet* **341**, 148-149
- Mills, J L , McPartlin, J M , Kirke, P N , Lee, Y J , Conley, M R , Weir, D G , and Scott, J M (1995) Homocysteine metabolism in pregnancies complicated by neural-tube defects *Lancet* **345**, 149-151
- Morrison, H I , Schaubel, D , Desmeules, M , and Wigle, D T (1996) Serum folate and risk of fatal coronary heart disease *JAMA* **275**, 1893-1896
- MRC Vitamin Study Research Group (1991) Prevention of neural tube defects Results of the Medical Research Council Vitamin Study *Lancet* **338**, 131-137
- Naughton, C A , Chandler, C J , Duplantier, R B , and Halsted, C H (1989) Folate absorption in alcoholic pigs in vitro hydrolysis and transport at the intestinal brush border membrane *Am J Clin Nutr* **50**, 1436-1441
- Naurath, H J , Joosten, E , Riezler, R , Stabler, S P , Allen, R H , and Lindenbaum, J (1995) Effects of vitamin B12, folate, and vitamin B6 supplements in elderly people with normal serum vitamin concentrations *Lancet* **346**, 85-89
- Neuhouser, M L , Beresford, S A A , Hickok, D E , and Monsen, E R (1998) Absorption of dietary folate and supplemental folate in women with prior pregnancies with neural tube defects and controls *J Am Coll Nutr* **6**, 625-630
- O'Broin, J D , Temperley, I J , Brown, J P , and Scott, J M (1975) Nutritional stability of various naturally occurring monoglutamate derivatives of folic acid *Am J Clin Nutr* **28**, 438-444
- O'Keefe, C A , Bailey, L B , Thomas, E A , Hofler, S A , Davis, B A , Cerda, J J , and Gregory, J F (1995) Controlled dietary folate affects status in nonpregnant women *J Nutr* **125**, 2717-2725
- Pancharuniti, N , Lewis, C A , Sauberlich H E , Perkins, L L , Go, R C , Alvarez, J O , Macaluso, M , Acton R T , Copeland, R B , Cousins, A L , Gore, T B , Cornwell, P E , Roseman, J M (1994) Plasma homocyst(e)ine, folate, and vitamin B-12 concentrations and risk for early-onset coronary artery disease *Am J Clin Nutr* **59**, 940-948

- Perry, J and Chanarin, I (1970) Intestinal absorption of reduced folate compounds in man *Br J Haematol* **18**, 329-339
- Perry, J and Chanarin, I (1973) Formylation of folates as a step in physiological folate absorption *Br Med J* **2**, 58-59
- Pfeiffer, C M , Rogers, L M , Bailey, L B , and Gregory, J F (1997) 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* **66**, 1388-1397
- Pietrzik, K and Remer, T (1989) [Bioavailability study of micronutrients] Zur Bioverfugbarkeitsprufung von Mikronahrstoffen *Z Ernahrungswiss* **28**, 130-141
- Potgieter, H, Ubbink, J B , Bissbort, S , Bester, M J , Spies, J H , and Vermaak, W J (1997) Spontaneous oxidation of methionine effect on the quantification of plasma methionine levels *Anal Biochem* **248**, 86-93
- Refsum, H , Ueland, P M , Nygard, O , and Vollset, S E (1998) Homocysteine and cardiovascular disease *Annu Rev Med* **49**, 31-62
- Reisenauer, A and Halsted, C H (1987) Human folate requirements *J Nutr* **117**, 600-602
- Reisenauer, A M , Buffington, C A T , Villanueva, J A , and Halsted, C H (1989) Folate absorption in alcoholic pigs in vivo intestinal perfusion studies *Am J Clin Nutr* **50**, 1429-1435
- Reisenauer, A M , Krumdieck, C L , and Halsted, C H (1977) Folate conjugase two separate activities in human jejunum *Science* **198**, 196-197
- Retief, F P (1969) Urinary folate excretion after ingestion of pteroylmonoglutamic acid and food folate *Am J Clin Nutr* **22**, 352-355
- Richardson, R E , Healy, M J , and Nixon, P F (1979) Folate of rat tissue Bioassay of tissue folylpolyglutamates and a relationship of liver folylpolyglutamates to nutritional folate sufficiency *Biochim Biophys Acta* **585**, 128-133
- Rimm, E B , Willett, W C , Hu, F B , Sampson, L , Colditz, G A , Manson, J E , Hennekens, C , and Stampfer, M J (1998) Folate and vitamin B6 from diet and supplements in relation to risk of coronary heart disease among women *JAMA* **279**, 359-364
- Rogers, L M , Pfeiffer, C M , Bailey, L B , and Gregory, J F (1997) 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* **127**, 2321-2327
- Rosenberg, I H and Godwin, H A (1971) The digestion and absorption of dietary folate *Gastroenterology* **60**, 445-463
- Rowland, M and Tozer, T N (1989) *Clinical Pharmacokinetics Concepts and Applications*, Philadelphia, Lea and Febiger
- Rubin, E and Farber, J L (1995) Developmental and genetic diseases In *Essential pathology*, 125-160 Edited by Rubin, E and Farber, J L , Philadelphia, J B Lippincott Company

