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Folate Forms Distribution in the Elderly Before and After B-Vitamins Supplementation Using LC-MS/MS

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Abbreviations

°C	degree Celsius
A_λ	absorbance
ABCC1	ATP-binding cassette, subfamily C, member 1
ADP	adenosine diphosphate
AICARTF	5-aminoimidazole-4-carboxamide ribonucleotide transformylase
ALAT	alanine aminotransferase
APCI	atmospheric-pressure chemical ionization
ATP	adenosine triphosphate
BADH	betaine aldehyde dehydrogenase
BHMT	betaine homocysteine methyltransferase
C	cytoplasm
c	concentration; cytoplasmic
CBS	cystathionine-β-synthase
CDH	choline dehydrogenase
cDNA	copy deoxyribonucleic acid
CGL	cystathionine-γ-lyase
CoA	coenzyme A
CRP	c-reactive protein
CV	coefficient of variation
CVD	cardiovascular disease
Cys	cystathionine
d	day
DC	direct current
DFE	dietary folate equivalent
DGE	Deutsche Gesellschaft für Ernährung
DHF	dihydrofolate
DHFR	dihydrofolate reductase
DMG	dimethylglycine
DMGDH	dimethylglycine dehydrogenase
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dTMP	deoxythymidine monophosphate
DTT	dithiothreitol
dUMP	deoxyuridine monophosphate
E	error
EDTA	ethylenediaminetetraacetate
ESI	electrospray ionization
FA	folic acid
FAD	flavin adenine dinucleotide
FBP	folate-binding protein
FDH	10-formylTHF dehydrogenase
FGCP	folyl-γ-polyglutamate carboxypeptidase
FIGLU	*N*-formiminoglutamic acid
FMT	methionyl-t-RNA formyl transferase
FPGS	folylpoly-γ-glutamate synthase
FR	folate receptor
FS	Folsäure
FTCD	5-formiminoTHF cyclodeaminase
FTHFS	10-formylTHF synthase
g	gram; gravitational constant
GARTF	glycinamide ribonucleotide transformylase

GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GCPII	glutamate carboxypeptidase II
GCS	glycine cleavage system
GFR	glomerular filtration rate
GGH	γ-glutamyl hydrolase (folylpoly-*γ*-glutamyl hydrolase)
Gly	glycine
GNMT	glycine-*N*-methyltransferase
h	hour
HC	haptocorrin
Hct	hematocrit
Hcy	homocysteine
HDL	high density lipoprotein
HHCY	hyperhomocysteinemia
HILIC	hydrophilic interaction chromatography
HoloTC	holotranscobalamin
HPLC	high-performance liquid chromatography
i	intercept
i.d.	internal diameter
IDL	instrumental detection limit
IQL	instrumental quantification limit
IU	international unit
IUPAC	International Union of Pure and Applied Chemistry
K_m	Michaelis constant
K_d	dissociation constant
L	liter
l	length
LC	liquid chromatography
LC-MS	liquid chromatography coupled to mass spectrometry
LC-MS/MS	liquid chromatography with tandem mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
m	milli; mass; slope
M	mitochondrium
MAT	methionine adenosyltransferase
MBDSTFA	*N*-methyl-*N*-tert-butyldimethylsilyltrifluoro-acetamid
MCM	*L*-methylmalonyl CoA mutase
MDL	method detection limit
Met	methionine
MFT	mitochondrial folate transporter
MMA	methylmalonic acid
MQL	method quantification limit
MRM	multiple reaction monitoring
MS	methionine synthase
MS/MS	tandem mass spectrometry
MTHFC	5,10-methenylTHF cyclohydrolase
MTHFD	5,10-methyleneTHF dehydrogenase
MTHFR	5,10-methyleneTHF reductase
MTHFS	5,10-methenylTHF synthase
m/z	mass-to-charge ratio
n	number
n.d.	not determined; not detected
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NHANES	National Health and Nutritional Examination Survey

NP-HPLC	normal-phase high-performance liquid chromatography
NTD	neural tube defect
p	p value
*p*ABA	*p*-aminobenzoic acid
*p*ABG	*p*-aminobenzoyl-*L*-glutamic acid
PCFT/HCP1	proton-coupled folate transporter/heme carrier protein 1
PCR	polymerase chain reaction
PEMT	phosphatidylethanolamine methyltransferase
RBC	red blood cell, erythrocyte
RDA	recommended dietary allowance
RF	radio frequency
RFC	reduced folate carrier
RMSE	root mean square error
RNA	ribonucleic acid
RP-HPLC	reversed-phase high-performance liquid chromatography
R	coefficient of correlation
R_s	resolution
s	seconds
SAH	*S*-adenosyl homocysteine
SAHH	*S*-adenosyl homocysteine hydrolase
SAM	*S*-adenosyl methionine
Ser	serine
SD	standard deviation
SDH	sarcosine dehydrogenase
SHMT	serine hydroxy methyltransferase
SIL	stable isotope labeled
S/N	signal-to-noise ratio
S/N:PtP	signal-to-noise:peak-to-peak ratio
SNP	single nucleotide polymorphism
SPE	solid-phase extraction
T	transmittance
TC	transcobalamin
TFOL	total folate measured by methods that do not distinguish between different folate forms
THF	tetrahydrofolate
tHcy	total homocysteine
TIC	total ion chromatogram
t_R	retention time
$tRNA_f^{Met}$	formylated methionyl transfer RNA
TS	thymidylate synthase
UPLC	ultra performance liquid chromatography
U.S.	United States
USA	United States of America
UV	ultra violet
V	volt
v	volume
VB	Vollblut
w_b	widths at the base of peak
WB	whole blood
y	response (area analyte/area internal standard)

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Abstract

Folates act as co-enzymes in the *de novo* synthesis of purines and thymidylates, in the formation of *S*-adenosyl methionine (SAM), and in the metabolism of amino acids. Deficiencies of folates and alterations in the folate forms distribution may have severe pathologic consequences. We aimed at investigating the folate forms distribution in serum and whole blood (WB), as well as the status of related vitamins and metabolites in non-supplemented older German subjects before and after the supplementation with B-vitamins. Therefore we performed two randomized and double-blind studies. In the short-term supplementation study (duration 3 – 4 weeks) we orally supplemented the participants with 400 µg/day folic acid (FA) or 400 µg FA, 8 mg vitamin B_6, and 10 µg vitamin B_{12} /day. In the long-term supplementation study (duration 12 months) we orally supplemented the participants with 500 µg FA, 500 µg vitamin B_{12}, 50 mg vitamin B_6, 456 mg calcium, and 1,200 IU vitamin D /day or 456 mg calcium, and 1,200 IU vitamin D /day.

We developed ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) methods for the quantification of folate forms in serum and WB, of the methylation markers SAM and *S*-adenosyl homocysteine in plasma, and of betaine and the related metabolites choline and dimethylglycine in plasma. We observed that the baseline serum concentrations of the sum of folates and of 5-methyltetrahydrofolate (5-methylTHF), and the 5-methylTHF content (as % of sum of folates) in serum were age- but not gender-dependent. The vitamin B_{12} status influences the concentrations of the folate forms in serum, as well as of that of the related metabolites. Unmetabolized FA in serum was present at baseline and after the supplementation with B-vitamins. Subjects co-supplemented with the vitamins B_6 and B_{12} had significantly lower amounts of unmetabolized FA, which might reflect a higher turnover of the vitamin. Oral B-vitamins supplementation led to a steady-state and a saturation of the red blood cells with folate between 6 and 12 months. Although the 5,10-methyleneTHF reductase *C677T* polymorphism had no influence on the baseline folate forms distribution of fasting subjects, we found significant differences after the short-term and long-term supplementation.

Compared to the immunoassay or other methods only measuring the total folate, the UPLC-MS/MS method provides additional information concerning the folate forms distribution. This is especially of interest in populations with a low vitamin B_{12} status, such as the elderly, pregnant women, or vegetarians. We therefore strongly recommend the use of (UP)LC-MS/MS methods for the quantification of folates in clinical studies in the future.

Zusammenfassung

Folate spielen eine wichtige Rolle als Koenzyme bei der *de novo* Synthese von DNA, der Bildung von *S*-Adenosylmethionin (SAM) sowie dem Kreislauf von Aminosäuren. Folatmängel und Änderungen der Folatformverteilung können schwere Konsequenzen haben. Ziel der Studie war die Untersuchung der Folatformverteilung in Serum und Vollblut (VB) sowie von verwandten Metaboliten in nicht-supplementierten, älteren Probanden vor und nach Supplementierung mit B-Vitaminen. Aus diesem Grund wurden zwei randomisierte und doppel-blinde Studien durchgeführt. In der kurzzeitigen Supplementationsstudie (Dauer: 3 – 4 Wochen) wurden die Probanden täglich entweder mit 400 µg Folsäure (FS) oder 400 µg FS, 8 mg Vitamin B_6 und 10 µg B_{12} supplementiert. In der langzeitigen Supplementationsstudie (Dauer: 12 Monate) wurden die Probanden täglich entweder mit 500 µg FS, 500 µg Vitamin B_6, 500 µg B_{12}, 456 mg Ca und 1.200 IE Vitamin D oder mit 456 mg Ca und 1.200 IE Vitamin D supplementiert.

