Dietary folate:

Bioavailability studies in humans

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Proefschrift

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Abstract

Low intake of the B vitamin folic acid, or folate, causes hyperhomocysteinaemia, which may be a risk factor for cardiovascular disease. In the Netherlands, the recommended daily allowance (RDA) for folate is 300 μg/d. It is not known to what extent folate in the diet is bioavailable, i.e. is absorbed and used or stored in the body. In food, folate is often conjugated to a polyglutamate chain, which has to be removed enzymatically by folylpoly γ-glutamyl carboxypeptidase (FGCP), encoded by the *GCPII* gene, before absorption can take place. Limitation or inhibition of this process may limit the bioavailability of polyglutamate folate.

The aim of the present research was to quantify the effect of the polyglutamate chain on folate bioavailability. This included the modulating effect of the 1561C>T polymorphism in the *GCPII* gene. Furthermore, we aimed to establish the minimal amount of folate that lowers plasma homocysteine concentrations adequately.

The average intake of dietary folate in a representative Dutch population sample of 2,435 men and women aged 20-65 y was 210 μg/d. About two thirds of dietary folate intake was in the polyglutamate form. In a 12-w randomized trial, 180 subjects aged 50-75 y consumed daily either a low dose of heptaglutamyl folic acid or monoglutamyl folic acid, or a placebo capsule. The bioavailability of heptaglutamyl folic acid relative to that of monoglutamyl folic acid was found to be 66%. This implies that the bioavailability of folate from the diet is maximally 77% due to the presence of two thirds of folate as polyglutamate. No differences in bioavailability were found between *GCPII* 1561C>T genotypes. In order to reduce the sample size and duration of future studies on folate bioavailability, a new dual isotopic labelling method was developed. This method comprises that low quantities of two diferentially isotope labelled folate compounds are simultaneously administered to volunteers during several days. The ratio of isotopically labelled folate in plasma then reflects the bioavailability of one compound relative to the other. This method provides a sensitive, accurate and efficient method for measuring folate bioavailability. In a 12-w randomized trial with 316 subjects aged 50-75 y, we established that the amount of supplemental folic acid required to reduce plasma homocysteine concentrations adequately is at least 400 μg/d.

In conclusion, folate bioavailability from the diet is reduced by approximately 23% due to the presence of folate in the polyglutamate form. The average intake of folate in the Netherlands is currently lower than the RDA. Moreover, a substantial increase of folate intake is required to reduce plasma homocysteine concentrations adequately, provided that such lowering of plasma homocysteine concentrations is desirable.

Keywords*: Folate; Folic acid; Dietary folate intake; Folate bioavailability; Polyglutamyl folic acid; Homocysteine; GCPII 1561C>T polymorphism; Stable isotopes; Dose-finding.*

"Je kunt het", zei hij.
En ze heeft het gedaan!

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Abbreviations

CI	confidence interval
CV	coefficient of variation
DHF	dihydrofolate
EPIC	European Prospective Investigation into Cancer and Nutrition
et al.	et alii (and others)
10-FDHF	10-formyldihydrofolate
FGCP	folylpoly γ-glutamyl carboxypeptidase
5-FTHF	5-formyltetrahydrofolate
GCPII	glutamate carboxypeptidase II gene
HDL	high density lipoprotein
HPLC	high performance liquid chromatography
LC-MS/MS	liquid chromatography - mass spectrometry
LOQ	limit of quantification
LOD	limit of detection
µg/d	microgram per day
mL	milliliter
MPa	megapascal
MRM	multiple reaction monitoring
5-MTHF	5-methyltetrahydrofolate
5,10-MeTHF	5,10-methyltetrahydrofolate
nmol/L	nanomol per liter
NMR	nuclear magnetic resonance
pmol	picomol
$PteGlu_1$	pteroyl monoglutamic acid, monoglutamyl folic acid
$PteGlu_n$	pteroyl polyglutamic acid, polyglutamyl folic acid
RDA	recommended daily allowance
SD	standard deviation
THF	tetrahydrofolate
w	week, weeks

Chapter 1

Introduction

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1.1 Introduction to folate bioavailability

Folic acid is a B vitamin (B_{11}) that is essential for many aspects of human health. In its various forms, it is present in our daily diet in foods such as liver, vegetables, bread, dairy products and fruit. Most people consume less than the recommended daily allowance of folic acid. Moreover, some of the ingested vitamin is not absorbed from the gut and thus is not bioavailable. Bioavailability is defined as the proportion of the ingested vitamin that is used and stored in the body. The work described in this thesis aims to obtain a better understanding of the processes that determine the bioavailability of folic acid. A better understanding of these processes will help to predict whether some foods are better sources of folic acid than others.

Throughout this thesis, 'folic acid' refers to the oxidized form of the vitamin, mostly used as a supplement or in food fortification. 'Folate' refers to the reduced derivatives, as present in foods and in the human body. The predominant reduced derivatives are dihydrofolate (DHF), tetrahydrofolate (THF), 5-methyltetrahydro-folate (5-MTHF), 5,10-methylenetetrahydrofolate (5,10-MeTHF), and 5-formyltetra-hydrofolate (5-FTHF). Folate may contain more than one glutamate residue. In **Figure 1.1** the molecular structure of folic acid and its derivatives is shown.

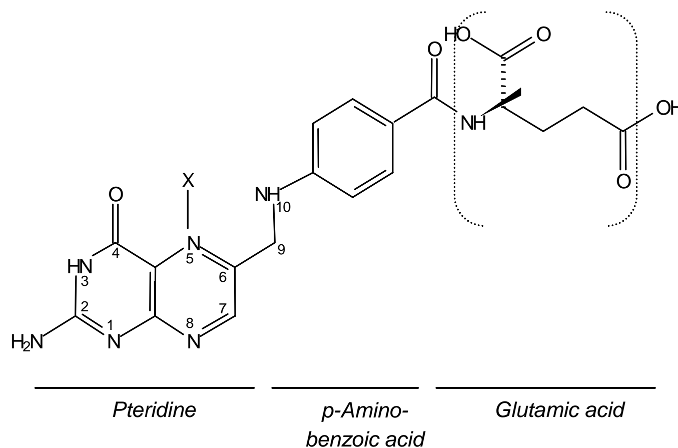

Figure 1.1 Molecular structure of monoglutamyl folic acid (PteGlu). Double bonds at positions 5, 6, 7, and 8 can be reduced to 5,6-dihydrofolate (DHF) or 5,6,7,8-tetrahydrofolate (THF). At position X the following one-carbon groups can be bound: methyl (CH_3): 5-MTHF, mehylene (CH_2): 5,10-MeTHF, or formyl (CHO): 5-FTHF. Monoglutamyl folic acid and its derivatives can be bound to a polyglutamyl chain ($PteGlu_n$).

In this introductory Chapter, background information is given that is required to understand the context of the research described in this thesis. First the importance of folate for our health is explained and how much of it is required each day. Then, a description follows of how the vitamin is absorbed from the small intestine and transported through the body, and how folate status can be assessed. Thereafter, an overview of all factors that influence bioavailability of folate is given. Several factors are discussed in more detail. Dietary folate often has more than one glutamate moiety[1,2], and the effect of a polyglutamate chain on bioavailability is addressed. In addition, the effects of variation in a gene, involved in the conversion of folate to the absorbable form, and the amount of folate ingested are discussed. This Chapter concludes with the rationale and outline of the thesis.

1.2 Health importance

Folate plays an important role in purine and pyrimidine nucleotide biosynthesis; serine and glycine metabolism; DNA-methylation; and methionine biosynthesis (**Figure 1.2**).[3] The clinical manifestation of folate deficiency is megaloblastic anaemia.[4,5] During the nineties, it became evident that low folate status plays an important role in the development of neural tube defects.[6-8] Furthermore, low folate status may cause neurocognitive dysfunction[9-12] and certain types of cancer[13-16].

Low folate intake causes mild hyperhomocysteinaemia - elevation of plasma homocysteine concentrations[17] - which is a putative risk factor for cardiovascular disease[18]. Prospective studies indicate that an increment of 5 μmol/L in plasma homocysteine concentrations is associated with a 20-30% increase in risk of cardiovascular disease.[19] There is increasing evidence that there is a causal relationship between hyperhomocysteinaemia and the risk of cardiovascular disease.[19-24] Various mechanisms have been suggested for that relationship[25-27] but high homocysteine concentrations could also merely reflect underlying metabolic malfunction.[28,29] Currently, clinical trials are being carried out to examine the effect of lowering of plasma homocysteine levels on cardiovascular disease, or markers of such disease. The results are expected to become available in several years. Supplementation with folic acid, even at low doses, leads to a significant reduction of plasma homocysteine concentrations in healthy subjects.[30-34] Also, increased intakes of folate from diets rich in vegetables and fruits are effective in lowering plasma homocysteine levels.[35]

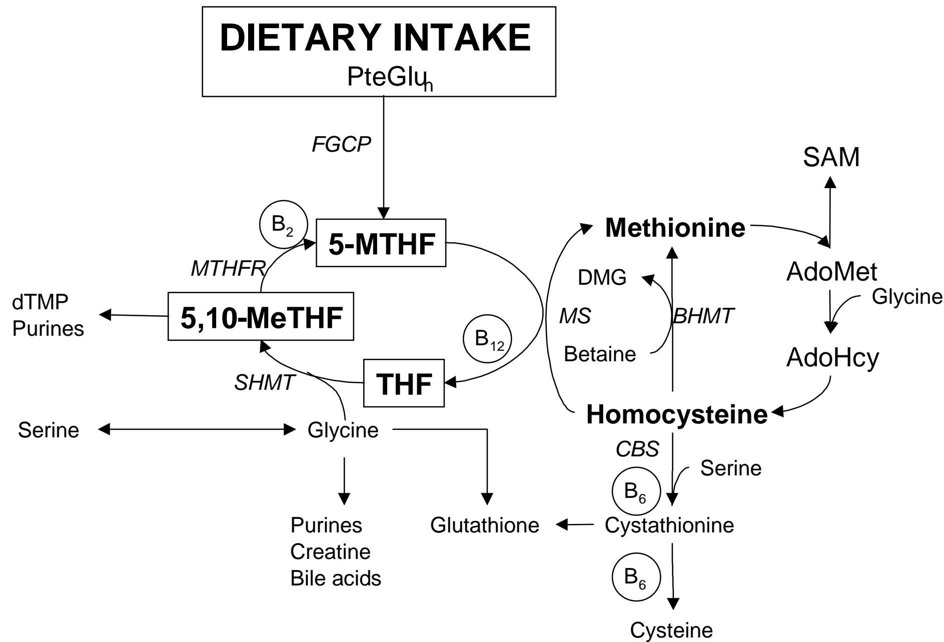

Figure 1.2 Overview of folate metabolism. Folate is derived from the diet, mainly as a polyglutamate ($PteGlu_n$). Folate γ-glutamyl carboxypeptidase (FGCP) converts it to the monoglutamate form. Ingested folate is metabolized to its derivatives 5-methyl-tetraydrofolate (5-MTHF), tetrahdrofolate (THF) and 5,10-methylene-tetrahydrofolate (5,10-MeTHF). MTHFR = methylene-tetrahydrofolate reductase; SHMT = Serine hydroxymethyltransferase; MS = methionine synthase; DMG = dimethylglycine; BHMT = betaine homocysteine methyltransferase; AdoMet = adenosyl methionine; AdoHcy = adenosyl homocysteine; SAM = S-adenosyl methionine; CBS = cystathionine β-synthase; B_2 = vitamin B_2 (riboflavin); B_6 = vitamin B_6 (pyridoxal 5'-phosphate); B_{12} = vitamin B_{12} (methylcobalamin).

1.3 Estimated and recommended intake

In the Netherlands, the recommended daily allowance (RDA) of dietary folate is 300 μg for adults.[36] This advice is based on the prevention of megaloblastic anaemia and not on the prevention of neural tube defects, cardiovascular disease or other chronic diseases. For the prevention of hyperhomocysteinemia, the requirement for folate might be higher than the RDA. In order to prevent neural tube defects, Dutch women who wish to become pregnant are advised to take a supplement of 400 μg folic acid per day starting at least four weeks before anticipated conception until eight weeks thereafter. For the same reason, several countries, including Hungary, Bolivia, Colombia, Mexico, Venezuela, South Africa and the USA, have introduced mandatory fortification of flour with folic acid. However, fortification of food with folic acid is not allowed in the Netherlands.[37]

According to the 1992 Dutch National Food Consumption Survey the dietary intake of folate was 251 ± 97 µg/d (mean ± SD) in adults.[38] Vegetables, bread and dairy products appear to be the most significant sources of folate in the diet. Compared to western countries, folate intake in developing countries is usually much lower, although data are scarce.[39-42]

With intakes of 15 mg/d, mild toxic effects of folic acid have been reported. However, this has not been confirmed in other studies.[43] Folic acid intake from supplements can mask the diagnosis of neurological damage caused by vitamin B_{12} deficiency.[44] This is why the US Institute of Medicine set the tolerable upper intake level (UL) from supplements at 1 mg/d. This argument was followed by the Health Council of the Netherlands.[36,37]

The quality of data on folate intake depends on the quality of measurement of food intake but probably more importantly on the quality of food composition analysis.[45] In addition, the actual amount of folate taken up by the body depends on its bioavailability.

1.4 Absorption and transport

When present, the polyglutamate chain of folate needs to be removed by folylpoly γ-glutamyl carboxypeptidase (FGCP) before folate can be absorbed as monoglutamate.[46,47] Removal of the polyglutamate chain is referred to as deconjugation. Limitation or inhibition of this process would decrease the absorption of folate present as polyglutamate. FGCP is present in the brush border of the jejunum of the small intestine and is encoded for by the glutamate carboxypeptidase II gene (*GCPII*). Folate present as the monoglutamate does not require deconjugation. Folate absorption from the small intestine involves a pH-dependent folate transporter, encoded by the reduced folate carrier gene (*RFC-1*).[48] At higher concentrations (>10 µM) absorption can also take place through a non-saturable mechanism involving passive diffusion.[49]

Prior to entry into the portal vein, folic acid is reduced to 5,6,7,8-tetrahydrofolate (THF) and either methylated or formylated (see Figure 1.1).[50] This process has been found to be saturable because supplementation with folic acid above ~250 µg/d results in unreduced folic acid entering the blood circulation.[51] In body tissues such as the liver and red blood cells, folate can again be conjugated and used as a substrate for various enzymes. For example, 5-MTHF polyglutamate serves as a substrate for methionine synthase. This enzyme methylates homocysteine to methionine, thereby lowering plasma homocysteine concentrations.

Folate is transported throughout the body via the blood mainly as 5-MTHF and is cleared from the body by the kidneys and excreted in the urine.[49] The liver and red blood cells are the main storage tissues for folate.

1.5 Biochemical parameters of folate status

In research, folate status is measured using various parameters. The concentration of folate in serum or plasma is used as an indicator of short-term folate intake (normal values > 7 nmol/L[43]), while long-term intake is reflected by the concentration in erythrocytes (normal values > 305 nmol/L[43]).[52] Excretion of folate and its degradation products in urine is also used as an indicator of folate turnover.[53] Folate concentrations can be measured in various ways. The classical method is the microbiological assay. Other assay methods include radio-immune and immuno-binding assays. In the past few years, high performance liquid chromatography (HPLC) techniques have been developed and optimized for measurement of serum folate concentrations.[54-56] Unlike with other methods, individual folate derivatives (Figure 1.1) can be measured by HPLC.

Since folate is required for the methylation of homocysteine (Figure 1.2), high homocysteine concentrations in plasma can be used as a marker of cellular folate deprivation. Thus, another indicator, or biomarker, of folate status is the total concentration of homocysteine in plasma, which can be measured by HPLC. There is no consensus on normal values for plasma homocysteine concentrations. However, the plasma homocysteine concentrations in most people is < 15 µmol/L.[57]

1.6 Factors determining bioavailability

Bioavailability of folate is defined as the fraction of ingested folate that is absorbed from the small intestine and that can be used for metabolic processes or storage in the body. Bioconversion is the proportion of ingested folate that is metabolised into its derivatives, whereas bioefficacy is defined as the fraction of ingested folate that has a favourable effect on another biomarker, such as plasma homocysteine concentrations.[58] The bioavailability of folate from a mixed diet is only about 50%.[35,59] Factors influencing folate bioavailability, bioconversion and bioefficacy can be summarized in the acronym SLAMENGHI, which stands for: Species of folate; molecular Linking; Amount of folate; Matrix in which it is entrapped; Effect modifiers; Nutrient status; Genetic factors; Host-related factors; and mathematical Interactions.[60] **Table 1.1** summarizes these factors.[58]

In this thesis, studies are presented on quantifying the effect of the linking of folate to a polyglutamate chain on bioavailability and bioefficacy. Furthermore, data on the effect of a genetic variant on the deconjugation process and of the amount of folic acid intake on bioavailability and bioefficacy are presented.

Table 1.1 Factors affecting folate bioavailability, bioconversion and bioefficacy.[58]

S	*Species of folate* Bioavailability, bioconversion and bioefficacy of reduced forms of folate (5-MTHF, DHF, THF, 5-FTHF, 5,10-methylene-THF) may differ from the oxidized form (folic acid). Some studies show higher bioavailability of the reduced forms,[50,71-73] others show no difference.[62,74,75]
L	*Linking to a polyglutamate chain* Some studies show lower bioavailability of polyglutamyl relative to monoglutamyl folic acid,[53,64,66-69] others show no difference.[62,63,65,70] The effect of a polyglutamate chain on bioconversion and thus bioefficacy is not known.
A	*Amount of folate* The relative response of serum folate and erythrocyte folate concentrations to low doses (<200 μg/d) of folic acid is higher than that to higher doses.[76-78] Doses >500 μg provide no further effect in lowering of homocysteine concentrations.[30]
M	*Matrix of the food (encapsulation and binding)* Sauberlich *et al.* in 1987[59] found that the bioavailability of folate from a mixed diet was 50%. When a diet rich in vegetables and fruits was consumed, bioavailability ranged from 60 to 98%, depending on the parameter measured.[35] Disruption of the vegetable matrix, by chopping or enzymatically, increases folate bioavailability from spinach.[79,80]
E	*Effect modifiers* Bioavailability of folate is reduced by food constituents, such as dietary fibre.[62,64,81,82] The activity of folylpoly γ-glutamyl carboxypeptidase (FGCP) is pH dependent (pH optimum: 6.5-7.0)[47,83] and can be inhibited by tomatoes and orange juice,[84] and by citrate.[85] Alcohol may inhibit folate absorption.[86-91] Since FGCP requires zinc for activity,[92] bioavailability of dietary polyglutamate folate is dependent on the amount of zinc in the diet.[93]
N	*Nutrient status of the body* Folate deficiency can alter the partition of folate between body tissues[94-97] but it is not clear whether this affects bioefficacy of folate. Low vitamin B_{12} status affects the bioconversion of folate in the body because it is required for the activity of methionine synthase, that transfers the methyl group of 5-MTHF to homocysteine to form methionine.[44,98] Zinc status influences the absorption of polyglutamate folates.[93,99]
G	*Genetic factors* The reduced folate carrier gene (*RFC-l*) regulates folate absorption.[48,100] Polymorphism in the glutamate carboxypeptidase II gene (*GCPII*) has been reported to reduce FGCP activity.[101] A common polymorphism in the gene encoding for methylene tetrahydrofolate reductase (*MTHFR* 677C>T) affects the bioconversion of folate derivatives in the body and thereby its bioefficacy.[102,103]
H	*Host-related factors* Age has no influence on folate bioavailability.[68] Folate requirements are increased periconceptionally to prevent neural tube defects[7,8] and during pregnancy[104,105]. Folate bioavailability has been shown to be impaired in women who have previously had a child with a neural tube defect,[106] but other studies have not shown this.[107,108] Folate absorption has been shown to be lower in gastro-intestinal disease.[109]
I	*Mathematical Interactions* The combined effect of factors summarised above may be different than the product of the effect of single factors.

1.7 Bioavailability: role of polyglutamate chain

Up until now, various researchers have studied the bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid (see the **Appendix** at the end of this Chapter). Usually in such studies, several human subjects receive single doses of polyglutamyl and monoglutamyl folic acid orally, intravenously or by jejunal perfusion mostly in cross-over designs. At various time intervals up to several hours or days, folate concentrations in blood, urine, or jejunal perfusate are measured. From such studies the bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid has been found to vary from 50 to 100% depending on the study design.[61] Studies have been carried out using either unlabelled folic acid, or folic acid labelled with stable or radio-isotopes. In the next sections, all studies of which we are aware (n=10) are described in detail in order to provide a background to the design of the studies described in this thesis.

1.7.1 Studies with unlabelled folic acid

Four cross-over studies have been carried out with single doses of unlabelled monoglutamyl and polyglutamyl folic acid. All were performed with folic acid doses >400 µg.[62-65] In two studies, subjects were saturated with high doses of monoglutamyl folic acid prior to dosing[62,64] in order to ensure an adequate response. In three studies, no significant difference in the bioavailability of polyglutamate relative to that of monoglutamate folate was found.[62,63,65] One study found that the bioavailability of heptaglutamyl relative to monoglutamyl folic acid was 54-63%.[64]

1.7.2 Studies with isotope-labelled folic acid

Six studies have been performed. Three involved jejunal perfusion[66-68] in which total doses of 360 to 1350 µg of folic acid were given over a period of 40 to 100 min. In these three studies, bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid was 70-80%. In the other three studies, single doses of approximately 300 µg of folic acid were administered in a cross-over design.[53,69,70] In two of these studies, pre-loading doses were given, while in the other, an intramuscular flushing dose was given 4 h after dosing. Two studies found that the relative bioavailability of polyglutamate folic acid was 63-79%,[53,69] while one study found no difference in bioavailability between polyglutamate and monoglutamate folate.[70]

1.7.3 Polymorphism of the GCPII gene

The 1561C>T polymorphism in the *GCPII* gene that codes for FGCP may affect folate bioavailability. *In vitro*, the T allele-coded enzyme exhibits only 50% of the

activity of the wild-type C-allele coded enzyme.[101] In a healthy population, the serum concentrations of folate have been found to be lower and concentrations of homocysteine higher in subjects with the CT genotype, although the number of subjects studied was small (n= 69 CC and 6 CT subjects).[101] This negative association of the T allele with folate status has not been confirmed in several larger studies.[110-112]

1.7.4 Conclusions on the role of the polyglutamate chain

As pointed out above, the results of studies performed on the effect of linking of folate to a polyglutamate chain on its bioavailability are inconclusive. This may be due to several limitations in study design, including:

- In all studies relatively high single doses were used, which may not mimic the usual pattern of intake of folate from the diet. A single dose, as opposed to multiple doses, may not result in upregulation of the gene encoding for intestinal FGCP.
- Pre-loading, which has been used in many studies, may provide erroneous results because it may alter absorption and subsequent metabolism of folate.

It is not clear whether the 1561T allele of the *GCPII* gene reduces bioavailability from polyglutamate folate. Summarizing, at present it is not known to what extent the lower bioavailability of dietary folate can be attributed to the polyglutamate chain. This can be overcome by applying study designs in which subjects are not pre-loaded such as is possible when subjects are given multiple doses of folic acid.

1.8 Bioefficacy: role of amount of folate intake

The first studies that were carried out to examine the effect of folic acid on homocysteine concentrations in plasma used regimens in which high doses of folic acid, often >5 mg/d, were administered.[30] However, it has become clear that doses of folic acid ranging from 400 µg/d to 5 mg/d have similar effects on plasma homocysteine concentrations.[30] Little is known about the effect of doses of folic acid <400 µg/d. Because vitamin B_{12} deficiency can be masked by high intakes of folic acid,[44] it is of great interest to determine the lowest dose that can lower homocysteine concentrations adequately. Moreover, it would be important to know if the lowest effective dose would still be in the range of what can be reached with the diet.

It has been reported that 800 µg folic acid per day is required in patients with ischemic heart disease to obtain the maximum lowering effect on plasma homocysteine concentrations.[113] However, the dependency of the reduction of plasma homocysteine concentrations on initial homocysteine concentrations was not taken into account. In another study in healthy males, the effect of a dose of folic acid of 200 µg/d was found not to be different from that of 400 µg/d.[31]

Unfortunately, this study did not include a placebo group and the sequential design may have led to carry-over effects.

In conclusion, the minimum dose of folic acid required in healthy adults to lower plasma homocysteine concentrations maximally is not known. The appropriate dose could be determined in a study using a range of doses from zero to 800 μg/d, in which treatment groups should be stratified by initial plasma homocysteine concentrations.

1.9 Rationale and outline of the thesis

This thesis aims to quantify the effect of several factors on folate bioavailability. First of all, we studied the role of the polyglutamate chain in determining the bioavailability of folate. We chose to study this factor because the results of studies up until now have been inconclusive. The study designs used have had several limitations such as those arising from the use of single relatively high doses and of pre-loading of subjects with folic acid. We argued that this could be overcome by carrying out a longer term study in which multiple low doses would be administered. A genetic variant in the GCPII gene was also studied in this respect. Secondly, we aimed to quantify the effect of the amount of folate on the bioefficacy of folate, i.e. the reduction of plasma homocysteine concentrations. The rationale for this was that if high doses of folate would be required, this could possibly mask the damaging effects of vitamin B_{12} deficiency. Moreover, in order to find the right strategies for adequate folate intake it is of interest to see if the adequate dose would still be in the range of what can be reached by intake from the diet. Knowledge of these aspects would help to find new ways of improving the folate status of the general population.

This thesis comprises two parts. **Part I** deals with studies on the dietary intake of folate and food processing methods that can potentially increase bioavailability of folate from vegetables. **Part II** presents results of intervention studies in which the effects of the polyglutamate chain and of the amount of folic acid in the diet on bioavailibility and bioefficacy were investigated. In the next section, the specific research questions within each part are described in more detail. **Figure 1.3** provides a schematic overview of the issues studied.

1.9.1 Research questions Part I: Folate in the diet

What is the daily intake of monoglutamate folate and polyglutamate folate from the diet and how does such intake relate to folate status?

In **Chapter 2**, the dietary intake of folate, expressed as monoglutamate and polyglutamate, in a representative sample (n=2435) of the Dutch population, aged

20-65 y, is described. Furthermore, associations between intake of each folate form and plasma folate concentrations are presented.

Can vegetables be processed in such a way that the content of monoglutamate folate is increased?

In **Chapter 3**, results from various processing experiments that aimed to convert endogenous polyglutamate folate to the monoglutamate form in three different vegetables are described.

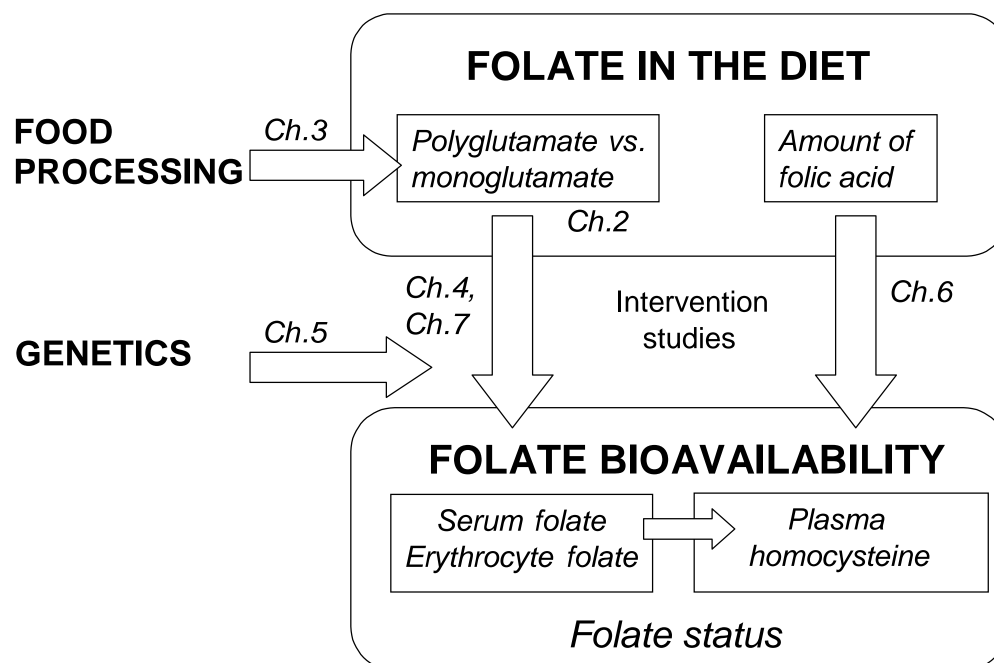

Figure 1.3 Schematic overview of the folate bioavailability issues studied in this thesis.

1.9.2 Research questions Part II: Folate bioavailability

To what extent does the required deconjugation of polyglutamyl folic acid limit the bioavailability of folate?

In order to study this, (**Chapter 4)** a randomized placebo-controlled double-blind intervention trial was carried out with 180 subjects aged 50-75 y. Subjects consumed each day a capsule containing a low dose of monoglutamyl folic acid, a low dose of polyglutamyl folic acid, or a placebo for 12 w. Bioavailability was calculated from the relative response of folate in serum and erythrocytes, while the changes in plasma homocysteine concentrations were used to calculate bioefficacy.

Does polymorphism of the GCPII 1561C>T gene affect folate bioavailability?

In **Chapter 5**, we compared the bioavailability of heptaglutamyl folic acid relative to that of monoglutamyl folic acid among *GCPII* 1561C>T genotypes. This was done using data obtained from the intervention study described in Chapter 4.

Can we develop a dual-label stable isotope method in order to obtain reliable data *on folate bioavailability in humans using a limited number of subjects?*

In contrast to carrying out studies involving large numbers of subjects, such as that described in Chapter 4 with 180 subjects, we have developed a method to measure folate bioavailability using very low doses of [$^{13}C_{11}$]-monoglutamyl folic acid and [$^{13}C_6$]-hexaglutamyl folic acid. The study involved 20 subjects aged 20-50 y and lasted 28 days.

How much folic acid is required to lower plasma homocysteine concentrations adequately?

To answer this question, we conducted a 12-w dose-finding study on folic acid and adequate lowering of plasma homocysteine concentrations among 316 men and women aged 50-75 y (**Chapter** 7).

The final Chapter of this thesis (**Chapter 8**) provides a general discussion, conclusions and implications from the studies described. Furthermore, suggestions for future research are made, because in science *'the answer to one question raises many new ones'*.

Appendix. Bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid.

Reference	Subjects and design	Findings	Bioavailability
Studies using unlabelled folic acid			
Tamura & Stokstad, 1973[62]	Subjects: 6 males Doses:[a] 750 µg - 2.0 mg $PteGlu_3$, $PteGlu_7$ or $PteGlu_1$ Design: Cross-over (pre-loading: 2-10 mg $PteGlu_1$) Outcome: Urinary folate excretion (24 h)	Bioavailability: $PteGlu_3$, 85%; $PteGlu_7$, 90%	85-90%
Bailey *et al*, 1988[63]	Subjects: 9 adults Doses:[a] 750 µg $PteGlu_1$ or $PteGlu_7$ added to bran cereal, spinach or water Design: Cross-over Outcome: Plasma folate Area Under Curve (AUC), 8 h	No difference in water and spinach $PteGlu_7 < PteGlu_1$ with bran;	100%
Keagy *et al*, 1988[64]	Subjects: 7 males Doses:[a] 500 µg $PteGlu_1$ or $PteGlu_7$ added to a formula meal, white beans, or wheat bran (9 d). Design: Cross-over (constant loading: 500 µg $PteGlu_1$/d). Folate absorption tests on alternate days during last four days of each 9-d period Outcome: Urinary excretion (24 h) and serum folate AUC (1-2 h)	Bioavailability: Urine, 63%; serum, 54%	54-63%
Konings *et al*, 2002[65]	Subjects: 12 healthy ileostomy subjects Doses:[a] A) 436 µg spinach folate (60% polyglutamates); B) 362 µg spinach folate (100% monoglutamates); or C) 500 µg $PteGlu_1$ Design: Cross-over Outcome: Serum folate AUC (9 h); ileostomy folate excretion (24-h)	Ileostomy excretion: A, 73%; B, 85%; C, 91% Bioavailability: A, 81%; B, 82%	100%
Studies using isotope labelled folic acid			
Godwin & Rosenberg, 1975[69]	Subjects: 11 adults Doses:[a] ~265 µg $[^3H]PteGlu_1$ or $[^{14}C]PteGlu_7$ in 300 mL water. Design: Cross-over (intramuscular flushing dose $PteGlu_1$ 4 h after dosing) Outcome: Urinary excretion (48 h)	Urinary recovery: $PteGlu_1$, 71±13%; $PteGlu_7$, 56±11%; $P<0.05$	80%

Continuation of appendix

Reference	Subjects and design	Findings	Bioavailability
Halsted *et al*, 1975[66]	<u>Subjects</u>: 5 adults <u>Doses</u>:[a] 360 µg [^{3}H]$PteGlu_1$ or [^{14}C]$PteGlu_7$ in 300 mL water <u>Design:</u> Jejunal perfusion <u>Outcome</u>: Luminal isotope recovery	$PteGlu_1$, 75%; $PteGlu_7$, 53%; $P<0.001$	71%
Halsted *et al*, 1978[67]	<u>Subjects</u>: 10 adults (4 patients with coeliac sprue) <u>Doses</u>:[a] 400 µg [^{3}H]$PteGlu_1$ or [^{14}C]$PteGlu_6$ in solution <u>Design</u>: Jejunal perfusion (intramuscular flushing dose: 15 mg $PteGlu_1$) <u>Outcome</u>: Urinary isotope recovery (48 h).	Urinary recovery of $PteGlu_1$ > $PteGlu_7$ < $PteGlu_1$ $PteGlu_7$, $P<0.001$	
Bailey *et al*, 1984[68]	<u>Subjects:</u> 13 males (8 elderly, 5 young) <u>Doses</u>:[a] 1350 µg [^{3}H]$PteGlu_1$ or [^{14}C]$PteGlu_7$ in solution <u>Design</u>: Jejunal perfusion (intramuscular flushing dose: 15 mg $PteGlu_1$) <u>Outcome</u>: Luminal disappearance and urinary recovery (48 h)	*Elderly:* $PteGlu_1$, 63%; $PteGlu_7$, 51% *Young*: $PteGlu_1$, 67%; $PteGlu_7$, 48%	Elderly, 81%; young, 72%
Gregory *et al*, 1991[53]	<u>Subjects</u>: 7 males <u>Doses</u>:[a] 300 µg d_2-$PteGlu_1$ or d_2-$PteGlu_6$ in apple juice + intravenous injection with 250 µg d_4-$PteGlu_1$. <u>Design</u>: Cross-over (pre-loading: 2 mg $PteGlu_1$/d) <u>Outcome</u>: Urinary isotope excretion (48 h)	Excretion ratios: $PteGlu_1$ (d_2/d_4), 1.45; $PteGlu_6$ / $PteGlu_1$, 0.67	46%
Wei *et al*, 1996[70]	<u>Subjects</u>: 7 males <u>Doses</u>:[a] ~300 µg d_4-$PteGlu_1$ or d_2-$PteGlu_6$ in water; orange juice; tomatoes; lima beans; citrate solution; water again <u>Design</u> Cross-over (pre-loading: 2-10 mg $PteGlu_1$/d) <u>Outcome</u>: Plasma folate concentrations; urinary isotope excretion.	$PteGlu_6$ /$PteGlu_1$: ~1.0 for the control, tomato, lima bean and citrate buffer ~0.67 for orange juice	In water, tomato, lima bean, and citrate buffer, 100%; in orange juice, 67%

[a] $PteGlu_n$ = pteroylglutamic acid, or folic acid; n = number of glutamate residues.