- Russell, R M , Rosenberg, I H , Wilson, P D , Iber, F L , Oaks, E B , Giovetti, A C , Otradovec, C L , Karwoski, P A , and Press, A W (1983) Increased urinary excretion and prolonged turnover time of folic acid during ethanol ingestion *Am J Clin Nutr* **38**, 64-70
- Sauberlich, H E , Kretsch, M J , Skala, J H , Johnson, H L , and Taylor, P C (1987) Folate requirement and metabolism in nonpregnant women *Am J Clin Nutr* **46**, 1016-1028
- Savage, D G and Lindenbaum, J L (1995) Folate-cobalamin interactions In *Folate in health and disease*, 237-285 Edited by Bailey, L B , New York, Marcel Dekker
- Sayers, G M , Hughes, N , Scallan, E , and Johnson, Z (1997) A survey of knowledge and use of folic acid among women of child-bearing age in Dublin *J Public Health Med* **19**, 328-332
- Schorah, C J , Devitt, H , Luccock, M , and Dowell, A C (1998) The responsiveness of plasma homocysteine to small increases in dietary folic acid a primary care study *Eur J Clin Nutr* **52**, 407-411
- Schrijver, J , Speek, A J , and Schreurs, W H P (1981) Semi-automated fluorimetric determination of pyridoxal-5'-phosphate (vitamin B6) in whole blood by high-performance liquid chromatography (HPLC) *Int J Vitam Nutr Res* **51**, 216-222
- Scriver C R , Gregory, D M , Sovetts, D and Tissenbaum, G (1985) Normal plasma free amino acid values in adults the influence of some common physiological variables *Metabolism* **34** 868-873
- Selhub, J , Brin, H , and Grossowicz, N (1973) Uptake and reduction of radioactive folate by everted sacs of rat small intestine *Eur J Biochem* **33**, 433-438
- Selhub, J , Dhar, G J , and Rosenberg, I H (1983) Gastrointestinal absorption of folates and antifolates *Pharmacol Ther* **20** 397 418
- Selhub, J , Jacques, P F , Bostom, A G , D'Agostino, R B , Wilson, P W , Belanger, A J , O'Leary, D H , Wolf, P A , Schaefer, E J , and Rosenberg, I H (1995) Association between plasma homocysteine concentrations and extracranial carotid-artery stenosis *N Engl J Med* **332**, 286-291
- Shane, B (1995) Folate chemistry and metabolism In *Folate in health and disease*, 1-22 Edited by Bailey, L B , New York, Marcel Dekker, Inc
- Smithells, R W , Seller, M J , Harris, R , Fielding, D W , Schorah, C J , Nevin, N C , Sheppard, S , Read, A P , Walker, S , and Wild, J (1983) Further experience of vitamin supplementation for prevention of neural tube defect recurrences *Lancet* **i**, 1027-1031
- Stampfer, M J , Malinow, M R , Willett, W C , Newcomer, L M , Upson, B , Ullmann, D , Tishler, P V , and Hennekens, C H (1992) A prospective study of plasma homocyst(e)ine and risk of myocardial infarction in US physicians *JAMA* **268**, 877-881
- Steegers Theunissen R P M , Boers, G H J , Blom, H J , Nijhuis, J G , Thomas, C M G , Borm, G F and Eskes, T K A B (1995) Neural tube defects and elevated homocysteine levels in amniotic fluid *Am J Obstet Gynecol* **172**, 1436-1441
- Steegers Theunissen, R P M , Boers, G H J , Trijbels, F J , and Eskes, T K A B (1991) Neural-tube defects and derangement of homocysteine metabolism *New Engl J Med* **324**, 199-199

- Steegers Theunissen, R P M , Boers, G H J , Trijbels, F J , Finkelstein, J D , Blom, H J , Thomas, C M , Borm, G F , Wouters, M G A J , and Eskes, T K A B (1994) Maternal hyperhomocysteinemia a risk factor for neural-tube defects? *Metabolism* **43**, 1475-1480
- Stichting NEVO (1993) *Dutch Nutrient Data Base*, The Hague, Voorlichtingsbureau voor de Voeding
- Stites, T E , Bailey, L B , Scott, K C , Toth, J P , Fisher, W P , and Gregory, J F (1997) Kinetic modeling of folate metabolism through use of chronic administration of deuterium-labeled folic acid in men *Am J Clin Nutr* **65**, 53-60
- Strain, J J and West, C E (1999) The challenge of bioavailability Proceedings of the Bioavailability meeting *Eur J Clin Nutr* (In Press)
- Strum, W B (1979) Enzymatic reduction and methylation of folate following pH-dependent carrier-mediated transport in rat jejunum *Biochim Biophys Acta* **554**, 249-257
- Tamura, T (1995) Nutrient interaction of folate and zinc In *Folate in health and disease*, 287-312 Edited by Bailey, L B , New York, Marcel Dekker, inc
- Tamura, T and Stokstad, E L R (1973) The availability of food folate in man *Br J Haematol* **25**, 513-532
- Tamura, T , Freeberg, L E , and Cornwell, P E (1990) Inhibition of EDTA of growth of *Lactobacillus casei* in the folate microbiological assay and is reversal by added manganese or iron *Clin Chem* **1993**,
- Tamura, T , Shane, B , Baer, M T , King, J C , Margen, S , and Stokstad, E L R (1978) Absorption of mono- and polyglutamyl folates in zinc-depleted man *Am J Clin Nutr* **31**, 1984-1987
- te Poele-Pothoff, M T , van den Berg, M , Franken, D G , Boers, G H , Jakobs, C , de Kroon, I F , Eskes, T K A B , Trijbels, J M , and Blom, H J (1995) Three different methods for the determination of total homocysteine in plasma *Ann Clin Biochem* **32**, 218-220
- Thorand, B , Pietrzik, K , Prinz, L R , Hages, M , and Holzgreve, W (1996) Maternal and fetal serum and red blood cell folate and vitamin B12 concentrations in pregnancies affected by neural tube defects *Z Geburtshilfe Neonatol* **200**, 176-180
- Toiher, B , Roy, K , and Sirotnak, F M (1998) Structural analysis of the human RFC-1 gene encoding a folate transporter reveals multiple promoters and alternatively spliced transcripts with 5' end heterogeneity *Gene* **211**, 331-341
- Truswell, A S and Kounnavong, S (1997) Quantitative responses of serum folate to increasing intakes of folic acid in healthy women *Eur J Clin Nutr* **51**, 839-845
- Tucker, K L , Selhub, J , Wilson, P W , and Rosenberg, I H (1996) Dietary intake pattern relates to plasma folate and homocysteine concentrations in the Framingham Heart Study *J Nutr* **126**, 3025-3031
- Ubbink, J B (1997) The role of vitamins in the pathogenesis and treatment of hyperhomocyst(e)inaemia *J Inherit Metab Dis* **20**, 316-325