Wir haben Ultra-Performance-Flüssigkeitschromatographie-Tandem-Massenspektrometrie (UPLC-MS/MS) Methoden für die empfindliche und zuverlässige Quantifizierung von Folatformen in Serum und VB sowie von den Methylierungsmarkern SAM und *S*-Adenosylhomocystein und von Betain und dessen verwandten Metaboliten Cholin und Dimethylglycin entwickelt. Die Serumkonzentrationen von der Summe der Folate und von 5-Methyltetrahydrofolat (5-MethylTHF) sowie vom 5-MethylTHF Anteil (% von der Folatsumme) im Serum zu Studienbeginn war alters- aber nicht geschlechtsabhängig. Der Vitamin B_{12} Status hat sowohl die Konzentration der Serum-Folatformen als auch die von anderen Metaboliten beeinflusst. Unmetabolisierte FS wurde zu Studienbeginn und nach der Supplementation im Serum festgestellt. Jedoch hatten die Probanden mit Vitamin B_6 und B_{12} Kosupplementierung geringere Serumkonzentrationen, was auf einen höheren Vitaminumsatz schließen lässt. Die orale Supplementierung mit B-Vitaminen hat zu einem Steady-State und einer Sättigung der Erythrozyten mit Folat zwischen 6 und 12 Monaten geführt. Obwohl der 5,10-Methylentetrahydrofolatreduktase *C677T* Polymorphismus keinen Einfluss auf die Folatformverteilung bei nüchternen Probanden zu Studienbeginn hatte, konnten wir signifikante Unterschiede nach der Supplementierung feststellen.

Verglichen mit dem Immunoassay oder anderen Methoden, die nur das Gesamtfolat messen, liefert die UPLC-MS/MS zusätzliche Informationen über die Verteilung der Folatformen. Dies ist besonders in Populationen von Interesse, die einen geringen Vitamin B_{12}-Status haben, wie ältere Menschen, Schwangere und Vegetarier. Aus diesem Grund empfehlen wir für die Zukunft in klinischen Studien den Einsatz von (UP)LC-MS/MS-Methoden für die Bestimmung von Folaten.

1 Introduction

Folates represent a large family of water-soluble B-vitamins, which were first recognized by Lucy Wills in 1931 as a hematopoietic factor in yeast and liver extracts (246). In 1940, Snell *et al.* described a factor that is essential for the growth of *Lactobacillus casei* (208). This factor was isolated, characterized, and named later as folate. The term "folic acid" from Latin "folium" or "leaf" was marked by Mitchell in 1941, who extracted the substance from spinach leaves (157). The synthesis of folic acid (FA; pteroylmonoglutamic acid) in pure crystalline form was accomplished by Stokstad *et al.* in 1943 and Angier *et al.* in 1945 (3). In 1952, Welch and Nichol had cleared most of the tasks of FA in the transfer of one-carbon units (244).

Since the 1980s folate is in the focus of scientific research as its decisive function in embryonic development became apparent. Over the last decades, the role of folate in several biological pathways has been recognized. Folates act as essential cofactors in many cellular functions including the *de novo* synthesis of purines and thymidylates and the metabolism of amino acids. Folate metabolism intersects with the methionine (Met) cycle, as well as the choline pathway. An adequate function of the one-carbon metabolism depends upon availability of the B-vitamins (folate, vitamin B_6, and B_{12}) and the normal function of the enzymes involved in several interacted pathways. Folate deficiency causes hyperhomocysteinemia (HHCY) and megaloblastic anemia and it has been related to the risk of neural tube defects (NTDs), cardiovascular diseases (CVDs), and cancer (120).

1.1 Folate chemistry, physiology, and biology

1.1.1 Chemical structure of folates and folic acid

FA is the synthetic and the most stable form of the vitamin. FA is fully oxidized and consists of a 2-amino-4-hydroxy-pteridine ring linked at the C-6 position to a *p*-aminobenzoic acid (*p*ABA), and *L*-glutamic acid. In its pure form, FA is an orange-yellow crystalline, taste- and odorless powder. Therefore, FA is the preferred form to be used in dietary supplements and fortified foods (108). Naturally occurring folate forms consist of derivatives of 5,6,7,8-tetrahydropteroyl-γ-glutamate (THF) that are fully reduced at the 5, 6, 7, and 8 positions of the pyrazine ring. The molecular structure of FA, THF, and the reduced folate forms are shown in **Figure 1**. Natural folate forms are polyglutamates. The polyglutamate side chain may contain up to eleven glutamate residues and varies between mammalian species with five to eight glutamate residues prevailing (46).

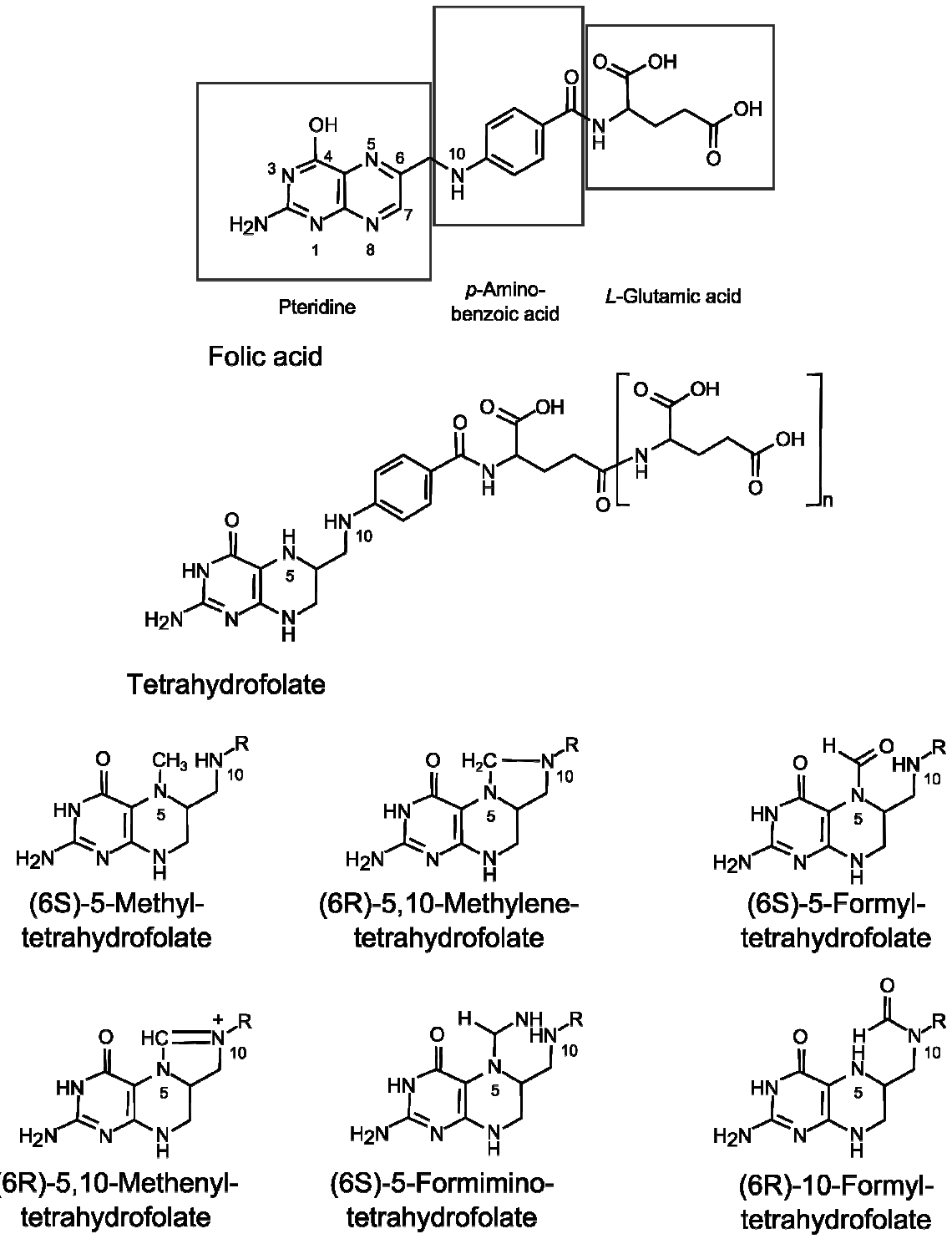

Figure 1: Molecular structure of folic acid, 5,6,7,8-tetrahydrofolate, and reduced folate forms. Bonds at positions 5, 6, 7, and 8 can be oxidized to 7,8-dihydrofolate. One carbon units can be accepted by THF at N-5 and/or N-10 positions of the pteridine ring.

Folates can have a variety of one-carbon units at the N-5 and/or N-10 positions, which can be transferred by means of certain enzymes. Reduced folates function as acceptors and donors of one-carbon units at three different oxidation levels: methanol (5-methylTHF), formaldehyde (5,10-methyleneTHF), and formic acid (5,10-methenylTHF, 5-formiminoTHF, 5-formylTHF, and 10-formylTHF) (237). The oxidation levels differ from each other by the gain (reduction) or the loss (oxidation) of two electrons.

There is a considerable number of theoretical folate forms. In 1983, Krumdieck *et al.* detected 100 different vitamers in biological tissues (131). Tannenbaum *et al.* stated in 1985, that if the number of glutamate residues is limited to six there might be about 140 possible forms (220). In summary, folates differ in three aspects: first, the hydrogenation of pteridines, which can be either oxidized (FA), di-(7,8-dihydrofolate; DHF), or tetrahydrated (THF). Second, the degree of substitution at atoms N-5 and N-10. Third, the number of glutamyl residues that are linked by γ-peptide bonds (177). These chemical differences are strongly related to bioavailability, cellular distribution, and functions. The spectral properties (19;178) of FA and its derivatives are shown in **Table 1**.