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Part I.
Folate in the diet

Chapter 2

Dietary monoglutamate and polyglutamate folate are both associated with plasma folate concentrations in Dutch men and women aged 20-65 y

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ABSTRACT Dietary folate consists of monoglutamate and polyglutamate folate species. In the small intestine, folate polyglutamate is deconjugated to the monoglutamate form before absorption takes place. This enzymatic deconjugation might limit the bioavailability of polyglutamate folate. Until now, no data are available on dietary intake of both folate forms and their associations with folate status. Therefore we estimated the intake of monoglutamate and polyglutamate folate in the Dutch population, and studied whether the association with plasma folate is different for these two folate forms. Dietary intake of monoglutamate and polyglutamate folate from non-fortified foods was estimated for 2,435 subjects (1,275 men; 1,160 women) aged 20 to 65 years. The intake of monoglutamate folate was about one third of total folate intake, derived mainly from bread (~20%) and meat (~18%), while two thirds consisted of polyglutamates, derived mainly from vegetables (~25%). The predictive power of the regression model with total folate intake as the independent variable adjusted for age, smoking, and alcohol intake, did not increase when including the ratio of monoglutamate to polyglutamate folate intake. In addition, linear regression models showed that both monoglutamate and polyglutamate folate intake were associated positively with plasma folate levels. However, in men the monoglutamate folate form appeared to be a three-fold stronger determinant of plasma folate levels than polyglutamate folate, while in women both folate forms were equally strong determinants. This might be explained by different food intake patterns of men and women, including alcohol intake. At present, it does not seem necessary to distinguish between food folate forms in advice for increasing folate intake from non-fortified foods.

***Key words**: Dietary folate intake; Bioavailability; Polyglutamate folate; Monoglutamate folate; Plasma folate.*

2.1 Introduction

Folate is an important nutrient in the daily diet. For several decades it has been known that deficiency of this B vitamin can lead to megaloblastic anemia.[1,2] More recently, folic acid has been acknowledged in the prevention of neural tube defects.[3,4] Folate has been associated with risk of colon cancer[5] and it is considered a major determinant of plasma homocysteine concentration, a potential risk factor for cardiovascular disease.[6,7] It is estimated that a 5-µmol/L increase of plasma homocysteine concentration is associated with a 20-30% higher risk of cardiovascular disease.[8]

Intake of supplemental folic acid effectively decreases plasma homocysteine concentrations.[9,10] High dietary folate intake is also associated with lower homocysteine levels.[11-13] However, there is debate on whether increased folate intake from non-fortified foods is effective in lowering homocysteine levels in the general population as dietary folate has a lower bioavailability than supplemental folic acid.[14-17]

Bioavailablity is defined as the proportion of a nutrient ingested which becomes available to the body for metabolic processes or storage. Bioavailability of dietary folate may be hampered by the polyglutamate chain to which most of the natural folate is attached. This polyglutamate chain needs to be removed, except for the proximal glutamate moiety, by the enzyme folylpoly γ-glutamyl carboxypeptidase (FGCP), which is present in the human brush border. After that, folate can be absorbed and transported as a monoglutamate into the portal vein. Synthetic folic acid used for supplements and fortification consists of the monoglutamate form only. The relative bioavailability of dietary folate is estimated to be only 50% compared to synthetic folic acid.[18]

In the past decades, several attempts have been made to assess the bioavailability of folate polyglutamate compared to the monoglutamate form.[19-24] The data available suggest that the polyglutamate form is 60-80% bioavailable compared to the monoglutamate form.[25] Definite conclusions cannot be made, however, due to study designs with single high doses, short periods of effect measurement, pre-saturation protocols and small groups of subjects, combined with a high between-person variation.

Until now, no data have been published on intake of monoglutamate and polyglutamate folate from non-fortified sources. Such figures are necessary in order to assess the need to include this particular bioavailability aspect in the dietary reference intake for the general population, especially for countries where folate fortification is not allowed, such as the Netherlands.

With data from a Dutch survey we calculated the intake of monoglutamate and polyglutamate folate from non-fortified foods and assessed the main food sources of both forms of the vitamin. Furthermore, we related monoglutamate and polyglutamate intake data to plasma folate concentrations. We hypothesized that the association between monoglutamate folate intake with plasma folate concentrations would be stronger than that of polyglutamate folate because of the assumed lower bioavailability of the polyglutamate form.

2.2 Subjects and methods

2.2.1 Study population

Subjects were randomly sampled from the population of the 'Monitoring Project on Risk factors for Chronic Diseases' (MORGEN). The general purpose of this survey is to determine the prevalence of risk factors for chronic diseases in a representative sample of the Dutch population. The MORGEN study was approved according to the guidelines of the Helsinki Declaration by the external Medical Ethical Committee of the TNO Toxicology and Nutrition Institute.

The MORGEN study population consists of men and women aged 20-65 y living in three Dutch towns (Amsterdam, Doetinchem and Maastricht). Data were collected from 1993 to 1997. Of the total number of 19,066 subjects that entered the MORGEN study until 1996, 14,356 persons were eligible for our analyses. This implies that we had completed questionnaires, enough blood available for all aimed biochemical analyses, such as plasma homocysteine and plasma folate, and recorded time of drawing and centrifugation of blood from these persons. Storage of whole blood at room temperature may increase plasma homocysteine concentrations artificially.[26,27] Therefore, from the total of 14,356 subjects, 7,992 were excluded because their blood had been stored at room temperature for more than one hour. Of the 6,364 subjects left, plasma homocysteine and plasma folate levels were determined for a random subsample of 3,025 subjects, stratified for age and sex. This sample did not differ from the total study population with respect to established cardiovascular risk factors such as total and HDL cholesterol level, blood pressure, socio-economic status, body mass index (BMI), and smoking. De Bree *et al.* (2001) recently described the relation between intake of B vitamins and the distribution of plasma homocysteine concentrations in this population.[11]

2.2.2 Data collection

Respondents filled out two questionnaires and underwent a medical examination. One of the questionnaires contained general questions on items such as age, sex, presence of chronic diseases and risk factors for chronic diseases such as smoking habits and alcohol consumption. Food intake was determined by a

semi-quantitative food frequency questionnaire (EPIC food frequency questionnaire) on dietary habits over the last 12 months, containing about 178 food and supplement intake items with pictures of portion sizes. The food frequency questionnaire and its reproducibility have been described in more detail by Ocké *et al.*[28,29]

Recently, the monoglutamate and polyglutamate folate content of 125 foods that account for 90% of folate intake in the Dutch population was determined by an HPLC method.[30] During food preparation, folate can be lost from foods by destruction or by leakage. Furthermore, enzymatic conversion of folate polyglutamate to the monoglutamate form may occur. Therefore, foods were prepared according to normal household practice before analysis.[31] With these figures, the monoglutamate and polyglutamate folate intake of our study population was calculated. For food items for which total folate content was known but for which we did not know the monoglutamate proportion, we estimated this proportion making use of a closely comparable food item for which we did know the monoglutamate proportion (e.g. green and white cabbage). For a few food items we could not find a comparable food item (e.g. mushrooms). These items, which accounted for only 3% of total folate intake, were excluded in the data analyses.

Alcohol intake was assessed with the food frequency questionnaire by asking respondents how many glasses of beer, wine, liqueur and strong drinks per day, week, month or year they drank over the previous 12 months. Each glass of alcoholic beverage was assumed to contain 10 g of alcohol.

2.2.3 Blood sampling

Blood sampling took place between 9.00 and 14.00 h at the Municipal Health Centers in each of the 3 towns, after participants had signed an informed consent form. Venous blood (30 mL) was collected from non-fasting subjects. Blood samples were centrifuged and separated, and stored within 1 hour at -20°C at the Health Centers. Samples were transported to the National Institute of Public Health (Bilthoven, the Netherlands) within three weeks and stored at -80°C until further analysis.

2.2.4 Laboratory analysis

Plasma folate concentrations were determined by a *L. casei* microbiological assay according to the method described by Molloy and Scott[32] in the laboratory of the Department of Pharmacology, University of Bergen, Norway. Intra-assay and interassay variability were 4.3% and 7.0%, respectively.

2.2.5 Data analysis

Users of B vitamins were excluded from all statistical analyses (n=588), as were 2 persons with incomplete data; data of 2,435 persons (1,275 men and 1,160

women) were left for analysis. Plasma folate and folate intake data were all log-transformed in order to obtain normally distributed data. Residual energy adjustment was performed for nutrient intake according to the method described by Willett *et al.*[33] In order to be able to interpret nutrient intake data, the individual residual nutrient intakes as calculated from the regression line of total energy intake on total nutrient intake were added to the nutrient intake at the mean energy intake (9,391 kJ/d) in the study population.

The monoglutamate versus polyglutamate folate content of the diet was expressed as a ratio calculated by dividing the monoglutamate folate intake by the polyglutamate folate intake (MP-ratio). Linear regression models were used to describe trends in plasma folate levels (dependant variable) when associated with intake of monoglutamate, polyglutamate, or total folate (independent variables). Intake of both monoglutamate and polyglutamate folate were strongly correlated to total folate intake and could therefore not be fitted in the same model together with total folate. Total folate intake and MP-ratio were non-collinear (Pearson correlation coefficient of 0.19 in men and 0.09 in women) and could therefore be fitted in the same model. Monoglutamate and polyglutamate folate intake were also considered as non-collinear (r=0.38 in men and 0.51 in women).

Continuous models were used to compare the differences in plasma folate concentrations at a 50 µg higher intake of either monoglutamate or polyglutamate folate. Plasma folate levels were related to folate intake by using the geometric means of quintiles of intake of monoglutamate or polyglutamate folate as independent variables in the models to visualize trends. Multivariate models were adjusted for major explanatory factors of folate status, like age, smoking habits and alcohol intake.[34] BMI was not included in the models because it was not a confounder in the association between folate intake and plasma folate. Because we expected differences in dietary intake patterns we carried out all analyses for men and women separately. All data-analyses were performed with the Statistical Application Software for PC, version 6.12 (SAS, SAS Institute Inc., Cary, NC).

2.3 Results

In **Table 2.1**, the dietary folate intake and plasma folate levels in the study population are shown. Total folate intake was 232 µg/d in men, of which 38% was provided by the monoglutamate form, and 186 µg/d in women, of which 33% was provided by the monoglutamate form.

The MP-ratio was significantly higher for men (mean 0.60; 95% CI 0.59 to 0.61) than for women (mean 0.48; 95% CI 0.47 to 0.49). The most important sources of monoglutamate in the Dutch diet were bread and meat, for both men and women (**Figure 2.1**). For men, alcoholic beverages also were an important source of

Table 2.1 General characteristics, folate intake and folate status of Dutch men and women.[a]

	Men (n=1,275)	Women (n=1,160)
Age (y)	40.5 ± 12.1	40.8 ± 12.5
BMI (kg/m^2)	25.4 ± 3.5	24.9 ± 4.3
Alcohol intake (g/d)	17 ± 22	5 ± 10
Smokers (%)	35	37
Plasma folate (nmol/L)	8.4 ± 4.2 (7.6)	8.1 ± 4.0 (7.3)
Total folate intake (μg/d)	232 ± 71 (198)	186 ± 52 (200)
Monoglutamate folate intake (μg/d)	87 ± 37 (70)	60 ± 22 (63)
Polyglutamate folate intake (μg/d)	145 ± 41 (126)	126 ± 34 (134)
MP *ratio*[b]	0.60 ± 0.19 (0.58)	0.48 ± 0.14 (0.46)

[a]*Arithmetric* means ± SD (Geometric means; nutrient intakes are energy-adjusted). [b]*MP*-ratio is defined as monoglutamate/ polyglutamate folate intake.

monoglutamate folate intake, followed by eggs, milk, vegetables and fruit. For women, alcoholic beverages were a less important source of monoglutamate folate, while eggs, milk, vegetables and fruit were equally important sources. Vegetables were the most important source of polyglutamate folate for both men and women. Bread, potatoes and fruits delivered a substantial amount of polyglutamate folate to the diet as well, while meat and milk contributed less.

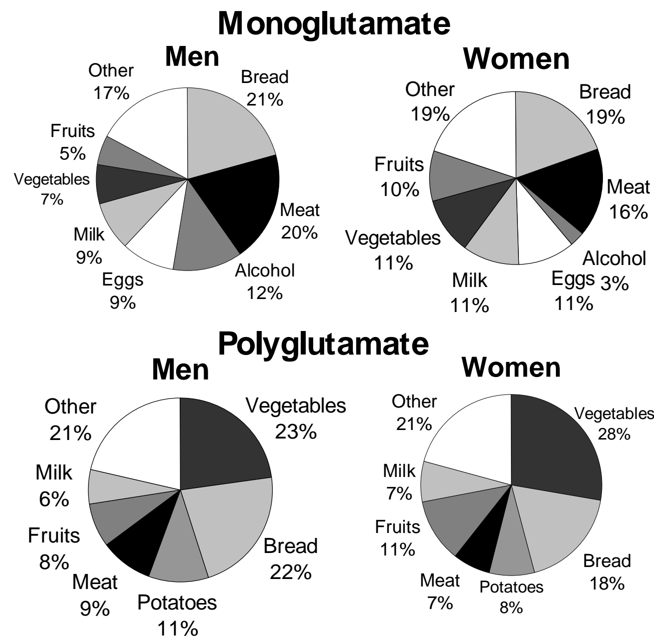

Figure 2.1 Percentage of monoglutamate and polyglutamate folate intake from food sources.

Table 2.2 Univariate and multivariate linear regression models between total folate intake and plasma folate levels, with and without introducing MP-ratio in the models.

	Men			Women		
	Coefficient[a]	P-value	Adj-R^2	Coefficient[a]	P-value	Adj-R^2
Univariate model						
Total folate intake[b]	0.71	<0.001	0.08	0.59	<0.001	0.07
Multivariate models						
Total folate intake[b]	0.62	<0.001	0.17	0.53	<0.001	0.10
Age,y	0.01	<0.001		0.00	<0.3	
Smoking, yes/no	-0.05	<0.05		-0.07	<0.01	
Alcohol intake, medium/low[c]	0.07	<0.05		0.03	<0.4	
high/low[d]	0.28	<0.001		0.19	<0.001	
Total folate intake[b]	0.58	<0.001	0.17	0.53	<0.001	0.10
MP-ratio	0.23	<0.01		-0.00	<1.0	
Age, y	0.01	<0.001		0.00	<0.3	
Smoking, yes/no	-0.06	<0.05		-0.07	<0.01	
Alcohol intake, medium/low[c]	0.07	<0.05		0.03	<0.4	
high/low[d]	0.23	0.0001		0.20	0.0001	

[a] Coefficients represent changes per variable unit in log-transformed plasma concentration (ln nmol/L).
[b] Intake in µg/d, log-transformed and energy-adjusted.
[c] Medium alcohol intake is 0-20 g/d for men and 0-10 g/d for women.
[d] High alcohol intake is > 20 g/d for men and > 10 g/d for women.

2

Table 2.3 Univariate and multivariate linear regression models between monoglutamate and polyglutamate folate intake and plasma folate levels in Dutch men and women aged 20-65 y.

	Men				Women			
	Coefficient[a]	95% CI	P-value	Adj-R^2	Coefficient[a]	95% CI	P-value	Adj-R^2
Univariate model								
Monoglutamate folate intake[b]	0.55	0.47-0.63	<0.001	0.13	0.36	0.27-0.45	<0.001	0.05
Polyglutamate folate intake[2]	0.35	0.23-0.47	<0.001	0.03	0.48	0.36-0.59	<0.001	0.06
Multivariate models								
Monoglutamate folate intake[b]	0.38	0.28-0.47	<0.001	0.18	0.17	0.06-0.27	<0.01	0.10
Polyglutamate folate intake[2]	0.18	0.05-0.31	<0.01		0.35	0.21-0.48	<0.001	
Age, y	0.01		<0.001		0.00		<0.3	
Smoking, yes/no	-0.06		<0.05		-0.08		<0.01	
Alcohol intake, medium/low[c]	0.06		<0.05		0.06		<0.05	
high/low[d]	0.23		<0.001		0.32		<0.001	

[a] Coefficients represent changes per variable unit in log-transformed plasma concentration (ln nmol/L).
[b] Intake in µg/d, log-transformed and energy-adjusted.
[c] Medium alcohol intake is 0-20 g/d for men and 0-10 g/d for women.
[d] High alcohol intake is > 20 g/d for men and > 10 g/d for women.

In **Table 2.2**, the univariate and multivariate linear regression models for the association between total folate and plasma folate are shown. When MP-ratio was brought into the model as an explanatory variable, a significant coefficient was seen in men, but not in women. This indicates that in men the MP-ratio of the dietary folate intake was an explanatory factor for plasma folate levels but not in women. However, addition of the MP-ratio to the models did not increase their explanatory capacity because the adjusted-R^2 remained 0.17 for men and 0.10 for women, respectively.

When exploring this further, univariate linear regression analysis of the continuous data showed that monoglutamate folate intake explained 13% and 5% of plasma folate levels for men and women, respectively, while polyglutamate folate intake explained 3% and 6%, respectively (**Table 2.3**). The multivariate model, in which intake of both monoglutamate and polyglutamate folate were included as independent variables as well as the confounding variables age, smoking, and alcohol intake, could explain 18% of plasma folate levels in men and 10% in women. The associations in men were stronger for the monoglutamate than for the polyglutamate form, while in women this was the other way around. The coefficients for monoglutamate folate intake in men and women differed statistically significant from each other (mean 0.38, 95% CI 0.28 to 0.47 for men; mean 0.17, 95% CI 0.06 to 0.27 for women). The results of the regression analyses are visualized in **Figure 2.2.**

In both men and women, high alcohol intake was a strong determinant of plasma folate concentrations. In our regression models, alcohol intake showed a slight interaction with the intake of monoglutamate folate in men, although not significant (data not shown). When exploring this further, a trend of increasing plasma folate levels with quartiles of alcohol intake, adjusted for total folate intake, age and smoking was found in men. This association was not seen in women, which may be explained by the lower alcohol intake range in this group (men: 17 ± 22 g/d; women: 5 ± 10 g/d). This is in line with the finding that alcohol intake and homocysteine levels were inversely associated in men from the same study population.[35]

From the multivariate model, we calculated that in men a 50 µg/d higher intake of monoglutamate folate was associated with 27% higher plasma folate levels while a 50 µg/d higher intake of polyglutamate folate intake was associated with a higher plasma folate level of only 8%. This suggests that, in men, monoglutamate folate was a three times stronger determinant of plasma folate levels than polyglutamate folate. For women, a 50-µg higher intake of monoglutamate folate was associated with 11% higher levels of plasma folate, while this was 16% for a 50-µg higher polyglutamate folate intake.

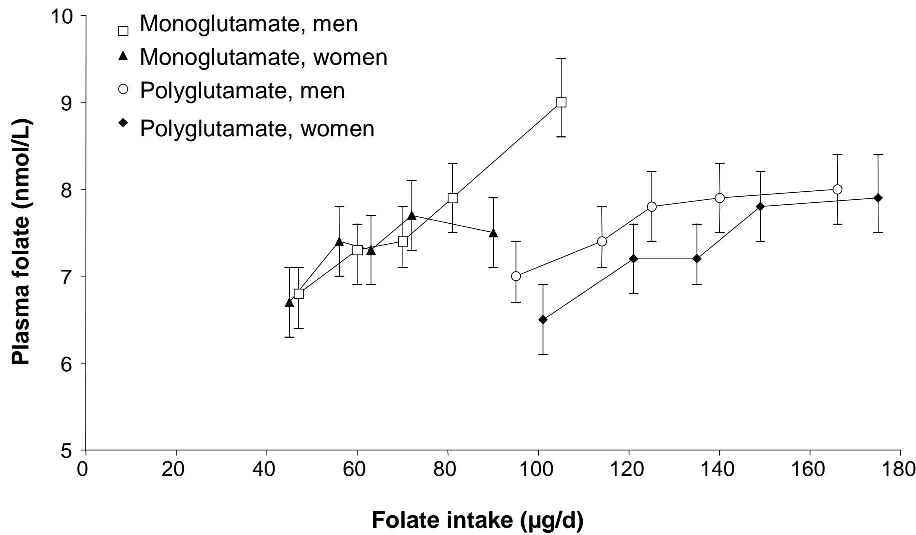

Figure 2.2 Plasma folate concentration (nmol/L) by quintiles of intake of monoglutamate and polyglutamate folate in Dutch men and women aged 20-65 years. Data points are geometric quintile means with 95% CI. The models were adjusted for intake of either polyglutamate or monoglutamate folate and for alcohol intake, age and smoking.

2.4 Discussion

For the first time, population-based folate intake data that distinguish between the monoglutamate and polyglutamate form of the vitamin are presented. We found that in the Dutch diet about one third of folate intake is in the monoglutamate form and two thirds in the polyglutamate form. Bread, meat, eggs, and milk are important sources of the monoglutamate form, while plant foods such as vegetables, potatoes, bread, and fruit are important intake sources of the polyglutamate form.

Intake of both monoglutamate and polyglutamate folate were positively associated with plasma folate levels. In men, intake of monoglutamate folate was a three-fold stronger determinant of plasma folate levels than polyglutamate folate, which is in line with our prior hypothesis. In women, monoglutamate and polyglutamate folate appeared to be about equally strong determinants of plasma folate. In line with these findings, the coefficient for the MP-ratio in the multivariate models was significant only for men, although the variance explained by the model did not increase after addition of the MP-ratio to the model.

The present study can only give a rough estimate of real bioavailability issues due to 1) bias in dietary data collection; 2) variation in the monoglutamate and polyglutamate folate content of foods; and 3) the assumption that one

measurement of non-fasting plasma folate reflects folate status well enough to find valid associations. The bias in the collection of dietary data for the questionnaire used, was studied using 12 monthly 24 h-recalls as a reference to the EPIC food frequency questionnaire.[29] For important folate sources like bread, potatoes, fruits, meat, eggs and milk, relative validity figures of 0.41 to 0.78 were reported. For vegetable intake, a lower relative validity (0.25-0.36) was observed, leading to more measurement error in the estimation of vegetable intake than for that of other folate sources. Another issue is the estimation of intake of monoglutamate and polyglutamate folate. The food composition data we used, for which foods were prepared by standard household practice, approach actual intakes of monoglutamate and polyglutamate folate as closely as possible. Seasonal and regional variation in folate content of foods appeared to be low. Analytical variance might however have introduced some error.[30] As known, erythrocyte folate concentrations give a better reflection of long-term folate status than plasma folate. Because whole blood was not collected we could not determine folate concentrations in erythrocytes. The proportion of variance in plasma folate levels explained by our models are in line with those found by other researchers.[34]

The different results obtained in men and women may be explained by distinct patterns of food intake. While men obtain 12% of their monoglutamate folate from fruit and vegetables, women obtain 21% from these sources. The food matrix of fruit and vegetables might reduce folate bioavailability. The food matrix can be described as the cell structure that encapsulates a nutrient and complexes it to proteins and fibre.

Little is known about the role of the food matrix and its interaction with different chemical folate forms in folate bioavailability. Van het Hof *et al* [36] and Castenmiller *et al*[37] compared the effects of consumption of whole leaf spinach with that of chopped spinach on plasma folate levels. Both groups concluded that disruption of the vegetable matrix resulted in higher folate bioavailability.

Another explanation for the better bioavailability of monoglutamate folate in men than in women might be differences in alcohol intake. Brussaard *et al*.[34] also reported a consistent positive relationship between alcohol consumption in the general Dutch population and serum folate concentrations independent of folate intake, as we have found in the present study. Intake of monoglutamate folate from beer may partly explain this association although the effect remained after adjustment for total folate intake. A direct positive effect of low doses of ethanol on plasma folate concentrations cannot be excluded. So far, research has been focused on the effect of high doses of alcohol. An inverse relationship between alcohol intake and folate status has been shown in alcoholics.[38,39] In vitro studies showed that alcohol could cleave folate, thereby destroying its vitamin activity.[40] Also, alcohol intake was shown to decrease FGCP activity thereby affecting the

absorption of polyglutamate folate.[41,42] Up till now, only one intervention study looked at the effects of lower alcohol doses on folate levels.[43] In this study no effect was found of either red wine, beer or spirits on plasma folate concentrations as compared to water. Dosage of alcohol in this study however still might have been too high (40 g/d).

In conclusion, intake of polyglutamate folate is twice that of monoglutamate folate in this Dutch population. The positive trends in plasma folate levels found for intake of both monoglutamate and polyglutamate folate intake indicates that both folate forms contribute favourably to plasma folate levels in both men and women. The present study suggests that folate bioavailability in men is reduced by the polyglutamate chain, which supports our initial hypothesis, but not in women. This discrepancy might be due to differences in dietary patterns leading to differences in folate bioavailability caused by the food matrix in which the folate is contained. Therefore, it may not be the polyglutamate chain that reduces dietary folate biavailability but rather the matrix in which dietary folate is incorporated. Alcohol intake appeared to be a strong determinant of folate status and might also explain the different findings in men and women. Because of possible uncontrollable bias, the data must be interpreted with some caution. To study the specific factors involved in folate bioavailability, well designed intervention studies are required. At present, it does not seem necessary to distinguish between the intakes of monoglutamate and polyglutamate folate in making recommendations on increasing folate intake from the diet.

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Chapter 3

Influence of processing on total, monoglutamate and polyglutamate folate content of leeks, cauliflower, and green beans

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ABSTRACT Bioavailability of dietary folate might be impaired by the polyglutamate chain to which about 70% of dietary folates are bound. This chain must be removed enzymatically in the intestine before folate is absorbed as a monoglutamate. To increase formation of monoglutamate folate in vegetables we subjected these to various processing treatments. Treatments included freezing (–8°C, 16h) and thawing (4°C, 24 h), and hydrostatic high-pressure treatment (200 megapascals, 5 min). Both freezing and thawing, and high-pressure treatment increased the proportion of folate in the monoglutamate form in leeks, cauliflower and green beans two- to threefold. However, loss of total folate after these treatments was over 55%. We conclude that conversion of folate polyglutamate to the monoglutamate form in vegetables is possible by certain processing treatments. Potentially this could lead to vegetables with higher folate bioavailability. However, to prevent folate loss into processing water, processing in a closed system should be applied.

***Keywords**: Folate; Monoglutamate; Polyglutamate; Processing; Freezing; High-pressure treatment; Blanching; Steaming; Vegetables.*

3.1 Introduction

Vegetables and fruits are rich sources of dietary folate, which is the natural form of folic acid. This is a B vitamin that has proven capacity to decrease the risk of neural tube defects in newborns[1,2] and can lower plasma homocysteine concentrations in humans. High plasma homocysteine concentrations are associated with cardiovascular diseases.[3] Currently the impact of lowering plasma homocysteine levels by folic acid on disease risk is under study.[4] Folate intake is also studied in relation to certain forms of cancer.[5]

Various studies have shown that the bioavailability of dietary folate is lower than that of synthetic folic acid.[6,7] One of the main factors impairing the bioavailability of dietary folate may be that food folate is present mainly as polyglutamate conjugate.[8,9] Before folate is absorbed from the gut, all but the proximal glutamate moiety must be removed by the enzyme folylpoly γ-glutamyl carboxypeptidase (FGCP, also known as γ-glutamyl hydrolase or folate conjugase), present in the brush border of the small intestine.[10] Synthetic folic acid, used as a supplement and in food fortification, is a monoglutamate and this may explain why it is absorbed more easily.[11] Study results vary however with study designs and some studies show hardly any difference in absorption rate of polyglutamate compared to monoglutamate folate[9,12]. Other important factors that can impair dietary folate bioavailability are the matrix of the food, intestinal pH, fibre content, and the presence of organic acids or folate binding protein in the food.[8,9]

In vegetables, folate is present in various vitamers, including tetrahydrofolate (THF) and 5-methyltetrahydrofolate (5-MTHF). These vitamers exist to a small extent as monoglutamates but are mainly conjugated to a chain of 2-8 glutamate moieties. FGCP is also present in vegetables. A few decades ago it was noted that during homogenization preceding analysis of fresh vegetables, spontaneous conversion of folate polyglutamate to the monoglutamate form occurs.[13] Although this process interfered with the determination of the natural monoglutamate folate content of vegetables, we speculated that it could be used to produce vegetables rich in monoglutamate folate thereby increasing the bioaccessibility of folate from vegetables. Bioaccessibility is defined as the proportion of an ingested nutrient, which is presented to the intestinal brush border in an absorbable form.

It is not clear where FGCP is located within plant cells. Although in animals it is known to be lysosomal[14-16], in plants it is probably located mainly in the cytosole and extracellularly.[17,18] We hypothesized that folate bioaccessibility from vegetables could then be increased by disruption of the cell structure. Cell disruption would allow contact between polyglutamate folate and the enzyme thereby stimulating hydrolysis of folate polyglutamate to the monoglutamate form.

Thus, the aim of this study was to process vegetables in such a way that the endogenous γ-glutamyl hydrolase activity would be stimulated, resulting in vegetables with an increased content of monoglutamate folate. In a later stage, folate bioavailability from such vegetables could then be tested in humans and be compared to that from vegetables processed in the same way (similar matrix, pH, etc.) but with a low proportion of folate in the monoglutamate form.

3.2 Materials and methods

3.2.1 Materials

From a list of products that contribute >75% to the folate intake of the Dutch population[19], we selected several vegetables with a high folate content, a high proportion of polyglutamate folate, and with different plant structures (color, type of tissue). The vegetables chosen were leeks, cauliflower, and green beans. The processing conditions used were chosen because of their ability to damage cell structure. In addition, the vegetables were blanched either before or after processing to destroy enzymatic activity thereby preventing any further conversion of folate polyglutamate to the monoglutamate form.

3.2.2 Pilot study

Leeks (20 kg) were purchased from a local supermarket, cut into 5-mm rings and washed, and subjected to various experimental treatments as described in **Table 3.1**. High-pressure treatment is a novel processing technique used to preserve food products in a mild way. Because this technique affects membrane porosity it can affect the enzymatic conversion of folate polyglutamate to the monoglutamate form. High-pressure treatment at 100-200 megapascal (MPa) results in crystallization of phospholipids in cell membranes, resulting in permeabilization of membranes.[20] Freezing also damages the plant cell structure by expansion of the intracellular fluid. In theory, slow freezing causes more damage to cell structure because of the larger ice crystals formed in comparison to rapid freezing at lower temperatures. Therefore we applied both freezing at –18 °C and at –80 °C.

All treatments were started on the same day to prevent changes in folate content caused by pre-treatment storage. After treatment, vegetables were stored in the refrigerator to allow enzymatic activity to take place. After blanching, draining and airtight packed cooling at room temperature, samples (n=1) were stored in freezer bags at –80 °C until analysis.

High-pressure treatment was carried out at the industrial test plant of the Agrotechnologic Research Institute (ATO), Wageningen. All other treatments were performed at the Division of Human Nutrition, Wageningen University.

Table 3.1 Description of the processing treatments used in the pilot study with leeks.