- Ubbink, J B , Vermaak, W J , van der Merwe, A , and Becker, P J (1993) Vitamin B-12, vitamin B-6, and folate nutritional status in men with hyperhomocysteinemia *Am J Clin Nutr* **57**, 47-53
- Ubbink, J B , Vermaak, W J , van der Merwe, A , Becker, P J , Delport, R , and Potgieter, H C (1994) Vitamin requirements for the treatment of hyperhomocysteinemia in humans *J Nutr* **124**, 1927-1933
- Ueland, P M , Refsum, H M , and Brattstrom, L (1993) Plasma homocysteine and cardiovascular disease In *Atherosclerotic cardiovascular disease, hemostasis, and endothelial function*, 183-236 Edited by Boyer Francis Jr , R , New York, Marcel Dekker, Inc
- US Department of Health and Human Services Food and Drug Administration (1996) Food standards amendment of the standards of identity for enriched grain products to require addition of folic acid *Fed Regist* **61**, 8781-8807
- US Public Health Service (1992) Recommendations for the use of folic acid to reduce the number of cases of spina bifida and other neural tube defects *Morbidity and Mortality Weekly Report* **41**, RR-14
- van den Berg, M , Franken, D G , Boers, G H J , Blom, H J , Jakobs, C , Stehouwer, C D , and Rauwerda, J A (1994) Combined vitamin B6 plus folic acid therapy in young patients with arteriosclerosis and hyperhomocysteinemia *J Vasc Surg* **20**, 933-940
- van der Put, N M J , Eskes, T K A B , and Blom, H J (1997a) Is the common 677C-T mutation in the methylenetetrahydrofolate reductase gene a risk factor for neural tube defects? A meta-analysis *QJM* **90**, 111-115
- van der Put, N M J , Steegers Theunissen, R P M , Frosst, P , Trijbels, F J , Eskes, T K A B , Heuvel van den, L P , Mariman, E C M , den Heijer, M , Rozen, R , and Blom, H J (1995) Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida *Lancet* **346**, 1070-1071
- van der Put, N M J , Thomas, C M G , Eskes, T K A B , Trijbels, F J , Steegers Theunissen, R P M , Mariman, E C , De Graaf-Hess, A , Smeitink, J A , and Blom, H J (1997b) Altered folate and vitamin B12 metabolism in families with spina bifida offspring *QJM* **90**, 505-510
- Varela-Moreiras, G and Selhub, J (1992) Long-term folate deficiency alters folate content and distribution differentially in rat tissues *J Nutr* **122**, 986-991
- Voedingsraad (1992) *Nederlandse voedingsnormen 1989*, The Hague, Voorlichtingsbureau voor de Voeding
- Wagner, C (1995) Biochemical role of folate in cellular metabolism In *Folate in health and disease*, 23-42 Edited by Bailey, L , New York, Marcel Dekker, Inc
- Ward, G J and Nixon, P F (1990) Modulation of pteroylpolyglutamate concentration and length in response to altered folate nutrition in a comprehensive range of rat tissues *J Nutr* **120**, 476-484
- Ward, M , McNulty, H , McPartlin, J , Strain, J J , Weir, D G , and Scott, J M (1997) Plasma homocysteine, a risk factor for cardiovascular disease, is lowered by physiological doses of folic acid *QJM* **90**, 519-524

- Weekes, E W , Tamura, T., Davis, R.O., Birch, R., Vaughn, W.H., Franklin, J.C., Bargarier, C., Cosper, P , Finley, S C., and Finley, W.H. (1992) Nutrient levels in amniotic fluid from women with normal and neural tube defect pregnancies *Biol Neonate* **61**, 226-231.
- Wei, M M , Bailey, L B., Toth, J P. and Gregory, J F. (1996) Bioavailability for humans of deuterium-labeled monoglutamyl and polyglutamyl folates is affected by selected foods *J Nutr* **126**, 3100-3108.
- Wei, M M. and Gregory, J.F. (1998) Organic acids in selected foods inhibit intestinal brush border pteroylpolyglutamate hydrolase in vitro: Potential mechanism affecting the bioavailability of dietary polyglutamyl folate. *J Agric Food Chem* **46**, 211-219.
- Whitehead, A S., Gallagher, P., Mills, J.L., Kirke, P.N., Burke, H., Molloy, A.M., Weir, D.G., Shields, D C , and Scott, J M. (1995) A genetic defect in 5,10-methylenetetrahydrofolate reductase in neural tube defects. *QJM* **88**, 763-766.
- Yates, A A , Schlicker, S.A., and Sutor, C.W. (1998) Dietary reference intakes: the new basis for recommendation for calcium and related nutrients, B vitamins and choline. *J Am Diet Assoc* **98**, 699-706
- Zettner, A., Boss, G R , and Seegmiller, J.E. (1981) A long-term study of the absorption of large oral doses of folic acid *Ann Clin Lab Sci* **11**, 516-524.