Table 1: Absorption wavelengths and molar extinction coefficients of folates.

Analyte	Wavelength λ, nm	Molar extinction coefficient ε
5-MethylTHF	290	31,700
5-FormylTHF	285	37,200
10-FormylTHF	253	15,300
5,10-MethenylTHF	352	25,000
5,10-MethyleneTHF	295	25,000
THF	297	29,100
DHF	282	22,400
Folic acid	282	27,600

1.1.2 Folate sources for humans

Sources of folates in the human diet are wheat germ, yeast, liver, and leafy vegetables. Although mammals can synthesize the pteridine ring they can not connect it with other components and therefore depend on the uptake of preformed folates from food (211). Müller *et al.* determined in 1993 the amount of folate in vegetables and fruit (165), as well as in foods of animal origin (166) using high-performance liquid chromatography (HPLC). The foods with the highest total folate sum of folates concentrations in 100 g fresh weight are: beef liver with 963 µg (consisting of 398 µg THF, 476 µg 5-methylTHF, and 53 µg 5-formylTHF), bovine kidney with 410 µg (198 µg THF, 172 µg 5-methylTHF, 28 µg 5-formylTHF), and spinach with 145 µg (4.6 µg THF, 106.5 µg 5-methylTHF, 40.7 µg 5-formylTHF) sum of folates content (165;166). The main dietary folate forms are 5-methylTHF and formylTHF in polyglutamate forms (152).

In addition to folates supplied by the diet, folates can be synthesized by the microflora of the large intestine. The synthesized folates can be absorbed in the large intestine (58) and were believed earlier to be insignificant for vitamin supply (187). Recently, Aufreiter *et al.* suggested that the amount of folate absorbed in the colon is responsible for 5% of the average folate requirements for healthy adults, assuming the potential to influence the folate status (9). The intake and absorption of dietary folates depend on the bioavailability of the folate form. This is of interest for the determination of the Recommended Dietary Allowance (RDA) especially in countries applying no fortification of staple foods with FA. The bioavailability of reduced folates is estimated by the comparison to the bioavailability of FA. The availability of folate depends on a number of factors. First, the intestinal deconjugation of polyglutamates, second, the food matrix, third, the stability of the folate forms, and fourth, the presence of other food constituents or additives that may influence the folate stability (e.g. ascorbic acid, salts). The bioavailability of polyglutamates is significantly lower (≈ 65 – 70%) than those of folate monoglutamates (118;154). Tamura and Stokstad found different availabilities of the native reduced folates for humans (219). If the bioavailability of FA is set to 100%, THF has a bioavailability of 104.7%, 5-methylTHF of 120.8%, and 5-formylTHF of 70% (219). Long-term dietary intervention studies estimated the folate bioavailability between 30 – 98% of that of FA (28;83;247). However, there is a general agreement that the bioavailability of natural food folates is less than that of FA (151) especially when it is estimated depending on changes in erythrocyte (RBC) folate concentrations (249). Recent studies suggest the use of 5-methylTHF as the reference folate for studying the bioavailability (249).

1.1.3 The recommended dietary allowance of folate

The RDA recommended by the U.S. Institute of Medicine in 1998 represents the average daily intake that is sufficient to meet the nutrient requirements of nearly all (97 – 98%) healthy individuals in each age and gender group (109). The recommendation for food folate was based on a study by Sauberlich *et al.*, which stated that the bioavailability of food folates was no more than 50% of that of FA (190;247). The RDA for folate is 400 µg/d of dietary folate equivalents (DFE) for adult males and females and 600 µg/d for pregnant and lactating women, whereas 1 µg of DFE is defined as 0.6 µg FA (56;109). The upper tolerable limit for synthetic FA for adults is 1 mg/d. There is currently little, if any, evidence that high intake of reduced folates can be harmful to humans. **Table 2** summarizes the RDA and upper intake limits of dietary folate according to the U.S. Institute of Medicine (109).

Table 2: Recommended dietary allowance at dietary folate equivalents and upper intake limit of folic acid by the U.S. Institute of Medicine (109).

Group	Recommended dietary allowance [µg/d]	Upper intake limit of FA [µg/d]
Young infants (0 – 6 months)	65	n.d.
Older infants (7 – 12 months)	80	n.d.
Children (1 – 3 years)	150	300
Children (4 – 8 years)	200	400
Children (9 – 13 years)	300	600
Adolescents (14 – 18 years)	400	800
Adults (19 – 50 years)	400	1,000
Adults (> 50 years)	400	1,000
Pregnant women (14 – 50 years)	600	1,000[a]
Lactating women (14 – 50 years)	500	1,000[a]

[a]: Upper intake level = 800 µg/d for 14 – 18 years and 1,000 µg/d for > 19 years of age.
n.d.: not determined.

1.1.4 Blood concentrations of folate

Serum concentration of folates range from ~ 10 – 50 nmol/L, with 5-methylTHF predominating (82 – 93% of the sum of folates) (126;178). In blood, most of the folate is unspecifically bound to low-affinity proteins (e.g. α_2-macroglobulin, albumin (K_d ~ 1 mmol/L), and transferrin) (145) or specifically to high-affinity folate-binding proteins (FBPs) (K_d ~ 1 nmol/L) (97). The concentration of FBPs in human serum is approximately 0.6 nmol/L, whereas there is a 10 – 30fold higher molar concentration of folate in serum (18). Although FBPs have a high binding affinity, they can bind only very low amounts of folate. Therefore it can be assumed that the serum FBPs are fully saturated (17). The liver is responsible for the maintenance of the serum folate concentration (145;211). This is ensured by the reversible formation and depletion of the intracellular non-methylated folate polyglutamates.

Folates are incorporated into developing RBCs during erythropoiesis. FBPs are expressed on early stage hematopoietic cells and are involved in the folate uptake. Although they do not transport great amounts of folates the reduced folate carrier (RFC) is likely necessary for the folate transport in erythroid cells (255). RBC folates are mainly 5-methylTHF and formylTHF in their polyglutamate form, mostly penta- and hexaglutamates. Under normal conditions, RBC folate is 30fold higher than the serum folate concentration. Similar to serum, the RBC folates are unspecifically bound to proteins whereas hemoglobin might be one possible binding protein (84). Matured RBCs have a life span of approximately 120 days and are impermeable for folylpolyglutamates (145;211). This leads to an intracellular retention of the folates with a half-life of about 100 days (130). Therefore, RBC folate represents a long-term marker for the folate status (34;95). This marker is not affected by recent changes in the dietary intake (199).

1.1.5 Absorption, transport, and compartmentalization of folates

Dietary folates and FA enter the circulation via absorption in the small intestine. Upon ingestion, dietary folylpolyglutamates must be enzymatically hydrolyzed into monoglutamates at the brush border cells of the duodenum and jejunum. The responsible enzyme for this hydrolysis is the glutamate carboxypeptidase II (GCPII; EC 3.4.17.21), which is present mainly in the area of the proximal mucosa of the small intestine. The brush border GCPII acts as an exopeptidase and has a pH optimum in the neutral range (pH 6.5). GCPII has the same affinity for polyglutamates with different glutamate residue chain length. In addition, there is the exopeptidase γ-glutamate hydrolase (GGH; EC 3.4.19.9) also known as folyl-γ-polyglutamate carboxypeptidase (FGCP) (240). The intracellular (lysosomal) form of GGH is found in the chyle. Both forms of the enzyme are pH-dependent and saturable (177). In contrast, GGH dissolved in the chyle has an optimum at pH 4.5. However, the role of GGH in the intestinal ingestion of folates is unknown (65). The intestine plays a central role in regulating the folate homeostasis. When absorbed, the monoglutamates are converted intracellularly to polyglutamates by the folylpoly-γ-glutamate synthase (FPGS; EC 6.3.2.17) (200). The preferred substrate for FPGS is THF and the affinity for 5-methylTHF is very poor. In order to prevent cellular efflux of folates they must exist in at least triglutamate form (160). FPGS activity is highest in the liver thus making the liver a major storage organ for folates. FPGS is absent or only present in negligible amounts in muscle tissue and mature blood cells (161).

Intracellular folylpolyglutamates represent the natural substrates for the folate-metabolizing enzymes that exhibit high affinity and low K_m for these folates (192). Only the monoglutamates can be effectively transported across cell membranes (96). Consequently, polyglutamates are efficiently retained within cells allowing them to concentrate folates at much higher levels than in the extracellular compartments (199). The chain length of the polyglutamates differ from one cell type to another even within different organelles of a single cell (221). The polyglutamate side chain of the folates has an additional role in regulation of enzymatic reactions. They can affect the K_m values of the coenzymes and substrates in the reaction. This effect is translated into higher binding of folate to the enzymes and the ability of the enzyme to downregulate certain reactions via negative feedback mechanisms (134).