	Treatment	Description
A	Blanching	Submersion of the cut and washed vegetables in boiling water (1 L water/kg fresh vegetable weight) for 10 min using household utensils on an electric stove.
B	Freezing (-80°C), thawing	Freezing at -80°C for 16 h, followed by 24 h thawing in the refrigerator at 4°C, and then blanching (treatment A).
C	Freezing (-18°C), thawing	Freezing at -18°C for 16 h, followed by 24 h thawing in the refrigerator at 4°C, and then blanching (treatment A).
D	Freeze-drying	Freeze-drying for 120 h, followed by re-addition of the evaporated water, storage in the refrigerator at 4°C for 6 h, and then blanching (treatment A).
E	High-pressure treatment	High-pressure treatment at 50, 100, 150, and 200 MPa for 5 min. Pressure was established by compression of glycol surrounding the vegetable, which was vacuum, packed in a plastic bag. The treatment was followed by storage for 6 h in the refrigerator at 4°C, and then blanching (treatment A).

3.2.3 Main study

From the pilot study, the best treatments for conversion of folate polyglutamate to the monoglutamate form were selected. Criteria were firstly the best results in converting polyglutamate folate into monoglutamate folate and secondly the applicability of the processing method on a larger scale. These included freezing at −18 °C followed by thawing at 4°C for 24 h, and hydrostatic high-pressure treatment at 200 MPa for 5 min, followed by 24 h storage at 4°C (in the pilot study the period was 6 h). For high-pressure treatment, the start temperature was set at 22°C and the maximum temperature during the treatments was 30°C. Both freezing and thawing, and high-pressure treatment were either preceded or followed by blanching for enzyme inactivation. This was done to produce vegetables either high in polyglutamate folate or high in monoglutamate folate, but not different in other respects. The separate effects of 24 h refrigerated storage or blanching on folate vitamer content were studied, as well as the effect of hydrostatic high-pressure treatment without subsequent storage. The effects of steaming the vegetables instead of blanching were also studied. All treatments applied during the main study are summarized in **Table 3.2**.

Freshly cut and washed leeks (rings, 5 mm), cauliflower (florets, 2-4 cm), and green beans (pieces, 5 cm) were purchased in batches of 5 kg each from a wholesale greengrocer. After thorough mixing, random samples of 200 g were taken from these vegetable batches, and processed. Five of these samples were packed in freezer bags and immediately frozen at −80°C, until analysis. Folate vitamer content after each of the treatments G to N (Table 3.2) was compared to the mean content of these 5 raw samples. All treatments started on the same day.

Table 3.2 Description of the processing treatments used in the main study with leeks, cauliflower and green beans.

	Treatment	Description
F	Raw	No treatment.
G	Storage	Storage for 24 h in a refrigerator at 4 °C.
H	Blanching	Blanching in an industrial blanching kettle (10 L water/ 200 g fresh vegetable weight) for 5 min (leeks), 8 min (cauliflower), or 6 min (green beans).
I	Steaming	Steaming in a steaming sieve of 200 g vegetable above 1 L boiling water for 5 min (leeks), 7 min (cauliflower), or 6 min (green beans).
J	High-pressure treatment	High-pressure treatment at 200 MPa for 5 min. Pressure was established by compression of water surrounding the vegetables.
K	Freezing, thawing, blanching	Freezing at -18°C for 16 h, followed by thawing during storage (treatment G), and then blanching (treatment H).
L	High-pressure treatment, blanching	High-pressure treatment (treatment J) followed by storage (treatment G), and then blanching (treatment H).
M	Blanching, freezing, thawing	Blanching (treatment H) followed by freezing at -18°C for 16 h and thawing during storage (treatment G).
N	Blanching, high-pressure treatment	Blanching (treatment H) followed by high-pressure treatment (treatment J) and storage (treatment G).

The time needed for blanching cauliflower and green beans was established by placing 5 thermocouples to the innermost part of the vegetables. The vegetables were put in boiling water and the temperature was measured every second using a datalogger until a temperature of > 90 °C was reached. For leeks, from which the thermocouples always became detached, it was assumed that the time required for heating the vegetable internally would be shorter than for cauliflower and green beans because of the higher water content and smaller cross section. In order to inactivate enzymes and micro-organisms effectively, blanching periods were calculated as the time required for heating the innermost part of the vegetable plus 4 minutes. For leek, cauliflower and green beans blanching periods of 5, 8, and 6 min were used, respectively. To establish steaming periods, the same approach was used: leek, cauliflower and green beans were steamed for 5, 7, and 6 min, respectively. After blanching or steaming, the vegetables were drained in a colander for a few seconds. Vegetables were immediately packed in freezer bags, cooled on ice water, and stored in the freezer at –80 °C until analysis.

3.2.4 Analysis

Frozen samples were homogenized each with ca. 1 L of liquid nitrogen in a 4 L Waring Blender for 3-5 min until a homogeneous powder was developed. Samples of ~100 g were put in duplicate 100 mL plastic pots and stored at -80 °C.

Homogenization was done within one week after storage of the processed vegetables.

Samples from the pilot study were transported on dry ice for analysis to the Inspectorate of Health Protection and Veterinary Public Health. Samples from the main study were analyzed at the RIKILT, Wageningen. Both institutes used the same analysis method. In short, after thawing of the vegetable powder, folates were extracted from the samples by further homogenization in a 50 mM Ches/Hepes buffer (pH 7.8), containing 2% ascorbic acid and 2M 2-mercaptoethanol as antioxidants, with an Ultra Turrax homogenizing under nitrogen for 15 sec. This homogenate was subjected to heat treatment (10 min under nitrogen in a boiling water bath) and homogenized again with an Ultra Turrax under nitrogen for 15 sec. A first aliquot was analyzed without addition of any enzymes (treatment 1) to estimate the monoglutamate folate content of the samples. In a second aliquot, folate concentrations were quantified after addition of rat plasma conjugase for conversion of folate polyglutamate to the monoglutamate form, as well as protease and amylase (treatment 2). In this way, the sum of monoglutamates and polyglutamates was established. The difference between the amount of folate assayed in treatments 1 and 2 were assumed to represent the folate polyglutamate content. Deconjugation was 100% complete for each sample as checked by external addition of triglutamate folic acid. After purification by affinity chromatography, folate monoglutamates were determined using a HPLC method with fluorescence and diode array detection.[21] This procedure was used to quantify the levels of several folate forms naturally present, including THF, 5-MTHF, 5-FTHF, and 10-FDHF. The method showed recoveries of 81-87% with externally added 5-MTHF for raw samples of leek, cauliflower and green bean.

3.3 Results

3.3.1 Pilot study

The pilot study with leeks showed that all processing treatments applied (treatments B to E) yielded higher monoglutamate folate content in leeks compared to blanching (treatment A; **Figure 3.1**; n=1). Based on relative monoglutamate folate yield, theoretically expected larger ice crystals after slow freezing, and applicability of the processing method on a larger scale, we chose freezing at -18°C (treatment C) and high-pressure treatment at 200 MPa for 5 min (treatment E4) as the best methods for our further experiments.

3.3.2 Main study

In **Table 3.3**, the total, monoglutamate, and polyglutamate folate content of raw leeks, cauliflower, and green beans are shown. Variations in total folate within

5 samples, expressed as standard deviations, were small in all vegetables (leeks, 49.6 ± 4.4 µg/100 g; cauliflower, 53.3 ± 8.2 µg/100 g, green beans, 39.1 ± 5.1 µg/100 g). In raw leeks, 29.5% of the folate was in the monoglutamate form. For raw cauliflower and green beans this was 8.5% and 27.8%, respectively. 5-MTHF was the predominant folate form present in all vegetables. THF was present only in the polyglutamate fraction of the vitamin.

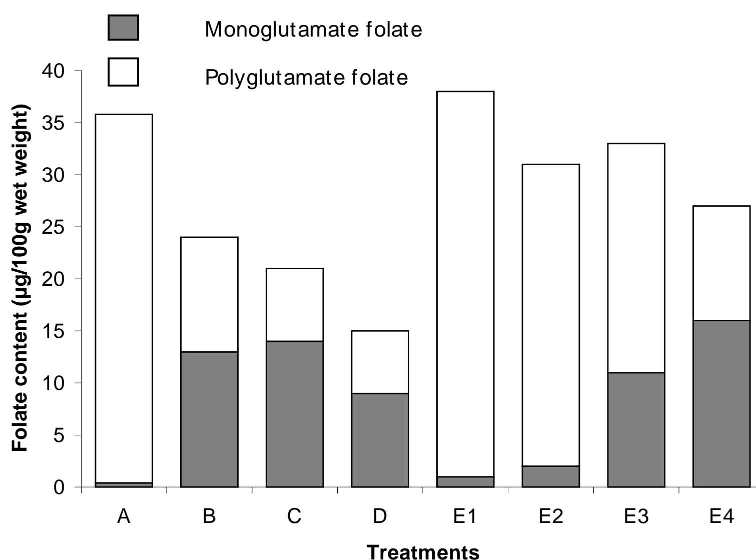

Figure 3.1 Folate content (total, monoglutamate and polyglutamate) of leeks after various processing treatments used in the pilot study (n=1). Polyglutamate content was calculated as the total folate content (after deconjugation) minus the monoglutamate folate content (before deconjugation). See Table 3.1 for a detailed description of treatments: A. Blanching; B. Freezing (-80 °C), thawing; C. Freezing (-18 °C), thawing; D. Freeze-drying; E. High-pressure treatment: E1) 50 MPa; E2) 100 MPa; E3) 150 MPa; E4) 200 MPa.

The effect of the processing treatments on the total, monoglutamate, and polyglutamate folate content of the vegetables, based on dry weight, is shown in **Table 3.4** and visualized in **Figure 3.2**.

Effects on total folate – Storage of the cut and washed vegetables for 24 h in the refrigerator (treatment G) resulted in folate losses of 0 to 25%. Blanching (treatment H) caused folate losses of 10 to 28%, while for steaming (treatment I) this ranged from 8 to 26%. Folate loss in green beans was less after steaming (10%) than after blanching (21%). High-pressure treatment (treatment J) led to greater losses of folate in all vegetables, ranging from 47 to 81%. Freezing and thawing or high-pressure treatment followed by blanching (treatments K and L, respectively) resulted in folate losses over 55%, while processing preceded by blanching

Table 3.3 Folate vitamer content of raw vegetables (µg/100 g), based on wet and dry weight (Mean ± SD, n=5)

	Total	**Monoglutamate**			**Polyglutamate**[a]	
		5-MTHF	THF	Total mono as % of total folate	5-MTHF	THF
Wet weight:						
Leeks	49.6 ± 4.4	16.1 ± 3.3	<DL[b]	29.5 ± 8.0	27.9 ± 6.6	5.6 ± 1.3
Cauliflower	53.5 ± 8.2	4.8 ± 1	<DL	8.5 ± 1.2	45.7 ± 6.7	3.0 ± 1.1
Green beans	39.1 ± 5.1	12.7 ± 0.4	<DL	27.8 ± 4.7	19.2 ± 4.0	7.3 ± 1.8
Dry weight:						
Leeks	580 ± 56	187 ± 38	<DL	32.8 ± 9.4	326 ± 78	66 ± 16
Cauliflower	696 ± 111	62 ± 11	<DL	9.0 ± 1.3	595 ± 95	39 ± 14
Green beans	526 ± 65	170 ± 7	<DL	32.9 ± 5.1	257 ± 52	98 ± 23

[a] Polyglutamate content was calculated as the total folate content (after deconjugation) minus the monoglutamate folate content (before deconjugation). [b] <DL= Under detection limit.

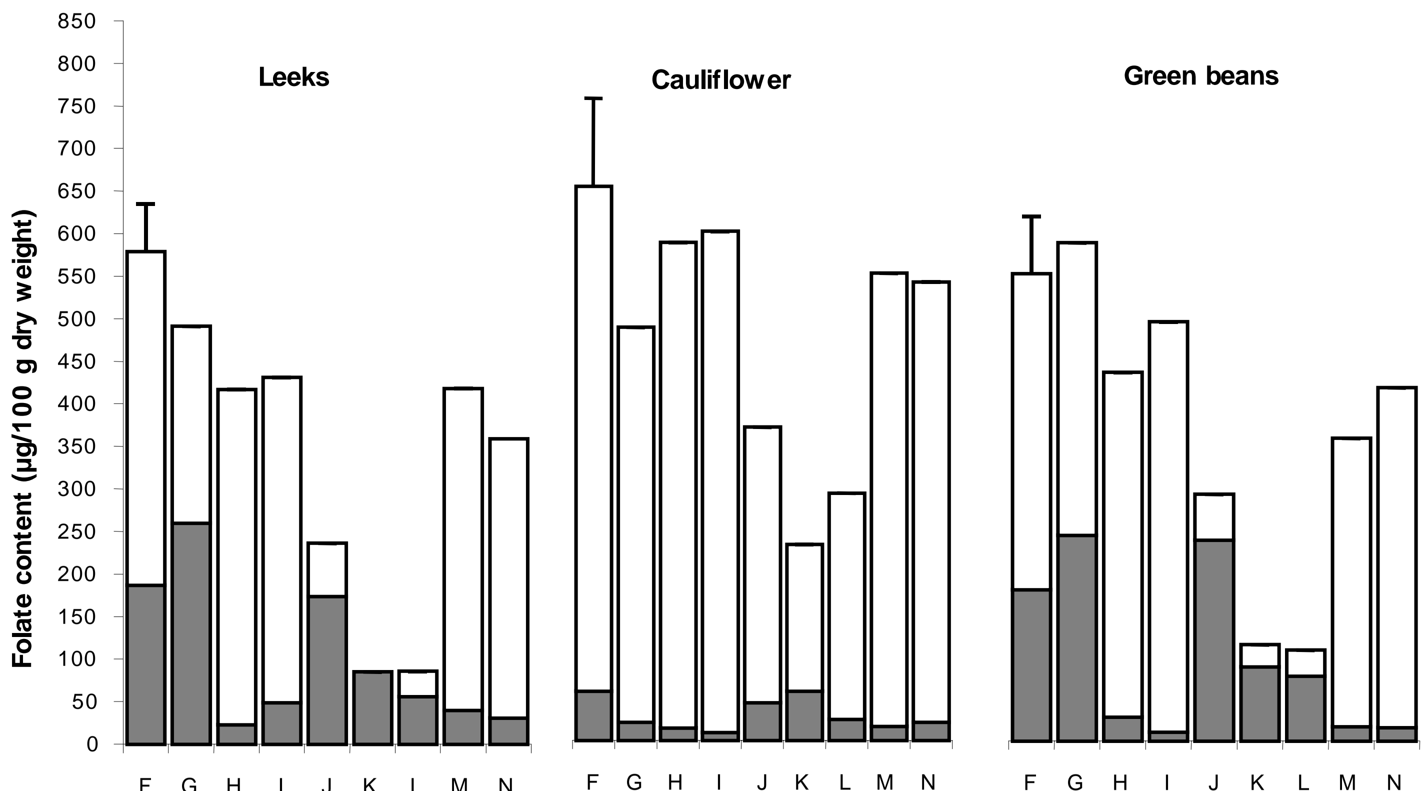

Figure 3.2 Folate content (total, monoglutamate and polyglutamate) of leeks, cauliflower and green beans before and after processing treatments used in the main study, based on dry weights. Values for raw vegetables are shown as mean (n=5) with SD in error bars. See Table 3.2 for a description of treatments. Polyglutamate content was calculated as the total folate content (after deconjugation) minus the monoglutamate folate content (before deconjugation). F. Raw; G. Storage; H. Blanching; I. Steaming; J. High-pressure treatment; K. Freezing, thawing, blanching; L. High-pressure treatment, blanching; M. Blanching, freezing, thawing; N. Blanching, high-pressure treatment.

Table 3.4 Folate content (total, monoglutamate and polyglutamate) of vegetables after various processing treatments.[a]

Treatments[b]	Total			Monoglutamate		Polyglutamate
	Dry matter %	µg/ 100 g dry weight	% loss	µg/ 100 g dry weight	% of total	µg/ 100 g dry weight
Leeks						
Raw (F)	8.6 ± 0.1	580 ± 56	0	187 ± 38	33 ± 9	392 ± 81
Storage (G)	8.6	491	15	260	53	231
Blanching (H)	6.5	417	28	23	6	394
Steaming (I)	8.7	431	26	49	11	382
High-pressure treatment (J)	6.5	236	61	174	74	62
Freezing, thawing, blanching (K)	5.6	85	85	85	100	0
High-pressure treatment, blanching (L)	5.3	86	85	56	65	30
Blanching, freezing, thawing (M)	6.5	418	28	40	10	378
Blanching, high-pressure treatment (N)	5.6	359	38	31	9	328
Cauliflower						
Raw (F)	7.7 ± 0.3	696 ± 111	0	62 ± 11	9 ± 1	634 ± 105
Storage (G)	7.7	519	25	23	4	496
Blanching (H)	6.9	626	10	16	3	610
Steaming (I)	7.7	640	8	10	2	630
High-pressure treatment (J)	6.8	394	43	48	12	346
Freezing, thawing, blanching (K)	6.7	246	65	62	25	184
High-pressure treatment, blanching (L)	6.3	311	55	27	9	284
Blanching, freezing, thawing (M)	6.7	587	16	18	3	569
Blanching, high-pressure treatment (N)	6.1	576	17	23	4	553

Continuation of Table 3.4

Treatments[b]	Total			Monoglutamate		Polyglutamate
	Dry matter %	µg/ 100 g dry weight	% loss	µg/ 100 g dry weight	% of total	µg/ 100 g dry weight
Green beans						
Raw (F)	7.4 ± 0.2	526 ± 65	0	170 ± 7	33 ± 5	355 ± 71
Storage (G)	7.4	560	-7	231	41	329
Blanching (H)	7.9	414	21	27	7	387
Steaming (I)	7.8	471	10	10	2	461
High-pressure treatment (J)	7.4	277	47	226	82	51
Freezing, thawing, blanching (K)	8.2	108	79	83	77	25
High-pressure treatment, blanching (L)	7.4	102	81	73	72	29
Blanching, freezing, thawing (M)	8.5	340	35	16	5	324
Blanching, high-pressure treatment (N)	8.1	397	24	15	4	382

[a] Values for raw vegetables expressed as mean ± SD (n=5). All other data are based on single measurements. [b] See Table 2 for a description of treatments. [c] Polyglutamate content was calculated as the total folate content (after deconjugation) minus the monoglutamate folate content (before deconjugation).

3

(treatments M and N, respectively) resulted in no more loss of folate than blanching alone (<35%).

Effects on monoglutamate and polyglutamate folate – Refrigerated storage (treatment G) showed a moderate increase in monoglutamate folate in leeks (from 33% to 53%) and green beans (from 33% to 41%), but not in cauliflower. Compared to raw vegetables, the proportion of folate in the monoglutamate form was lower after blanching (treatment H) and steaming (treatment I): in leeks, 6% and 11%, respectively; in cauliflower, 3% and 2%, respectively; and in green beans, 7% and 2%, respectively. Both blanching and steaming did not cause changes in the absolute amount of polyglutamate folate in the vegetables, while the absolute amount of monoglutamate folate decreased strongly. The proportion of folate in the monoglutamate form was increased after high-pressure treatment (treatment J) to 74% in leeks, 12% in cauliflower and to 82% in green beans. After freezing and thawing or after high-pressure treatment, both followed by blanching (treatments K and L, respectively), the proportion of folate as monoglutamate was increased: in leeks to 100% and 65%, respectively; in cauliflower to 25% and 9%, respectively; and in green beans to 77% and 72%, respectively. Blanching followed by processing (treatments M and N, respectively) resulted in a low percentage of folate in the monoglutamate form, namely for leeks, 10% and 9%, respectively; for cauliflower, 3% and 4%, respectively; and for green beans, 5% and 4%, respectively.

3.4 Discussion

In the present study we were able to stimulate the hydrolysis of folate polyglutamate to the monoglutamate form in vegetables by various processing treatments. We saw a marked increase in the proportion of monoglutamate folate relative to total folate after freezing and thawing, and also after high-pressure treatment. However, both processing treatments also led to substantial loss of total folate.

The high folate losses are probably caused by leakage of folate from the vegetables during freezing and high-pressure treatment, and subsequent loss into the liquid during blanching. Blanching and steaming led to major losses of monoglutamate folate, while the amount of polyglutamate folate in the vegetables remained practically unchanged. Freezing and thawing or high-pressure treatment enhanced loss of folate during subsequent blanching. This implies that folate in the monoglutamate form may be lost preferentially when the vegetable is in direct contact with water. Destruction of the vitamin by exposure to heat might also have led to loss of folate. However, Petersen[22] showed that blanching of broccoli for 5 min in a closed system results in only minor losses of folate. Therefore, leakage of folate mainly in the monoglutamate form must have been the most important

reason for loss of folate in our experiments. Because of the preferential leakage of monoglutamate folate, our data most probably give an underestimation of the real conversion of polyglutamate to monoglutamate by stimulated deconjugase activity due to treatment K and L.

From the pilot study with leeks, we concluded that the best way of converting folate polyglutamate to the monoglutamate form was to freeze it at –18°C, or to use high-pressure treatment. Freezing at –18°C showed similar results than freezing at –80°C. In theory, slow freezing is assumed to cause greater damage to cell structures than rapid freezing where the small ice crystals formed impart less.

Loss of total folate in leeks was greater in the main study as compared to the pilot study. This is probably due to the difference in the proportion of blanching water used in the two studies: 1 L/kg vegetable in the pilot study versus 50 L/kg in the main study. The latter proportion of water was chosen keeping in mind scaling up of the process at a later stage. Furthermore, in the pilot study we used a hydrostatic high-pressure apparatus with a small sample container for which glycol was used as pressure medium. For the use of this apparatus the vegetable samples were packed in vacuum plastic bags. In our main study we intended to do the same with larger amounts of vegetables in an apparatus with a larger container and with water as pressure medium. However, we did not succeed in keeping the vacuum plastic bags from leaking, and therefore we decided to carry out the experiments without prior packaging. This might also have led to greater losses of folate during high-pressure treatment in the main study compared to those in the pilot study. We considered analyzing the folate content in the processing water after high-pressure treatment and blanching. However, calculations revealed that the folate concentration would be far below the detection limit.

To our knowledge no earlier studies have reported on processing of vegetables with the aim of converting polyglutamate folate to the monoglutamate form. However, data have been published on folate retention in vegetables during storage or processing. Storage of blanched vegetables in the frozen state decreases folate retention by 15%, while fresh vegetables lose about 25% of folate when stored frozen.[23,24] As yet, few data are available on the impact of refrigerated storage on the folate content of vegetables. Chen *et al.*[23] report an increase of the monoglutamate folate content of 30% after refrigerated storage of whole leaf spinach for seven days. We found increases in the same range for cut leeks and green beans after refrigerated storage for only one day. Recently, Konings *et al.*[19] also reported increases in the monoglutamate folate content of chopped spinach stored at room temperature. Storage up to 60 min increased the amount of monoglutamate folate two-fold. Chen *et al.*[23] found an increase in the monoglutamate folate content of 57% in whole leaf spinach stored at room temperature for 10 h.

The retention of folate for steaming that we found (75-90%) is in accordance with others.[24] For blanching, we found that more folate was retained (70-90%) than did others (30-70%).[23,24] Differences in vegetables and methods make it difficult to draw conclusions. In general, the longer vegetables are exposed to heat or water, the lower the folate retention will be. Therefore, processing techniques such as processing in a closed system, microwave or vacuum packed high-pressure treatment, by which direct or prolonged exposure to heat or water is avoided, are preferred.

The results from this study are based on analysis of single samples, except for the raw samples in the main study. However, all samples were taken from the same vegetable batches that were carefully homogenized before sampling. The small standard deviations of the mean folate content in five samples of raw vegetables show that our sampling strategy has been successful.

In conclusion, freezing as well as high-pressure treatment are promising approaches for improving the bioaccessibility of folate from vegetables. Potentially this could lead to production of vegetables with higher folate bioavailability. However, leakage of folate in the processing water, especially of the monoglutamate form, counteracts these positive effects. To prevent loss of folate into the processing water, processing in a closed system should be applied. The effect of refrigerated storage of cut vegetables on the conversion of polyglutamate folate to the monoglutamate form deserves further research.

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Part II.

Folate bioavailability

Chapter 4

Comparison of bioavailability of heptaglutamyl folic acid with monoglutamyl folic acid in healthy adults

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ABSTRACT *Background:* Bioavailability and bioefficacy of dietary folate is estimated to be 50 to 60% that of synthetic folic acid. Linkage to a polyglutamate chain is one of the determinants of lower folate absorption from food but it is not yet known to what extent this reduces folate bioavailability and bioefficacy. *Objective***:** Our goal was to quantify the relative bioavailability and bioefficacy of low doses of polyglutamyl folic acid relative to monoglutamyl folic acid. *Design*: In total, 180 men and women (50-75 y) ingested daily capsules containing 323 nmol heptaglutamyl or 262 nmol monoglutamyl folic acid, or placebo capsules in a randomized double-blind placebo-controlled parallel trial. Serum and erythrocyte folate concentrations, and plasma homocysteine concentrations were determined after an overnight fast at baseline and after 2 and 12 weeks of intervention. *Results***:** Serum and erythrocyte folate concentrations increased less in the polyglutamyl (6.1 and 155 nmol/L, respectively) than in the monoglutamyl folic acid group (11.8 and 282 nmol/L, respectively). Differences remained statistical significant (P<0.05) after correction for the difference in test doses. Plasma homocysteine concentrations decreased similarly in both groups (polyglutamyl group: -12.1%; monoglutamyl group: -14.1%). The relative bioavailability of polyglutamyl folic acid, corrected for the difference in test doses, was 64% (95%-CI 52 to 75) based on serum folate and 68% (95%-CI 51 to 84) based on erythrocyte folate. Bioefficacy, determined by changes in plasma homocysteine concentrations, was found to be 106% (95%-CI 77 to 134). *Conclusion***:** Increasing the amount of monoglutamate folate in the food supply may be an appropriate strategy to improve folate status in a population.

Key words: *Polyglutamyl folic acid; Monoglutamyl folic acid; Folic acid; Bioavailability; Bioefficacy; Human; Serum folate; Erythrocyte folate; Plasma homocysteine.*

4.1 Introduction

Folate is an essential B vitamin in the human diet. Folic acid is the synthetic form of the vitamin, whereas folate refers to the natural forms. Low folate intake is a common cause of mild hyperhomocysteinemia, which is a potential risk factor for cardiovascular disease.[1,2] Supplementation with folic acid leads to a significant reduction of plasma homocysteine levels in healthy subjects, even at low doses.[3-5] An increased intake of dietary folate from vegetables and fruits lowers plasma homocysteine levels as well.[6]

The bioavailability and bioefficacy of dietary folate appear to be less than that of folic acid.[7] Bioavailability is defined as the fraction of folate that is absorbed and can be used for metabolic processes or storage as measured by changes in folate status, whereas bioefficacy, or more correctly functional bioefficacy, is defined as the fraction that has a positive effect on a functional parameter, for instance lowering of homocysteine concentrations.[8]

One of the most important determinants of dietary folate bioavailability, and thus of bioefficacy, is the linkage to a polyglutamate chain. In the Netherlands, where no food fortification with folic acid is allowed, two thirds of dietary folate intake is ingested in the polyglutamate form, mainly derived from vegetables, bread and fruits.[9] Before absorption of polyglutamated folates takes place, the enzyme folylpoly γ-glutamyl carboxypeptidase (FGCP), present in the brush border of the jejunum, cleaves glutamate moieties from the folate molecule. Subsequently, folate is absorbed mainly in the monoglutamate form. Limitation or inhibition of this enzymatic process might decrease the absorption of folate from polyglutamate sources. Knowledge of the extent to which linkage to a polyglutamate chain decreases folate bioavailability would indicate whether it is necessary to find methods to improve the bioavailability of dietary folate.

Up until now, many studies on the bioavailability of polyglutamyl relative to that of monoglutamyl folic acid have been carried out.[10-18] However, results are equivocal and have resulted in estimates of the bioavailability of polyglutamyl relative to monoglutamyl folic acid varying from 50 to 100% depending on study design. In our view, it is not yet clear to what extent the lower bioavailability of dietary folate relative to that of folic acid can be attributed to the polyglutamate chain. In addition, the bioefficacy of polyglutamyl folic acid relative to monoglutamyl folic acid for reduction of plasma homocysteine concentrations has not been studied previously.

The present study was carried out to quantify the bioavailability and bioefficacy of low doses of polyglutamyl relative to monoglutamyl folic acid in a long-term study in people aged 50-70 y.

4.2 Subjects and Methods

4.2.1 Recruitment of subjects

Subjects were recruited from a database of volunteers maintained at the Division of Human Nutrition, Wageningen University. Men and post-menopausal women aged 50 to 70 y were eligible for our study. Exclusion criteria were: hematological diseases and chronic diseases including cancer, renal insufficiency, liver disease, and diagnosed gastro-intestinal disorders; use of anti-inflammatory, antacid or anticonvulsant drugs; and chronic consumption of aspirin or B vitamin supplements, or of other drugs or dietary supplements interfering with folate or homocysteine metabolism. Subjects with low serum vitamin B_{12} concentrations (<160 pmol/L), high serum creatinine concentrations (>125 μmol/L) or high plasma homocysteine concentrations (>26 μmol/L) were also excluded from participation. A letter explaining the research and a medical questionnaire were sent to 957 persons. The questionnaire provided information on the health criteria that subjects had to fulfill for inclusion in the study, and subjects were asked to return the questionnaire only when they believed all criteria were met. From the 957 questionnaires, 387 were completed and returned. Based on the questionnaire, 290 subjects were eligible for admission to the study. Forty-one persons withdrew from participation before the start of the study for logistic, medical or personal reasons. Because the aim was to recruit about 180 subjects, we excluded 61 persons randomly. Thus, the population invited for biochemical screening comprised 188 apparently healthy adults aged 50-70 y. The research protocol was approved by the Medical Ethical Committee of Wageningen University. Subjects gave written informed consent.

4.2.2 Study design

The study comprised a screening visit, a five-week run-in period, a baseline visit, a 12-w intervention period and two follow-up visits after 2 and 12 w of intervention. At the screening visit, a fasting blood sample was taken to determine serum vitamin B_{12}, serum creatinine, and plasma homocysteine concentrations. Furthermore, weight and height were measured. All subjects took part in a 5-w run-in period during which they took placebo capsules. In the meantime the biochemical analyses were carried out. The serum vitamin B_{12} concentration was too low in one subject and another subject had a too high plasma homocysteine concentration. These subjects were therefore excluded from participation. A further four subjects reported start of medication interfering with folate or homocysteine metabolism between the screening and first baseline visit and were therefore excluded. The remaining 182 subjects entered the intervention phase of the study which had a randomized, double-blind, placebo-controlled parallel design with

three groups and lasted 12 w. In order to guarantee similar distributions of homocysteine concentrations in all groups at baseline, randomized blocks based on screening homocysteine concentrations were used for the assignment of subjects to the three groups. Subjects sharing one household (21 couples) were allocated to the same intervention group to minimize the possibility of the wrong capsules being taken.

The three intervention groups received 323 nmol monoglutamyl folic acid (~145 μg), 262 nmol heptaglutamyl folic acid (~320 μg), or placebo in the form of one capsule per day. Throughout the study, subjects were asked not to consume liver because of the high folate content. In addition, consumption of liver products, such as pâté and liver paste, was not allowed for three days prior to drawing of blood. Compliance was checked by counts of capsules that were returned after the study. Furthermore, subjects kept a diary for compliance monitoring, illnesses, use of drugs or dietary supplements and departure from restriction of consumption of liver and liver products.

After the run-in period, blood was drawn from all subjects at three points in time: at baseline, and after 2 and 12 w of intervention. At each time point, blood was collected twice from all subjects on two separate days each three days apart. Measurements in the blood samples taken three days apart were averaged in order to improve the statistical power of the study.[19] Blood was drawn at the Division of Human Nutrition after subjects had fasted overnight. For determination of vitamin B_{12}, creatinine and folate concentrations in serum, 5 mL blood was taken in a serum separator tube and, after standing for 30 min at room temperature, centrifuged for 10 min at 2600 g. For the determination of folate concentrations in whole blood, 4 mL blood was taken in EDTA tubes and placed on a roller bank for 10 min to ensure thorough mixing. From this sample, 0.4 mL was diluted with 1.6 mL ascorbic acid (0.1% w/v) and homogenized on a vortex. For the determination of plasma homocysteine concentrations, blood samples (5 mL) were collected into EDTA tubes which were immediately placed on ice water and centrifuged within 30 min for 10 min at 2600 g. Serum, plasma and whole blood samples were stored at –80°C until analysis.

4.2.3 Capsules

Monoglutamyl folic acid was obtained from Merck & Co., Inc. (Whitehouse Station, NJ , USA) and heptaglutamyl folic acid from Schircks (Jona, Switzerland) as the ammonium salt (chemical purities: >98%). Identical capsules containing monoglutamyl folic acid or polyglutamyl folic acid, with a target amount of 450 nmol per capsule, and placebo capsules were produced manually at the pharmacy of the Gelderse Vallei Hospital, Ede, the Netherlands. The folic acid content of the capsules was determined by HPLC with fluorescence and diode array detection.[20]

Polyglutamyl folic acid was hydrolyzed to monoglutamyl folic acid by incubation with rat plasma before injection to the HPLC. Six batches of each type of capsules were analyzed with each batch consisting of 20 randomly chosen capsules. The folic acid content expressed as nmol/capsule was as follows: placebo capsules, 0 (range: 0-0 nmol), monoglutamyl folic acid capsules, 323 (219-373), and polyglutamyl folic acid capsules, 262 (249-297). Thus, the actual content of the monoglutamyl folic acid capsules was 71% and of the polyglutamyl folic acid capsules 58% of the targeted dose (450 nmol per capsule). Coefficients of variation of the folic acid content in the capsule batches did not exceed 6%.