Summary

An elevated total plasma homocysteine concentration is a risk factor for women for neural tube defects and for men and women for cardiovascular disease. The intervention studies described in this thesis focused on the effects of synthetic folic acid and natural dietary folate on plasma total homocysteine concentrations and folate status.

In the first study (Chapters 2 and 3) we investigated whether low doses of folic acid every other day could decrease plasma total homocysteine concentrations and improve the folate status in healthy volunteers. In a randomised study with 144 healthy women, we provided subjects with either 500 µg of folic acid every day, or 500 µg of folic acid every other day, or a placebo tablet daily for four weeks. Both folic acid intervention strategies significantly improved plasma folate and red blood cell folate concentrations. Intervention with 500 µg of folic acid each day decreased plasma total homocysteine concentrations by 22% and intervention with 500 µg of folic acid every other day decreased the concentrations by 11%. The study showed that the washout period after four weeks of intervention with folic acid is more than 8 weeks.

Folic acid supplementation is most likely to affect the remethylation reaction from methionine into homocysteine. Therefore, supplementation with folic acid can be expected to not only decrease plasma total homocysteine concentrations, but also to increase plasma methionine concentrations. However, four weeks of intervention with low doses of folic acid (500 µg/d or 500 µg/2d) did not influence fasting plasma methionine concentrations in healthy volunteers (Chapter 3).

In Chapter 2 subjects in one group took tablets containing 500 µg of folic acid every other day and placebo tablets on alternate days. Therefore, the second study (Chapter 4) examined whether supplementation with 500 µg of folic acid every other day has a similar homocysteine lowering capacity as supplementation with 250 µg of folic acid each day. Although the difference in change in homocysteine between the groups (0.64 µmol/L) was not significant, the results of the study suggested that the 250-µg dose each day had more homocysteine lowering capacity than the 500-µg dose every other day. This suggests that not all folic acid of the higher dose can be metabolised to the 5-methyltetrahydrofolate form that serves a methyl donor for the remethylation of homocysteine to methionine.

The third study (Chapter 5) firstly addressed the question whether spina bifida patients have an impaired folate and homocysteine status. Secondly, the study investigated how effective supplementation with 500 µg of folic acid for four weeks

decreased homocysteine concentrations and improved the folate status in spina bifida and disabled control patients. No indications for an impaired homocysteine or folate metabolism in patients with a spina bifida were shown. In addition, folic acid supplementation seemed at least as effective in spina bifida patients as in control patients. Therefore, other factors, such as a higher need for folic acid by the mother, or a defect in the intrauterine transport from mother to child are probably more important causes for neural tube defects than a defect in the child itself.

In the fourth study (Chapter 6) we investigated whether a high daily dose of folate from vegetables and citrus fruit decreases plasma total homocysteine concentrations and improves the folate status. In a dietary controlled intervention study subjects received either a diet with a normal folate content (on average 210 µg/d) and a placebo tablet, or the same diet in combination with every other day a 500 µg folic acid tablet, or a diet high in dietary folate from vegetables and citrus fruit (560 µg/d) and a placebo tablet. The intervention with the dietary folate from vegetables and citrus fruit decreased plasma total homocysteine concentrations and improved the folate status. The bioefficacy of the dietary folate from vegetables and citrus fruit compared to folic acid was 60% in this study. The bioavailability was 78% when change in plasma folate was used as the determinant of bioavailability and 98% when change in red blood cell folate was used. The fact that intervention with folic acid was accomplished by supplying the tablets every other day may have overestimated the effect of the dietary folate compared to the folic acid. However, the study clearly showed that dietary folate from vegetables and citrus fruit decreased plasma total homocysteine concentrations and increased the folate status in healthy volunteers.

Chapter 7 reviewed the literature with respect to factors influencing bioavailability of folate and folic acid. Two factors stand out in effect: food matrix and amount of folic acid ingested. The studies in the review also suggest that glutamate chain length plays a role as well.

In conclusion, daily doses as low as 250 µg of folic acid in addition to the habitual diet of healthy volunteers decrease plasma total homocysteine concentrations and improve the folate status. An impaired folate or homocysteine metabolism in patients with a spina bifida seems not an important risk factor for the occurrence of the spina bifida. Supplementation with 500 µg of folic acid each day seems comparable effective in spina bifida patients as in control subjects. Finally, not only synthetic folic acid, but also a high intake of dietary folate from vegetables and citrus fruit decreases plasma total homocysteine concentrations and improves the folate status in healthy volunteers.