High extracellular folate concentrations $> 10\,\mu mol/L$ are subject to non-saturable passive diffusion (4). Additionally, folylmonoglutamates are transported across cell membranes via a saturable process involving both receptor-mediated and carrier-mediated transport mechanisms, which are variably expressed in diverse tissues (77). Currently three independent types of membrane transport systems are known to internalize folates at physiological concentration: the membrane folate receptor (FR) in the form of FBPs, the RFC-1, and the recently described

proton-coupled folate transporter/heme carrier protein 1 (PCFT/HCP1). The FR has a high affinity for folates (K_m ~ 1 nmol/L) and mediates unidirectional transport across the cell membrane at neutral pH via receptor-mediated endocytosis (203). FR prefers FA over reduced folate forms. In comparison with transmembrane carriers (RFC-1 and PCFT/HCP1) the transport via FR is a relatively slow process and the extent to which this receptor is involved in folate transport is not well established. RFC-1 bidirectionally transports the folate with a higher affinity for 5-methylTHF than for FA and functions optimally at physiological pH (pH 7.5) (87). The PCFT/HCP1 system prefers acidic pH (pH 4.5 – 5.5) but has a residual activity at physiological pH indicating the role as the major intestinal folate transporter (181). The unidirectional working carrier protein has a high affinity for folate (K_m ~ 1.7 µmol/L) and prefers oxidized FA to reduced folate. The transport into mucosal cells appears to occur primarily via PCFT/HCP1.

The expression of RFC-1 and PCFT in tissue is ubiquitous. FR is only expressed in certain tissues such as kidney, placenta, spleen, and thymus but not in the intestine (64). Both RFC-1 and PCFT are not saturated by reduced folate monoglutamates under physiological conditions even after the uptake of high doses of folate, which is in contrast to the FR. The transport into peripheral tissues occurs primarily via the RFC-1 (253). Transport of folates into the cerebrospinal fluid occurs in the choroid plexus where 5-methylTHF is transported across the blood-brain barrier by FRα in the adult or FRβ in the fetal brain. PCFT is ubiquitously expressed in human brain where it functions in concert with FRα and FRβ, or might export folates after FRα-mediated endocytosis (212;254). In kidney, organic anion transporters are involved in nonspecific folate transport across the apical membranes (148). The role of these transport mechanisms in intestinal and renal folate uptake is not completely understood (44). In **Figure 2** influx and efflux of folates and FA into hepatic cells is shown.

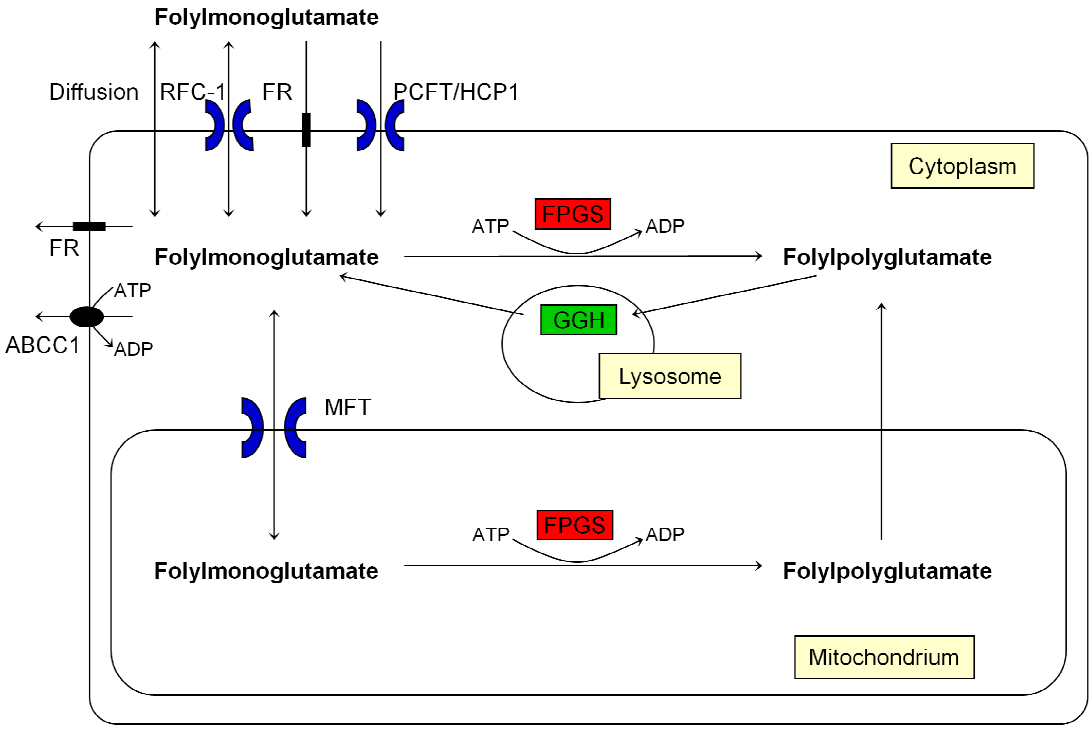

Figure 2: Influx and efflux of folates and folic acid into a hepatic cell. Folylmonoglutamates can be actively transported across cell membranes by the bidirectional RFC-1, the unidirectional FR, and the PCFT/HCP1 system. The ABCC1 is involved in folate efflux. Mitochondrial transport of folylmonoglutamate is carried out by MFT. Intracellularly, FPGS forms folate polyglutamates, which can be processed by folate-catabolizing enzymes. The lysosomal GGH has similar enzymatic function as FGCP. ABCC1: ATP-binding cassette, subfamily C, member 1; MFT: mitochondrial folate transporter.

Folylpolyglutamates are important regulators of one-carbon metabolism (5;192). Polyglutamate chains increase the affinity of folates for folate-dependent enzymes (except DHF reductase (DHFR; EC 1.5.1.3)), enhance the cellular retention of folates, and permit metabolic channeling of folates among folate-dependent enzymes (192). The expression of FPGS activity can therefore regulate the level and the enzymatic conversion of folates in cells (75;200).

Folates and folate-metabolizing enzymes are compartmentalized primarily between cytosol and mitochondria with small amounts in the nucleus (5). In mitochondria, one-carbon units in the form of formate are generated through catabolism of serine, glycine, and choline (121) and initiator transfer RNA ($tRNA_f^{Met}$) is produced for protein biosynthesis (15) (**Figure 3**). In cytoplasm, the nucleotide *de novo* synthesis and the remethylation of homocysteine (Hcy) occur. In nucleus thymidylates are generated. Compartmentalization can differ among tissues and during development (38). The distribution of folate forms in cytoplasm and mitochondria varies. In cytosol of rat liver cells, folate consists of 45% methylTHF, 30% formylTHF, and 25% THF, whereas in the mitochondria the predominant forms are methylTHF (7%), formylTHF (44%), and THF (48%) (102).

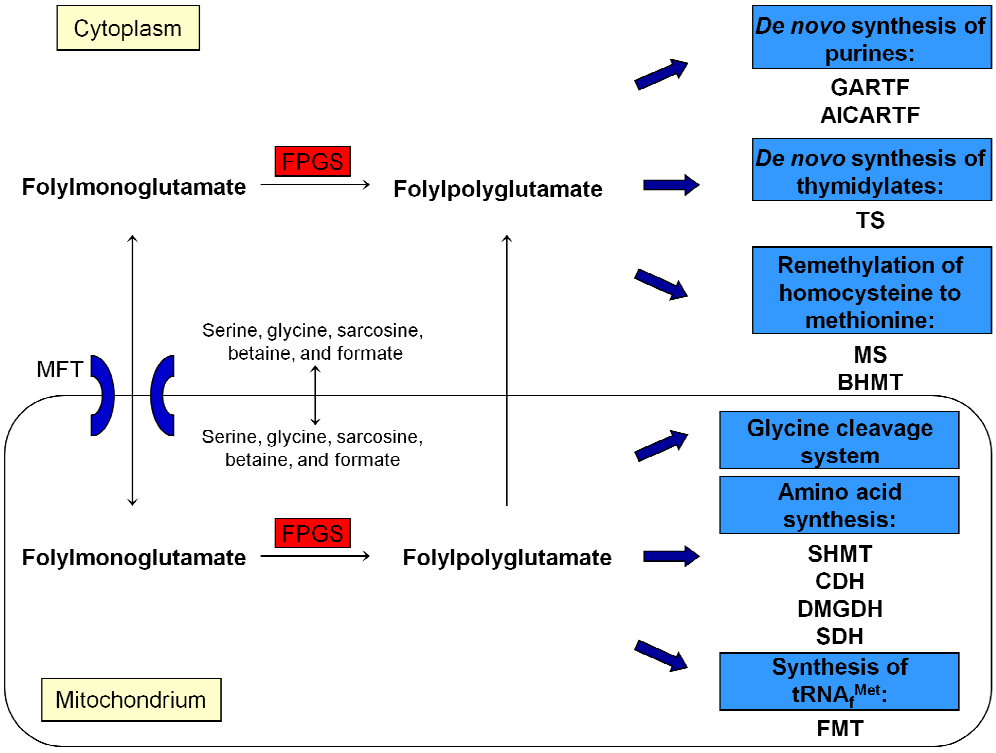

Figure 3: Intracellular compartmentalization of folates and important folate related reactions. In the cytoplasm of hepatic cells the *de novo* synthesis of purines and thymidylates occurs, as well as the re-methylation of homocysteine to methionine. Amino acid synthesis, synthesis of $tRNA_f^{Met}$, and the glycine cleavage takes place in the mitochondria. AICARTF: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; BHMT: betaine homocysteine methyltransferase; CDH: choline dehydrogenase; DMGDH: dimethylglycine dehydrogenase; FMT: methionyl-$tRNA_f^{Met}$ formyltransferase; FPGS: folylpoly-γ-glutamate synthase; GARTF: glycinamide ribonucleotide transformylase; MFT: mitochondrial folate transporter; MS: methionine synthase; SDH: sarcosine dehydrogenase; SHMT: serine hydroxy methyltransferase; $tRNA_f^{Met}$: initiator transfer RNA; TS: thymidylate synthase.