4.2.4 Biochemical analyses

The concentrations of serum vitamin B_{12}, serum folate, and whole blood folate were determined with a commercial chemiluminescent immuno-assay (Immulite 2000, Diagnostic Products Company, Los Angeles, CA). For determination of folate in whole blood, samples were diluted further with a concentrated human protein-based matrix (Immulite 2000 diluent) before measurement. Folate concentrations were measured at the clinical laboratory (CKCL) of the University Medical Centre, Nijmegen (intra-assay variation of <5% for serum and <14% for whole blood folate, respectively). Creatinine concentrations were measured with a kit (DuPont Dimension) based on the kinetic Jaffé reaction. Concentrations of vitamin B_{12} and of creatinine were assessed at the General Practitioner's Laboratory at Larenstein (Velp, the Netherlands). Plasma total homocysteine concentrations were measured by HPLC with fluorimetric detection at the Division of Human Nutrition and Epidemiology, Wageningen University (intra- and interassay coefficient of variation 2% and 7%, respectively). For the determination of serum folate, whole blood folate and plasma homocysteine concentrations, all samples of each person sampled at weeks 0, 2, and 12 were measured in the same run to eliminate interassay variation.

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4.2.5 Calculations and statistics

Power calculations were based on expected changes in concentrations of folate in serum and homocysteine in plasma. We expected an increase of 9.5 nmol/L in serum folate concentrations in the group receiving monoglutamyl folic acid and, assuming a relative bioavailability of polyglutamyl folic acid of 75%, an increase of 7.1 nmol/L in the group receiving polyglutamyl folic acid. Likewise, for plasma homocysteine concentrations, we expected a decrease of 2.5 µmol/L (20%) in the group receiving monoglutamyl folic acid and 1.9 µmol/L (16%) in the group receiving polyglutamyl folic acid. Thus 54 subjects per group would be required to detect a difference in the effect of the two forms of folic acid on serum folate

concentrations and 45 per group to detect a difference for plasma homocysteine concentrations with 80% power and α=0.05.

Erythrocyte folate concentrations were calculated using the following formula: (W-[S x (100-H)/100)] x (100/H), where W equals whole blood folate concentration, S equals serum folate concentration and H equals the hematocrit. Mean changes in serum folate, erythrocyte folate and plasma homocysteine concentrations in the groups receiving either monoglutamyl or polyglutamyl folic acid were computed and corrected for changes in the placebo group. Differences between the folic acid groups were tested by ANOVA (SAS, SAS Institute Inc., Cary, NC). Bioavailability was calculated as:

$$\frac{\Delta[C]_{poly} - \Delta[C]_{placebo}}{\Delta[C]_{mono} - \Delta[C]_{placebo}} * \frac{Dosage_{mono}}{Dosage_{poly}},$$

where $\Delta[C]$ represents the change in serum or erythrocyte folate concentrations over time in the monoglutamyl folic acid, polyglutamyl folic acid or the placebo group. Likewise bioefficacy was calculated using the decrease in plasma homocysteine concentrations expressed as a percentage.

4.3 Results

Data from two subjects were excluded from analyses because of use of a drug that was on the exclusion list. Therefore, all analyses are based on data from 180 subjects. During the intervention period, one person dropped out just before the final measurements because of illness (lung cancer). The average compliance to intervention based on counts of returned capsules was 99% (all subjects >80%). Study groups were very similar in age, BMI, sex, smoking behavior, and blood values of homocysteine, vitamin B_{12} and creatinine at screening. Characteristics of the study population are shown in **Table 4.1**.

Table 4.1 Characteristics of the study population on admission to the study.[a]

	Placebo n=60	Mono n=59	Poly n=61
Age (y)	61± 5	60± 6	59 ± 5
Body mass index (kg/m^2)	26.7± 3.9	25.4 ± 3.0	26.2 ± 2.9
Sex (% M/F)	62/38	53/47	61/39
Smokers (%)	14	17	18
Plasma homocysteine (µmol/L)	10.3± 2.0	10.4 ± 2.2	10.5 ± 2.4
Serum vitamin B_{12} (pmol/L)	321± 94	315 ± 83	307 ± 89
Serum creatinine (µmol/L)	85± 13	81± 12	85 ± 12

[a]Values are means ± SD. There were no statistically significant differences among groups.

Table 4.2 Serum folate, erythrocyte folate, and homocysteine concentrations during intervention, and changes from baseline at 2 and 12 w of intervention.

	Placebo n=60	Monoglutamyl folic acid n=59[a]	Polyglutamyl folic acid n=60	Difference between folic acid groups
Serum folate (nmol/L)				
Week 0[b]	14.2 ± 3.9	15.2 ± 4.1	14.4 ± 5.4	
Week 2[b]	13.6 ± 3.7	18.8 ± 4.7	15.8 ± 4.6	
Week 12[b]	12.9 ± 3.8	25.7 ± 7.4	19.3 ± 5.6	
Change at week 2 (nmol/L)[c]		3.9 (3.3 to 4.6)	1.8 (1.0 to 2.6)	2.1 (1.0 to 3.1)[d]
Change at week 12 (nmol/L)[c]		11.8 (10.3 to 13.3)	6.1 (5.3 to 7.0)	5.6 (3.9 to 7.4)[d]
Erythrocyte folate (nmol/L)				
Week 0[b]	685 ± 208	737 ± 211	800 ± 278	
Week 12[b]	636 ± 215	969 ± 254	905 ± 311	
Change at week 12 (nmol/L)[c]		282 (246 to 318)	155 (122 to 188)	127 (78 to 176)[d]
Plasma homocysteine (µmol/L)				
Week 0[b]	10.4 ± 1.7	10.4 ± 2.1	11.0 ± 2.6	
Week 2[b]	10.5 ± 1.8	10.0 ± 1.9	10.3 ± 2.3	
Week 12[b]	10.6 ± 1.9	9.0 ± 1.7	9.7 ± 2.0	
Change at week 2 (%)[c,e]		-3.9 (-5.9 to –2.0)	-5.8 (-8.1 to –3.0)	1.9 (-1.3 to 5.1)
Change at week 12 (%)[c,e]		-14.1 (-16.3 to –11.9)	-12.1 (-14.8 to –9.3)	2.1 (-1.5 to 5.6)

[a] In week 2 values of one subject are missing, n=58. [b] Values are means ± SD. [c] Changes are means (95% CI), corrected for change in placebo group. [d] Statistically significant, P<0.001. [e] Changes in homocysteine concentrations were expressed as percentages to correct for the differences in baseline values and because absolute changes were not normally distributed.

4

In **Table 4.2** the changes in concentrations of folate in serum and erythrocytes, and of plasma homocysteine are shown. Serum folate concentrations in the group receiving monoglutamyl folic acid increased by 27% at 2 w and 81% at 12 w of intervention after correction for changes in the placebo group. In the group receiving polyglutamyl folic acid, increases in serum folate concentrations were 16% and 46% after 2 and 12 w, respectively. Erythrocyte folate concentrations increased by 41% in the monoglutamyl folic acid group and by 21% in the polyglutamyl folic acid group after 12 w of intervention. Correction for the difference in capsule content did not alter the statistical significant differences in folate concentrations between groups to any extent. Decreases in plasma homocysteine concentrations were not different in both folic acid groups, namely 4% and 14% in the monoglutamyl folic acid group, and 6% and 12% in the polyglutamyl folic acid group after 2 and 12 w, respectively. This remained the same after correction for the difference in molar folic acid content in the capsules.

The bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid based on changes in serum and erythrocyte folate concentrations were very similar, namely 64% (95%-CI 52 to 75%) and 68% (51 to 84%), respectively, after 12 w of intervention. Bioefficacy of polyglutamyl folic acid as compared to monoglutamyl folic acid based on plasma homocysteine concentrations was 106% (95%-CI 77 to 134) after 12 w of intervention **(Table 4.3)**.

Table 4.3 Bioavailability and bioefficacy of heptaglutamyl compared to monoglutamyl folic acid.

		After 2 w	95% CI	After 12 w	95% CI
Bioavailability %:	Serum folate	57	30 to 83	64	52 to 75
	Erythrocyte folate	-		68	51 to 84
Bioefficacy %:	Plasma homocysteine	183	64 to 302	106	77 to 134

4.4 Discussion

In the present study we found that the bioavailability of polyglutamyl folic acid is 66% of that of the monoglutamyl form of the vitamin based on both serum folate and erythrocyte folate concentrations after 12 w of intervention. We could not detect a difference in bioefficacy between the two forms of the vitamin as measured by plasma homocysteine concentrations.

A number of studies have found that the bioavailability of polyglutamyl folic acid as compared to monoglutamyl folic acid is of the same order of magnitude as we have found.[11,12,16] However, a similar number of studies showed no difference between the bioavailability of polyglutamyl and monoglutamyl folic acid.[10,15,18] Protocols with single doses encountered the problem that relatively high doses or a high pre-load dose were needed to gain a measurable folate response in blood. Such protocols may not be appropriate for folate bioavailability research in the dosis

range of dietary folate intake. The use of isotopically labelled folates improved study designs importantly because lower dosages could be used. But still, exposure to polyglutamate folates may affect its bioavailability by regulation of the GCPII enzyme activity in the brush border of the small intestine and therefore study protocols of several hours or days with a single exposure may be too short.

The present study is the first long-term intervention study with large groups in which differences in bioavailability of daily low dosages of polyglutamyl and monoglutamyl folic acid could be quantified adequately. Folate intake was restricted to the habitual diet and the supplements supplied during the study. Since food fortification with folic acid is not allowed in the Netherlands and subjects did not use any supplements containing B vitamins, there were no other sources of folic acid that could interfere with the study. Furthermore, groups were well randomized and sufficiently large to quantify bioavailability within narrow confidence limits.

Analysis of the monoglutamyl folic acid and heptaglutamyl folic acid capsules showed that the folate content of the materials were lower than expected (71% and 58% respectively). This is probably due to a compounding error at the pharmacy, or else some uptake of water from the air or undetectable analytical error. The differences in dose between the monoglutamyl and polyglutamyl folic acid capsules were taken into account when the bioavailability and bioefficacy were calculated. This correction did not change the results to any extent.

The bioavailability of food folate is generally assumed to be only 50% of that of synthetic folic acid.[7] Our results allow us to calculate how much of this difference in bioavailability might be explained by the polyglutamate chain. In the Netherlands, about two thirds of dietary folate is provided by polyglutamates and one third by monoglutamates based on a cross-sectional study in 1275 men and 1160 women, aged 20-65 y.[9] If the bioavailability of polyglutamate folate is 66% of that of monoglutamate folate, as suggested by our findings, then the bioavailability of folate from a mixed diet would be (2/3 x 0.66 +1/3)*100%= 77% as compared to synthetic folic acid, assuming that chain length is the only determinant of bioavailability. This implies that polyglutamated folates decrease the bioavailability of food folate from a mixed diet by 23%, which is almost half of the assumed 50% lower bioavailability of food folate relative to that of folic acid. The remaining 27% could be due to other factors, such as the food matrix or to compounds in foods such as organic acids that can attenuate bioavailability.

For all our calculations, we set the bioavailability of monoglutamyl folic acid at 100%. The US Institute of Medicine.[21] has set the bioavailability of folic acid consumed in the fasted state at 100%, and that of folic acid consumed with a meal at 85%, either as a supplement or in a fortified food. We did not give any instructions to our subjects concerning whether or not the capsules should be consumed with a meal. If all subjects ingested their capsules with a meal, this

would impy that the relative bioavailability of polyglutamyl folic acid would be higher than the 66% we report here, namely 78% (66/85*100%).

In this study, we attempted to measure the bioefficacy of polyglutamyl folic acid relative to monoglutamyl folic acid based on reduction in plasma homocysteine concentrations. The estimated relative bioefficacy of polyglutamyl folic acid after 12 w intervention was found to be 106%, although the 95% CI was wide (Table 4.3). This is somewhat surprising as the mean estimate of the relative bioavailability of polyglutamyl folic acid, based on changes in the concentrations of folate in serum and erythrocytes over 12 w, was 66%. As folate status is an important determinant of circulating homocysteine, one would expect a lower relative bioefficacy of polyglutamyl folic acid as well. It should be realized that the bioefficacy is likely to depend on the doses that are tested. Recently we reported the results of a dose-finding study with folic acid on homocysteine concentrations in comparable subjects (Chapter 7) and found that doses of at least 400 μg/d are needed to reach stable plasma homocysteine concentrations. At lower doses the dose response relationship of folic acid and homocysteine is fairly linear.[22] This implies that at high doses the estimate of bioavailability is expected to go towards 100%. It is highly unlikely, however, that the maximum decrease in homocysteine concentrations had been reached already in our study since we used doses far lower than 400 μg.

Several other factors complicated the interpretation of our bioefficacy data. Firstly, the observed reductions in homocysteine concentrations – and hence the statistical power to detect a difference between the groups ingesting monoglutamyl and polyglutamyl folic acid – was lower than anticipated, mainly because of the lower folic acid content of the capsules. Secondly, we were confronted with a somewhat higher mean baseline homocysteine concentration in the polyglutamyl folic acid group than in the monoglutamyl folic acid group, despite the careful randomization at screening. Due to the phenomenon of regression to the mean – i.e. the fact that high initial concentrations will generally be lower at a second measurement time – the reduction in homocysteine levels in the polyglutamyl folic acid group may have been slightly exaggerated. Nevertheless, when we excluded two subjects with high baseline homocysteine values from the polyglutamyl folic acid group, which resulted in a mean baseline homocysteine concentration close to that of the monoglutamyl folic acid group, the estimate for the bioefficacy was virtually unchanged.

In conclusion, the relative bioavailability of polyglutamyl folic acid is 66% of that of monoglutamyl folic acid based on serum and erythrocyte folate concentrations. Taken that about two thirds of dietary folate is in the polyglutamate form, the presence of the polyglutamate chain may explain about half of the lower bioavailabiltiy of dietary folate as compared to synthetic folic acid. Thus, in order to

improve the folate status of the population, the amount of monoglutamate folate in the food supply could be increased by a variety of food based approaches. These include giving dietary advice to select products with a high monoglutamate folate content[23], through specific food processing techniques[24], biotechnology, plant breeding, or simply by fortification of foods with monoglutamyl folic acid.

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Chapter 5

Bioavailability of polyglutamyl relative to mono-glutamyl folic acid within glutamate carboxy-peptidase II 1561C>T genotypes

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ABSTRACT The B vitamin folate has many essential functions in the body. Dietary folate is ingested mainly as polyglutamate folate. In the intestinal brush border, the enzyme folylpoly-γ-glutamate carboxypeptidase (FGCP) cleaves ingested polyglutamate folates to monoglutamates, a necessary step before folate absorption. The 1561T allele in the glutamate carboxypeptidase II gene (*GCPII*), coding for FGCP, was found to decrease enzyme activity and may therefore impair bioavailability of polyglutamate folate. Our aim was to study the bioavailability of polyglutamyl relative to monoglutamyl folic acid in subjects with different *GCPII* 1561C>T genotypes. In this randomized double blind study, 180 healthy adults (50-75 y) received 323 nmol monoglutamyl (n=59) or 262 nmol heptaglutamyl folic acid (n=61), or placebo (n=60) for 12 w. Concentrations of serum folate, erythrocyte folate and plasma homocysteine were determined before and after intervention, and changes and genotypes were assessed *post hoc*. Bioavailability of heptaglutamyl relative to that of monoglutamyl folic acid was calculated by dividing the changes in serum folate concentrations in the treatment groups, after these were corrected for changes in the placebo group and for the administered dose. No subjects with the TT-genotype were observed. At baseline, serum and erythrocyte folate levels were higher in CT (geometric means [95% CI] 16.3 [13.7 to 19.3] and 863 [735 to 1012] nmol/L, n=19) than in CC subjects (13.7 [13.1 to 14.3] and 685 [652 to 721] nmol/L, n=161, $P<0.05$). Homocysteine concentrations did not differ between genotypes. Bioavailability of heptaglutamyl relative to monoglutamyl folic acid, was 64% (95% CI: 52 to 76) in CC and 70% (49 to 91) in CT subjects. In conclusion, bioavailability of polyglutamyl relative to monogluamyl folic acid is not different for CT and CC subjects. The T allele in the *GCPII* 1561C>T gene is associated with higher folate status. This might be explained by expression of the *GCPII* gene in body tissues other than the gut.

Keywords: *Folate bioavailability; Polyglutamyl folic acid; Serum folate; Erythrocyte folate; Plasma homocysteine; GCPII 1561C>T polymorphism; Genetics.*

5.1 Introduction

The B vitamin folate is essential in the human diet. Folate deficiency, or sub-optimal folate intake, is associated with various pathologies, such as anaemia[1], neural tube defects[2-4], hyperhomocysteinaemia and cardiovascular disease[5-10], cancer[11], and neurocognitive dysfunction[12-14]. Generally, folic acid refers to the oxidized forms, present in supplements and fortified foods, whereas folate refers to the reduced forms, present in the diet and in biological tissues.

Folate bioavailability from food may be only 50%.[15] Bioavailability is defined as the proportion of an ingested nutrient that is absorbed and becomes available for use and storage in the body. In several studies it was found that conjugation of folate to a polyglutamate chain, which is the case for about two thirds of dietary folate[16], impairs folate bioavailability.[17-23] Polyglutamate folate requires enzymatic deconjugation to the monoglutamate form before it can be absorbed. Monoglutamate folate does not require such deconjugation before absorption. Incomplete deconjugation of polyglutamate folate could lead to lower bioavailability.

The enzyme folylpoly-γ-glutamate carboxypeptidase (FGCP), encoded by the glutamate carboxypeptidase II gene (*GCPII*) and present in the brush border of the human small intestine, is responsible for the deconjugation of polyglutamate folate.[24] In 2000, a 1561C>T polymorphism in this gene was reported.[25] *In vitro* experiments with COS-7 cells, revealed that the T allele was associated with 50% reduced activity of the FGCP enzyme as compared to the wild-type C allele. Furthermore, in 75 healthy subects, the CT genotype (n=6) was associated with significant lower serum folate and higher plasma homocysteine concentrations compared with the CC genotype.

In the present study we aimed to assess the bioavailability of polyglutamyl relative to monoglutamyl folic acid in subjects with different *GCPII* 1561C>T genotypes. Our hypothesis was that the bioavailability of polyglutamyl relative to monoglutamyl folic acid would be impaired in subjects with the CT compared with the CC genotype.

5.2 Subjects and methods

5.2.1 Subjects

The present study was conducted within a 12-w randomized double blind placebo controlled trial in which we studied the bioavailability of heptaglutamyl relative to monoglutamyl folic acid in 180 healthy subjects (Chapter 4). The study protocol was approved by the Medical Ethical Committee of Wageningen

University. Details on recruitment of subjects and the methodology issues are reported in Chapter 4[23] and are therefore only briefly summarized here. Subjects were 180 healthy older adults (50 to 75 y). They were screened on the basis of a medical questionnaire, and vitamin B_{12} concentrations had to be >130 pmol/L, serum creatinine <125 μmol/L, and plasma homocysteine concentrations <26 μmol/L. Subjects had no previous history of cardiovascular diseases and did not suffer from chronic diseases such as cancer, renal disease, or gastro-intestinal disorders. Use of drugs that are known to interfere with folate and homocysteine metabolism, such as anti-epileptics, and use of B vitamin supplements were also reasons for exclusion. All subjects were informed in writing and orally about the study and gave their written consent.

5.2.2 Design

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

5.2.3 Biochemical measurements

Blood samples intended for analysis of plasma homocysteine concentrations were immediately placed on ice and centrifuged at 2600 g for 10 min within half an hour after venapuncture. Homocysteine concentrations in the plasma samples were measured by HPLC with fluorescence detection at the laboratory of the Division of Human Nutrition (intra-assay CV: 2%, Wageningen University).[26,27] Separate blood samples were collected in serum separator tubes and centrifuged for determination of serum folate concentrations. A third blood sample was used for the analysis of folate concentrations in erythrocytes. For this, after assessment of haematocrit values, whole blood samples were diluted with four volumes of ascorbic acid (10 g/L), and, before analysis, further diluted with Immulite 2000 diluent (Diagnostic Products Company, Los Angeles, CA). Serum and erythrocyte folate concentrations were measured with a chemiluminescent immunoassay analyzer (Immulite 2000, Diagnostic Products Company, Los Angeles, CA) at the clinical laboratory (CKCL)

of the University Medical Centre, Nijmegen (intra-assay CV <5% for serum and <14% for erythrocyte folate).

5.2.4 Genotyping

The *GCPII* genotypes were determined in DNA isolated from whole blood samples. The 1561C>T variant changes a histidine to a tyrosine residue and creates an *Acc*I site. Mutation analysis was performed with PCR and subsequent restriction enzyme analysis with *Acc*I. PCR was done in a total volume of 50 µl containing 50 ng of the forward primer 5'-CATTCTGGTAGGAATTTAGCA-3' and 50 ng of the reverse primer 5'-AAACACCACCTATGTTTAACA-3', 200 µM of each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM $MgCl_2$ and 1 unit *Taq* polymerase (Life Technologies). The PCR conditions were set at an initial denaturation step of 3 min at 92°C followed by 35 cycles of 92°C/30 sec (denaturation), 55°C/30 sec (annealing), and 72°C/30 sec (extension), and a final extension of 7 min at 72°C. The amplified PCR fragment of 244 basepairs (bp) was digested with the restriction enzyme *Acc*I followed by gel electrophoresis analysis on a 2% agarose gel. After restriction enzyme analysis the 1561TT genotype results in two fragments of 141 and 103 bp, respectively, while the 1561CC genotype shows only a fragment of 244 bp.[25]

5.2.5 Calculations and Statistics

Geometric means of folate concentrations in serum and erythrocytes and of plasma homocysteine concentrations were calculated at baseline for each *GCPII* (1561C>T) genotype. Changes in these concentrations after 12 w of intervention were calculated for the monoglutamyl and polyglutamyl folic acid groups and corrected for changes in the placebo group. Within each of the folic acid treatment groups, we tested whether the changes in blood parameters between genotypes were statistically significantly different by using Pearson's *t* test. All statistics were performed with SAS (SAS Institute Inc., Cary, NC). Relative bioavailability of polyglutamyl as compared to monoglutamyl folic was calculated as for each genotype as follows:

$$\frac{\Delta[C]poly - \Delta[C]placebo}{\Delta[C]mono - \Delta[C]placebo} * \frac{Dosage_{mono}}{Dosage_{poly}}$$

In this equation, *[ΔC]* stands for the change in serum or erythrocyte folate concentrations after 12 w.

5.3 Results

The three study groups did not differ from each other with respect to age, BMI, serum vitamin B_{12}, serum creatinine and plasma homocysteine concentrations (**Table 5.1**). From the 180 subjects, 161 (90%) had the *GCPII* 1561CC genotype and

19 (10%) had the CT genotype. No homozygous TT subjects were found in this study population.

Table 5.1. Baseline characteristics of the study population by treatment group.[a]

	Placebo (n=60)	Mono (n=59)	Poly (n=61)
Age, y	61 ± 5	60 ± 6	59 ± 5
BMI, kg/m^2	26.7 ± 3.9	25.4 ± 3.0	26.2 ± 2.9
Male/female, %	62/38	53/47	61/39
GCPII 1561 CC/CT/TT, n	52/8/0	54/5/0	55/6/0
Plasma homocysteine, µmol/L[a]	10.3 ± 2.0	10.4 ± 2.2	10.5 ± 2.4
Serum vitamin B_{12}, pmol/L[a]	321 ± 94	315 ± 83	307 ± 89
Serum creatinine, µmol/L[a]	85 ± 13	81 ± 12	85 ± 12

[a]Values are means ± SD. There were no statistically significant differences among groups.

At baseline, serum folate and erythrocyte folate concentrations were, 19% and 26% higher in CT than in CC subjects (P≤ 0.05), respectively. Plasma homocysteine concentrations were slightly lower in CT than in CC subjects, however this difference was not statistically significant (**Table 5.2**).

In **Table 5.3**, the changes in serum folate, erythrocyte folate and plasma homocysteine concentrations in CC and CT subjects are shown after 12 w of intervention with either 323 nmol monoglutamyl folic acid or 262 nmol heptaglutamyl folic acid. Changes in the two treatment groups were corrected for changes in the placebo group. Changes in the placebo group over the 12-w intervention period were -1.3 nmol/L for serum folate; -59 nmol/L for erythrocyte folate; and +0.1 µmol/L for plasma homocysteine in CC subjects. These changes were -1.3, +17, and +0.2 in CT subjects, respectively. In both treatment groups, there were similar changes in serum folate among the genotypes, but responses to polyglutamyl folic acid were generally smaller than to monoglutamyl folic acid. However, increases in erythrocyte folate concentrations were much smaller among CT than CC subjects in the polyglutamyl folic acid group, due to their higher initial erythrocyte folate concentrations. This is also demonstrated in **Figure 5.1** for percentual changes in erythrocyte concentrations.

Table 5.2. Serum folate, erythrocyte folate and plasma homocysteine concentrations at baseline by *GCPII* 1561C>T genotype.[a]

	CC (n=161)	95% CI	CT (n=19)	95% CI	*P* value[b]
Serum folate, nmol/L	13.7	13.1 to 14.3	16.3	(13.7 to 19.3)	0.05
Erythrocyte folate, nmol/L	685	(652 to 721)	863	(735 to 1012)	0.01
Plasma homocysteine, µmol/L	10.4	(10.1 to 10.7)	10.2	(9.6 to 10.7)	0.38

[a]Values are geometric means (95%-CI) [b]Differences between genotypes were tested with Pearson's *t* test.

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Table 5.3. Changes in serum folate, erythrocyte folate and plasma homocysteine concentrations after 12 w of intervention with 323 nmol monoglutamyl or 262 nmol heptaglutamyl folic acid daily by *GCPII* 1561C>T genotype.[a]

	Monoglutamyl folic acid n=59			Polyglutamyl folic acid n=61		
	CC (n=54)	CT (n=5)	*P value*[b]	CC (n=55)	CT (n=6)	*P value*[b]
Serum folate, nmol/L						
Before intervention	14.9 ± 3.9	17.8 ± 6.1		13.8 ± 5.0	19.3 ± 7.0	
After intervention	25.6 ± 7.3	26.8 ± 9.0		18.8 ± 5.4	23.8 ± 6.0	
Change[c]	11.9 (10.3 to 13.5)	10.2 (7.1 to 13.3)	0.4	6.2 (5.4 to 7.0)	5.8 (1.7 to 9.9)	0.9
Erythrocyte folate, nmol/L						
Before intervention	732 ± 216	790 ± 164		756 ± 224	1188 ± 428	
After intervention	973 ± 257	931 ± 240		873 ± 281	1193 ± 441	
Change[c]	300 (262 to 339)	123 (35 to 211)	0.01	176 (144to 208)	-12 (-158 to 134)	0.05
Plasma homocysteine, µmol/L						
Before intervention	10.3 ± 2.2	10.7 ± 1.3		11.1 ± 2.7	10.0 ± 0.6	
After intervention	8.9 ± 1.8	9.6 ± 1.4		9.7 ± 2.1	9.3 ± 0.5	
Change[c]	-1.6 (-1.9 to -1.3)	-1.4 (-2.2 to -0.6)	0.5	-1.5 (-1.9 to -1.1)	-0.9 (-1.6 to -0.2)	0.3

[a]Values are means ± SD (95% CI) [b]*Differences* between genotypes were tested with Pearson's *t* test. [c]*Changes* were corrected for changes in the placebo group.

Bioavailability of heptaglutamyl relative to monoglutamyl folic acid based on serum folate concentrations was 64 (95% CI 52 to 76)% in CC and 70 (49 to 91)% in CT subjects. Based on erythrocyte folate concentrations, this was 72 (42 to 103)% for CC subjects. Because of the different starting concentrations of CT subjects in the two treatment groups we did not calculate bioavailability based on erythrocyte folate concentrations for this group.

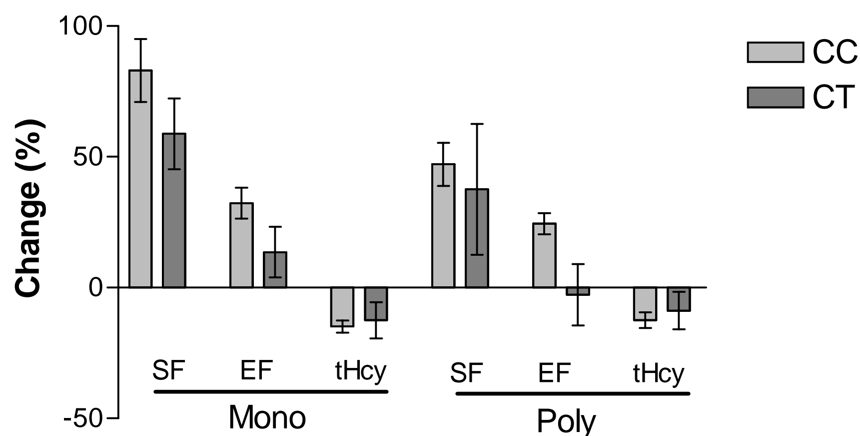

Figure 5.1. Percentual changes from baseline in the monoglutamyl and polylgutamyl folic acid groups by *GCPII* 1561C>T genotype. SF = serum folate; EF = erythrocyte folate; tHcy = plasma homocysteine. Changes were corrected for changes in the placebo group.

5.4 Discussion

We found that the bioavailability of polyglutamyl relative to monoglutamyl folic acid is about 65-70% and does not differ between *GCPII* 1561CT and CC subjects. Baseline and 12-w folate concentrations in serum and erythrocytes of CT subjects, however, were 20-25% higher than those of subjects with the CC genotype. Plasma homocysteine concentrations did not differ between genotypes. These findings are in accordance with published data of other cross-sectional studies, in which the 1561T allele in the *GCPII* gene was associated with higher folate concentrations. One study did not find such associations, which may be attributed to higher folate intake levels due to mandatory food fortification with folic acid.[28-31]

In our study population, consisting of 180 healthy subjects, we found that 10% carried the T allele, but there were no homozygous TT subjects. Others also reported prevalences of ~10% for the CT genotype and ~0.5% for the TT.[25,28] Therefore, polymorphism in the *GCPII* gene is rare in comparison to some other

polymorphisms, for instance the MTHFR 677C>T polymorphism (11% TT, 43% CT and 46% CC).[10]

In CT subjects, responses in erythrocyte folate concentrations were smaller compared with CC subjects after treatment with polyglutamyl folic acid and - unexpectedly - also with monoglutamyl folic acid. However, subjects carrying the T allele already had higher erythrocyte folate concentrations from the start. Their initial values were comparable to values after 12 w of supplementation with doses of 200-400 µg of folic acid.[32] Lower responses in these subjects can probably be attributed to their higher initial concentrations. Moreover, erythrocyte folate concentrations of CT subjects in the polyglutamyl folic acid group were far higher than those of CT subjects in the monoglutamyl folic acid group. Therefore, calculation of the relative bioavailability of polyglutamyl folic acid based on changes in erythrocyte folate concentrations in these groups was not justifiable.

The similar bioavailability of polyglutamyl relative to that of monoglutamyl folic acid among *GCPII* 1561C>T genotypes suggests that the FGCP activity of the T allele-coded enzyme is not reduced. Although others have shown lower activity of the T-allele coded enzyme *in vitro*[25], it needs not necessarily to be the same for the *in vivo* situation. From a metabolic point of view, our findings on the *GCPII* 1561C>T polymorphism are puzzling. Why would folate status be higher in CT subjects than in CC subjects, even though bioavailability of folate is similar? It may be that the *GCPII* 1561C>T polymorphism is expressed in other body tissues thereby affecting enzymes that are similar or linked to brush border FGCP, such as glutamate deconjugase in the liver, erythrocytes or in blood plasma. Further investigation in this area is needed to address these issues in order to obtain a better understanding of folate metabolism in general.

In conclusion, we found that bioavailability of polyglutamyl relative to monoglutamyl folic acid does not differ between *GCPII* 1561C>T genotypes. At baseline, and after 12 w of supplementation with folic acid, the 1561CT genotype was associated with 20-25% higher folate concentrations in serum and erythrocytes. This suggests that the GCPII 1561C>T polymorphism may be expressed in other body tissues or is linked to other genes. Further research is needed to investigate the mechanism behind these findings.