Samenvatting

Foliumzuur is een B-vitamine dat van belang is voor de gezondheid van de mens. Dit proefschrift beschrijft enkele aspecten van het foliumzuurmetabolisme. Onderzoek naar foliumzuur is belangrijk, omdat er verband lijkt te zijn met aangeboren neurale-buisafwijkingen (bijv. een spina bifida = open ruggetje) en met hart- en vaatziekten. Wij hebben met name de invloed van het innemen van extra foliumzuur op het homocysteïnegehalte en de biologische beschikbaarheid van folaat uit voedsel bestudeerd. We spreken van foliumzuur als het om de synthetische vorm gaat en van folaten als het om de natuurlijke vormen van dit vitamine gaat. De folaatstatus bij de mens wordt bepaald door meting van folaten in rode bloedcellen en in het bloedplasma. Homocysteïne ontstaat als het aminozuur methionine in ons lichaam afgebroken wordt. Aminozuren zijn bouwstenen van eiwitten. Methionine is een aminozuur dat het lichaam niet in voldoende mate kan aanmaken en dat daarom met de voeding moet worden ingenomen. De omzetting van methionine naar homocysteïne wordt beïnvloed door foliumzuur. Inname van hoge doses foliumzuur in aanvulling op de gebruikelijke voeding kan het homocysteïnegehalte verlagen.

Uit eerdere onderzoeken is gebleken dat het innemen van extra foliumzuur in de periode rond de bevruchting de kans op het krijgen van een kindje met een neurale-buisafwijking aanzienlijk verkleint. Bovendien hebben vrouwen die eerder een kindje met een neurale-buisafwijking hebben gehad gemiddeld een hoger homocysteïnegehalte in het bloedplasma dan vrouwen die een gezond kind hebben gehad. Niet alleen vrouwen die eerder een kind met een neurale-buisafwijking hebben gehad, maar ook mensen die aan hart- en vaatziekten lijden hebben vaker een verhoogd homocysteïnegehalte dan gezonde mensen.

Bij de start van ons onderzoek was reeds bekend dat het mogelijk is het homocysteïnegehalte te verlagen door inname van foliumzuur in doses boven de 650 µg per dag in aanvulling op de normale voeding. Over het effect van inname van lagere doses foliumzuur op de folaatstatus en homocysteïnegehalten in het bloed was minder bekend. Ook was nog onduidelijk of het mogelijk is om met extra natuurlijk folaat uit de voeding de folaat- en homocysteïnegehalten gunstig te beïnvloeden. Voorts was nog niet duidelijk op welke wijze extra foliumzuur de kans op het krijgen van een kind met een neurale-buisafwijking verkleint. Het onderzoek dat beschreven is in dit proefschrift ging daarom uit van de volgende vragen:

- Is het mogelijk om bij gezonde vrijwilligers de folaatstatus te verbeteren en het homocysteïnegehalte te verlagen door het verstrekken van foliumzuur in doses tussen de 250 en 500 µg per dag in aanvulling op de gebruikelijke voeding?

- Is bij patiënten met een open rug de folaat- en homocysteïnestofwisseling afwijkend ten opzichte van controlepatiënten? Wat is bij patiënten met een open rug het effect van het verstrekken van extra foliumzuur op de folaat- en homocysteïnestatus?
- Is het bij gezonde vrijwilligers mogelijk om met natuurlijke folaten uit groenten en fruit de folaatstatus te verbeteren en het homocysteïnegehalte te verlagen en hoe verhoudt dit effect zich tot het effect van synthetisch foliumzuur?

Om de eerste vraag te beantwoorden werd een studie uitgevoerd waaraan 144 gezonde jonge vrouwen deelnamen (hoofdstuk 2). Deze vrouwen werden ingedeeld in drie groepen en slikten gedurende vier weken, of 500 µg foliumzuur per dag, of 500 µg foliumzuur om de dag, of een placebotabletje iedere dag. Daarnaast consumeerden de vrouwen hun normale dagelijkse voeding, die gemiddeld 280 µg folaat per dag bevatte. De placebotabletjes bevatten slechts vulmiddel en geen vitamine, maar waren niet te onderscheiden van de foliumzuurtabletjes. In de beide groepen die foliumzuur ontvingen stegen de folaatgehalten in bloedplasma en in rode bloedcellen en daalden de homocysteïnegehalten in het plasma. De interventie met foliumzuur had geen effect op de gehalten aan het aminozuur methionine in het bloedplasma (hoofdstuk 3). Acht weken nadat de interventieperiode was afgelopen werden de gehalten nogmaals gemeten. De folaatgehalten in de groepen die foliumzuur hadden gekregen waren nog steeds verhoogd ten opzichte van de placebogroep en de homocysteïnegehalten waren ook nog steeds verlaagd. Dit betekent dat het gunstige effect van extra foliumzuur nog tenminste enkele weken door blijft werken.

In deze eerste studie hebben we aan een van de groepen om de dag een tabletje met 500 µg foliumzuur verstrekt. We hebben daarom in een volgende studie onderzocht of het effect van 500 µg foliumzuur om de dag vergelijkbaar is met het effect van 250 µg foliumzuur iedere dag (hoofdstuk 4). De dosis van 250 µg foliumzuur per dag leek effectiever het homocysteïnegehalte in het bloedplasma te verlagen dan de dosis van 500 µg foliumzuur om de dag. Dit suggereert dat bij een dosis van 500 µg niet al het foliumzuur beschikbaar is voor de verlaging van het homocysteïnegehalte.