Polyglutamates can not be transported into mitochondria but they can effluxed into the cytosol without prior hydrolysis (215). Although the exchange of free folates between cytosol and mitochondria is limited (224), both compartments are metabolically connected by transport of one-carbon donors (serine, glycine, betaine, and formate) (38;221). Titus and Moran isolated a gene encoding a protein that facilitates the transport of cytosolic folate monoglutamates into mitochondrial matrix of mammalian cells – the mitochondrial folate transporter (MFT) (223). In **Table 3** the folate-dependent enzymes and their intracellular location and function are shown.

Table 3: Folate-dependent enzymes: intracellular location and function in one-carbon metabolism in hepatic cells.

Enzyme	EC	Cofactors	Function	Location
Folypoly-γ-glutamate synthase (FPGS)	6.3.2.17		addition of glutamate moieties	C/M
Serine hydroxy methyltransferase (SHMT)	2.1.2.1	B_6, NAD	THF + Ser + NAD^+ ↔ 5,10-methyleneTHF + Gly + H_2O	C/M
5,10-MethenylTHF cyclohydrolase (MTHFC)	3.5.4.9		10-formylTHF + H^+ ↔ 5,10-methenylTHF + H_2O	C/M
5,10-MethyleneTHF dehydrogenase (MTHFD)	1.5.1.5	NADP	5,10-methenylTHF ↔ 5,10-methyleneTHF	C/M
10-FormylTHF synthase (FTHFS)	6.3.4.3		THF + formate + ATP ↔ 10-formylTHF + ADP+P_i	C/M
5,10-MethenylTHF synthase (MTHFS)	6.3.3.2		5-formylTHF + ATP → 5,10-methenylTHF + ADP+P_i	C/M
10-FormylTHF dehydrogenase (FDH)	1.5.1.6	NADP	10-formylTHF → THF + CO_2	C/M
Dihydrofolate reductase (DHFR)	1.5.1.3	NADP	folic acid → DHF; DHF → THF	C/M
5,10-MethyleneTHF reductase (MTHFR)	1.5.1.20	FAD, NADP	5,10-methyleneTHF → 5-methylTHF	C
Methionine synthase (MS)	2.1.1.13	B_{12}	5-methylTHF + Hcy → THF + Met	C
Glycinamide ribonucleotide transformylase (GARTF)	2.1.2.2		10-formylTHF → purine + THF	C
5-Aminoimidazole-4-carboxamide ribonucleotide transformylase (AICARTF)	2.1.2.3		10-formylTHF → purine + THF	C
Thymidylate synthase (TS)	2.1.1.45		5,10-methyleneTHF + dUMP → DHF + dTMP	C
Glutamate formiminotransferase	2.1.2.5		THF + formiminoglutamate → 5-formiminoTHF + glutamate	C
5-FormiminoTHF cyclodeaminase (FTCD)	4.3.1.4		5-formiminoTHF + H_2O → 5,10-methenylTHF + NH_3	C
Glycine cleavage system (GCS)	2.1.2.10 1.4.4.2 1.8.1.4	B_6, FAD, NAD	THF + Gly → 5,10-methyleneTHF + CO_2 + NH_4^+	M
Dimethylglycine dehydrogenase (DMGDH)	1.5.99.2	FAD	THF + DMG → 5,10-methyleneTHF + sarcosine	M
Sarcosine dehydrogenase (SDH)	1.5.99.1	FAD	THF + sarcosine → 5,10-methyleneTHF + Gly	M
Methionyl-t-RNA formyl transferase (FMT)	2.1.2.9		10-formylTHF + $tRNA^{Met}$ → THF + $tRNA_f^{Met}$	M

C: cytoplasm; DMG: dimethylglycine; dTMP: deoxythymidine monophosphate; dUMP: deoxyuridine monophosphate; FAD: flavin adenine dinucleotide; Gly: glycine; M: mitochondrium; Met: methionine; NAD: nicotinamide adenine dinucleotide; NADP: nicotinamide adenine dinucleotide phosphate; Ser: serine; $tRNA_f^{Met}$: formyl-methionyl transfer RNA.

1.1.6 Biological functions of folates

Adequate folate intake is crucial for cell division and homeostasis (13). Folates act as essential coenzymes in many biological pathways including purine and thymidylate biosynthesis, deoxyribonucleic acid (DNA) methylation (61), and amino acid metabolism (13).

1.1.6.1 Folate metabolism

The folate metabolism intersects with the Met cycle and the choline pathway (**Figure 4**). Apart from DNA synthesis, important functions are the methylation of Hcy and the formation of *S*-adenosyl methionine (SAM), which is the most important methyl donor in various reactions, including DNA methylation – important in cellular differentiation and genomic imprinting. Folate metabolism is mostly located in hepatocytes (hepatic folate levels range from 10 – 35 µmol/L).

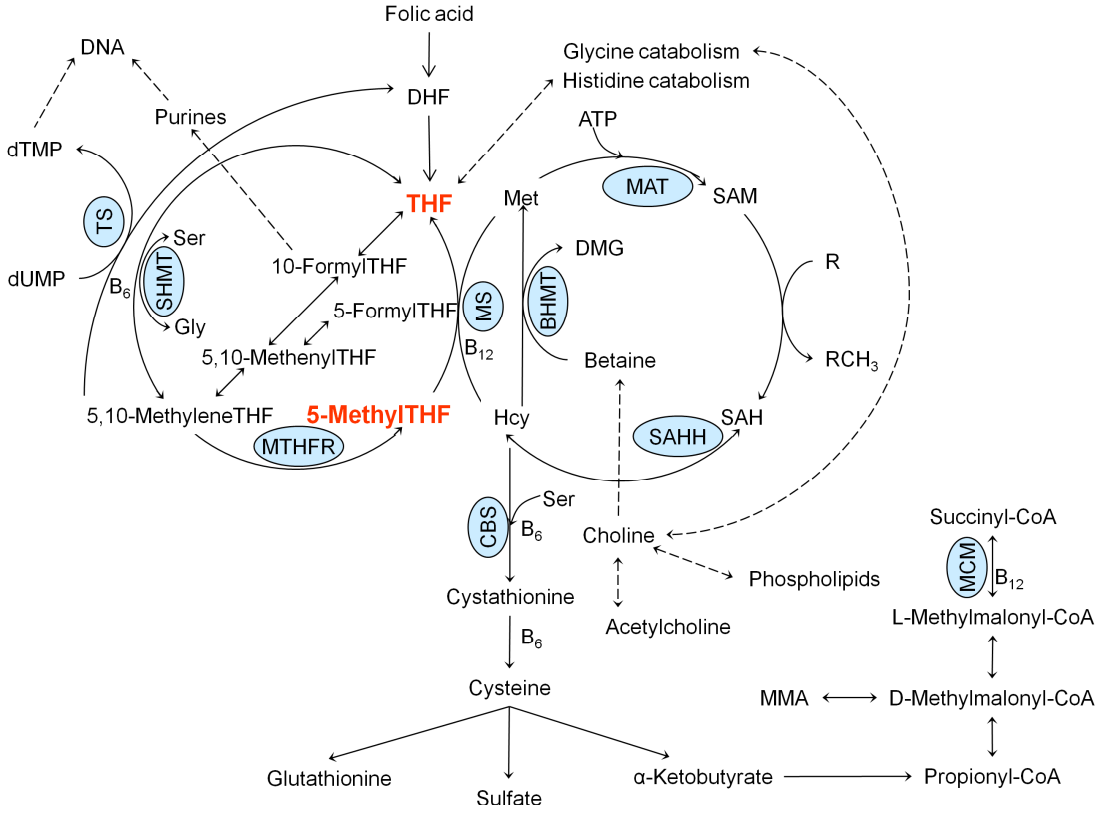

Figure 4: Folate, methionine, and choline metabolism. THF is a key folate and 5-methylTHF links folate and methionine cycles. BHMT: betaine homocysteine methyltransferase; CBS: cystathionine-*β*-synthase; DMG: dimethylglycine; dTMP: deoxythymidine monophosphate; dUMP: deoxyuridine monophosphate; Gly: glycine; MS: methionine synthase; MAT: methionine adenosyltransferase; MCM: *L*-methylmalonyl CoA mutase; MMA: methylmalonic acid; MTHFR: 5,10-methyleneTHF reductase; SAH: *S*-adenosyl homocysteine; SAHH: *S*-adenosyl homocysteine hydrolase; SAM: *S*-adenosyl methionine; Ser: serine; SHMT: serine hydroxy methyltransferase; TS: thymidylate synthase.

1.1.6.2 Tetrahydrofolate – the active form of cellular folate

THF is the active form of folate and is build from DHF in a reaction catalyzed by DHFR. The same enzyme catalyzes the formation of DHF from FA. THF acts as one-carbon acceptor and serine, glycine, betaine, and formate are the principle one-carbon donors. After attachment of one-carbon units to THF the newly developed folate form contributes in folate metabolism. The cytosolic folate metabolism consists of three interconnected cycles. One cycle starts from 10-formylTHF and produces purines, two cycles use 5,10-methyleneTHF to form deoxy thymidine monophosphate (dTMP) and Met. In all three cycles THF is returned to the folate pool where in the next step one-carbon units are added from three sources. First, formate comes from mitochondria and cytosolic sources to form 10-formylTHF. Second, serine is added from the cytosolic serine hydroxy methyltransferase (SHMT; EC 2.1.2.1) to form 5,10-methyleneTHF. Third, the C-2 of histidine originates from *N*-formiminoglutamic acid (FIGLU) and forms 5,10-methenylTHF (46).