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Chapter 6

An innovative dual isotopic labelling method for studying the bioavailability of hexaglutamyl relative to monoglutamyl folic acid in humans

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To be submitted

ABSTRACT *Background:* Folic acid is an essential nutrient and various diseases are associated with low folate intake. Folate bioavailability can be low. One of the factors that limits folate bioavailability is that folate in the diet has more than one glutamate moiety. These glutamate moieties need to be removed before folate is absorbed as monoglutamate from the small intestine. Results from previous studies on the bioavailability of polyglutamyl relative to that of monoglutamyl folic acid have been inconsistent, possibly because of the single high-dose designs used in the studies. *Objective:* The aim of the study was to develop a method in which monoglutamyl folic acid and polyglutamyl folic acid, each specifically labelled with ^{13}C, would be administered in multiple low doses to humans. Once the relative isotopic enrichment in plasma folate of the two specifically labelled folic acid compounds has reached a plateau, the ratio of the enrichment from the two sources would provide an estimate of the bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid. We also aimed to establish the time to reach plateau isotopic enrichment and between-person variation in the estimate of bioavailability. In addition, the performance of LC-MS/MS methods for measuring the isotopic enrichment were also examined. *Methods:* Each day, for 28 d, 20 subjects aged 20-50 y consumed two capsules that were planned to contain ~ 50 nmol of [$^{13}C_6$]-hexaglutamyl and [$^{13}C_{11}$]-monoglutamyl folic acid. The degrees of enrichment in plasma 5-methyltetrahydrofolate (5-MTHF) with [$^{13}C_6$] and [$^{13}C_{11}$] were measured by LC-MS/MS on days 0, 1, 2, 4, 8, 15, and 28. The ratio of [$^{13}C_6$] to [$^{13}C_{11}$] in plasma 5-MTHF were calculated and the time to reach a plateau was estimated visually. The ratio at 28 d was then corrected for the [$^{13}C_{11}$] to [$^{13}C_6$] ratio of folic acid compounds in the capsules in order to calculate the bioavailability of hexaglutamyl folic acid relative to that of monoglutamyl folic acid. The degree of isotopic enrichment was measured by MS/MS in positive ionisation mode after separation on a reversed phase column, and by MS/MS in negative ionisation mode after separation on a minibore HILIC column. *Results:* The ratio

of [$^{13}C_6$] to [$^{13}C_{11}$] in plasma 5-MTHF reached a plateau on day 4 and was 0.66 (95% CI 0.58 to 0.74) on day 28. The ratios determined by the two LC-MS/MS methods were similar from day 2 to 28. Due to difficulties in measuring the [$^{13}C_{11}$] to [$^{13}C_6$] ratio in the folate compounds administered in the capsules it is not yet possible to calculate the bioavailability of hexaglutamyl relative to that of monoglutamyl folic acid. *Conclusions:* This dual-label isotopic labelling method provides a sensitive, accurate and efficient method for measuring folate bioavailability. When the relative degree of enrichment in the capsules is known, it will be possible to provide an estimate of the bioavailabiltiy of hexaglutamyl folic acid relative to that of monoglutamyl folic acid within a 95% CI of ± 12%. It is expected that the analytical problems will be overcome shortly. Because the ratio of isotopic enrichment reaches a plateau within 4 days, future studies will only need to last this long.

Key words: *Polyglutamyl folic acid; Monoglutamyl folic acid; Bioavailability; Stable isotopes; Mass spectrometry; Plasma folate; Human.*

6.1 Introduction

Deficiency of folate leads to anaemia[1], neural tube defects[2], and possibly to chronic diseases such as cardiovascular disease[3], colon cancer[4] and neurocognitive dysfunction[5] in humans. Folate status is not only determined by the intake of folate but also by its bioavailability. Bioavailability is defined as the proportion of ingested folate that is absorbed and available for metabolic processes and storage.[6] Bioavailability of folate from the diet is assumed to be about 50%[7], while bioavailability of folic acid, used in supplements and as a food fortificant, is estimated to range from 76 to 97% in humans.[8] Folic acid is the oxidized form of the vitamin, whereas folate refers to reduced forms, such as 5-methyltetrahydrofolate (5-MTHF), as present in foods and in biological tissues.

Folate from the diet is to a large extent conjugated to a polyglutamate chain.[9-11] Before absorption, the polyglutamate chain is deconjugated by the enzyme folylpoly γ-glutamate carboxypeptidase (FGCP) in the jejunum of the small intestine. Folate is subsequently absorbed and transported in the body as a monoglutamate. Limited deconjugation capacity might be a cause of lower bioavailability of folate from foods.[12]

Estimates of bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid in humans vary widely between persons and between studies.[13] This may be due to several limitations in the way that studies have been designed. One of the limitations up until now has been the relatively high doses, i.e. >400 μg, which have been administered as single doses.[14-16] However, folate from the diet is usually ingested in small quantities spread over the day. It may be that small doses of polyglutamate folate, e.g. 25-50 μg, can be readily deconjugated by FGCP, while deconjugation capacity is not sufficient for single doses as high as the recommended daily allowance (RDA, 300 μg/d[17]). Another limitation of previous studies has been that subjects have often been given one or more doses of folic acid >1 mg prior to the test dose in order to saturate body tissues.[14,16,18,19] This enables a response to the test doses to be elucidated. Otherwise, increased concentrations of folic acid in serum or urine would not be observed. However, such pre-loading probably perturbs normal folate metabolism.

Use of isotopically labelled folic acid enables lower doses of folic acid to be administered. Furthermore, by using two folate compounds labelled in such a way that they can be subsequently tracked enables the bioavailability of one compound relative to that of the other to be measured.[18-22] However, one of the limitations up until now has been the sensitivity of the analytical methods used for the detection of labelled folates in biological samples. Therefore, it was necessary to use relatively high doses of the order of 300 μg.[18-20,23]

In order to address these limitations, we have explored the possibility of developing a method in which specifically ^{13}C-labelled monoglutamyl folic acid and specifically ^{13}C-labelled polyglutamyl folic acid would be administered in multiple low doses over a period of time. The plasma concentrations of folate carrying the specific tags from each of the two labelled compounds would reach a plateau. It is assumed that the folate mixes completely with folate in other body pools. The ratio of isotope labels in plasma folate, corrected for the ratio of the two labelled compounds administered would provide a measure of the bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid.[24] The length of time required to reach a plateau in the degree of enrichment and the between-person variation in the estimate of bioavailabiltiy were examined. In addition, the performance of liquid chromatography - mass spectrometry (LC-MS/MS), used for measuring the degree of isotopic enrichment, was also studied.

6.2 Subjects and Methods

The first steps in the development of the methods were to synthesise the specifically ^{13}C-labelled monoglutamyl folic acid and polyglutamyl folic acid and to develop a LC-MS/MS method to measure the isotopic enrichment in plasma folate at relatively low concentrations.

6.2.1. Labelled compounds

[$^{13}C_{11}$]-Monoglutamyl folic acid and [$^{13}C_6$]-hexaglutamyl folic acid were synthesised specifically for our studies by ARC, Apeldoorn. The ^{13}C atoms were incorporated into the *p*-aminobenzoic acid structure (6 ^{13}C atoms) and for the monoglutamyl variant also in the glutamyl moiety (5 additional ^{13}C atoms), as shown in **Figure 6.1**. We chose to incorporate at least 6 ^{13}C atoms in order to overcome interference from natural ^{13}C abundance which gives rise to $^{13}C_1$ and $^{13}C_2$ labelling. The procedure was based on the method of Maunder *et al.*[25] The chemical purity of the labelled folic acid was >96% as determined by nuclear magnetic resonance (NMR), optical rotation and element counting, and the isotopic incorporation was >98% as determined by mass spectrometry.

6.2.2 LC-MS/MS procedure

We aimed to develop an LC-MS/MS method that would be sensitive enough to measure concentrations of folate at a level of 1% of that present in plasma. This would enable us to measure an isotopic enrichment of 1%. Taken that the average plasma folate concentration is 10 nmol/L, the limit of detection (LOD) of the method should be 100 pmol/L. For this purpose, an LC-MS/MS method was developed that could measure [$^{13}C_6$]- and [$^{13}C_{11}$]-5-MTHF) in the presence of abundant unlabelled [$^{13}C_0$]-5-MTHF in plasma in the required ranges (RIKILT,

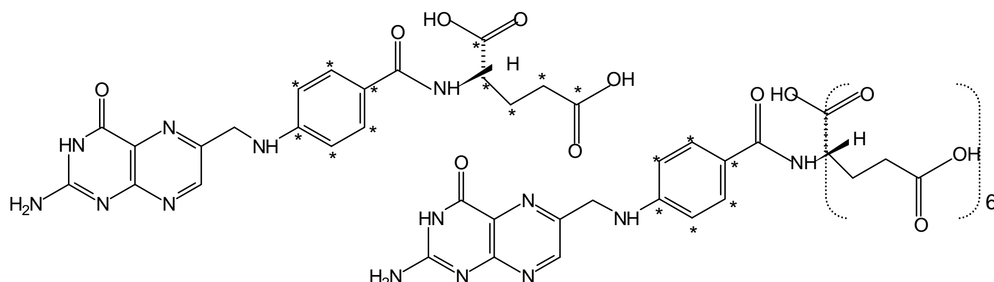

Figure 6.1. Chemical structure of [$^{13}C_{11}$]-monoglutamyl (left) and [$^{13}C_6$]-hexaglutamyl folic acid (right).

Wageningen). This method is referred to as the Wageningen LC-MS/MS method. We decided to limit measurements to 5-MTHF because this is the predominant folate compound in plasma[26], and concentrations of other labelled folate compounds were assumed to be under the LOD.

In short, liquid chromatograhpy separation of folates was performed using a reversed phase LUNA C18 column (150 mm x 2.0 mm ID, d_f =10 µm; Phenomenex, Torrance, CA, USA). The HPLC system was directly coupled to a Quattro Ultima triple quadrupole mass spectrometer (Micromass, Manchester, UK). An electrospray interface was used in positive ionisation mode. Data acquisition was done by multiple reaction monitoring (MRM) of major characteristics of fragmentation reactions. MRM transitions inclucted m/z 466 →319 for [$^{13}C_6$]-5-MTHF, *m/z* 471 → 319 for [$^{13}C_{11}$]-5-MTHF, *m/z* 460 → 313 for [$^{13}C_0$]-5-MTHF, and *m/z* 465 → 313 for the internal standard [$^{13}C_5$]-5-MTHF labelled at the glutamyl moiety. LOD of this method was 20-40 pmol/L and the limit of quantification (LOQ) was 60-80 pmol/L. The intra-assay CV was <5% and recoveries were between 65 and 80%.

Several pilot studies were carried out in order to test the ability of the LC-MS/MS method to determine 5-MTHF enrichments in plasma in the required ranges. In order to evaluate the accuracy of the method, a different LC-MS/MS method using negative ionisation mode was developed for comparison of results (College of Pharmacy, University of Illinois, Chicago, USA). This method is referred to as the Chicago LC-MS/MS method. A detailed description of both LC-MS/MS methods, pilot studies and comparison of methods is given in the Appendix at the end of this Chapter.

6.2.3 Subjects

The study protocol was approved by the Medical Ethical Committee of Wageningen University. Subjects were recruited from staff and students of Wageningen University by e-mail messages and poster advertisements. In total, 20

healthy subjects aged 18-50 y were included in the study. Exclusion criteria comprised chronic diseases such as renal insufficiency, liver disease or gastro-intestinal diseases, use of drugs that interfere with folate metabolism, and low serum vitamin B_{12} concentrations (<130 pmol/L). Subjects were informed about the design and purpose of the study both in writing and orally and gave their written informed consent before the study commenced.

6.2.4 Study design

Both labelled folic acid compounds were encapsulated at a target amount of 50 nmol (~23 µg) [$^{13}C_{11}$]-monoglutamyl folic acid and 50 nmol (~55 µg) [$^{13}C_6$]-hexaglutamyl folic acid per capsule (Pharmacy of the Gelderse Vallei Hospital, Ede, the Netherlands). Exact amounts of hexaglutamyl and monoglutamyl folic acid in the capsules were examined by analyzing capsule samples (n=5), consisting of three capsules per sample, by HPLC[27] (RIKILT, Wageningen, the Netherlands) and by microbiological method using *L. casei* (TNO Food and Nutrition Research, Zeist). The ratio of [$^{13}C_{11}$]:[$^{13}C_6$]-folic acid in capsules was measured by the Wageningen LC-MS/MS method. Before these analyses, hexaglutamyl folic acid in the capsules was deconjugated to the monoglutamyl form. The ratio of labelled folic acid in the capsules was also determined by matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) without prior deconjugation of hexaglutamyl folic acid using three different matrices in Chicago.

Subjects consumed one capsule with breakfast and one with dinner each day for 4 w. Blood was collected in EDTA vials by venapuncture in the morning after an overnight fast on days 0, 1, 2, 4, 8, 14, and 28 of intervention and 1 and 4 w thereafter.

Dietary folate intake was assessed by a 24-h recall method. A trained dietician contacted all participants by telephone on five days of the last two weeks of the study and asked them about their food intake on the previous day. Folate intake was calculated with the current Dutch Food Composition Table.[28]

6.2.5 Biochemical measurements

Samples of whole blood were centrifuged for 10 min at 2600 g within 30 min after blood being drawn. Plasma was separated in 2 vials of 1 mL and stored immediately at -80°C until further analysis. Plasma samples were transported on dry ice to laboratories and were still frozen upon arrival. LC-MS/MS measurements were performed as described in the Appendix. Serum vitamin B_{12} concentrations were assessed by a chemiluminescent immunoassay analyzer (Immulite 2000, Diagnostic Products Company, Los Angeles, CA) at the General Practitioner's Laboratory in Velp.

6.2.6 Calculations and statistics

From the plasma concentrations of [$^{13}C_0$]-5-MTHF, [$^{13}C_{11}$]-5-MTHF, and [$^{13}C_6$]-5-MTHF measured, for each individual at each time point [$^{13}C_6$]:[$^{13}C_{11}$]-5-MTHF ratios were derived. Bioavailability of hexaglutamyl folic acid relative to that of monoglutamyl folic acid was calculated as follows:

$$\frac{[^{13}C_6]}{[^{13}C_{11}]} \text{- 5 - MTHF ratio}_{day\ 28} * \frac{\text{Dose of } [^{13}C_{11}] \text{ - monoglutamyl folic acid}}{\text{Dose of } [^{13}C_6] \text{ - hexaglutamyl folic acid}}.$$

Enrichment levels were calculated as the concentration of the labelled compound divided by the total of labelled and unlabelled folate concentrations and expressed as a percentage. Means and 95% CI or standard errors of all variables and at each point in time were calculated for the group as a whole.

The calculations described above were done with data sets obtained with both LC-MS/MS methods, and the ratios of [$^{13}C_6$]:[$^{13}C_{11}$]-5-MTHF obtained with both methods were compared. Values were considered to differ significantly when 95% CIs were not overlapping, and not to be significantly different when standard errors were overlapping. Results of this comparison of methods are reported in the Appendix at the end of this Chapter.

6.3 Results

Nineteen subjects completed the study and their general characteristics are shown in **Table 6.1**. Data from one subject were discarded because of use of anti-inflammatory medication which may interfere with folate metabolism. All subjects had normal serum vitamin B_{12} and plasma folate levels. Mean dietary folate intake was 239 ± 60 (SD) µg per day.

Table 6.1 General characteristics of the study population (n=19)[a]

Characteristic	Mean ± SD
Age, y	25 ± 4.7
Male/female, n	5/14
BMI, kg/m^2	23.0 ± 3.1
Vitamin B_{12}, pmol/L	285 ± 77
Dietary folate intake, µg/d	239 ± 60
Plasma folate concentration at baseline, nmol/L	9.2 ± 4.1

[a]Values are means ± SD.

Results of capsule content analyses yielded inconsistent results. According to HPLC analyses, the content of hexaglutamyl folic acid was about 30% lower than we aimed at. Microbiological analysis also revealed a lower content of hexaglutamyl folic acid in capsules but variability in duplicate samples was high (CV ~25%). The mean ratio of [$^{13}C_{11}$]:[$^{13}C_6$]-folic acid measured by LC-MS/MS was close to 1.0, but

varied considerably between samples (n=5, SD=0.36). MALDI-TOF-MS analysis revealed ratios of [$^{13}C_{11}$]:[$^{13}C_6$]-folic acid from 1.74 to 5.35. These ratios need further quantification using standards of [$^{13}C_{11}$]-monoglutamyl folic acid and [$^{13}C_6$]-hexaglutamyl folic acid because the ionisation efficiencies of the two compounds varied depending on the matrix.

At day 0, no [$^{13}C_{11}$]- or [$^{13}C_6$]-5-MTHF was detected in the plasma samples. Concentrations of [$^{13}C_{11}$]- and [$^{13}C_6$]-5-MTHF in plasma increased over time until concentrations of 690 pmol/L (95% CI 580 to 800) and 440 pmol/L (360 to 520) respectively were reached on day 28 (**Figure 6.2**). Labelled folate was still present at 1 w but not 4 w after cessation of supplementation. The levels of folate enrichment in plasma were 6.3% (95% CI 5.6 to 7.0) for [$^{13}C_{11}$]-5-MTHF and 4.0% (3.5 to 4.5) for $^{13}C_6$-5-MTHF on day 28.

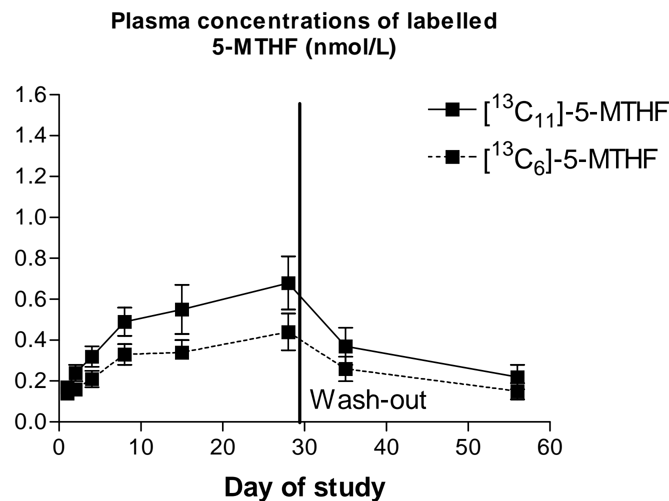

Figure 6.2 Concentrations of [$^{13}C_{11}$]- and [$^{13}C_6$]-5-MTHF in plasma as measured by LC-MS/MS (means ± 95% CI).

As can be seen from **Figure 6.3**, a constant level of the ratio of [$^{13}C_6$]:[$^{13}C_{11}$]-5-MTHF concentrations was established by day 4 and remained stable over time. On day 28, the mean ratio was 0.66 (95% CI, 0.58 to 0.74; SE 0.04).

Comparison of these results using the Wageningen LC-MS/MS with those obtained with the Chicago LC-MS/MS method (see Appendix at the end of this Chapter) shows that the two methods gave different results for the measurement of concentrations of labelled and unlabelled 5-MTHF in plasma. However, the ratios of [$^{13}C_6$]:[$^{13}C_{11}$]-5-MTHF were similar from day 2 up until 28. The ratio on day 28 obtained by the Chicago LC-MS/MS method was 0.73 (95%-CI, 0.63 to 0.83; SE, 0.05).

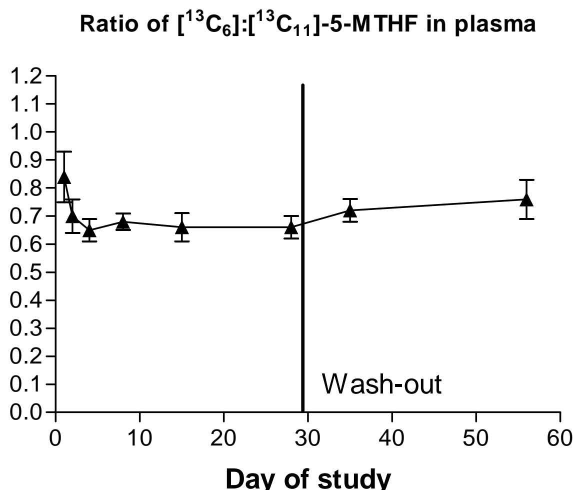

Figure 6.3 Ratios of [$^{13}C_6$]:[$^{13}C_{11}$]-5-MTHF in plasma as measured by LC-MS/MS (means ± SE).

To enable calculation of the bioavailability of polyglutamyl relative to that of monoglutamyl folic acid, the ratio of the amount of each compound administered needs to be known. Up until now we have been unable to determine this with sufficient precision. Therefore, we are unable to estimate the bioavailability of hexaglutamyl folic acid relative to that of monoglutamyl folic acid at this stage.

6.4 Discussion

Our data show that the ratio of [$^{13}C_6$]:[$^{13}C_{11}$]-5-MTHF in plasma could be measured within narrow confidence limits and results were similar between the two laboratories using widely different methods. Therefore, the dual isotopic labelling method presented here, in which multiple low doses of the two forms of folic acid were administered for a longer period, can be used as a sensitive and accurate method for measuring folate bioavailability.

Unfortunately we had underestimated how difficult it would be to analyse the low amounts of monoglutamyl and hexaglutamyl folic acid in the capsules. This is the reason that we could not finalize the quantification of bioavailability of hexaglutamyl relative to that of monoglutamyl folic acid. We aimed at an amount of 50 nmol of each of the labelled folic acid compounds in the capsules. We used several approaches to examine the contents of folic acid in the capsules. Firstly, from the pharmacy report could be calculated that the amount of monoglutamyl folic acid used for compounding the capsules may have exceeded the required amount by about 10%. Secondly, HPLC and microbiological analyses indicated a lower amount of hexaglutamyl folic acid in the capsules than aimed for. And

thirdly, LC-MS/MS indicated no relative differences in the amounts of labelled folic acid, whereas MALDI TOF MS indicated a relatively lower amount of labelled hexaglutamyl folic acid in the capsules.

The discrepancies between methods might be explained by several causes. Firstly, the folic acid in the capsules may have been partially unlabelled revealing different results when measuring labelled (LC-MS/MS) or total (HPLC, microbiological) amounts of folic acid. However, no significant signals were seen for unlabelled or partially labelled monoglutamyl or hexaglutamyl folic acid in LC-UV-MS/MS spectra. Secondly, deconjugation of hexaglutamyl folic acid preceding HPLC and microbiological analysis might have been incomplete. However, deconjugase activity was 100% as assessed by measurement of a known amount of triglutamyl folic acid. The third explanation is that the filler material of the capsules has disturbed analyses. This could explain the large variation found among the MS measurements, which is a problem also encountered previously during HPLC measurements. The analytical problems need to be overcome before the bioavailability of hexaglutamyl folic acid relative to that of monoglutamyl folic acid can be quantified reliably. It is expected that the problems will be overcome shortly.

Several groups of researchers have previously reported the use of a dual-label stable isotope method for studying folate kinetics and bioavailability in humans. The group of Gregory evaluated a dual-label stable isotope method, in which the enrichment in plasma folate in response to an oral dose of 1010 nmol [$^{13}C_5$]-folic acid was compared to that of an intravenous dose of 226 nmol [$^{2}H_2$]-folic acid.[29] Despite the high oral dose, plasma isotopic enrichment was 15 to 20- fold greater for injected folic acid. Therefore, it was concluded that plasma kinetics would be of limited usefulness in assessing the relative bioavailability of nutritionally relevant oral doses of labelled folic acid in single dose experiments. In the same study, it was found that oral administration of equimolar oral doses of labelled folic acid yielded equivalent urinary excretions of each dose.[29] The latter approach has been applied for studying the bioavailability of folate from fortified cereal-grain products and of supplemental folic acid.[30] However, between-subject variation was high compared with previous methods in which subjects were loaded with folic acid prior to study. Another research group used an oral/ intravenous dual-label stable isotope method to determine the bioavailability of folic acid from fortified cereal grain foods.[31,32] They concluded that bioavailability could not be assessed because labelled folic acid compounds were handled differently in the body due to the route of administration.

With the dual isotopic labelling method we presented here these limitations have been overcome. The two labelled folic acid compounds were both administered orally and the administered dose of polyglutamyl folic acid was only ~15% of the RDA (=300 μg/d) and spread in two portions over the day. Therefore,

disturbance of normal folate metabolism has been minimal. Pre-loading of subjects was not necessary and yet between-person variability was small. The use of multiple doses even allowed for any possible upregulation of FGCP activity due to a slightly higher daily supply of polyglutamyl folic acid.

Until recently, techniques for folate analysis lacked the sensitivity to detect folate concentrations in plasma as low as that required for our study.[33,34] Our prior target was to be able to measure enrichment levels of 1%. However, plasma enrichments of both labelled compounds were well above this target. Furthermore, ratios of [$^{13}C_6$]:[$^{13}C_{11}$]-5-MTHF reached a plateau by 4 d. Therefore, a shorter intervention period and halving of the administered doses would be sufficient to obtain reliable data.

An important assumption we made is that the physical, chemical and metabolic behaviour of the 5-MTHF derived from two labelled compounds is equal. If this were not the case, this could give rise to differential metabolism of the two isotopic species of 5-MTHF in the body. However, such isotope effects have not been reported for [$^{13}C_6$]-folic acid, labelled in the PABA structure, or for [$^{13}C_5$]-folic acid labelled in the glutamyl structure.[35] Another problem could be that the labelled moiety of the molecule would be lost or exchanged with unlabelled molecules. In one of the pilot studies (see Appendix, § 3.2) we checked whether the [$^{13}C_{11}$]-labelled folic acid remained intact in vivo. We did not detect isotopic species of 5-MTHF in plasma other than [$^{13}C_{11}$]-5-MTHF.

The methodology we used is based on the principle that the two differentially labelled compounds in plasma are in equilibrium with other tissues and that the degree of enrichment reaches a plateau following repetitive consumption of low doses. Since the half-life of folate is estimated to be ~100 d[36], steady state cannot be reached in 28 d. Our data show indeed that plasma concentrations increased gradually over the whole intervention period. However, ratios already reached a plateau after 4 d of folic acid administration. This supports the validity of the assumption that there are no differences in post-absorptive metabolism between the two labelled compounds.

The application of the dual isotopic labelling method we presented is not restricted to studying the bioavailability of polyglutamyl relative to monoglutamyl folic acid. Van Lieshout *et al.* used this method in a slightly different way for studying the bioavailability, bioconversion and bioefficacy of carotenoids from mixed diets.[24,37] Their approach was to dilute plasma enrichments by replacing a low carotenoid diet by a diet high in carotenoids after a certain period of time. Subsequently, the extent of dilution can be used as a measure of bioavailability. Many other applications of the method can be thought of, such as quantifying the bioavailability of any carbon containing nutrient from mixed diets or for studying

the effects of food-related factors, e.g. the food matrix, on the bioavailability of nutrients.

In conclusion, the dual isotopic labelling method that we described here provides a sensitive, accurate and efficient method for measuring folate bioavailability. As yet, no estimates can be made of the bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid at this time because of problems associated with measurements in the capsules. However, we expect these data to become available shortly.

Acknowledgements

We thank all people who participated in this trial. We are grateful to Prof Lugtenburg from Leiden University and Dr Rob van der Steen and Marcel Bartels from ARC Laboratories, for their ingenuity in producing the labelled compounds. We thank Prof Frans Russel for his input in the mathematical modelling. The late Peter van de Bovenkamp and Truus Kosmeyer are thanked for their help with the HPLC analyses, and Els Siebelink and Annelies Rotteveel for their effort in performing the 24h-recalls and calculating dietary folate intake.

Appendix

Comparison of two LC-MS/MS methods for measuring the ratio of differentially ^{13}C-labelled folate compounds in plasma of humans.

1. Introduction

In order to evaluate the accuracy of LC-MS/MS measurements performed for the bioavailability study, as described in Chapter 6, two LC-MS/MS method were developed. In this Appendix, detailed descriptions of both methods are given. Further, the results of several pilot experiments carried out for validation purposes are described. In the last part of this Appendix, data from the two LC-MS/MS methods (Chapter 6) are compared.

2. Description of LC-MS/MS procedures

2.1 LC-MS/MS method developed at RIKILT, Wageningen

Sample preparation - Plasma (500 µL) was mixed with 1 mL buffer (1% w/v ascorbate, 100 mM ammonium acetate). Sample preparation was carried out using Solid Phase Extraction (SPE) on an Oasis® MAX cartridge (Waters, Milford, MA, USA). The column was conditioned with 1 mL methanol followed by 1 mL buffer (1% w/v ascorbic acid, 100 mM ammonium acetate). Diluted plasma samples were loaded on the SPE cartridge and the SPE cartridge was rinsed with 2 mL 50% v/v methanol, vacuum dried and rinsed again with 2 mL ethyl acetate. After a second vacuum drying, the SPE cartridge was eluated with 2 mL 3% (v/v) formic acid in methanol. The methanol fraction was dried at 50°C under a stream of nitrogen and reconstituted in 200 µL buffer (1% w/v ascorbate, 100 mM ammonium acetate). Aliquots of 25 µL were used for LC-MS/MS analysis.

Liquid Chromatography - HPLC separation of differentially labelled 5-MTHF was performed on a reversed phase LUNA C18 column (150 mm x 2.0 mm ID, d_f = 10 µm; Phenomenex, Torrance, CA, USA). An acetonitrile-5 mM formic acid eluent was used with a 10-min linear gradient from 0 to 80 vol.% acetonitrile, starting 2 min after injection; the flow-rate was 250 µL/min.

Mass Spectrometry - The HPLC system was directly coupled to a Quattro Ultima triple quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray (ESI) interface, using positive ionisation mode. The mass spectrometer was operated with ion source settings optimised for maximum sensitivity. [$^{13}C_5$]-5-MTHF, labelled in the glutamyl moiety (Eprova, Schaffhausen, Switzerland), was used as an internal standard. Data acquisition was done by multiple reactin monitoring (MRM) of the major fragmentation reactions characteristics. The MRM transitions selected for quantitative LC-MS/MS analysis were as follows: *m/z* 466 $\rightarrow$ 319 for [$^{13}C_6$]-5-MTHF, *m/z* 471 $\rightarrow$ 319 for [$^{13}C_{11}$]-5-MTHF, *m/z* 460 $\rightarrow$ 313 for [$^{13}C_0$]-5-MTHF (endogenous, non-labelled folate) and *m/z* 465 $\rightarrow$ 313 for the internal standard [$^{13}C_5$]-5-MTHF

Quality control - The limit of detection (LOD) of this method was 20-40 pmol/L, the limit of quantification (LOQ) 60-80 pmol/L, and the within-run CV <5%. Recoveries were between 65 and 80%.

2.2 LC-MS/MS method developed at University of Illinois, Chicago

Sample preparation - After thawing, 2 mL of each plasma sample was vortex mixed with 100 µL of antioxidant solution containing 0.1 mg/mL ascorbic acid and 0.01 mg/mL 2-mercaptoethanol. This was done in order to prevent oxidation and decomposition of endogenous folate. For extraction of both free and protein-bound folates while precipitating the plasma proteins, 8 mL of 25 mM ammonium acetate in acetonitrile/water (9:1; v/v) solution was added followed by sonication for 1 min and vortex mixing for 1 min. The resulting mixture was then centrifuged for 5 min at ~10500 *g* at 4 °C. The resulting supernatant solution was removed and passed through a 3 mm-OD x 0.2-µm pore size PTFE syringe filter. The

6

filtered extract was evaporated to dryness under vacuum at room temperature. The residue was then reconstituted in 100 µL of 25 mM ammonium acetate in acetonitrile/water (9:1; v/v) for LC-MS/MS analyses.

Liquid chromatography - HPLC separation was carried out during LC-MS/MS using a minibore HILIC column (The Nest Group, Southborough, MA, USA) consisting of polyhydroxyethyl aspartamide on silica (150 mm x 1.0 mm I.D., 100Å-pore size, 5 µm particle size).

Mass Spectrometry - Product ion MS/MS scans of deprotonated molecules of the various folate species were obtained following argon collision induced dissociation (CID) to identify abundant diagnostic fragment ions for subsequent multiple reaction monitoring (MRM). The MS/MS instrument used was a ThermoFinnigan (San Jose, CA, USA) TSQ Quantum triple quadrupole mass spectrometer. The MRM transitions selected for quantitative LC-MS/MS analysis were as follows: *m/z* 464 $\rightarrow$ 335 for [$^{13}C_6$]-5-MTHF, *m/z* 469 $\rightarrow$ 335 for [$^{13}C_{11}$]-5-MTHF, *m/z* 458 $\rightarrow$ 329 for [$^{13}C_0$]-5-MTHF and *m/z* 463 $\rightarrow$ 329 for the internal standard [$^{13}C_5$]-5-MTHF (Eprova, Schaffhausen, Switzerland). The use of the TSQ Quantum MS/MS instrument not only facilitated the accurate quantification of labelled and unlabelled 5-MTHF, but also the less abundant folate species 5-formyltetrahydrofolate, 10-formyltetrahydrofolate, dihydrofolate, and tetrahydrofolate could be measured. The concentrations of these less abundant species were 5-17% of that of 5-MTHF.

Quality control - Folate concentrations were obtained by interpolation of their peak area ratios (folate/internal standard) from the calibration curve. The LOD was 52 – 78 pmol/L and the LOQ was 170 – 226 pmol/L. The linear range of the calibration curve was 65 pmol/L – 22 nmol/L (mean r^2 of standard curves = 0.984). The recovery of 5-MTHF was > 95% over this concentration range. The intra-day (RSD, n=5) and interday (RSD, n=8) precision for 5-MTHF were 2.1% and 7.2%, respectively.

3. Pilot studies

Approval of the Medical Ethical Committee of Wageningen University was obtained prior to carrying out all pilot studies. All subjects were informed in writing and orally about the study, and all gave written informed consent. Measurements for the pilot studies as described here were all obtained by using the LC-MS/MS method developed at RIKILT in Wageningen.