In de derde studie hebben we allereerst onderzocht of patiënten met een spina bifida een afwijkend folaat- en homocysteïnemetabolisme hebben in vergelijking met controlepatiënten (hoofdstuk 5). Vervolgens hebben we gedurende vier weken onderzocht hoe goed 500 µg foliumzuur per dag de folaatgehalten verbetert en de homocysteïnegehalten verlaagt in spina bifida patiënten en bij

controlepersonen met een handicap. De resultaten gaven geen aanwijzingen dat het folaat- of homocysteinemetabolisme bij patienten met een spina bifida afwijkend zou zijn ten opzichte van controlepatienten. Bovendien bleek de inname van 500 µg foliumzuur per dag bij spina bifida patienten minstens zo effectief de folaatstatus te verbeteren en de homocysteïnegehalten te verlagen als bij gehandicapte controlepersonen. Een afwijking in het folaat- en homocysteinemetabolisme van het kind zelf lijkt dus op grond van dit onderzoek dus geen belangrijke risicofactor voor het ontstaan van een neurale-buisafwijking. Echter, van bepaalde genetische afwijkingen in het folaatmetabolisme bij het kind is wel bekend dat ze een groter risico geven op een neurale-buisafwijking. Andere oorzaken, zoals een hogere behoefte aan folaat van de moeder, of een afwijking in het transport van folaat van moeder naar kind, zouden belangrijkere redenen kunnen zijn waarom extra foliumzuur de kans op het krijgen van een kind met een neurale-buisafwijking verkleint.

Onze eerste studies hebben aangetoond dat het mogelijk is om de folaatstatus te verbeteren en het homocysteïnegehalte te verlagen door inname van doses synthetisch foliumzuur van slechts 250 µg per dag. Dit bood perspectief voor een interventie met natuurlijke folaten uit de voeding. Immers, indien het mogelijk is om met lage doses foliumzuur in aanvulling op de normale voeding de folaatstatus te verbeteren en homocysteïnegehalten te verlagen, dan is het misschien ook haalbaar om dit effect te bereiken door de inname van extra voedingsfolaat. In de vierde studie (hoofdstuk 6) hebben we dan ook onderzocht of een hoge dagelijkse dosis folaat, afkomstig uit groenten en citrusvruchten, de folaatstatus kan verbeteren en de homocysteïnegehalten kan verlagen. We hebben hiervoor gebruik gemaakt van een zogenaamde gecontroleerde voedingsproef. In deze gecontroleerde voedingsproef kregen de proefpersonen dagelijks 90 procent van hun dagelijkse inname aan energie van hun voeding verstrekt. Zij aten op doordeweekse dagen tussen de middag een warme maaltijd op de afdeling Humane Voeding en Epidemiologie in Wageningen en kregen de overige voedingsmiddelen (ook voor het weekend) mee naar huis. Alle voedingsmiddelen werden afgewogen. Bovendien werden gedurende de proef monsters van de voeding genomen om precies te meten hoeveel folaat de voeding bevatte. De deelnemers kregen, of een voeding met een normaal folaatgehalte (gemiddeld 210 µg folaat per dag) en een placebotabletje, of een voeding met hetzelfde folaatgehalte en om de dag 500 µg foliumzuur uit een tabletje, of een voeding die van nature rijk is aan folaat afkomstig van groente en citrusvruchten (560 µg folaat per dag) en een placebotablet. De voeding met de folaatrijke groenten en citrusvruchten verbeterde de folaatstatus en verlaagde de

homocysteïnegehalten van deze gezonde vrijwilligers. Het homocysteïne-verlagende effect van het folaat uit groenten en fruit was 60% ten opzichte van het synthetische foliumzuur. Het relatieve effect op het folaatgehalte in plasma was 78% en op het folaatgehalte in rode bloedcellen was 98%. Deze studie toont dus duidelijk aan dat voedingsfolaat afkomstig van groenten en citrusvruchten de folaatstatus verbetert en homocysteïnegehalten verlaagt.

Hoofdstuk 7 geeft tenslotte een overzicht van de literatuur met betrekking tot de factoren die het effect van folaat en foliumzuur op de folaatstatus (biobeschikbaarheid) en homocysteïnegehalten (biologische effectiviteit) kunnen beïnvloeden. De matrix van het voedsel en de hoeveelheid foliumzuur die wordt ingenomen lijken de belangrijkste factoren die de biologische beschikbaarheid van folaat en foliumzuur kunnen beïnvloeden.

We kunnen concluderen dat een afwijkend folaat- en homocysteïnemetabolisme bij patiënten met een spina bifida geen belangrijke oorzaak lijkt te zijn voor het ontstaan van de spina bifida. Bovendien leidde interventie met 500 µg foliumzuur per dag bij spina bifida patiënten tot een vergelijkbare verbetering van de folaatstatus en verlaging van homocysteïnegehalten als bij gehandicapte controlepersonen. Derhalve geeft dit onderzoek geen aanleiding om patiënten met een spina bifida van een speciaal advies te voorzien.

Zowel een dagelijkse dosis van slechts 250 µg synthetisch foliumzuur per dag in aanvulling op de gebruikelijke voeding van gezonde vrijwilligers als een hoge inname van voedingsfolaat afkomstig van groenten en fruit verbeterde de folaatstatus en verlaagde de homocysteïnegehalten. Verlaging van homocysteïnegehalten leidt mogelijk tot een kleinere kans op het krijgen van een kindje met een neurale-buisafwijking en tot een kleinere kans op het krijgen van hart- en vaatziekten. Dit geeft een extra ondersteuning voor het advies van het Voorlichtingsbureau voor de Voeding om dagelijks tenminste 200 gram groenten en 2 stuks fruit te eten. Echter, vrouwen die van plan zijn zwanger te worden moeten daarnaast nog steeds iedere dag 400 tot 500 µg synthetisch foliumzuur tot zich nemen. Dit, omdat extra voedingsfolaat mogelijk het risico op het krijgen van een neurale-buisafwijking verkleint, maar uit eerdere onderzoeken is gebleken dat extra foliumzuur dit zeker doet.