1.1.6.3 5-MethylTHF – the predominant folate form

5-MethylTHF is the predominant folate form, comprising 82 – 93% of the sum of folates in the human blood (178). After the cellular uptake, 5-methylTHF is converted into THF. This conversion reaction is carried out by the vitamin B_{12}-dependent Met synthase (MS; EC 2.1.1.13). MS links the folate and the Met cycles and represents the only enzyme utilizing 5-methylTHF as a substrate. The Met cycle is an important pathway for the conversion of Hcy to Met and the formation of SAM. Elevated serum concentrations of total Hcy (tHcy) can be caused by deficiencies of B-vitamins (folate, vitamin B_6, and B_{12}) or genetic defects (29). 5-MethylTHF is generated from 5,10-methyleneTHF by the flavoprotein 5,10-methyleneTHF reductase (MTHFR; EC 1.5.1.20) in an irreversible and $FADH_2$ and NADPH-dependent reaction (81) (**Figure** 5).

The so called “methyl trap hypothesis” explains why vitamin B_{12} deficiency often results in a functional folate deficiency (90). The vitamin B_{12}-dependent enzyme MS is inactive in vitamin B_{12} deficiency or after exposure to nitrous oxide (35). Because of the irreversible reaction of the enzyme MTHFR folate is “trapped” as 5-methylTHF and can neither be converted to THF via MS nor back to 5,10-methyleneTHF via MTHFR. This results in the trap of the cellular folates in the 5-methylTHF form. As 5-methylTHF is a poor substrate for FPGS, the polyglutamate synthesis ceases, which limits the pool of monoglutamates (197). The *de novo* synthesis of purines and thymidylates grinds to a halt (211). Vitamin B_{12} supplements can terminate the inhibition of MS and the folate cycle will not be interrupted.

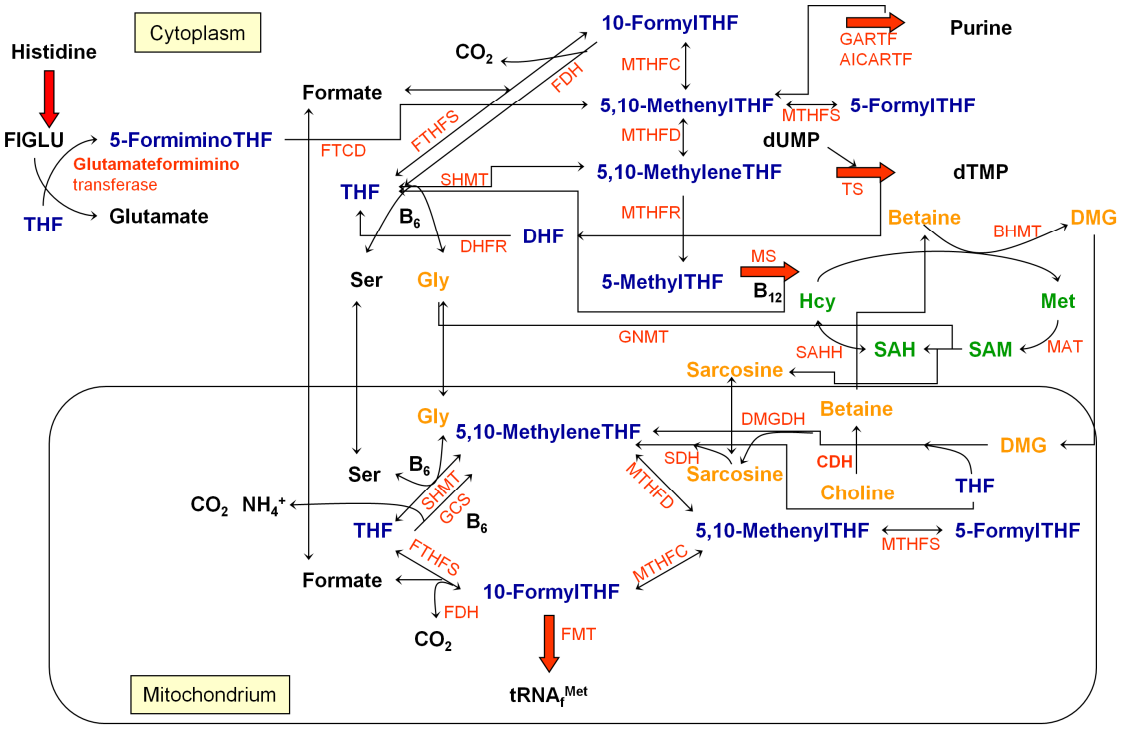

Figure 5: Folate metabolism in a hepatic cell with intersecting homocysteine and choline cycles. AICARTF: 5-aminoimidazole-4-carboxamide ribonucleotide transformylase; BHMT: betaine homocysteine methyltransferase; CDH: choline dehydrogenase; DMG: dimethylglycine; DMGDH: dimethylglycine dehydrogenase; dTMP: deoxythymidine monophosphate; dUMP: deoxyuridine monophosphate; FDH: 10-formylTHF dehydrogenase; FIGLU: formimino glutamate; FMT: methionyl-t-RNA formyl transferase; FTCD: 5-formiminoTHF cyclodeaminase; FTHFS: 10-formylTHF synthase; GARTF: glycinamide ribonucleotide transformylase; GCS: glycine cleavage system; Gly: glycine; GNMT: glycine-*N*-methyltransferase; MAT: methionine adenosyltransferase; MS: methionine synthase; MTHFC: 5,10-methenylTHF cyclohydrolase; MTHFD: 5,10-methyleneTHF dehydrogenase; MTHFS: 5,10-methenylTHF synthase; SAHH: *S*-adenosyl homocysteine hydrolase; SDH: sarcosine dehydrogenase; SHMT: serine hydroxy methyltransferase; $tRNA_f^{Met}$: initiator transfer RNA; TS: thymidylate synthase.

1.1.6.4 DNA synthesis

Purine and thymidylate syntheses involve five enzymes. SHMT provides 5,10-methyleneTHF. 5-Aminoimidazole-4-carboxamide ribonucleotide transformylase (AICARTF; EC 2.1.2.3) and glycinamide ribonucleotide transformylase (GARTF; EC 2.1.2.2) are required for the purine synthesis. The thymidylate synthase (TS; EC 2.1.1.45) and the DHFR are needed for the thymidylate synthesis. The vitamin B_6-dependent enzyme SHMT catalyzes the reversible and simultaneous conversions of serine to glycine and THF to form 5,10-methyleneTHF (191;211). This reaction introduces the *β*-carbon of serine into the one-carbon pool at the formaldehyde level and is an important step in the one-carbon metabolism (145). The produced 5,10-methyleneTHF is crucial for many other reactions in folate metabolism (82), including the function as substrate for TS and 5,10-methyleneTHF dehydrogenase (MTHFD; EC 1.5.1.5, which is important for nucleotide synthesis.

TS catalyzes the conversion of deoxyuridine monophosphate (dUMP) to dTMP, transferring formaldehyde to 5´-position of the dUMP. This is a key reaction in the thymidylate synthesis and limits the entire DNA synthesis. The allosteric enzyme DHFR inhibits MTHFR. This ensures that the priority lies with nucleotide biosynthesis instead of Met formation (145). Both, TS and DHFR are important targets in anti-cancer therapy. Antifolates, such as methotrexate blocks DHFR. Pemetrexed and raltitrexed inhibit TS, causing an arrest of thymidylate synthesis, of DNA replication, and leads eventually to cell death. In case of DHFR inhibition, the active key folate THF can not be regenerated from DHF. Therefore important reactions in folate metabolism are reduced or inhibited likewise resulting in cell death or an arrest of cell proliferation.

Initiation of translation of protein biosynthesis requires a formylated methionyl tRNA ($tRNA_f^{Met}$) species (234). The formylation of $tRNA^{Met}$ is catalyzed by the mitochondrial enzyme methionyl $tRNA_f^{Met}$ formyltransferase (FMT; EC 2.1.2.9) in a reaction that requires 10-formylTHF as a formyl-donor (234). In this process 10-formylTHF is converted to THF.

1.1.6.5 Methionine metabolism

Met is an essential proteinogenic amino acid that can not be synthesized by humans. Since the translation start codon is always AUG, Met is used in all proteins as first amino acid at the *N*-terminus where it is usually processed in subsequent steps or split off. Hcy is a non-proteinogenic amino acid. The single source of Hcy in the body is the demethylation of Met via SAM and *S*-adenosyl homocysteine (SAH). In the liver, three overlapping pathways use Hcy as a substrate: the remethylation pathway via 5-methylTHF as a methyl donor, the remethylation pathway via betaine as a methyl donor, and the transsulfuration pathway. The latter irreversibly cleaves Hcy first to cystathionine (Cys) and then to cysteine. This happens in a vitamin B_6 and serine-dependent reaction using the enzymes cystathionine-β-synthase (CBS; EC 4.2.1.22) and cystathionine-γ-lyase (CGL; EC 4.4.1.1) (73) (**Figure 4**). Betaine-dependent remethylation and transsulfuration occur mainly in liver and kidney (73). The transsulfuration pathway may be limited in brain (73), as CBS is expressed but CGL is scarce (235).