3.1 Capability to detect ^{13}C labelled 5-MTHF in human plasma

Objectives - 1) To test the capability of the LC-MS/MS method to detect labelled 5-MTHF in human plasma; and 2) to assess the level of folate enrichment, i.e. the amount of labelled folate relative to that of unlabelled folate, in plasma.

Materials – Capsules, each containing 100 nmol [$^{13}C_5$]-5-MTHF, labelled in the glutamyl moiety (Eprova, Schaffhausen, Switzerland), were prepared (Gelderse Vallei Hospital, Ede).

Subjects - Three adults, - one male and two females - consumed one capsule at breakfast and one at dinner each day for 3 w. Samples of fasting plasma were collected on days 1, 2, 5, 8, 11, 17 and 21 of the intervention, and 3 d and one month after the intervention period.

Results - On day 21, concentrations of 5-MTHF in plasma ranged from 2.0 to 27.1 nmol/L for [$^{13}C_0$]-5-MTHF, and from 300 to 800 pmol/L for [$^{13}C_5$]-5-MTHF. Mean CV% of duplicate measurements (one duplicate per subject) were 39% for [$^{13}C_0$]-5-MTHF and 16% for [$^{13}C_5$]-MTHF concentrations. In **Figure 1** the [$^{13}C_5$]-enrichment levels of plasma 5-MTHF over time can be seen.

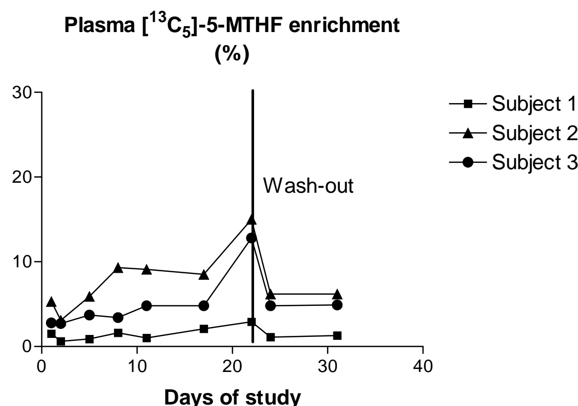

Figure 1 Individual [$^{13}C_5$]-5-MTHF enrichments (%) in plasma as measured by LC-MS/MS.

Conclusion - Plasma concentrations of [$^{13}C_5$]-5-MTHF could be measured and were well above the LOD. Enrichment levels turned out to be favourably higher than the target of 1% that was set initially. However, variation between duplicate measurements was high. Modifications to the analytical method was required to reduce this variability.

3.2 Stability of [$^{13}C_{11}$]-folic acid in vivo

Objective - To assess loss or exchange of the labelled glutamyl moiety from [$^{13}C_{11}$]-folic acid *in vivo.* If such loss or exchange should occur [$^{13}C_6$]-5-MTHF would be seen in addition to [$^{13}C_{11}$]-5-MTHF in plasma. Since we aimed to administer [$^{13}C_6$]-hexaglutamyl folic acid simultaneously, exchange of the $^{13}C_5$-glutamyl moiety with unlabelled glutamic acid would render the proposed method unusable.

Materials – Capsules each containing 100 nmol [$^{13}C_{11}$]-folic acid (ARC, Apeldoorn, the Netherlands) were prepared.

Subjects - Three adults - one male and two females - consumed one capsule with breakfast and one with dinner for 12 d. Fasting samples of plasma were collected on days 4, 7 and 12 of intervention for analysis of 5-MTHF.

Results - On day 12, enrichment of [$^{13}C_{11}$]-5-MTHF ranged from 2.3 to 3.4%. No [$^{13}C_6$]-5-MTHF was detected. The CV% of 5 replicate samples from each subject on day 12 was 27%.

Conclusion – No exchange or loss of labelled glutamyl from [$^{13}C_{11}$]-folic acid occurred (< LOD of 20 pmol/L).

3.3 Simultaneous measurement of [$^{13}C_6$]- and [$^{13}C_{11}$]-5-MTHF in human plasma

Objective - To quantify two differentially labelled 5-MTHF compounds in blood plasma with sufficient sensitivity.

Materials – Capsules each containing 100 nmol each of [$^{13}C_{11}$]-monoglutamyl and [$^{13}C_6$]-hexaglutamyl folic acid (ARC, Apeldoorn, the Netherlands) were prepared.

Subjects - Three subjects - one male and two females - consumed two capsules each day for 10 days. Fasting samples of plasma were collected on days 3, 6 and 10.

Results - Plasma concentrations of [$^{13}C_0$]-5-MTHF on day 10 ranged from 3.9 to 8.8 nmol/L. Concentrations of [$^{13}C_{11}$]-5-MTHF ranged from 490 to 890 pmol/L, and of [$^{13}C_6$]-5-MTHF from 350 to 630 pmol/L. The enrichment of 5-MTHF with [$^{13}C_{11}$]- and [$^{13}C_6$], and the [$^{13}C_6$]:[$^{13}C_{11}$] ratio in 5-MTHF are shown in **Figure 2.**

The within-run coefficient of variation (CV) of the measurements was 6%. The individual ratios of [$^{13}C_6$]:[$^{13}C_{11}$] in 5-MTHF were 0.65, 0.71 and 0.71.
Conclusion – Each of the two labelled 5-MTHF compounds in plasma could be measured and their concentrations were both well above the limit of detection. Analytical variation had been reduced to an acceptable level.

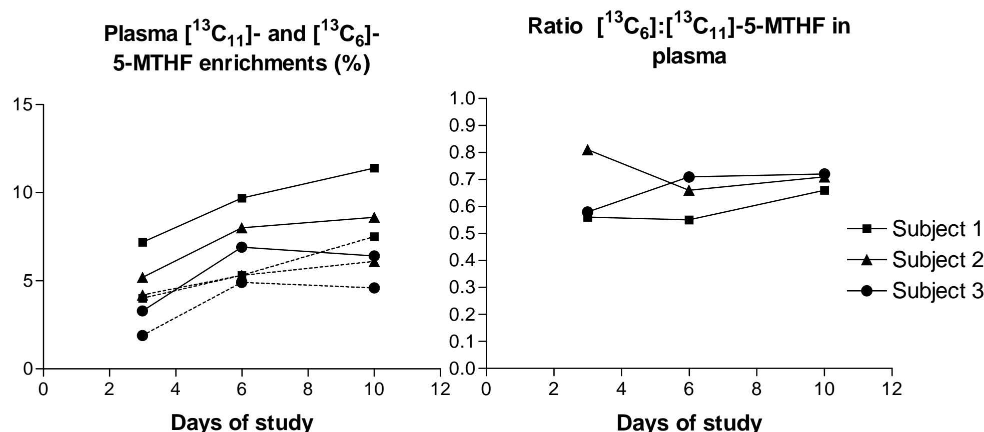

Figure 2 [$^{13}C_{11}$]- and [$^{13}C_6$]-5-MTHF enrichments (%) (left) and ratios of [$^{13}C_6$]:[$^{13}C_{11}$]-5-MTHF (right) in plasma.

4. Comparison of LC-MS/MS and HPLC methods

Objective - To assess the accuracy of the LC-MS/MS procedure used to quantify the bioavailability of [$^{13}C_6$]-hexaglutamyl folic acid relative to [$^{13}C_{11}$]-monoglutamyl folic acid.
Subjects and Materials - See Chapter 6.
Biochemical measurements – LC-MS/MS measurements were performed as described in § 2 of this Appendix. Additionally, total 5-MTHF in plasma was measured by HPLC with fluorescence detection at the Division of Human Nutrition, Wageningen University. Ascorbic acid (1%) was added to plasma as an anti-oxidant and perchloric acid (60%) was used for deprotonisation. Total plasma 5-MTHF concentrations – the sum of [$^{13}C_0$], [$^{13}C_{11}$]- and [$^{13}C_6$]-5-MTHF - as measured by LC-MS/MS methods were compared to total plasma 5-MTHF concentrations obtained by HPLC.
Results - Results of the LC-MS/MS method developed at the University of Illinois, Chicago, are reported in this Appendix. Results obtained by the LC-MS/MS method developed at RIKILT, Wageningen, are described in Chapter 6 and are reported here in italics within parantheses.
On day 0, [$^{13}C_{11}$]- and [$^{13}C_6$]-5-MTHF concentrations were under the LOD except for one subject. Concentrations of [$^{13}C_{11}$]- and [$^{13}C_6$]-5-MTHF in plasma increased rapidly until day 8 and then stabilized until concentrations of 1140 pmol/L (95%-CI 990 to 1290) and 810 (680 to 950) pmol/L were reached on day 28, respectively (**Figure 3**) *(Wageningen: 690 (580 to 800) and 440 (360-520) pmol/L, see* ***Figure 6.2*** *at page 96).*
Plasma 5-MTHF, both labelled and unlabelled as measured by HPLC, and the sums of [$^{13}C_0$]-, [$^{13}C_{11}$]-, and [$^{13}C_6$]-5-MTHF as measured by LC-MS/MS at RIKILT, Wageningen, were very similar. At the University of Illionois, Chicago, consistently higher concentrations were measured (**Figure 4**). The levels of folate enrichment in plasma were 6.3% (95%-CI 5.7to 6.9) for [$^{13}C_{11}$]-5-MTHF and 4.5% (3.9 to 5.1) for $^{13}C_6$-5'-MTHF at day 28. *(Wageningen: 6.3% (5.6 to 7.0) and 4.0% (3.5 to 4.5).*

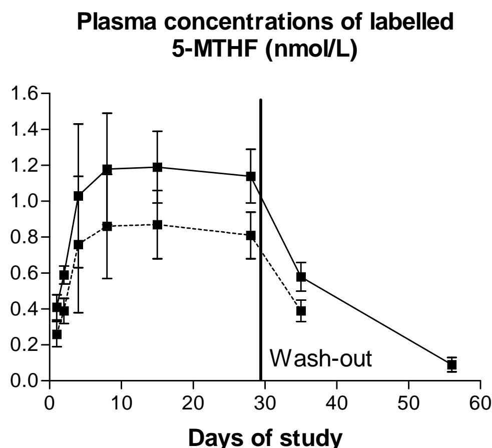

Figure 3 Plasma concentrations of [$^{13}C_{11}$]- and [$^{13}C_6$]-5-MTHF, measured in Chicago (means, 95% CI).

Ratios of [$^{13}C_6$]:[$^{13}C_{11}$]-5-MTHF in plasma obtained by the two laboratories were found to be similar as from day 2 (**Figure 5**).

Conclusion - Measurement of absolute concentrations of labelled and unlabelled 5-MTHF are consistently higher by the LC-MS/MS method developed in Chicago. This is probably due to the release of protein-bound folate (Figures 3 and 4). However, the ratio of the two labelled compounds in plasma measured by both methods are similar. Therefore, we conclude that the ratio of [$^{13}C_6$]:[$^{13}C_{11}$]-5-MTHF in plasma can be measured accurately by these two LC-MS/MS methods.

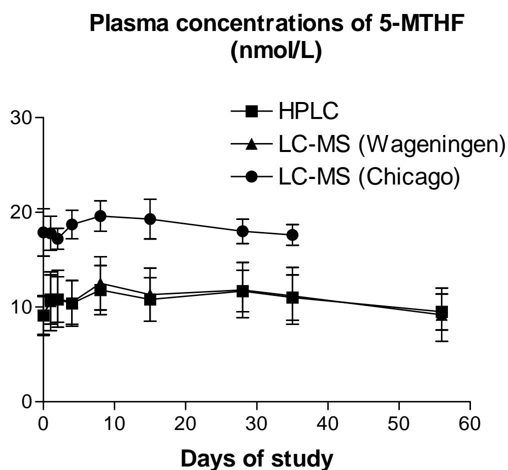

Figure 4 Plasma concentrations of 5-MTHF as measured by HPLC and by the two LC-MS/MS methods (means with 95% CI). Concentrations measured by LC-MS/MS methods were calculated as the sums of [$^{13}C_0$]-, [$^{13}C_{11}$]- and [$^{13}C_6$]-5-MTHF concentrations.

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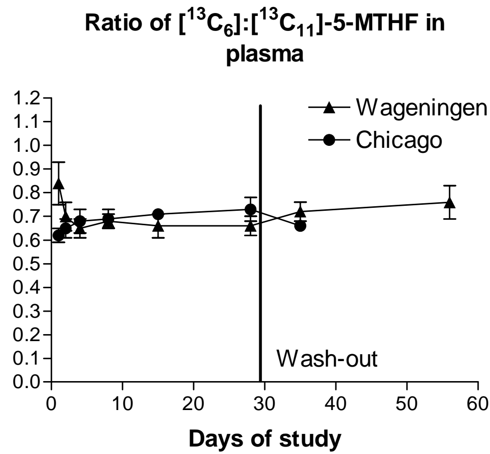

Figure 5 Ratios of [$^{13}C_6$]:[$^{13}C_{11}$]-5-MTHF in plasma as measured by LC-MS/MS at the RIKILT, Wageningen and at the University of Illinois, Chicago (means ± SE).

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Chapter 7

Folic acid and plasma homocysteine reduction in older adults: a dose finding study

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ABSTRACT *Background:* High homocysteine concentrations – a possible risk factor for cardiovascular disease - can be lowered effectively with folic acid. However, potential adverse effects of excessive doses of folic acid in vulnerable sub-groups give rise to concern. The minimum dose of folic acid that can lower homocysteine concentrations maximally is not yet known reliably. *Objective:* We aimed to determine the lowest folic acid dose that lowers plasma homocysteine concentrations adequately in healthy older adults. *Design:* A randomized, double-blind, parallel group, placebo-controlled dose-finding trial was carried out among 316 men and women (50 to 75 y). Subjects received daily one of six different folic acid doses, namely 50, 100, 200, 400, 600, and 800 µg/d or placebo for 12 w. The relative changes in plasma homocysteine concentration in response to increasing doses of folic acid were used to calculate the dose response curve. An adequate dose of folic acid was defined as the dose that induced at least 90% of the maximal reduction in homocysteine concentration. *Results:* The relative decrease in plasma homocysteine concentration was associated exponentially with increasing doses of folic acid. From the dose response curve, the adequate dose of folic acid was estimated to be 392 µg/d, which induced a reduction in plasma homocysteine concentrations of 22%. *Conclusions:* Daily intake of folic acid in older adults effectively lowered plasma homocysteine concentrations, with a dose of about 400 µg/d being the minimum dose required for adequate homocysteine reduction.

Keywords: *Folic acid; Homocysteine; Dose-finding; Food fortification; Human; Dose-response curve; Adults; Population.*

7.1 Introduction

Folic acid supplementation can reduce the risk of neural tube defects[1] and effectively lowers plasma total homocysteine concentrations[2], a possible risk factor for cardiovascular disease.[3] In the United States of America, mandatory fortification of flour with folic acid at a level of 140 µg/100 g was introduced in 1998[4], and resulted in almost a doubling in the mean daily intake of folic acid, substantial reduction in population mean blood total homocysteine concentrations[5-7] and reduced the incidence of neural tube defects by 19%.[8] In some countries even higher levels of fortification are being considered in order to reduce homocysteine concentrations in the population. The current British recommendations for instance are to fortify flour with folic acid at 240 µg/100 g.[9,10] But, there is concern about the potential adverse effects of excessive doses of folic acid in sub-groups with vitamin B12 deficiency, and individuals treated with anticonvulsants, or with antifolate drugs such as methotrexate.[11] There is real uncertainty about how best to maximize the intake of folic acid for women of child-bearing age and minimize the adverse effects of excessive intakes of folic acid for the elderly and other groups in the population.[9]

The minimum dose of folic acid that maximally lowers homocysteine concentrations is not known reliably. The initial studies of folic acid to lower homocysteine concentrations used daily doses of 5 mg or greater.[12] A meta-analysis of twelve randomized trials of folic acid-based multivitamin supplements to lower homocysteine concentrations demonstrated that doses of folic acid from 0.4 mg/d to 5 mg/d were equally effective.[2] However, there is little or no available randomized evidence for the homocysteine lowering effects of daily doses of folic acid less than 0.4 mg in healthy adults.Trials comparing the homocysteine lowering effects of one dose of folic acid with another at doses below 0.5 mg/day have either used a sequential design[13], or have been carried out in young populations,[14,15] or in people with cardiovascular disease.[10]

Reliable data on the minimum dose of folic acid required for a maximal decrease in homocysteine concentrations in older populations could inform the debate on the level of folic acid to use for food fortification. In addition, the inclusion of doses in the range of normal dietary folate intake could provide indirect evidence on whether improvement in the bioavailability of folate in foods or advice to increase daily dietary intake of folate could substantially reduce homocysteine concentrations. This information may have even greater relevance if the ongoing trials of folic acid supplementation in patients with cardiovascular disease[16] can demonstrate that lowering homocysteine can reduce the risk of cardiovascular disease.

The aim of this dose-finding trial was to estimate the adequate dose of folic acid, defined as the dose that induces 90% of the maximal decrease in plasma total homocysteine concentration. We compared the effects on plasma total homocysteine concentrations of supplementation with folic acid at daily doses of 50, 100, 200, 400, 600, or 800 µg with that of placebo.

7.2 Subjects and Methods

7.2.1 Subjects

We recruited healthy adults aged 50-75 years either from a random sample of people living in the community near Wageningen in the Netherlands, or through a database of volunteers who had previously indicated interest in participating in such studies by Wageningen University. **Figure 7.1** shows a summary of the recruitment procedures.

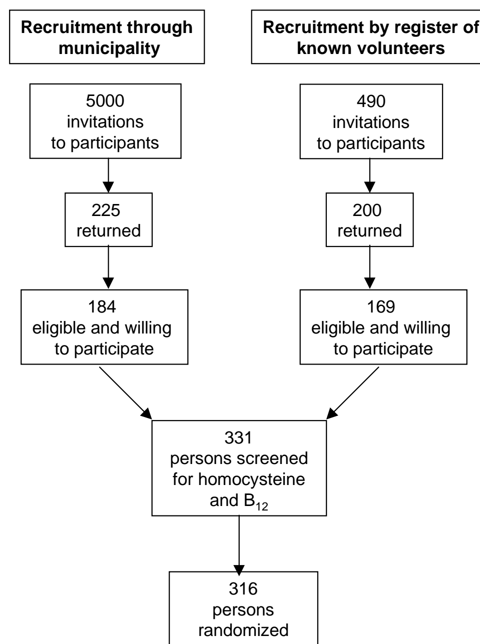

Figure 7.1. Flow schedule of recruitment procedure

Individuals with a history of cardiovascular diseases were excluded, as were individuals with any chronic disease that might interfere with folate or homocysteine metabolism (e.g. renal disease, thyroid disease), or individuals who used medication known to interfere with folate or homocysteine metabolism (e.g. methotrexate or malaria profylactics). All women were post-menopausal and only

one used hormone replacement therapy, which was permitted if participants used this at least 3 months prior to screening and intended to continue the therapy for the duration of the trial. People who used dietary supplements containing B vitamins or yeast extracts within three months prior to the study were excluded. Individuals with plasma homocysteine concentrations >26 µmol/L or serum vitamin B_{12} concentrations <160 pmol/L, or serum creatinine concentrations >125 µmol/L, were also excluded. Among the 316 individuals who were randomized 311 subjects completed the trial. Reasons for dropout included medical complication or use of medication that interferes with folate or homocysteine metabolism (n=4), or personal reasons (n=1). All participants gave written informed consent to a protocol that was approved by the Medical Ethical Committee of Wageningen University.

7.2.2 Study design

After the screening visit, individuals commenced a run-in period of 3 to 4 w, during which they used placebo capsules to assess compliance with study procedures. The run-in period was immediately followed by an intervention period of 12 w. A sample of blood was collected at screening visit, randomization visit, and at 4, and 12 w after start of treatment. Individuals were randomized to receive one out of seven treatments, namely placebo or, 50, 100, 200, 400, 600, or 800 µg folic acid daily. The randomisation procedure took account of the pre-treatment plasma homocysteine concentration by stratification of the treatment allocation by quartiles of homocysteine concentrations at screening. At the baseline visit (week 0) subjects received all supplements for the 12 w of intervention. We asked subjects to maintain their regular diet, but to avoid use of liver, yeast extracts, or supplements containing B vitamins during the trial, and avoid consumption of liver products within three days prior to blood sampling. Fortification of foods with folic acid is not allowed in the Netherlands, therefore the dietary intake of folate of the subjects is restricted to the natural content of folate in foods.

7.2.3 Data collection

At the screening visit height and weight were measured. All subjects kept a diary throughout the study in which they reported the daily intake of capsules, illnesses experienced, and use of medication. Smoking habits were also recorded.

We took fasting venous blood samples at screening, at the start of the intervention period (baseline), and at 4 and 12 w after the start of the intervention period. Total plasma homocysteine, serum vitamin B_{12} and serum creatinine concentrations were measured in the blood collected at screening. Total plasma homocysteine and serum folate concentrations were measured in the blood

samples obtained at baseline, at 4 w, and at 12 w of intervention. We assessed the concentration of folate in erythrocytes at baseline and at 12 w of intervention.

7.2.4 Laboratory procedures

Blood samples were drawn into EDTA-containing evacuated tubes. Samples for the determination of plasma homocysteine concentrations were immediately placed on ice and the plasma was separated from blood cells within 30 minutes. Samples for serum vitamin B_{12}, creatinine, and folate determinations were placed in the dark and stored at room temperature for at least 30 minutes before centrifugation for 10 minutes at 2600 g at 4°C. We assessed hematocrit values immediately and diluted whole blood with four volumes of sodium ascorbate (10 g/L), for the determination of folate concentrations erythrocytes. All samples were stored at -80°C.

Samples collected from one subject at baseline, after 4 and after 12 weeks of intervention were analyzed in the same batch to minimize variability. Total plasma homocysteine concentrations were measured by HPLC with fluorimetric detection at the Division of Human Nutrition, Wageningen University (intra- and interassay coefficient of variation 2% and 7%, respectively).[17,18] We measured folate concentrations in serum and in erythrocytes as well as serum vitamin B_{12} concentrations with a commercial chemiluminescent immunoassay analyzer (Immulite 2000, Diagnostic Products Company, Los Angeles, CA, USA). The samples for erythrocyte folate analysis were further diluted with a concentrated human protein-based matrix (Immulite 2000 diluent) before measurement. Folate concentrations were measured at the clinical laboratory (CKCL) of the University Medical Centre, Nijmegen. The intra-assay coefficient of variation of the serum folate assay was <10% and for erythrocyte folate <9%. Creatinine concentrations were measured with a modification of the kinetic Jaffé reaction (DuPont Dimension). All laboratory staff was blinded for the treatment allocation.

We used an HPLC method with fluorescence and diode array detection to measure the folic acid content of the capsules for treatment.[19] The mean and range of folic acid content in the capsules were 0 (0 to 0), 49 (48 to 52), 99 (95 to 102), 198 (192 to 205), 408 (396 to 431), 633 (619 to 655), and 872 (839 to 898) µg/capsule, in placebo and increasing doses of folic acid. The folic acid content of the capsules did not vary by more than 6%.

7.2.5 Statistical analysis

We calculated absolute changes in homocysteine after 12 w of intervention compared to baseline concentrations. Furthermore, we calculated individual relative changes by dividing each subject's absolute change in plasma homocysteine concentration at 12 w by his or her baseline plasma homocysteine concentration.

Curve fitting by non-linear regression was used to assess the adequate folic acid dose: the mean relative changes in plasma homocysteine concentration were plotted by dose of folic acid, and non-linear regression was used to find the best-fit curve through the relative decreases in homocysteine concentration. The adequate dose was arbitrarily defined as the dose that induces 90% of the maximal decrease of plasma homocysteine concentration as predicted by the asymptote of the best-fit curve (i.e. the decrease at infinite folic acid intake). Additionally, we calculated adequate doses based on 80% and 95% of the maximal decrease. The independent variable was the dose of folic acid as measured in the capsules. We used SAS statistical software (SAS Institute Inc., Cary, USA) for calculating means, and GraphPad Prism (GraphPad Software Inc., San Diego, USA) for the curve-fitting.

7.3 Results

All intervention groups were comparable with respect to baseline characteristics. The mean age of study participants was 60 ± 6 (SD) y, 59% (n=182) were male and 16% (n=48) were smokers. The mean body mass index was 27 ± 4 (SD) kg/m^2 and the mean serum vitamin B12 concentration was 315 ± 128 pmol/L. One person took the capsules of his partner who had been allocated to a different treatment and both were excluded from the analyses, as were the data for one subject because of poor compliance (42%). The remaining 308 subjects had a mean

Table 7.1 Mean serum and erythrocyte folate concentrations before and after 4 and 12 w of intervention; and the absolute changes after 12 w, by intervention group.

	N	Week 0	Week 4	Week 12	Change after 12 w (nmol/L)	
Serum folate (nmol/L)[a]						
Placebo	51	13.3 ± 3.6	12.9 ± 3.8	14.2 ± 4.2	1.0	(0.1 to 2.0)
50 µg/day	42	12.0 ± 3.1	14.7 ± 3.7	16.3 ± 4.4	4.3	(3.2 to 5.3)
100 µg/day	41	12.7 ± 4.6	17.4 ± 6.2	19.9 ± 7.6	7.2	(5.7 to 8.7)
200 µg/day	43	12.3 ± 4.2	19.8 ± 5.7	24.6 ± 7.5	12.3	(10.7 to 13.8)
400 µg/day	43	13.8 ± 5.3	31.9 ± 15.3	43.2 ± 21.0	29.4	(23.4 to 35.4)
600 µg/day	43	12.9 ± 4.9	41.4 ± 19.4	55.6 ± 24.5	42.7	(35.2 to 50.3)
800 µg/day	43	12.9 ± 3.6	53.4 ± 28.3	74.8 ± 43.3	61.9	(48.5 to 75.4)
Red blood cell folate (nmol/L)[a]						
Placebo	50	721 ± 257	-	733 ± 231	14	(-17 to 46)
50 µg/day	42	701 ± 223	-	755 ± 184	53	(21 to 86)
100 µg/day	39	722 ± 271	-	837 ± 242	120	(83 to 157)
200 µg/day	43	695 ± 251	-	979 ± 283	284	(245 to 322)
400 µg/day	42	836 ± 286	-	1381 ± 366	531	(466 to 595)
600 µg/day	38	679 ± 231	-	1350 ± 293	674	(604 to 745)
800 µg/day	42	761 ± 237	-	1593 ± 380	832	(750 to 914)

[a]Mean ± SD, or change (95% CI).

Table 7.2 Mean plasma homocysteine concentrations before and after 4 and 12 w of intervention, and absolute and relative changes after 12 w, by intervention group.

		Plasma homocysteine (μmol/L)[a]						
	N	Week 0	Week 4	Week 12	Change (μmol/L) 12 weeks		% Change 12 weeks	
Placebo	52	10.9 ± 2.3	11.3 ± 2.4	11.3 ± 2.7	0.4	(-0.05 to 0.8)	4.0	(0.1 to 7.9)
50 μg/d	43	11.5 ± 2.7	11.3 ± 2.6	11.1 ± 2.5	-0.4	(-0.9 to 0.03)	-2.9	(-7.0 to 1.2)
100 μg/d	41	11.7 ± 3.2	11.3 ± 3.1	10.8 ± 2.6	-0.9	(-1.5 to -0.3)	-6.1	(-10.1 to -2.0)
200 μg/d	43	11.8 ± 3.3	10.4 ± 2.5	10.0 ± 2.4	-1.8	(-2.4 to -1.3)	-14.0	(-17.4 to -10.7)
400 μg/d	43	12.0 ± 3.2	10.2 ± 2.7	9.6 ± 1.5	-2.4	(-3.2 to -1.7)	-17.3	(-21.5 to -13.2)
600 μg/d	43	11.8 ± 3.6	9.6 ± 2.3	8.8 ± 1.7	-3.0	(-3.7 to -2.2)	-22.1	(-26.6 to -17.6)
800 μg/d	43	11.5 ± 2.8	9.6 ± 2.2	9.1 ± 2.0	-2.4	(-2.9 to -1.9)	-19.9	(-22.9 to -16.8)

[a]Mean ± SD, or change (95% CI).

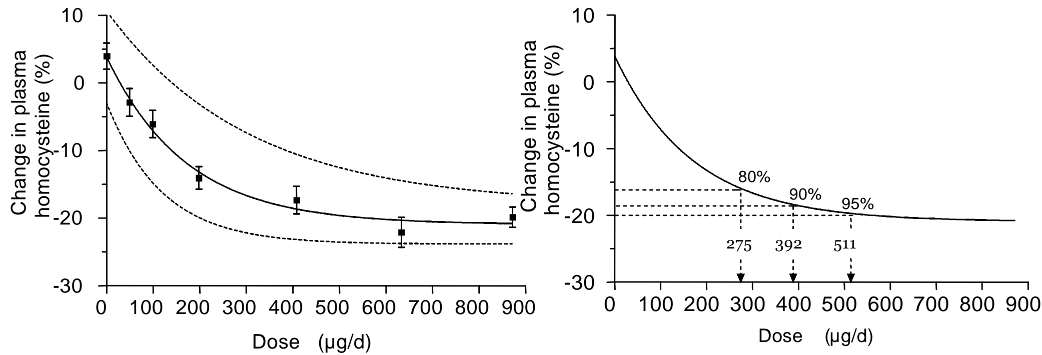

Figure 7.2 *Left panel* - The change in plasma homocysteine concentration (%) after 12 w of intervention by dose of folic acid (µg/d). The black boxes with error bars are the mean relative changes (%) and their 95% CIs. Solid line: the best fitting curve through data points; dotted lines: 95% CI for the curve. Goodness of fit: R^2=0.9997.

Right panel - The change in plasma homocysteine concentration (%) after 12 w of intervention by doses of folic acid (µg/d). The solid line indicates the best fitting curve. The dotted lines indicate the minimum dose of folic acid required to achieve 80%, 90%, or 95% of the maximal lowering of plasma homocysteine concentration.

compliance of 99% (all subjects ≥ 80%), as estimated by pill counting.

Table 7.1 shows that the baseline serum and erythrocyte folate concentrations in all treatment groups were well matched. Serum folate concentrations increased rapidly and linearly with increasing doses of folic acid, as shown by the observed increases after 4 weeks and the further increases in the following 8 w. There was a slight increase in serum folate concentrations in the placebo group after 12 w, which reflects random error. Folate concentrations in erythrocytes increased in all groups receiving folic acid, and did not change in the placebo group.

Table 7.2 shows that the plasma homocysteine concentrations were well matched among the treatment groups at baseline (week 0). The plasma homocysteine concentrations decreased in all folic acid groups during the first four weeks of intervention and decreased further between the fourth and the twelfth week of intervention. The decrease in plasma homocysteine concentrations after twelve weeks compared with baseline varied from 0.8 µmol/L (6.9%) to 3.4 µmol/L (26.9%) after correction for the 0.4 µmol/L (4%) increase in the placebo group.

Figure 7.2 (left panel) shows the best fitting dose-response curve plotted through the relative changes in homocysteine concentrations after 12 w of intervention. This dose-response curve was an exponential curve, and was described as:

Change in homocysteine after 12 w (%)=
24.8* exp(-0.0059 *folic acid intake in µg/d) -20.9

The curve had a $R^2$of 0.9997, which indicates a very good fit. The decrease at infinite folic acid intake (or the asymptote) was -20.9%, because the exponential term then equals to zero. When no folic acid is supplemented the change in plasma homocysteine equals 24.8-20.9=+3.9%, the estimated placebo effect. The maximum decrease (or decrease at infinite folic acid intake) in plasma homocysteine concentration corrected for placebo was therefore 24.8%. The figure clearly shows that the effect of the doses of 600 and 800 µg/d were almost similar to the decrease estimated at infinite folic acid intake. The lowest dose that achieved 90% of the maximal reduction was estimated to be 392 µg/day (95% CI 274 to 697 µg/d). At this dose homocysteine concentration was decreased by 22.3%.

Figure 7.2 (right panel) shows the same dose-response curve, but with dotted lines added to estimate the lowest dose required to achieve 80%, 90%, and 95% of the maximal decrease in plasma homocysteine concentration. This figure demonstrates that the adequate dose of folic acid varied from 275 µg/d for 80%, 392 µg/d for 90%, to 511 µg/d for 95% of the maximal decrease in plasma homocysteine concentration. When we used the absolute change in homocysteine concentration as response variable the adequate dose of folic acid was 374 µg/d.

7.4 Discussion

This trial demonstrated that a daily dose of folic acid of 392 µg, which decreased homocysteine concentrations by about 22%, was associated with 90% of the maximal decrease in plasma homocysteine concentration. Furthermore, supplementation with daily doses of folic acid as low as 50 µg/d and 100 µg/d resulted in a reduction in homocysteine concentrations of about 10%.

One of the strengths of this trial was the use of stratification for pre-treatment concentration of plasma homocysteine in quartiles prior to randomization to avoid any imbalance in homocysteine concentration in the treatment groups. This enabled the present trial to control for the confounding effect of the initial concentration of homocysteine on the observed reductions in homocysteine concentration achieved by different doses of folic acid.[13,14] We fitted a dose reponse curve based on the proportional change in homocysteine concentration after treatment relative to baseline homocysteine concentrations to estimate the adequate dose of folic acid to lower homocysteine. A similar conclusion about an adequate dose of folic acid was obtained by assessing absolute difference in homocysteine concentrations at 12 weeks compared with that at baseline.