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Ingeborg

Curriculum Vitae

Ingeborg Annemarie Brouwer was born on October 4, 1968, in Apeldoorn, the Netherlands. In 1987, she passed secondary school, gymnasium B, at the 'Christelijk Lyceum' in Apeldoorn. In the same year she started the study 'Human Nutrition' at the Wageningen Agricultural University. As part of that study she conducted research projects in Animal Nutrition (Department of Animal Sciences, State University Utrecht, Apr - Dec 1991), Toxicology (Department of Toxicology, Wageningen Agricultural University, Jan - Aug 1992) and Human Nutrition (Department of Applied Human Nutrition, University of Guelph, Canada, Sep 1992 - Mar 1993). In 1993 she received the MSc degree. In 1994 she worked as a research assistant on the project 'Vitamins, homocysteine and thrombosis' at the Department of Haematology, Leyenburg Hospital, The Hague. In March 1995 she started as a PhD-fellow in a collaborative project of the Department of Obstetrics and Gynaecology of the University Hospital Nijmegen St Radboud and the Division of Human Nutrition and Epidemiology of the Wageningen Agricultural University. She joined the education programme of the Graduate School VLAG (advanced courses in Food Technology, Agrobiotechnology, Nutrition and Health Sciences). She was the secretary of the PhD-council of this Graduate School from 1995 through 1998. She was a member of the PhD-excursion committee that organised a study tour to Scandinavia in 1997. She participated in international courses on 'Nutritional and lifestyle epidemiology (1995)' and 'Ecophysiology of the gastrointestinal tract (1996)'. In June 1997 she attended the Annual New England Epidemiology Summer Program at Tufts University, Boston, USA. She was selected to participate in the fifth European Nutrition Leadership Programme, March 1998, Luxembourg. Since April 1999 she works as a research associate at the Wageningen Centre for Food Sciences.

Publications

Research papers:

Beynen AC, **Brouwer IA**, Lemmens AG Dietary folate vs glucose does not influence iron status in the rat *Biological Trace Element Research* 1992, 35 89-92

Brouwer IA, Lemmens AG, Beynen AC Dietary fructose versus glucose lowers ferrous iron absorption in rats *British Journal of Nutrition* 1993, 70 171-8

den Heijer M, Bos GMJ, **Brouwer IA**, Gerrits WBJ, Blom HJ Variability of the methionine loading test no effect of a low protein diet *Annals of Clinical Biochemistry* 1996, 33 551-4

den Heijer M, **Brouwer IA**, Bos GMJ, Blom HJ, Spaans AP, Rosendaal FR, Thomas CMG, Haak HL, Wijermans PW, Gerrits WBJ Vitamin supplementation reduces blood homocysteine levels a controlled trial in patients with venous thrombosis and healthy volunteers *Arteriosclerosis Thrombosis and Vascular Biology* 1998, 18 356-61

Homocysteine Lowering Trialists' Collaboration Lowering blood homocysteine with folic acid based supplements meta-analysis of randomised trials *British Medical Journal* 1998, 316 894-8

Brouwer IA, van Dusseldorp M, Thomas CMG, Duran M, Hautvast JGAJ, Eskes TKAB, Steegers-Theunissen RPM Low-dose folic acid supplementation decreases plasma homocysteine a randomized trial *American Journal of Clinical Nutrition* 1999, 69 99-104

Brouwer IA, van Dusseldorp M, West CE, Meyboom S, Thomas CMG, Duran M, van het Hof KH, Eskes TKAB, Hautvast JGAJ, Steegers-Theunissen RPM Dietary folate from vegetables and citrus fruit decreases plasma homocysteine concentrations in humans in a dietary controlled trial *Journal of Nutrition* 1999 (In Press)

van het Hof KH, **Brouwer IA**, West CE, Haddeman E, Steegers-Theunissen RPM, van Dusseldorp M, Westrate JA, Eskes TKAB, Hautvast JGAJ Bioavailability of lutein from vegetables is five times higher than that of β -carotene *American Journal of Clinical Nutrition* 1999 (In Press)

Brouwer IA, van Dusseldorp M, Duran M, Thomas CMG, Hautvast JGAJ, Eskes TKAB, Steegers-Theunissen RPM Low-dose folic acid supplementation does not influence plasma methionine concentrations in young, non-pregnant women *British Journal of Nutrition* 1999 (In Press)

Castenmiller JM, van der Poll CJ, West CE, **Brouwer IA**, Thomas CMG, Van Dusseldorp M
The bioavailability of folate from processed spinach A controlled dietary intervention study
Submitted

Brouwer IA, van Rooij IALM, van Dusseldorp M, Thomas CMG, Blom HJ, Hautvast JGAJ, Eskes TKAB, Steegers-Theunissen RPM Homocysteine lowering effect of 500 µg of folic acid every other day versus 250 µg per day Submitted

Brouwer IA, van Dusseldorp M, Thomas CMG, van der Put NMJ, Gaytant MA, Eskes TKAB, Hautvast JGAJ, Steegers-Theunissen RPM Homocysteine metabolism and effects of folic acid supplementation in patients affected with spina bifida Submitted

Other publications:

Lans MC, **Brouwer I**, De Winden P, Brouwer A Different effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and Aroclor 1254 on thyroxine metabolism and transport Organohologen Compounds, 1993, Vol 13 Human Exposure, Toxicology, Epidemiology, 137-141