The first step in folate-dependent remethylation is the transfer of the methyl group of 5-methylTHF to the cofactor vitamin B_{12} by MS, producing methylcobalamin. Second, the methyl group is transferred from methylcobalamin to Hcy, forming Met and THF (200). Met is further converted to SAM by the ATP-dependent methionine adenosyltransferase (MAT; EC 2.5.1.6). After the transfer of the methyl group, the demethylated product of SAM, SAH, can be further hydrolyzed to Hcy and adenosine in a reversible reaction that is catalyzed by the SAH hydrolase (SAHH; EC 3.3.1.1). This reaction favors SAH formation in case of HHCY (51).

1.1.6.6 Role of betaine as an alternative methyl donor

Choline is an essential nutrient and plays a vital role as component of several major phospholipids such as phosphatidylcholine and sphingomyelin, which are important in cell membrane lipids, lipoproteins, bile lipids, and lung surfactants (252). Minor choline amounts are used to produce the neurotransmitter acetylcholine (59). The choline pathway intersects with the Met metabolism and choline acts as a precursor of betaine. Betaine is a major osmolyte.

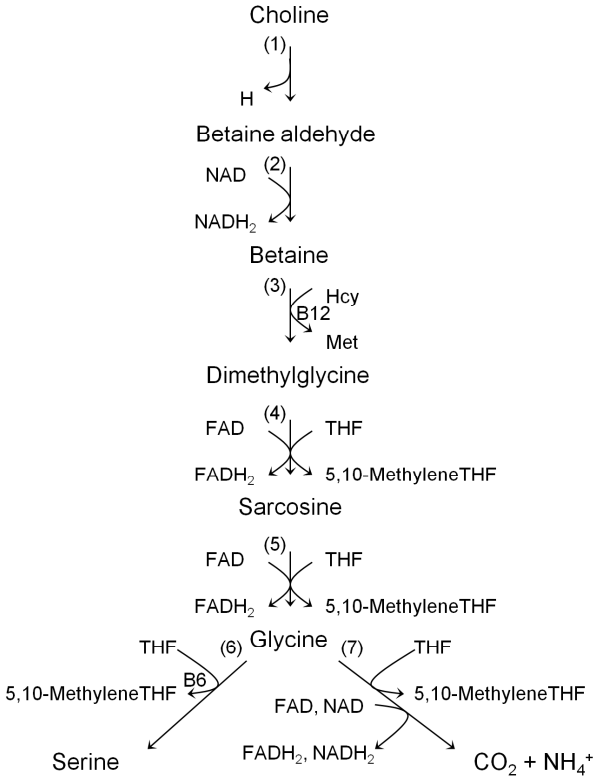

Figure 6: Choline oxidation pathway. Except from conversion from betaine to DMG (remethylation of Hcy), all steps are carried out in mitochondria of liver and kidney. 1: choline dehydrogenase (CDH); 2: betaine aldehyde dehydrogenase 3: betaine homocysteine methyltransferase; 4: dimethylglycine dehydrogenase (DMGDH); 5: sarcosine dehydrogenase (SDH); 6: serine hydroxy methyltransferase (SHMT); 7: glycine cleavage system (GCS). FAD: flavin adenine dinucleotide; NAD: nicotinamide adenine dinucleotide.

In mitochondrial choline metabolism THF receives an one-carbon unit forming 5,10-methyleneTHF (**Figure 6**). The choline oxidation pathway involves five enzymes to eventually form glycine from choline (enzymes 1 – 5 in **Figure 6**): choline dehydrogenase (CDH; EC 1.1.99.1), betaine aldehyde dehydrogenase (BADH; EC 1.1.1.8), betaine homocysteine methyltransferase (BHMT; EC 2.1.1.5), dimethylglycine dehydrogenase (DMGDH; EC 1.5.99.2), and sarcosine dehydrogenase (SDH; EC 1.5.99.1) (80). Except from BHMT, all enzymes are localized in the mitochondria of liver and kidney.

The choline oxidation comprises the conversion of choline to betaine aldehyde, which is mediated by CDH (138) (**Figure 6**). Betaine is formed in a subsequent step by BADH in mitochondria and then in cytoplasm. Betaine leaves the mitochondria and acts as an alternative methyl donor for Hcy where cytosolic BHMT catalyzes the betaine conversion to dimethylglycine (DMG). DMGDH transfers a methyl group from DMG to THF, generating sarcosine and 5,10-methyleneTHF (145). Sarcosine is methylated to glycine using SDH (16). SHMT catalyzes the simultaneous and reversible conversion of glycine and serine. The terminal glycine cleavage is tissue specific and is performed by the multi enzyme glycine cleavage system (GCS; EC 1.4.4.2, EC 2.1.2.10, EC 1.8.1.4).

1.1.6.7 Methylation reactions – the roles of S-adenosyl homocysteine and S-adenosyl methionine

SAM is the major methyl donor in all living organisms (42). Creatine synthesis is the major user of methyl groups from SAM, utilizing more SAM than all of the other methyltransferases combined, resulting in 75% of the Hcy formation (163;164). SAM is important for the methylation of DNA, as 5% of cytosines in the genome are converted by DNA methyltransferase to 5-methylcytosine (62). The binding of transcription factors is inhibited when the methylation of cytosine occurs in CpG islands in the promoter region (106). This leads to the turn-off of genes. SAM is needed for the methylation of histones, which acts as another epigenetic regulatory mechanism. SAM is necessary for the conversion of phosphatidylethanolamine into phosphatidylcholine, which is catalyzed by the liver specific phosphatidylethanolamine-*N*-methyltransferase (PEMT; EC 2.1.1.17) and requires three molecules of SAM. SAH is an effective competitive inhibitor for most cellular methyltransferases by binding to the active site of the enzymes. Under physiological conditions, SAH is hydrolyzed to Hcy. Elevated Hcy concentrations reverse the reaction. The SAM/SAH ratio is considered as an indicator for the cellular methylation capacity and is named the “methylation index”. Maintenance of the methylation index is important for the regulation of the enzymatic activity.

For the regulation of the methylation index, the enzyme glycine *N*-methyltransferase (GNMT; EC 2.1.1.20) is important. GNMT catalyzes the synthesis of sarcosine and SAH from SAM and glycine. The enzyme binds 5-methylTHF as endogenous ligand but catalyzes the reaction of sarcosine to glycine without participation of the folate (238). SAM regulates the MTHFR activity (high SAM levels downregulate MTHFR) by binding to an allosteric site of the enzyme (132). The inhibition can be reversed by SAH (72). Furthermore, SAM activates the CBS (196). At low SAM levels, 5-methylTHF production can occur without restrictions while increased SAM levels promote the transsulfuration of Hcy (73).

1.1.6.8 Role of B-vitamins (vitamins B_2, B_6, and B_{12}) as cofactors

For a proper function of the one-carbon metabolism the appropriate intake of B-vitamins (FA, vitamin B_6, vitamin B_{12}, and vitamin B_2) is crucial. Vitamin B_2 (riboflavin) serves as cofactor in the metabolism of fats, amino acids, carbohydrates, and vitamins (e.g. vitamin B_6). Vitamin B_2 deficiency manifests in mouth ulcers, dry and scaling skin, and cracked and red lips. In one-carbon metabolism, vitamin B_2 is required as a cofactor for the MTHFR enzyme, a flavoprotein. The coenzymic form of vitamin B_2 is FAD, its loss as a cofactor in *MTHFR 677 TT* polymorphism is responsible for the lower enzyme function. Vitamin B_2 serves as cofactor for the methionine synthase reductase (MTRR; EC 1.16.1.8), which activates the MS.

The water-soluble vitamin B_6 (pyridoxine, pyridoxal, pyridoxal-5-phosphate, etc.) functions as a coenzyme in more than 50 reactions, including the amino acid metabolism, the synthesis of neurotransmitters (serotonin, epinephrine, norepinephrine, and γ-aminobutyric acid), and of amine products (histamine). It plays an important role to reduce oxidative stress and affects cell proliferation. Vitamin B_6 deficiency manifests in various clinical conditions, e.g. seborrhoeic dermatitis, conjunctivitis, and neuropathy. In one-carbon metabolism, vitamin B_6 is necessary for many interconversions of folates and is a cofactor for CBS and CGL.

Table 4: RDA reference values for B-vitamins (DGE).

Vitamin	Males	Females
RDA reference values		
B_2	1.5 mg/d (15 – 25 years) 1.4 mg/d (25 – 51 years) 1.3 mg/d (51 – 65 years) 1.2 mg/d (> 65 years)	1.2 mg/d (adults) 1.5 mg/d (pregnant women (> 4th pregnancy month)) 1.6 mg/d (lactating women)
B_6	1.5 mg/d (adults)	1.2 mg/d (adults) 1.9 mg/d (pregnant and lactating women)
B_{12}	3 µg/d (adults)	3 µg/d (adults) 3.5 µg/d (pregnant women (> 4th pregnancy month)) 4.0 µg/d (lactating women)
Normal plasma concentrations[a] (67)		
B_2	19.2 (9.5 – 71.3) nmol/L	21.8 (10.9 – 77.0) nmol/L
B_6	69.5 (33.5 – 190.5) nmol/L	60.5 (31.1 – 288.5) nmol/L
B_{12}	274 (154 – 467) pmol/L	303 (170 – 517) pmol/L

[a]: The data are medians (5th – 95th percentiles). The normal plasma concentrations were determined using n = 1,105 males and n = 1,213 female.