The duration of the study was adequate since the life span of erythrocytes, which incorporate folate at erythropoiesis and retain it throughout their existence, is about 12 weeks.[20] Hence, after 12 weeks in the present trial, the body reserve of folate was likely to be optimal, and resulting reduction in homocysteine

concentration should have been maximal for any given dose of folic acid. While the maximum reduction in plasma homocysteine concentration was achieved more rapidly at higher doses of folic acid, the groups supplemented with 50 µg/d and 100 µg/d reached about 50% of the final (i.e. at 12 w) reduction within four weeks, which suggests that the maximum effect is likely to be attained by 12 weeks of treatment.

The doses of 50 µg/d to 100 µg/d lowered plasma homocysteine concentrations by 7% to 10%, relative to placebo. The magnitude of the decrease in the group with 100 µg/d is comparable with the observed effect of approximately three years of folic acid fortification (estimated to supply 100-200 µg of folic acid per day) on homocysteine concentration in middle aged and older adults in the USA.[5] Very low doses of folic acid can thus lower plasma homocysteine concentrations by about half of the maximal decrease. Based on our data we expect that improvement in the bioavailability of folate in foods or advice to increase daily dietary intake of folate could substantially contribute to reduction of homocysteine concentrations.

While the definition for an adequate dose of folic acid may vary, the definition adopted in the present trial took account of the observed change in the placebo group and – by using the relative changes – also the baseline concentrations. Nevertheless, from Table 7.2 we can conclude that the adequate dose of folic acid would not have been substantially different based on absolute change or final homocysteine measurement at week 12. The choice of a cut-off value of '90% of the maximal effect' was arbitrary, but the conclusion was not substantially different when alternative definitions (80% and 95%) for the estimation of the adequate folic acid dose were used.

In addition to the present study, one meta-analysis and three dose-finding studies have now sought to determine the lowest effective dose of folic acid to lower homocysteine levels. The meta-analysis of twelve randomized trials of folic acid-based multivitamin supplements to lower homocysteine concentrations showed that a dose of 400 µg/d was equally effective in lowering homocysteine as doses up to 5 mg/d, but no studies with doses less than 400 µg/d were available.[2] In a parallel three-month study with 151 patients with ischemic heart disease (mean age 65 years), Wald et al. compared the effects of doses of folic acid ranging from 200 µg/d to 1 mg/d on homocysteine concentration and concluded that the maximum reduction in homocysteine was achieved by a dose of 800 µg/d and no further reduction was achieved by supplementation with1 mg/d.[10] However, the failure to take account of differences in pre-treatment homocysteine concentration before randomization in that trial may have resulted in the imbalance observed in the initial homocysteine concentrations. Furthermore, participants in the latter trial had ischemic heart disease (and higher baseline homocysteine concentrations)

and may have required a higher dose of folic acid to reduce homocysteine concentrations. In a sequential-design study, carried out with 30 healthy men aged 34 to 65 years, supplementation with 400 µg of folic acid per day for 14 weeks was as effective as 200 µg/d for 6 weeks, whereas 100 µg/d for 6 weeks was less effective than either dose.[13] The sequential design without a placebo group is constrained in that each dose cannot be compared with a placebo and the observed effects cannot exclude the influence of any carry-over effect of the previous dose. In the present study, we stratified for the initial homocysteine concentration to ensure that it was similar in all groups before randomisation. Furthermore, the use of a parallel group design provided a more reliable assessment of the effects of the different doses of folic acid with that of a placebo group. Rydlewicz *et al.* recently published a dose finding study in a group of healthy elderly from which they conclude that daily supplementation with 600 µg of folic acid is the most effective therapy for homocysteine lowering.[21] However, where we aimed to find the lowest adequate dose of folic acid for reducing homocysteine concentrations, their aim was to find the adequate dose to bring 95% of the population below a plasma homocysteine cut-off value of 10 µmol/L. There is no consensus yet upon cut-off values for healthy homocysteine levels. Furthermore, variation between laboratory procedures with respect to sample collection and analysis of homocysteine concentrations makes it difficult to generalise conclusions. Therefore, we chose to show the dose response curve, allowing the reader to extrapolate the effect of any given dose on homocysteine reduction.

The present trial demonstrates that a daily dose of folic acid of 400 µg is the lowest dose that achieves the maximum reduction of homocysteine. This finding may have even greater relevance if the ongoing trials of folic acid supplementation in patients with cardiovascular disease can demonstrate that lowering homocysteine can reduce the risk of cardiovascular disease.

The lowest effective dose of folic acid is likely to be associated with a much lower risk of potential adverse effects of excessive intakes of folic acid. The increase of 400 µg/d can only be achieved by food fortification, but substantial benefits can be achieved by other food based approaches, such as improvement of the bioavailability of folate in foods, or changes in dietary habits.

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Chapter 8

General discussion

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8.1 Introduction

As described in Chapter 1, the main objective of this thesis was to quantify the effect of the polyglutamate chain on the bioavailability of folate. Further, we aimed to quantify the effect of the amount of folate in the diet on the bioefficacy of folate, i.e. reduction of plasma homocysteine concentrations. Knowledge of these aspects would help to find new ways of improving the folate status of the general population.

Based on data on dietary intake of folate, recommendations for the intake of folate are not met by most people in the Netherlands.[1-3] In this regard several issues should be taken into consideration. Firstly, estimates of folate intake depend on the validity of the method used for dietary assessment. Secondly, and more importantly, reliable food composition data are required. Folate intake based on newly developed HPLC methods is most probably underestimated by 25-40%.[4] Thirdly, folate requirements should be known, not only for the prevention of anaemia but also for the prevention of chronic diseases. In addition, requirements should be set not only for the general population but also for specific population groups. The fourth issue that has to be taken into consideration is the bioavailability of folate. Folate bioavailability varies considerably between foods.[5,6] Bioavailability of folate from whole diets is assumed to be 50% relative to that of folic acid* based on data from a depletion repletion study carried out in 1987.[7] However, this particular study was not designed in a way that such a quantitative conclusion could be drawn. Nevertheless, this figure is often cited and forms the basis for recommendations on folate intake. However, the bioavailability of folate from mixed diets is not known. Such knowledge is urgently required as a basis for recommended dietary intake of folate.

Before proceeding to the implications from the work described in this thesis (§8.5), we begin in this last Chapter by critically reviewing our data (§ 8.2 and 8.3). A brief overview of the research questions and main findings is given in **Table 8.1**. General conclusions are presented in § 8.4 and ideas for future research are generated in § 8.6. An epilogue to this Chapter summarizes the most important conclusions (§ 8.7).

* Folic acid is the oxidized form of the vitamin that is used for supplements and food fortification, whereas folate generally refers to the reduced forms as present in the diet and in biological tissues

Table 8.1 Overview of research questions and main findings

Part I. Folate in the diet	
Ch.2	*What is the daily intake of monoglutamate folate and polyglutamate folate from the diet and how does such intake relate to folate status?* Dietary folate intake: - 2/3 polyglutamate folate: main sources are vegetables, bread, fruits, potatoes - 1/3 monoglutamate folate; main sources are bread, meat, dairy, alcoholic beverages Associations with plasma folate: - Men: 3x stronger with intake of monoglutamate than with intake of polyglutamate folate - Women: no differences
Ch.3	*Can vegetables be processed in such a way that the content of monoglutamate folate is increased?* Processing (freezing and thawing, high-pressure treatment): - 2-3 fold increase in the proportion of monoglutamate folate in vegetables - High losses of total folate (>55%) Thus, processing in a closed system (vacuum-packed, microwave) is recommended
Part II. Folate bioavailability	
Ch.4	*To what extent does the required deconjugation of polyglutamyl folic acid limit the bioavailability of folate?* Bioavailability of heptaglutamyl relative to that of monoglutamyl folic acid: ~66% Thus, deconjugation of polyglutamyl folic acid limits folate bioavailability.
Ch.5	*Does polymorphism of the GCPII 1561C>T gene affect folate bioavailability?* *GCPII* 1561 CT genotype: 20-25% higher folate concentrations No evidence that the T allele-coded enzyme decreases FGCP activity *in vivo*
Ch.6	*Can we develop a dual-label stable isotope method in order to obtain reliable data on folate bioavailability in humans using a limited number of subjects?* Ratio of [$^{13}C_6$]:[$^{13}C_{11}$]-5-MTHF can be used as a sensitive and accurate measure for measuring folate bioavailability Ratio becomes stable after 4 d of intervention Data on capsule content still not available: thus bioavailability cannot yet be calculated.
Ch.7	*How much folic acid is required to lower plasma homocysteine concentrations adequately?* Dose of folic acid required for maximal lowering of homocysteine (↓22%): 400 μg/d Doses < 400 μg/d reduce plasma homocysteine concentrations substantially

8.2 Part I. Folate in the diet

8.2.1 Dietary intake of monoglutamate and polyglutamate folate

We found that total intake of folate from the average Dutch diet was 232 ± 71 µg/d (mean ± SD) for men and 186 ± 52 µg/d for women. One third of the intake was monoglutamate folate, mostly derived from bread, meat, dairy products, and alcoholic beverages. Two thirds of the intake was polyglutamate folate with vegetables, bread, fruits, and potatoes as most significant sources. In men, the association of intake of monoglutamate folate with plasma folate concentrations was three-fold stronger than for the polyglutamate form, while we found no such differences in women. This might be explained by differences in food intake patterns between men and women, e.g. high alcohol intake in men.

However, there are some sources of potential bias in our data. In the first place, the accuracy of estimated intake of folate depends on the quality of the food frequency questionnaire used. The questionnaire we used was not validated for estimating folate intake. Relative to 12 monthly 24-h recalls, the validity of the questionnaire ranged from 0.25-0.36 for vegetable intake.[8] Such validity is generally considered to be acceptable[2] and is comparable to that found by others.[8,9] However, vegetable intake was most probably somewhat overestimated.[10] Because vegetables are a significant source of polyglutamate folate, we may have therefore overestimated slightly the proportion of folate as polyglutamate in the diet. Since 75% of the intake of polyglutamate folate is from sources other than vegetables, such as bread, fruits and potatoes for which the relative validity of the questionnaire was good (relative validity from 0.41 to 0.78[8]), this constraint does not change our conclusion that intake of folate is mostly as polyglutamate.

Secondly, and probably more important, the reliability of estimates of folate intake depend to a large extent on the quality of methods for assessing the folate content of foods. We used data obtained using a newly developed HPLC method.[1,11] Such a method enables quantification of monoglutamate and polyglutamate forms of folate. Monoglutamate folate is measured as such, while the polyglutamate form requires deconjugation to the monoglutamate form prior to analysis. By using data based on HPLC analysis, we calculated, as have others[2,4], that the intake of folate is ~20% lower than that reported previously using data obtained using microbiological methods. This discrepancy may be due to difficulty in identifying the compound with folate activity.[4] Thus, we may have underestimated the total intake of folate. However, as explained, monoglutamate and polyglutamate folate are both measured in the monoglutamate form by HPLC. Therefore it is likely that the content of both monoglutamate and polyglutamate folate would have been underestimated equally in our data.

In the third place, random error may have been introduced in the associations presented by using concentrations of folate in plasma as an indicator of folate status. Plasma folate is sensitive to short-term changes in folate intake, whereas the concentration of folate in erythrocytes is a better estimate of long-term status[12]. Due to this, we may have underestimated the associations of habitual folate intake with folate status. It is unlikely that the associations with the intake of monoglutamate would be different to that with polyglutamate folate intake.

Associations of intake with folate status were different between men and women, which is probably due to differences in dietary patterns. This would imply that not only the polyglutamate chain but the source of folate in the diet would be a strong determinant of folate bioavailability, i.e. the matrix in which folate is incorporated. Not much is known about differences in folate bioavailability among food groups. Two studies have shown that disruption of the vegetable matrix from spinach can increase folate bioavailability.[13,14] This indicates that the food matrix of vegetables can hinder folate absorption. Folate from dairy products, on the other hand, may be better bioavailable because of the presence of folate binding protein (FBP). Folate that is bound to FBP is found to be less prone to destruction in the gastro-intestinal tract than unbound folate.[15,16] However on the other hand, FBP in the small intestine may inhibit the uptake of folate by the intestinal epithelium. Fibre is known to decrease folate bioavailability, in particular that of polyglutamate folate.[17] The effects of all these factors may have weakened the associations of folate intake with folate status.

In conclusion, data on the dietary intakes of monoglutamate and polyglutamate folate and their relationship with plasma folate concentrations are now being reported for the first time. Intake of folate in the Dutch population was found to be ~210 µg/d on average. This figure may be ~20% too low because the content of food was analysed by HPLC. Most folate in the diet - approximately two thirds - is in the polyglutamate form. Monoglutamate folate might be more bioavailable than polyglutamate folate as indicated by the three-fold stronger association with folate status in men.

8.2.2 Increasing monoglutamate folate content in vegetables by processing

An increased amount of food folate present in the monoglutamate form could potentially increase bioavailability of folate from such foods. We showed that the monoglutamate content in vegetables can be increased by stimulating endogenous deconjugase in vegetables. The proportion of monoglutamate folate increased 2-3 fold after freezing and thawing, and after hydrostatic high-pressure treatments of the vegetables. However, a large proportion of the folate present was lost during

treatment. This could be prevented by using a closed processing system in which vegetables do not come in direct contact with processing water.

The proportion of monoglutamate folate lost into processing water was greater than that of the polyglutamate form. Thus, our data are somewhat difficult to interpret. Although the proportion of monoglutamate folate relative to that of polyglutamate folate in the vegetables had increased after treatments (see Figure 3.2 in Chapter 3), the absolute amount of monoglutamate folate present was very low. However, in our pilot study, leakage of folate was prevented by vacuum packing of the vegetables and thus the amount of monoglutamate folate increased after treatment. Therefore, in our main study we probably underestimated the proportion of polyglutamate folate that was converted to the monoglutamate form.

Initially we aimed to carry out a long-term dietary controlled intervention trial in which bioavailability of folate from the processed vegetables would be assessed. Because of high loss of folate, we considered such a bioavailability study in humans not worthwhile. Others have reported that polyglutamate folate in spinach can be converted completely to monoglutamate folate after chopping and storage for 24h at 4 °C.[18] An intervention trial in humans using such spinach (see Appendix to Chapter 1) showed that the bioavailability of folate from treated spinach was not increased as compared to that of folate in untreated spinach. However, untreated spinach usually contains ~90% of folate as polyglutamate[1], whereas in the study described the proportion was only ~60%. Therefore, the contrast in the proportion of folate as monoglutamate in the two types of spinach may have been too small to see an effect. Furthermore, failure to correct for differences in the amount of folate provided, and also the small number of subjects, make it difficult to draw firm conclusions.

In conclusion, the amount of monoglutamate folate in vegetables can be increased by the processing methods we used. In this way, bioavailability of folate from vegetables can potentially be improved. This should be tested further in studies in humans. Because folate readily leaks out of vegetables during processing, a closed processing system should be used.

8.3 Part II. Folate bioavailability

8.3.1 Role of the polyglutamate chain

We studied whether deconjugation of polyglutamate folate limits the bioavailability of folate. We found that the bioavailability of heptaglutamyl relative to that of monoglutamyl folic acid is 66%. Bioefficacy - which is the fraction of ingested folate that is used to reduce plasma homocysteine concentrations - was found to be close to 100%.

As pointed out in Chapter 1, previous studies were designed mostly as small short-term studies with single exposures and with pre-loading of subjects. In several such studies, bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid was found to range from 55 to 80%.[19-21] Jejunal perfusion studies gave estimates of bioavailability from 70 to 80%.[22,23] In other studies, no difference in bioavailability between the two forms were found.[5,18,24,25] (see also Appendix to Chapter 1). Our study was designed as a long-term intervention trial with large groups using repeated low doses. Because of this approach, pre-loading of subjects with high doses of folic acid was not necessary, despite the low doses of folic acid used. Furthermore, the duration of the study would have allowed upregulation of the glutamate carboxypeptidase II gene over time. Thus, with this study design, several limitations of previous studies were overcome. Therefore, we regard our estimate of the bioavailability of polyglutamyl relative to that of monoglutamyl folic acid of 66% to be the most accurate estimate now available.

The dose of polyglutamyl folic acid administered was less than that of monoglutamyl folic acid on a molar basis. However, the smaller increase in the concentration of folate in serum and erythrocytes in the polyglutamyl folic acid group were not explained by this difference in dose. After correction, the bioavailability of heptaglutamyl folic acid relative to that of monoglutamyl folic acid was still 34% lower.

Unexpectedly, we found similar changes in plasma homocysteine concentrations in the two folic acid groups. However, estimates of these changes were not very robust since confidence limits were wide. We calculated the sample size for this study based on an expected decrease in plasma homocysteine concentrations of 20% for monoglutamyl folic acid. At present, we know from our dose-finding study (Chapter 6) that a daily dose of about 400 µg is required to induce such a decrease.[26] Therefore, changes induced by the folic acid doses of ≤ 145 µg/d that we gave were far smaller than initially anticipated in the power calculation. The similar changes in plasma homocysteine concentrations could also be due to the higher baseline concentrations in the group receiving polyglutamyl folic acid, despite stratification for screening levels. However, exclusion of two subjects resulted in similar baseline homocysteine concentrations in the groups but did not change the estimate of bioefficacy. Therefore, differences in baseline homocysteine concentrations were probably not responsible for the similar changes in plasma homocysteine concentrations that we observed. An alternative explanation could be that plasma homocysteine concentrations were already lowered maximally by the doses of folic acid given. However, as also mentioned above, folic acid doses of approximately 400 µg/d are needed for such an effect and the doses that we administered were much lower. Therefore, the discrepancy

between the relative bioavailability and the relative bioefficacy of polyglutamate folate when compared to monoglutamate folate remains unexplained.

In conclusion, this study overcame several limitations of previous studies, such as the use of single high doses and pre-loading of subjects. Therefore, the estimate of the bioavailability of polyglutamyl relative to that of monoglutamyl folic acid, namely 66%, is probably the best estimate available at this time. Taking all the evidence together we conclude that deconjugation of polyglutamate folate is a limiting step in determining folate bioavailability.

8.3.2 Polymorphism of the GCPII gene

Intestinal FGCP activity was 50% lower for the *GCPII* 1561T allele-coded enzyme *in vitro*. Thus, bioavailability of polyglutamyl folic acid could be impaired in subjects carrying a T allele. We found that the bioavailability of heptaglutamyl folic acid relative to that of monoglutamyl folic acid was not different between *GCPII* 1561C>T genotypes. At baseline, the CT genotype had 20-25% higher serum and erythrocyte folate concentrations in comparison with the CC genotype.

This is the first time that the effect of the *GCPII* 1561C>T polymorphism on folate bioavailability has been studied in an intervention trial. We found no evidence that this polymorphism of the *GCPII* gene affects polyglutamate folate bioavailability. This is in contrast with our hypothesis. Our hypothesis was based on results of others who found that, *in vitro*, the T allele-coded folylpoly γ-glutamyl carboxypeptidase (FGCP) exibited only 50% of the activity of the wild-type C allele-coded enzyme.[27] Apparently such an effect is not seen *in vivo*. Another explanation would be that the dose of polyglutamyl folic acid administered was so low that it could be metabolised readily despite the lower activity of FGCP in CT subjects. However, the bioavailability of polyglutamate folate relative to that of monoglutamate folate was also found to be lower in CC subjects with 'intact' FGCP activity. Therefore, if the activity of FGCP in CT subjects would indeed be 50% lower compared with that in CC subjects, we would expect that we would have been able to see such an effect.

There is one report that the CT genotype is associated with lower folate status in healthy subjects.[27] We found that folate status was increased in CT subjects which is in accordance with cross-sectional data from three European studies.[28-30] Data from a North American study showed no effects of the *GCPII* 1561C>T polymorphism on folate concentrations which may be attributed to the higher intake of folate from fortified foods in that region.[31] The higher folate status associated with the T allele observed in the European studies is as yet unexplained. It has been suggested that increased folate bioavailability plays a role[29] but as we have shown this is not the case. It may well be that the polymorphism affects the activity of FGCP in other tissues or affects other enzymes.

Concluding, bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid was similar between *GCPII* 1561C>T genotypes. Therefore, *in vivo* activity of FGCP in the brush border is probably not affected by this polymorphism. The 20-25% higher folate status of CT subjects at baseline suggests that the *GCPII* 1561C>T polymorphism may affect comparable enzymes in other body tissues, resulting in increased retention of folate in the body.

8.3.3 Development of a dual-label stable isotope method for studying folate bioavailability

In order to study folate bioavailability more efficiently, we explored the possibilities of a new dual-label stable isotope method. We found that the ratio of [$^{13}C_6$]- to [$^{13}C_{11}$]-5-MTHF in plasma can be measured accurately by LC-MS/MS and becomes stable after 4 d of intervention. Due to difficulties in determining the two forms of folate in the capsules, calculation of the bioavailability of polyglutamyl relative to that of monoglutamyl folic acid cannot be finalized at this time.

In the dual-label stable isotope study, much lower doses of labelled folic acid were used than in previous studies. The total amount of polyglutamyl folic acid administered was only ~15% of the RDA (<100 μg/d) divided into two portions each day, which amount is comparable to that obtained by drinking 1-2 glasses of orange juice extra each day. Other studies mostly have used single doses that exceeded the RDA (300 μg/d). Doses as low as possible were used in order to disturb folate metabolism as little as possible. Further, we applied repeated dosing to avoid the need for pre-loading of subjects. In addition, the relative long period of the study would enable any upregulation of the increased supply of polyglutamate folate, although minimal, to take place. We were able to use a relatively small number of subjects and calculation of bioavailability could be calculated within each person and within one time period, thus eliminating the need for a cross-over design. The ratio of [$^{13}C_6$]:[$^{13}C_{11}$]-5-MTHF in plasma could be measured accurately by LC-MS/MS with minimal analytical variability, as confirmed by the fact that similar results were obtained by the two laboratories involved. Moreover, after 28 d of intervention the variability in ratios between persons was only small which makes this ratio a sensitive measure of folate bioavailability.

As mentioned above, due to difficulties encountered in measuring folic acid content of the capsules, we are unable to finalize the calculation of the bioavailability of polyglutamyl relative to that of monoglutamyl folic acid as yet. It is expected that the analytical difficulties will be solved shortlly.

We assumed that there were no isotope effects, i.e. the labelled compounds were similar with respect to their physical, chemical and metabolic behaviour as the unlabelled compounds. This is confirmed by work of other researchers who found no evidence of isotope effects with [$^{13}C_5$]-folic acid labelled in the glutamate moiety,

or with [$^{13}C_6$]-folic acid labelled in the PABA structure[32]. Therefore, we do not expect that this phenomenon occurred in our study, although such isotope effects have been reported frequently for deuterium labelled compounds.

Several researchers have previously reported the use of a dual-label stable isotope design for studying folate bioavailability in humans.[33,34-36] These studies all used single doses and such studies have been useful for modeling the kinetics of folate metabolism in the body and for estimating body pool sizes. However, measurement of folate bioavailability was found to be more complicated. Folate administered intravenously as the reference dose was found to be metabolised differently to folate administered orally. Moreover, single administration of two doses of labelled folate orally resulted in wide between-person variation in the estimate of bioavailability. Such limitations have been overcome by the dual-label stable isotope protocol that we presented in this thesis.

Application of this design can be extended for studying other factors involved in folate bioavailability in a sensitive and accurate way, such as bioavailability of folate from mixed diets or from fortified foods. Except for studying the bioavailability of folate, it can also be extended to other nutrients. Van Lieshout *et al.* have already demonstrated previously how it can be used to study the bioavailability, bioconversion and bioefficacy of carotenoids.[37,38]

In conclusion, we found that the ratio of two differently labelled folate compounds in plasma can be used as a sensitive and accurate measure of folate bioavailability. With this study design, several limitations of other dual-label protocols are overcome, such as differences in kinetics caused by different administration routes, use of relatively high doses and wide between-person variation. As soon as the problems associated with the analysis of the capsules are overcome, we will be able to finalize the calculation of the bioavailability of hexaglutamyl folic acid relative to monoglutamyl folic acid.

8.3.4 Bioefficacy: role of amount of folate ingested

In our dose-response study we found that 400 µg of folic acid per day lowers plasma homocysteine concentrations adequately in subjects aged 50-75 y. A reduction in plasma homocysteine concentrations of 22% was achieved. Reductions in plasma homocysteine concentrations of about 10% were reached with doses of 100-200 µg of folic acid.

Due to the good study design with large groups per treatment, the inclusion of a placebo group, stratification for initial homocysteine concentrations and long term exposure, the mathematical model used fitted the data very well (goodness of fit: R^2=0.9997). Thus, we succeeded well in describing the association between folate intake and reduction of plasma homocysteine concentrations.

In previous research, the dose of folic acid required to reduce plasma concentrations adequately has been defined differently than we have done. This has resulted in estimates of the adequate dose of folic acid ranging from 200 to 800 μg/d.[39-41] Differences in determining the adequate dose may also have been caused by differences in the population group studied. Younger people[39] may require less folic acid to achieve maximal reduction of plasma homocysteine concentrations than do older people with ischemic heart disease.[40] There is no consensus yet on values for plasma homocysteine concentrations which could be regarded as healthy. Thus, the dose of folic acid required to obtain adequate or optimum lowering of plasma homocysteine concentrations remains somewhat arbitrary. It is clear, however that at least 400 μg/d is required to lower plasma homocysteine concentrations maximally in older adults.[26,41]

We questioned whether adequate lowering of plasma homocysteine concentrations could be achieved by increased folate intake from the diet. Under controlled conditions, an extra intake of 350-400 μg from the diet can be achieved.[42-44] However, others have found that dietary advice is not effective in improving folate status.[45] It is not likely that an extra intake of 400 μg/d from current diets without fortification can be achieved in the general population. Increasing the folate content of fruits and vegetables could be achieved by using new varieties developed by classical breeding techniques or genetic modification.

In conclusion, we found that an extra dose of at least 400 μg of folic acid per day is required in order to lower homocysteine concentrations maximally (~22%) in healthy older adults. To date, it is not known whether maximal reduction of homocysteine concentrations is desirable in order to minimize the risk of cardiovascular disease in the general population. Lower amounts of folic acid (100-200 μg/d) can reduce homocysteine concentrations already substantially.

8.4 General conclusions

8.4.1 Folate in the diet

Most folate in the average Dutch diet is in the polyglutamate form. In men, the association between monoglutamate folate intake and folate status was stronger than that for polyglutamate folate. This suggests that the monoglutamate form is more bioavailable. Although observational data such as these can provide new leads for further research, they cannot provide an accurate estimate of the bioavailability of polyglutamate relative to that of monoglutamate folate. In order to quantify the effect of the polyglutamate chain on folate bioavailability, intervention studies are required, such as that described in Chapter 4 of this thesis.

We have shown that it is possible to increase the amount of bioavailable folate in vegetables by processing methods that stimulate endogenous deconjugase

activity. Although some additional research is needed before methods can be scaled up, this provides a challenge to food industry to apply such methods in order to improve the folate status of consumers.

8.4.2 Folate bioavailability

We found that the bioavailability of polyglutamyl folic acid is 66% relative to that of monoglutamyl folic acid. This provides strong evidence that the intestinal deconjugation process required for polyglutamyl folic acid absorption limits its bioavailability. In contrast to most previous studies, we were able to quantify the bioavailability of polyglutamyl folate relative to that of monoglutamyl folic acid within narrow confidence limits. Bioavailability of folate from the diet is strongly determined by the polyglutamate chain. Thus, folate status can best be improved by increasing the intake of monoglutamate folate.

We found no evidence that the T allele in the *GCPII* 1561C>T polymorphism impairs the intestinal deconjugation process of polyglutamyl folic acid in vivo. In contrast, the CT genotype is associated with 20-25% higher folate concentrations in serum and erythrocytes in comparison with the CC genotype. To date this effect of the T allele on folate status is unexplained. Further investigation of this association can increase our understanding of folate metabolism in general.

The dual-label stable isotope protocol that we developed for studying folate bioavailability can be used as an efficient, sensitive and accurate method to study the bioavailability of folate. Previously, this methodology has already proven to be useful for studying bioavailability, bioconversion and bioefficacy of ß-carotene.[37,38] Therefore, the development of this protocol has a wider implication than for the research field of folate bioavailability only. Bioavailability of other carbon containing vitamins and food components, such as other B vitamins, flavonoids or phyto-estrogens could be studied likewise.

The reduction of plasma homocysteine concentrations by administering a supplement of folic acid reaches its maximum effect at doses of 400 µg/d. Increases in intake of this order cannot be achieved by increased dietary intake of folate. Supplementation or food fortification would be necessary. However, doses as low as 100-200 µg/d can reduce plasma homocysteine concentrations substantially.

8.5 Implications for public health

In order to establish and evaluate recommendations on dietary folate intake for the general population, three questions need to be answered. Firstly, how much folate is required? Secondly, how much folate is ingested? And thirdly, how much of the ingested folate is actually taken up by and stored in the body? We now describe how the work presented in this thesis contributes to each of these issues.

8.5.1 Folate requirements

Folate requirements, expressed as folic acid, had already been established for preventing anaemia (50-100 µg/d)[46], for keeping serum folate and erythrocyte folate concentrations within the normal ranges (200 µg/d)[7] and for the prevention of neural tube defects (400 µg/d)[47]. Up until now it was not known how much folic acid would be needed to reduce plasma homocysteine concentrations adequately in view of risk of cardiovascular disease. We found that, in addition to intake of folate from the diet, doses of folic acid of 400 µg/d are required to reduce plasma homocysteine concentrations maximally. Such amounts can reduce plasma homocysteine concentrations by approximately 25%. Extrapolation from the associated risk for cardiovascular diseases as indicated by prospective cohort studies[48] reveals that this would reduce risk by 10-15%.

Taking into account that bioavailability of dietary folate is lower than that of folic acid, the required amount of folate from the diet would be 600-1000 µg/d. A varied healthy diet according to the Dutch standards for healthy food intake delivers approximately 350 - 425 µg of folate per day. But in reality, most people do not eat such a varied healthy diet. We found an average folate intake of 210 µg/d in a representative sample of the Dutch population. This shows that the current recommendation of consuming 300 µg/d is not even met by most people and is not nearly enough to lower plasma homocysteine concentrations maximally.

The easiest and most effective way to increase folate intake in the general population would be to introduce mandatory fortification of certain foods with folic acid. Currently, food fortification with folic acid is not allowed in the Netherlands. Therefore, other food-based approaches should be explored and put into practice to improve folate status of the general population. These include advice on selection of foods with a high folate content and on household preparation methods.[49] It would also be possible to develop new varieties of fruits and vegetables with increased folate content and/or bioavailability. Such development could include traditional breeding methods or genetic modification[50,51], now referred to as biofortification. The agricultural and food industries can make major contributions to such dietary improvements. Another approach would be to increase the intake of folic acid from pharmanutrients, i.e. dietary supplements.

The question remains to what extent homocysteine reduction is desirable. If trials demonstrate that lowering of plasma homocysteine concentrations decreases risk of cardiovascular disease, goals for healthy plasma homocysteine concentrations in the general population should be set. Subsequently, policy makers have to decide what measures should be taken in order to reach such goals.

8.5.2 Dietary folate ingested

In order to obtain reliable data on habitual folate intake in the general population, questionnaires for dietary assessment should be validated for intake of the vitamin. This could be achieved by comparison of food frequency questionnaires with a more exact method of measuring folate intake, such as weighed records, or by relating folate intake to folate status. In general, the association of dietary folate intake with folate status is poorer in women than in men. Correlation coefficients usually range from 0.15 to 0.50.[3,52,53]

In addition to having valid methods for assessing intake of folate from foods, high quality data on the folate content of foods should become available. An important remark has to be made here. In the Netherlands, HPLC data on folate composition of foods have been included in the latest national food composition table. Therefore, folate intake of the general population appears to have decreased in recent years compared to previous data obtained with older food composition data. However, to a large extent this is due to analytical issues and not to lower intake levels of dietary folate. Like others, we have found that data on folate intake are ~20% lower when derived from HPLC analyses.[4] Despite this, even if we have underestimated folate intake by 20%, the average intake remains below the current recommendation for folate intake.

8.2.3 Folate bioavailability

The actual amount of folate ingested that is absorbed is determined by folate bioavailability. Although bioavailability of folate from mixed diets is often assumed to be 50%, reliable data are lacking. We found that, relative to monoglutamyl folic acid, only 66% of polyglutamyl folic acid becomes available for use and storage in the body (§ 8.3.1). Because most folate - about two thirds - from the diet is ingested as polyglutamate folate (§ 8.2.1), this will have a significant impact on the folate status of the population. As shown by the calculation in the text box below, the polyglutamate chain alone lowers dietary folate bioavailability by ~23%. Other factors can attenuate bioavailability even further, e.g. the food matrix.