Brouwer IA, Louwman M, Schuurman C, Loose N, Duran M, van Dusseldorp M, Steegers-Theunissen RPM Foliumzuur en homocysteine effect van foliumzuursuppletie in lage doses op homocysteine Voeding 1997, 58 33

de Bree A, van Dusseldorp M, **Brouwer IA**, van het Hof KH, Steegers-Theunissen RPM Folate intake in Europe recommended, actual and desired intake (review) European Journal of Clinical Nutrition 1997, 51 643-660

Brouwer IA. Meer foliumzuur voor wie, hoe en hoeveel? NVVL-Workshop Foliumzuur en hart en vaatziekten Voeding 1998, 59 11-14

Brouwer IA, van Dusseldorp M, de Vries JHM, Steegers-Theunissen RPM Aanbevolen dagelijkse hoeveelheid foliumzuur onvoldoende voor optimale homocysteïnespiegels (letter) Nederlandse Tijdschrift voor Geneeskunde 1998, 142 1473

Brouwer IA, van Dusseldorp M, West CE, Gregory III JF, Steegers-Theunissen RPM Bioavailability and efficacy of folate and folic acid Review of the literature In preparation

Abstracts:

den Heijer M, **Brouwer IA**, Blom HJ, Gerrits WBJ, Bos GMJ Lowering of homocysteine blood levels by means of vitamin supplementation Irish Journal of Medical Science 1995, 164 (suppl 15) 7

Brouwer IA, Steegers-Theunissen RPM, van Dusseldorp M, Thomas CMG, Duran M, Hautvast JGAJ, Eskes TKAB Daling homocysteinegehalte en verbetering folaatstatus door suppletie met lage dosis foliumzuur *Tijdschrift voor Sociale Gezondheidszorg* 1997, 75 15

Brouwer IA Efficacy of natural dietary folate, derived from vegetables and citrus fruits, in improving the folate and homocysteine status Report of the fifth seminar of the European Nutrition Leadership Programma 1998, 56

Brouwer IA, van Dusseldorp M, West CE, Meyboom S, Thomas CMG, Duran M, van het Hof KH, Eskes TKAB, Hautvast JGAJ, Steegers-Theunissen RPM High daily intake of dietary folate decreases homocysteine and improves folate status a controlled dietary trial in young healthy volunteers *Netherlands Journal of Medicine* 1998, 52 S41

Den Heijer M van der Put N, Segers MFG, **Brouwer IA**, Blom HJ, Thomas CMG Mutated methylenetetrahydrofolate reductase and the distribution of folate metabolites *Netherlands Journal of Medicine* 1998, 52 S48

Meyboom S, de Vries JHM, **Brouwer IA**, van Staveren WA, van Dusseldorp M Folate contents of diets calculated by use of food tables compared with those determined by a microbiological assay *Eur J Clin Nutr* 1998, 52 (supplement 2) S87

Siebelink E, Roosemalen K, de Vries JHM, Alles M, **Brouwer IA**, Castenmiller JJM, van Staveren WA Self-reported energy intake by a food frequency questionnaire compared with energy need to maintain body weight in 170 non-obese adults *European Journal of Clinical Nutrition* 1998, 52 S21

Brouwer IA, van Dusseldorp M West CE, Meyboom S, Thomas CMG, Duran M, Steegers-Theunissen RPM Folaat uit groenten en fruit verlaagt het plasmahomocysteinegehalte *Tijdschrift voor Sociale Gezondheidszorg* 1998, 76 53

Stellingen

1. Het gegeven dat een dagelijkse inname van een geringe dosis synthetisch foliumzuur het homocysteïnegehalte bij gezonde mensen kan verlagen kan er op duiden dat hun folaatstatus nog niet optimaal is (*dit proefschrift*).
2. Een verhoogde gemiddelde consumptie van groenten en citrusvruchten door de algehele bevolking zal leiden tot een lager gemiddeld homocysteïnegehalte en een verbeterde folaatstatus (*dit proefschrift*).
3. De uitgangswaarde van homocysteïne in plasma bepaalt of inname van extra foliumzuur dit niveau kan verlagen (*dit proefschrift*).
4. Bij gezonde mensen is het zinloos om ter verlaging van het homocysteïnegehalte foliumzuurdoses boven de 500 µg per dag te geven (zie Homocysteine Lowering Trialists' Collaboration BMJ 1998; 316: 894-8; Kelly *et al.* Am J Clin Nutr 1997; 65: 1790-5).
5. Verrijking van voedingsmiddelen verarmt de wetenschap.
6. Op verpakkingen van alle anti-conceptiemiddelen dient een duidelijk advies te komen voor vrouwen met zwangerschapswens om dagelijks extra foliumzuur in te nemen ter preventie van aangeboren afwijkingen.
7. De opsporing van jonge kinderen met coeliakie moet sterk verbeteren aangezien op dit moment van slechts één op de vijftien kinderen met coeliakie bekend is dat ze deze aandoening hebben (zie Csizmadia *et al.* Lancet 1999; 353: 813-4).
8. Uit het feit dat artsen hun werkkleding tijdens de lunchpauze aanhouden blijkt dat de 'doktersjas' nog steeds meer als statussymbool dan uit hygiënische overwegingen wordt gedragen.
9. Dankzij de Europese Gemeenschap worden verre vrienden goede burens.
10. Het rally-point systeem bij het volleybal heeft een negatieve invloed op zowel het speel- als kijkgenot.

Stellingen behorend bij het proefschrift:

'Folic acid, folate and homocysteine. Human intervention studies'

Ingeborg Brouwer, Nijmegen, 1 juli 1999.