Calculation of the impact of the polyglutamate chain on dietary folate bioavailability

Assumptions:

1) Chain length is the only determinant of folate bioavailability
2) Bioavailability of monoglutamate folate from the diet = 100%

Average dietary folate intake: 300 µg/d (1/3 monoglutamate, 2/3 polyglutamate)

Amount bioavailable:
100 µg monoglutamate = 100 µg
200 µg polyglutamate * 0.66 = 132 µg
232 µg

232/300 * 100% ~ 77%

An important question is how such information should be incorporated in recommendations for folate intake and in food composition tables. In the United States of America, the recommended daily allowance is expressed as dietary folate equivalents (DFE): 1 DFE equals 1 µg of dietary folate or 0.6 µg folic acid from fortified foods, taken that the bioavailability of dietary folate is 50% and of fortified foods 85% (50/85=0.6).[47] This approach has been adopted by the Health Council of the Netherlands.[54] However, the use of DFE is not logical and makes it difficult to take into account bioavailability among various non-fortified foods. Since factors for the bioavailability of folate in the diet change with our increasing knowledge and vary among different foods and diets, dietary folate should not be taken as a reference. The most comprehensible and sustainable way to incorporate folate bioavailability in the recommended daily allowances and in food composition tables is to express it relative to that of 1 µg of folic acid. In this way, recommendations on folate intake can still be made and bioavailability of folate from single fortified and non-fortified foods, and from total diets can be taken into account in food composition tables.

8.6 Recommendations for future research

8.6.1 Folate in the diet

HPLC methods for the analysis of folate in foods result in 20-40% lower estimates of folate intake compared with microbiological methods[4], probably in part due to the difficult quantification of separate peaks on the chromatogram. Therefore, HPLC methods for the measurement of food folate need to be improved and should be validated against microbiological assays. HPLC methods could be further adapted to enable measurement of the proportion of folate present as compounds containing different numbers of glutamate residues[55]. This may help us better understand the bioavailability of folate from the diet.

In this thesis we described several processing methods that have the potential to improve the bioavailability of folate from vegetables. However, since much folate was lost in the processes used, methods should be developed which allow the processing to take place in a closed systems, such as a microwave oven or a vacuum pack. Furthermore, new ways of improving folate content and bioavailability, such as by conventional breeding and genetic engineering[50,51], should be explored.

8.6.2 Folate bioavailability

Although many studies on folate bioavailability from selected foods have been carried out[5,6,56-61], surprisingly little is known on the bioavailability of folate from mixed diets. As mentioned before, bioavailability of dietary folate is often assumed to be 50%. This is based on one study that was not designed for drawing such a

quantitative conclusion.[7] In another study in which mixed diets high in vegetables and fruits were studied, estimates for bioavailability ranging from 60 to 98% were found, depending on the parameter measured.[43] Knowledge on this aspect should be improved in order to provide a better basis for establishing recommendations for folate intake. Therefore, data on the bioavailability of folate from mixed diets is urgently required. Another aspect that is worth studying further is the impact of food-related factors on folate bioavailability, such as the presence of organic acids or folate binding protein. The dual-label stable isotope design that we have developed provides an efficient, sensitive and accurate method to study these and other factors determining folate bioavailability.

From a mechanistic point of view, folate metabolism is not yet fully understood. For example, while bioavailability of polyglutamyl folic acid is lower, we found that plasma homocysteine concentrations were reduced equally with supplements containing either monoglutamyl folic acid or polyglutamyl folic acid. Others have found that 5-MTHF lowers plasma homocysteine concentrations better than does folic acid, although plasma folate concentrations were not different.[62] Further, the role of genetics in folate bioavailability is a largely unexplored area. Instead of measuring total folate concentrations in research, measurement of folate derivatives in body tissues and fluids, for instance in bioavailability studies, using HPLC and mass spectrometry methods may be the key to further understanding of folate metabolism.

8.7 Epilogue

It can be concluded that bioavailability of polyglutamyl relative to monoglutamyl folic acid is 66%. Translated to intake of folate from an average diet, about 23% of dietary folate is not bioavailable due to the presence of folate in the polyglutamate form. Other factors, such as the matrix in which food folate is incorporated, can attenuate folate bioavailability even more. Further research on the bioavailability of folate from mixed diets, food groups and individual foods is required in order to establish and evaluate recommendations for folate intake rightly. The dual-label stable isotope protocol that we developed provides an efficient, sensitive and accurate method to perform such studies.

With an average intake of 210 µg/d, the current recommended folate intake of 300 µg/d is not met by most people in the Netherlands. Moreover, an extra dose of folic acid of 400 µg/d would be required to achieve a maximal lowering of plasma homocysteine concentrations (~25%), thereby reducing the risk of cardiovascular disease. If such maximal lowering of plasma homocysteine concentrations is desirable, which should become clear from clinical trials within a few years, measures should be taken to increase the intake of folate substantially in the general population.

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Summary

Low intake of the B vitamin folic acid can be the cause of diseases such as anaemia and neural tube defects. Moreover, it is associated with hyperhomocysteinaemia, a putative risk factor for cardiovascular disease. In the Netherlands, the current recommended daily intake of folate is 300 µg/d. Although foods are fortified in a number of countries including the USA, fortification of foods with folic acid in the Netherlands is not allowed. The form of the vitamin used in supplements and as a food fortificant is folic acid, whereas the form of the vitamin present in foods and biological tissues is folate.

Not all folate ingested becomes available for use and storage in the body, i.e. the bioavailability is less than 100%. At present, data on the bioavailability of folate from average mixed diets, food groups and individual foods are not reliable. Such data are required in order to establish and evaluate recommended dietary intake of folate. Folate bioavailability is affected by various factors. The aim of the work described in this thesis was to quantify the effects of three factors that determine the bioavailability of dietary folate: the chemical *form* of folate, a *genetic variant* which may determine the rate of metabolism of one of these forms in the small intestine, and the *amount* of folate that is ingested.

Dietary folate is often present as a polyglutamate. The polyglutamate chain has to be removed – or deconjugated - by the enzyme folylpoly γ-glutamyl carboxypeptidase (FGCP) before folate can be absorbed as the monoglutamate. This enzyme is present in the brush border of the small intestine and is encoded by the *GCPII* gene. Folate present as a monoglutamate upon ingestion does not require such deconjugation. Limitation of the deconjugation process would decrease bioavailability of folate present as a polyglutamate.

In a representative sample of the Dutch population, we calculated the total dietary folate intake to be ~210 µg/d, of which two thirds was as polyglutamate (*Chapter 2*). Although total folate intake may be underestimated by ~20% due to the use of data obtained by HPLC, this would not affect the estimate for the proportion of polyglutamate folate present in the diet.

In previous studies, the bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid ranged from 50 to 100%. The variation in estimates may be due to several limitations of study designs, such as the administration of relatively high single doses, and the loading of subjects with high doses of folic acid prior to admnistration of the test dose. In order to overcome these limitations, we studied the bioavailability of polyglutamyl relative to monoglutamyl folic acid in a long-term (12 w) randomised placebo-controlled study, using low doses of folic acid (~150 µg/d), in 180 subjects aged 50-75 y (*Chapter 4*). Such a design mimics the

usual pattern of dietary folate intake better and makes pre-loading of subjects unnecessary. We estimated that the bioavailability of polyglutamyl relative to that of monoglutamyl folic acid is ~66%. We consider this to be the most accurate estimate so far.

In the same population we studied whether genetic variation in deconjugation activity modifies the bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid. The *GCPII* 1561C>T polymorphism has been reported to affect FGCP activity *in vitro*. Therefore, we expected the bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid to be decreased in subjects carrying the T allele. However, when we stratified our data for this genotype, we found no differences in bioavailability between *GCPII* 1561C>T genotypes (*Chapter 5*). Therefore, there is no evidence from our study that the activity of T allele-coded FGCP is lower *in vivo*.

In order to study folate bioavailability more efficiently, we explored a new dual-label stable isotope method for studying the bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid (*Chapter 6)* Low doses (~ 25 µg/d) each of [$^{13}C_6$]-hexaglutamyl folic acid and [$^{13}C_{11}$]-monoglutamyl folic acid were administered twice each day to 20 volunteers for 28 d. After reaching a plateau, the relative isotopic enrichments derived from the two compounds administered, corrected for the isotopic ratio in the administered compounds, provides an estimate of the relative bioavailability. The plateau was reached wtihin 4 d of intervention and the ratios measured by two independent methods based on liquid chromatography and mass spectrometry (LC-MS/MS) were almost identical. When data become available on the ratio of isotopic enrichment in the folic acid compounds becomes known, it will be possible to provide an estimate of the bioavailabiltiy of folic acid hexaglutamyl relative to that of monoglutamyl folic acid within confidence limits of ± 12%.

Another issue that we studied was the effect of the amount of supplemental folic acid on plasma homocysteine concentrations, i.e. folate bioefficacy. High intakes of folic acid can mask the damaging effects of vitamin B_{12} deficiency. In order to prevent such masking but yet to lower plasma homocysteine concentrations adequately, information on the lowest adequate dose of folic acid is required. Moreover, we wanted to know whether adequate lowering of plasma homocysteine concentrations could be achieved with folate intakes in the range of dietary intake. For this purpose, we carried out a 12-w randomised placebo controlled study in 316 volunteers, aged 50-75 y, in which doses of folic acid ranging from 50 to 800 µg/d were administered (Chapter 7). We found that, in addition to folate provided by the diet, a dose of folic acid of 400 µg/d is required to reduce plasma homocysteine concentrations maximally (~25%). Using a dietary approach, this would mean that diets would need to deliver folate in the range of 600-1000 µg/d. This would be

difficult to achieve. To date, it is not clear whether maximal reduction of homocysteine concentrations in the general population is desirable. Within a few years, clinical trials should reveal whether or not lowering of plasma homocysteine concentrations can prevent cardiovascular disease.

In conclusion, in one study in which unlabelled compounds were administered, we found that the bioavailability of polyglutamyl relative to that of monoglutamyl folic acid is 66%. We now have a dual-label stable isotope method which can obtain such data more readily and results will become available shortly. We calculated that because most of the folate in the diet is present as polyglutamate folate, folate bioavailability is ~23% lower than it would be if all folate was present as monoglutamate. Other factors, such as the matrix of the food in which folate is incorporated, can reduce folate bioavailability even further. More research is needed to quantify the effects of other factors on folate bioavailability from mixed diets, food groups and inidividual foods.

The current recommended folate intake of 300 µg/d is not met by most people in the Netherlands. It is important to note that folate intake may be underestimated by ~20% since new data based on analyses using HPLC on the folate content of foods have been incorporated into food composition tables. However, even when this is taken into account, folate intake from the diet is still sub-optimal in the general population. In order to reduce plasma homocysteine concentrations adequately, even more folate would be required. Since fortification of food with folic acid is not allowed in the Netherlands, other food-based approaches should be explored, such as advice on selection of foods with high folate content and on household preparation methods. Another approach would be to increase the content or the bioavailability of folate in foods by processing (*Chapter 3*), plant breeding, or genetic engineering.

Samenvatting

Een te lage inname van foliumzuur (vitamine B_{11}) kan de oorzaak zijn van verschillende ziekten, zoals bloedarmoede, open ruggetjes bij pasgeborenen en mogelijk ook van dikkedarmkanker en Alzheimer. Bovendien kan het leiden tot een te hoog gehalte aan homocysteïne in het bloed. Homocysteïne is een eiwitachtige stof die mogelijk de kans op het krijgen van hart- en vaatziekten vergroot. In Nederland is de aanbevolen inneming van foliumzuur met de voeding recentelijk verhoogd tot 300 microgram per dag. Dit is voldoende om bloedarmoede te voorkomen, maar waarschijnlijk niet genoeg om het homocysteïnegehalte in het bloed adequaat omlaag te brengen. Vanwege het risico op open ruggetjes worden in een aantal landen, bijvoorbeeld de Verenigde Staten, sommige voedingsmiddelen verrijkt met foliumzuur. In Nederland is dit echter niet toegestaan. Voor vrouwen die zwanger willen worden geldt hier een aparte aanbeveling, namelijk om per dag 400 microgram foliumzuur te slikken als supplement, vanaf vier weken voor tot en met acht weken na bevruchting. De vorm van vitamine B_{11} zoals die gebruikt wordt in supplementen wordt aangeduid met foliumzuur, terwijl de vormen aanwezig in voedsel en bijvoorbeeld in bloed worden aangeduid als folaat.

Niet al het folaat dat we met de voeding binnenkrijgen is beschikbaar voor de processen in het lichaam; slechts een gedeelte wordt daadwerkelijk geabsorbeerd en kan gebruikt worden. Met andere woorden: de biobeschikbaarheid van folaat in de voeding is minder dan 100%. Vergeleken met foliumzuur in een supplement wordt van folaat in de voeding misschien maar 50% opgenomen. De huidige kennis over de biobeschikbaarheid van folaat in de voeding, in voedselgroepen en in individuele voedingsmiddelen is echter beperkt.

De biobeschikbaarheid van folaat wordt beïnvloed door verschillende factoren. Het doel van het werk dat in dit proefschrift staat beschreven was om de effecten van twee verschillende factoren te quantificeren. Ten eerste bestudeerden we het effect van een bepaalde *chemische vorm* van folaat op de opname in het bloed. Bovendien keken we hierbij naar de invloed van een genetische variant. Ten tweede bestudeerden we het effect van de *hoeveelheid* foliumzuur die wordt geconsumeerd op het homocysteïnegehalte in het bloed. Deze informatie kan gebruikt worden om in te schatten welke voedingsmiddelen een goede bron van foliumzuur zijn, en om te bepalen hoeveel foliumzuur per dag nodig is om eventueel het risico op hart- en vaatziekten te verkleinen.

Allereerst hebben we dus gekeken naar het effect van de *chemische vorm* op de biobeschikbaarheid van foliumzuur. Folaat in de voeding is veelal gebonden aan een polyglutamaatketen, terwijl foliumzuur in supplementen als monoglutamaat aanwezig is. Folaat wordt in de bloedsomloop opgenomen als monoglutamaat. Dit betekent dat folaat dat in de polyglutamaatvorm in de dunne darm terechtkomt eerst enzymatisch omgezet moet worden tot monoglutamaat folaat. Dit proces

wordt deconjugatie genoemd. In dit onderzoek vroegen wij ons af of misschien slechts een deel van het polyglutamaat folaat dat we met de voeding binnenkrijgen wordt omgezet tot monoglutamaat folaat. Dit zou kunnen verklaren waarom voedingsfolaat minder biobeschikbaar is dan bijvoorbeeld foliumzuur uit een supplement.

Om dit te bestuderen hebben we allereerst berekend welke hoeveelheid van beide vormen met de dagelijkse voeding wordt ingenomen. Hiervoor hebben we gegevens gebruikt van een representatieve steekproef van de Nederlandse bevolking, bestaande uit 2435 personen in de leeftijd van 20-65 jaar. We berekenden dat de gemiddelde inname van folaat 210 microgram per dag is, waarvan tweederde uit polyglutamaat en de rest uit monoglutamaat folaat bestaat (Hoofdstuk 2). De gemiddelde folaatinname zou met 20% onderschat kunnen zijn vanwege de gebruikte techniek voor het meten van het folaatgehalte in voedingsmiddelen.

In een interventie-onderzoek met gezonde proefpersonen hebben we vervolgens de biobeschikbaarheid van polyglutamaat foliumzuur ten opzichte van monoglutamaat foliumzuur bestudeerd (Hoofdstuk 4). De resultaten van voorgaand onderzoek naar de biobeschikbaarheid van polyglutamaat foliumzuur ten opzichte van monoglutamaat foliumzuur waren uiteenlopend. Sommig onderzoek liet zien dat de biobeschikbaarheid van polyglutamaat foliumzuur lager is dan die van monoglutamaat foliumzuur, terwijl ander onderzoek geen verschil liet zien. De oorzaak hiervan zou kunnen liggen in de opzet van deze onderzoeken. Doordat meestal relatief hoge en eenmalige testdoses werden gebruikt, werd nogal sterk afgeweken van de gebruikelijke inneming met de voeding. Ook werden voorafgaand aan het onderzoek vaak hoge doses foliumzuur aan vrijwilligers gegeven, waardoor het normale folaatmetabolisme verstoord kan raken. Om deze beperkingen te vermijden hebben wij ervoor gekozen om herhaalde lage doseringen te gebruiken gedurende een langere onderzoeksperiode, namelijk 12 weken. Er deden 180 mensen in de leeftijd van 50 tot 75 jaar mee, die steekproefsgewijs in drie groepen van ongeveer 60 personen werden verdeeld. Alle groepen kregen dagelijks een supplement: de eerste groep kreeg een lage dosis polyglutamaat foliumzuur, de tweede groep kreeg een lage dosis monoglutamaat foliumzuur en de derde groep kreeg een placebo. Vervolgens werden de bloedwaarden voor en na het onderzoek tussen de verschillende groepen vergeleken. Uit dit onderzoek bleek dat de relatieve biobeschikbaarheid van polyglutamaat foliumzuur 66% is (ten opzichte van monoglutamaat foliumzuur). Dit betekent dat de biobeschikbaarheid van folaat uit de voeding, waarvan tweederde uit polyglutamaat folaat bestaat, maximaal 77% is.

In hetzelfde onderzoek hebben we ook gekeken naar het effect van een genetische variant op de relatieve biobeschikbaarheid van polyglutamaat foliumzuur (Hoofdstuk 5). Enkele jaren geleden werd een afwijking gevonden in het gen dat het enzym aanmaakt voor omzetting van polyglutamaat naar

monoglutamaat folaat. Eerder onderzoek had laten zien dat deze genetische variant voor een 50% lagere enzymactiviteit zou kunnen zorgen vergeleken met het normale type. Daarom verwachtten we dat de biobeschikbaarheid van polyglutamaat foliumzuur verminderd zou zijn bij personen met deze genetische variant. Echter, wij vonden geen verschil in de relatieve biobeschikbaarheid van polyglutamaat foliumzuur. Wel vonden we, tegengesteld aan de verwachting, dat bij deze personen het folaatgehalte in het bloed hoger was dan normaal. Mogelijk heeft het gen dat wij bestudeerden een andere, nog onbekende, functie in het lichaam.

Het hiervoor beschreven onderzoek werd uitgevoerd met 180 mensen gedurende 12 weken. Om onderzoek naar de biobeschikbaarheid van foliumzuur sneller, met minder mensen en nog beter te kunnen uitvoeren hebben we een nieuwe methode uitgetest. Hierbij werd gebruik gemaakt van foliumzuur dat is gelabeld met koolstof-13 (^{13}C) isotopen (Hoofdstuk 6). Zulk gelabeld foliumzuur is iets zwaarder dan gewoon foliumzuur en hierdoor kan het, met behulp van een weegmethode (massa-spectrometrie), in het bloed getraceerd worden. Voor dit onderzoek gebruikten we polyglutamaat foliumzuur gelabeld met 6 ^{13}C isotopen en monoglutamaat foliumzuur gelabeld met 11 ^{13}C isotopen. Dagelijks werden zeer lage doseringen [$^{13}C_6$]-polyglutamaat foliumzuur en [$^{13}C_{11}$]-monoglutamaat foliumzuur verstrekt aan 20 proefpersonen gedurende 28 dagen. Na verloop van tijd ontstond op die manier een vaste verhouding van isotoopconcentraties in het bloed. Deze vaste verhouding weerspiegelt de relatieve biobeschikbaarheid van polyglutamaat foliumzuur ten opzichte van de monoglutamaatvorm. Na 4 dagen was deze verhouding constant en de resultaten van metingen gedaan met twee verschillende massa-spectrometrie methoden waren vrijwel identiek. We concluderen dat deze methode een goede en efficiënte manier is om de biobeschikbaarheid van foliumzuur te meten. Vanwege analytische problemen kunnen we de biobeschikbaarheid van polyglutamaat foliumzuur ten opzichte van monoglutamaat foliumzuur uit dit onderzoek op dit moment echter nog niet berekenen.

Het tweede onderwerp dat we hebben bestudeerd is het effect van de *hoeveelheid* foliumzuur op de homocysteïne concentraties in plasma. In onderzoek worden veelal hoge doses gebruikt om het homocysteïnegehalte te verlagen. Echter, bij teveel inname van foliumzuur is een tekort aan vitamine B_{12} moeilijker vast te stellen, terwijl daardoor toch neurologische schade ontstaat. Daarom vroegen wij ons af met welke minimale hoeveelheid foliumzuur het homocysteïnegehalte adequaat verlaagd kan worden. Verder wilden we graag weten of deze minimale dosis binnen het bereik van de dagelijkse inneming met de voeding zou liggen. Hiervoor hebben we een 12-weeks interventie-onderzoek uitgevoerd bij 316 vrijwilligers in de leeftijd van 50-75 jaar (Hoofdstuk 7). Deze mensen werden verdeeld in 7 groepen, waarvan 6 groepen dagelijks een dosis foliumzuur als supplement kreeg, variërend van 50 tot 800 microgram. De 7e groep kreeg dagelijks een placebo. We vonden dat, naast de folaatinname met de voeding, een

dosis van 400 microgram nodig is om het plasma homocysteïnegehalte maximaal te reduceren (~25%). Rekening houdend met de lagere biobeschikbaarheid van folaat uit de voeding zou dit betekenen dat de dagelijkse voeding 600 tot 1000 microgram folaat zou moeten bevatten. Dit is echter moeilijk te bereiken met een normale gezonde voeding. Over een paar jaar zal uit de resultaten van onderzoek duidelijk worden of verlaging van het homocysteïnegehalte daadwerkelijk hart- en vaatziekten kan voorkomen. Tot op heden is het echter nog niet duidelijk of maximale reductie van het homocysteïnegehalte bij gezonde mensen wenselijk is.

Samenvattend: wij hebben gevonden dat de biobeschikbaarheid van polyglutamaat foliumzuur ten opzichte van monoglutamaat foliumzuur 66% is. Hierdoor is de biobeschikbaarheid van folaat uit de voeding ~23% lager dan wanneer alleen monoglutamaat folaat aanwezig zou zijn. We hebben ook een stabiele isotoopmethode ontwikkeld waarmee onderzoek naar de biobeschikbaarheid van foliumzuur beter en efficiënter uitgevoerd kan worden.

De huidige aanbeveling om per dag 300 microgram folaat met de voeding in te nemen wordt door de meeste mensen in Nederland niet gehaald. Om de folaatinname te verhogen kunnen verschillende benaderingen worden gebruikt, zoals bijvoorbeeld voedingsadvisering, verbetering van de biobeschikbaarheid van folaat uit de voeding (Hoofdstuk 3) of klassieke plantenveredeling. Om het homocysteïnegehalte adequaat te reduceren zou de inname van folaat echter hoger moeten zijn dan met een gezonde voeding haalbaar is. Om dat te bereiken zijn andere maatregelen nodig, zoals fortificatie, biofortificatie of het gebruik van voedingssuplementen.

Appendix

The optimal time interval between repeated blood sampling for measurements of total homocysteine in healthy subjects

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Background

Many studies have shown that elevated plasma concentrations of total homocysteine (tHcy) are associated with cardiovascular disease.[1] Like most biological variables, plasma tHcy levels fluctuate within a subject around a long-term mean.[2,3] Measurements of tHcy concentrations are often based on a single blood sample. Taking the mean of two or more repeated measurements will reduce the error due to within-subject biological fluctuations and reflect more closely a person's true mean tHcy value within a certain period. However, as the time interval between two repeated measurements becomes shorter the values become more similar and the error reduction diminishes. In that case the benefit of taking the mean of two measurements is not fully utilized. On the other hand, too long intervals (several weeks) may compromise the compliance of study participants and allow long-term seasonal changes.[4,5]

Several studies on the weekly and monthly biological variation as expressed by the coefficient of variation in plasma tHcy concentrations have been published (**Table 1**). From these studies, no conclusions can be drawn about the optimal time interval between two repeated samples to obtain a sufficiently precise estimate of the true tHcy concentration within a predefined period. The optimal time interval is reached when the interval-specific coefficient of variation does not further increase with increasing time intervals.

The aim of this study was to determine how many days between two times sampling within one week are needed to reach the maximum biological variation in tHcy levels. Our findings may be useful in studies involving estimates of a subject's true mean concentration of tHcy.

Table 1. Biological variation in plasma homocysteine concentrations according to several studies using weekly through monthly sampling strategies.

Design	Subjects (n)	Biological CV (%)	Reference
Weekly sampling during 5 weeks	24	8.1	6
Weekly sampling during 4 weeks	20	8.3	7
Sampling at 2-month interval during 1 year	96	8.9	8
Biweekly sampling at 4 visits	44	9.4	9
Weekly sampling during 4 weeks	20	7.0	10

Methodology

The participants in this study were 6 male and 9 female healthy volunteers aged 19 to 46 years. Use of medication or dietary supplements known to affect homocysteine or folate metabolism were contra-indications for participation. We applied strict criteria related to folate metabolism, since folate is a strong determinant of homocysteine metabolism. All screened subjects were eligible. Volunteers agreed to participate by signing an informed consent form. The Medical Ethical Committee of the Wageningen University approved the study.

On five sequential days, 5 mL of venous blood was collected from fasted (9-11 h) subjects between 8.00 and 10.00 h. The five visits of each subject occurred at the same time in the morning on each day. Each subject had his/her venipunctures in the same position, sitting or lying down, throughout the study. Samples were collected in EDTA-containing tubes. Plasma was separated within 30 minutes and stored at –80°C until analysis.

Plasma tHcy concentrations were measured within two weeks after the end of the study by HPLC with fluorescence detection at our laboratory.[11,12] All samples from one individual were analyzed in duplicate in one run, to avoid between-run analytical variation. The within-run analytical coefficient of variation (CV) based on these samples was 4.1%. The between-run CV in this laboratory was known to be 6.0%.

The intercorrelation of tHcy values within subjects over time, the semi-variance, was calculated for intervals of one, two, three, and four days. The semi-variance, $\gamma(d)$, is equal to half the average squared difference in concentration between any two samples that are d days apart; the sum of squares is divided by twice the number of pairs available at a specified interval.[4] Thus, it is an estimate of the

conditional variance of the second measurement, given the outcome of the first one. The formula for this calculation is:

$$\gamma(d) = \sum(x_t - x_{t+d})^2/2n$$

where d denotes the time-interval between any two observations, x denotes the measured values (here tHcy concentration), t denotes one point in time and n is the number of interval pairs in the summation.[4,5] As the value of d increases, the semi-variance gradually increases and will ultimately approach the full within-person variance, i.e. both analytical and biological variation. At this point the measurements are no longer correlated and the shortest time interval is found for which the maximum biological variation in tHcy levels is reached.[4]

If $\underline{x}_t$ is a random variate with $E(\underline{x}_t) = \mu$, $Var(\underline{x}_t) = \sigma^2$, and $Covar(\underline{x}_t, \underline{x}_{t+d}) = \sigma^2\rho^d$ for d=0, 1, 2 . . . , then ρ is called the autocorrelation.
For this model, the variance of the sum and the difference of two values are:

$$Var(\underline{x}_t + \underline{x}_{t+d}) = \sigma^2 + 2\sigma^2\rho^d + \sigma^2 = 2\sigma^2(1+\rho^d)$$

$$Var(\underline{x}_t - \underline{x}_{t+d}) = \sigma^2 - 2\sigma^2\rho^d + \sigma^2 = 2\sigma^2(1-\rho^d).$$

Thus it follows that the variance of the average of two measurements is:

$$Var\{(\underline{x}_t + \underline{x}_{t+d})/2\} = \tfrac{1}{4} \cdot 2\sigma^2(1+\rho^d) = \sigma^2(1+\rho^d)/2$$

and the semi-variance is:

$$\tfrac{1}{2}\{Var(\underline{x}_i - \underline{x}_{i+d})\} = \tfrac{1}{2} \cdot 2\sigma^2(1-\rho^d) = \sigma^2(1-\rho^d).$$

If repeated measurements are more correlated when they are taken at short time intervals than when they are far apart, a model such as the one described in the text box can be applied. The within-subject correlation coefficient ρ between two consecutive measurements, which is called autocorrelation, affects the variance of sums and differences of two consecutive measurements. For uncorrelated measurements (ρ = 0) it is a well-known property that the standard error of the mean becomes smaller by a factor $1/\sqrt{2}=0.71$. On the other hand, if the two measurements are completely correlated, i.e. identical, then there is obviously no reduction of variance at all. By plugging in some selected values of ρ and σ^2 in the model, and comparing the resulting curve with the empirical data, we found that values of the autocorrelation in the range of 0.5-0.8 were consistent with the data.

A gradual increase of SDs with increasing individual mean values of tHcy measured over five consecutive days in our data indicated that the usual assumptions of normality and constant variance were violated. Therefore, we performed a logarithmic transformation on tHcy. This made it possible to express the variability as a coefficient of variation (CV). For small through moderate values of the CV, i.e. up to about 20%, the (semi-) variance of 100×ln(tHcy) provides a fair

estimate of the squared CV as can be derived straightforward from the Taylor series:

$$\text{CV}=\sqrt{(semi-)Var\{100*\ln(tHcy)\}}\ .$$

This is the more natural model to use when within-subject standard deviations are proportional to the mean level of the variable as appeared to be the case in our data. All statistical analyses were performed with SAS (version 6.12 for Windows).

Results

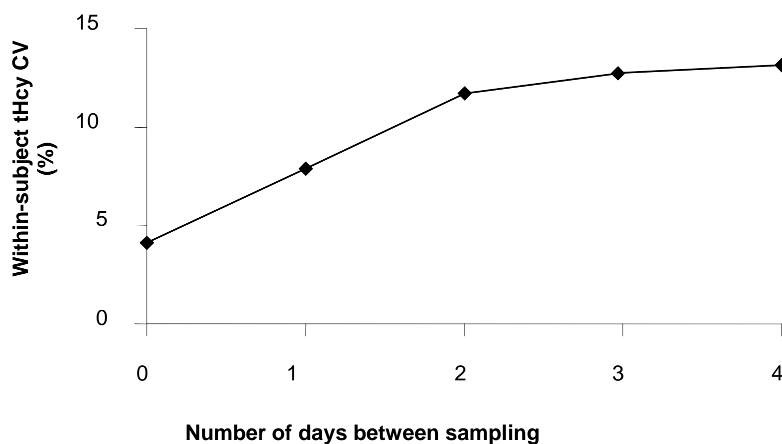
Three volunteers were not fasting on the first day but completed the rest of the study. Data from these volunteers were reported as missing on the first day. The plasma homocysteine concentrations in the 15 subjects throughout the five days ranged from 4.2 to 20.9 μmol/L with a mean of 9.2 (SD 2.8) μmol/L.

In **Figure 1**, the relationship between CV and sampling interval is shown. The values of the semi-variance for the different time intervals of one, two, three, and four days, based on ln(tHcy) values and expressed as a CV, were 7.9, 11.7, 12.7, and 13.1%, respectively, with corresponding number of observation pairs per interval of 57, 42, 27 and 12, respectively. The CV at the time interval of zero days refers to the analytical variation for duplicates of a single sample, i.e. the within-run analytical CV, which was 4.1 % in our study. As the figure shows, the CV increased monotonically at a decreasing rate when the interval increased from zero to four days. This confirms that measurements on consecutive days were correlated. Beyond the time interval of two days the CV hardly increased anymore.
Thus, sampling on consecutive days reduces the benefit of taking the mean of two measurements as a means of improving precision. In case of uncorrelated measurements the error would be reduced by a factor 0.71 when taking duplicate samples. We estimated that the autocorrelation for consecutive days was 0.6 in this study. Therefore, the error was only reduced by a factor $\sqrt{\{(1+\rho^d)/2\}}$ = 0.89 for a one-day interval and 0.83 or 0.78 for a two- or three-day interval, respectively. For a four-day interval the error was reduced by a factor 0.75 and thus almost reached the full error reduction of a factor 0.71 that would be achieved if no autocorrelation were present.

Discussion and conclusion

This study showed that the CV calculated from the semi-variances of two repeated tHcy measurements increases with an increasing sampling interval of one to four days. This implicates the presence of autocorrelation of repeated tHcy measurements on short time intervals. The variation hardly increased between intervals of two to four days. We therefore suggest that serial blood measurements

of homocysteine to assess a person's true mean average should be made at least two



days apart.

Figure 1. Coefficient of variation of total homocysteine concentrations within individuals as a function of time interval between repeat fasting blood collections in days. Day 'zero' refers to the within-run analytical variance for duplicate samples.

Thirup & Ekelund[13] also examined the day-to-day variation of plasma total homocysteine on 5 consecutive days, but calculated only one CV instead of interval-specific CVs. They reported a mean within-subject biological CV of 13%.[13] We calculated that the within-subject biological variation in our study was 12.4% at the four-day interval, which is close to their finding. Studies that used weekly through monthly sampling strategies found lower figures for the biological CV in plasma tHcy (Table 1). We used the method as described by Rotterdam *et al.*[5] for assessing the optimal time interval in days between repeated cholesterol measurements. Like them, we examined the biological variability as a function of time between repeated blood measurements, expressed as a semi-variance. An attractive feature of this approach is that it presents different CVs at various time intervals, rather than just one CV.

Because of the presence of autocorrelation, the extent to which the error of the mean can be reduced increases with the time interval between two repeated tHcy measurements. We found that a four-day interval almost yields the full benefit that can be achieved without the presence of autocorrelation.

In conclusion, this study shows that when the mean of two repeated homocysteine determinations is calculated to enhance precision, the measurements

should be taken at least two or, even better, three or four days apart to optimize error reduction.

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List of publications

Peer-reviewed papers:

Melse-Boonstra A, Rozendaal M, Rexwinkel H, Gerichhausen MJW, van den Briel T, Bulux J, Solomons NW, West CE. Determination of discretionary salt intake in rural Guatemala and Benin to determine the iodine fortification of salt required to control iodine deficiency disorders: studies using lithium-labeled salt. Am J Clin Nutr 1998;68:636-41.

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