Vitamin B_{12} (cobalamin) exists in many chemically related compounds (cyanocobalamin, methylcobalamin, adenosylcobalamin, etc.) and is essential for the normal brain function, DNA synthesis, and erythropoiesis. In humans, two vitamin B_{12} coenzyme-dependent enzyme reactions exist: MS and methylmalonyl-CoA mutase (MCM; EC 5.4.99.2; isomerization of methylmalonyl-CoA to succinyl-CoA) (**Figure 4**). In case of vitamin B_{12} deficiency, both

reactions are decelerated or stopped, leading to elevated tHcy and methylmalonic acid (MMA) concentrations. MMA is therefore a sensitive and specific marker for vitamin B_{12} deficiency. In addition, vitamin B_{12} deficiency results in the methyl trap of folate and can produce abnormalities in both DNA methylation and uracil incorporation (141). In **Table 4**, RDA reference values in conformity with the "Deutsche Gesellschaft für Ernährung" (DGE) are presented. Additionally, normal plasma concentrations of vitamins B_2, B_6, and B_{12} determined in a case-control study within the European Perspective Investigation into Cancer and Nutrition study are shown (67).

1.2 Clinical relevance

One-carbon metabolism can be impaired due to low folate concentrations, causing several clinical conditions. In addition, it can be disrupted by B-vitamins deficiencies other than folate and genetic mutations or polymorphisms. Folate deficiencies manifest in megaloblastic anemia (89). Low folate concentrations are associated with the development of NTDs (162) and may cause certain types of cancer (114;202) and neurocognitive dysfunction (147;169). The accumulation of tHcy is associated with many pathologic conditions (91).

1.2.1 Health importance of folate and folate deficiency

In countries without mandatory FA fortification, adequate dietary intake of folates and other vitamins is crucial. Flynn *et al.* summarized the intake of selected nutrients from foods, supplementation, and fortification in European countries (74). In Germany, the folate intake in adults from diet and diet plus supplements was below the recommended 400 µg/d. During 1997 – 1999 the mean daily folate intake in Germany of 2,267 adult women without supplementation was 241 µg, with supplementation 280 µg, and in 1,763 adult men 288 µg and 307 µg, respectively (74). This shows that deficiencies in folate are common even in wealthy countries.

Folate deficiencies are associated with megaloblastic anemia and development of NTDs. Moreover, low folate levels are associated with vascular disease via its influence on tHcy levels. Some studies found an inverse relationship between folate status and coronary heart disease (185;236). Most prospective cohort studies have found inverse associations between the folate status and stroke or other cerebrovascular outcomes (14;86). Merchant *et al.* found an inverse relationship between total folate intake and risk of peripheral arterial disease (156). After 12 years of follow-up, men with a mean folate intake of 840 µg/d had a ~ 30% lower risk (risk ratio = 0.67) of peripheral arterial disease compared to men with a mean folate intake of 244 µg/d. In addition, low folate status and resulting higher tHcy levels are associated with a higher prevalence of cognitive impairment or Alzheimer's disease (172).

Associations of folate intake and cancer risk, which are considered the result of genetic and epigenetic changes (e.g. global hypomethylation or promoter hypermethylation) have been studied extensively. The one-carbon metabolism is therefore capable of influencing the pathogenesis of human cancers. The folate metabolism is also a potential target for chemotherapy (antifolate treatment). Evidence indicates significant inverse associations between dietary folate intake and risk of breast cancer indicating a protective effect of the vitamin (135). However, one has to bear in mind that folate may provide protection early in carcinogenesis but it may promote carcinogenesis if administered later and at very high doses.

Folate deficiencies are often accompanied by the accumulation of tHcy, which causes HHCY (77). HHCY is classified into three types: moderate HHCY (tHcy = 12 – 30 µmol/L), intermediate HHCY (tHcy > 30 – 100 µmol/L), and severe HHCY (tHcy > 100 µmol/L) (111). Moderate HHCY is associated with several pathologic conditions, including cardiovascular and neurodegenerative diseases (91). Apart from oxidative stress, another main pathomechanism in HHCY is hypomethylation (30). Elevated tHcy concentrations can be lowered by supplementing low doses of FA in healthy subjects (27). In a meta-analysis, the Homocysteine Lowering Trialists' Collaboration stated that daily doses of ≥ 800 µg FA are required to achieve the maximal reduction in plasma tHcy concentrations (100). Lower doses of 200 and 400 µg FA are associated with 60% and 90% tHcy lowering, respectively, of this maximal effect. However, the effect of tHcy lowering on pathologic conditions is ambiguous. On the one hand, tHcy lowering prevents stroke (189) and cardiovascular events in hemodialysis patients (184), on the other hand the risk of major cardiovascular events in patients with vascular disease could not been lowered (143), cognitive performance could not be improved in healthy subjects (150), and lower tHcy had no beneficial effect on inflammatory markers associated with atherosclerosis in patients with stable coronary artery diseases (22).

1.2.1.1 Folates in pregnancy

Only recently FA attracted attention as it became evident that it plays a decisive role in embryonic development. Maternal folate and micronutrient requirements increase during pregnancy due to fetal demands for growth and development (40). Moreover, an increased cell division is associated with the rapidly growing placenta and the expansion of maternal RBC number. Due to the higher demands of folate, pregnant women are at higher risk of developing folate deficiencies and megaloblastic anemia (245). Inadequate folate intake and low serum folate concentrations are associated with poor pregnancy outcomes. RBC folate is especially important during the pregnancy due to the fact that RBC production increases in the course of the blood volume expansion. Additionally, folates are actively transported to the fetus. Despite the fact that

the transport mechanism from maternal to fetal circulation is still unknown, participation of chorionic FRs is evident. 5-MethylTHF is rapidly bound to FRs in the placenta and transported to the intervillous blood. This allows for fetal folate accumulation against a concentration gradient and results in markedly elevated folate concentrations in fetal and newborn blood compared to maternal circulation (88).

Folate deficiency during pregnancy favors the occurrence of NTDs, which are congenital malformations of the brain and the spinal cord caused by the incomplete closure of the neural tube between the days 21 – 28. Defects vary from spina bifida to anencephaly and also can lead to premature births. NTDs have an incidence of > 300,000 new cases a year and over 95% of them are first occurrence (239). There is evidence that the folate concentrations are associated with the occurrence of orofacial clefts (10) and congenital heart defects (24). In addition, low maternal folate concentrations and pregnancy complications such as placental abruption, preeclampsia, and spontaneous abortions are likely to be associated. In 1970, the U.S. Food and Nutrition Board proposed a FA intake of 200 – 400 µg/d for pregnant women, leading to a significant decline in folate deficiency symptoms (45). But only in the early 1990s it became apparent that there is a correlation between maternal folate status and the incidence of fetal malformations, especially of NTDs (218). Studies demonstrated prevention up to three fourths of NTDs with FA (48;162). This led to a mandatory food fortification of grain products in some countries, which resulted in a significant decline of NTD incidence (140;144;182). For the prevention of NTDs supplementation with FA is especially recommended for women of childbearing age prior and in the first trimester of the pregnancy. However, even in the presence of FA supplementation and fortification NTDs continue to occur (94).

1.2.1.2 Folate and elderly health

B-vitamins deficiency is common in elderly people. This can be partly explained by lower intake or bioavailability of the vitamins or by reduced resorption. Folate deficiency is a risk factor for many age-related diseases such as stroke, dementia, cancer, and coronary vascular disease (91). Elevated plasma tHcy has been found in patients with Parkinson's disease (21). Recently, age-dependent decreases in DNA methyltransferase levels due to low dietary micronutrients including folate and Met were found (136). Hooshmand *et al.* found in a longitudinal study that both tHcy and holotranscobalamin (HoloTC) may be involved in the development of Alzheimer's disease (101). Mild cognitive impairment has a prevalence of 14 – 18% in adults over 70 years (176) and approximately half of them will develop Alzheimer's disease or other forms of dementia within 5 years (54). In a recent study with six weeks of FA supplementation in older adults (60 – 90 years) it could be demonstrated that supplementation will improve the folate

status and lower the tHcy concentration for adults with low serum folate levels (1). Moreover, tHcy-lowering by B-vitamins (800 µg FA, 500 µg cyanocobalamin, and 20 mg pyridoxine HCl) slows the rate of accelerated brain atrophy in mild cognitive impairment (205).

Malabsorption of food bound vitamin B_{12} is often based on an atrophic gastritis. HCl and pepsin synthesis are reduced but intrinsic factor, which is important for the receptor-mediated uptake of vitamin B_{12} is sufficiently produced. Once the intrinsic factor is not longer available in sufficient concentrations the receptor-mediated uptake by the enterocytes is limited. B-vitamins deficiencies are associated with elevated tHcy concentrations and therefore pose a higher risk of developing Hcy associated diseases. Plasma tHcy concentrations strongly depend on age. The age-related increase in tHcy concentration can be partly explained by the physiological decline in the renal function (92). The elevation of tHcy is linear until the age of 60 – 65 years and subsequently increases in speed. The average tHcy concentration increases about 10% or 1 µmol/L per decade (49).

1.2.2 Supplementation and fortification

Considering the low intake of folate from food and the risk that is associated with reduced folate status, it is not surprising that supplementation with FA is recommended. In some countries like the U.S., Canada, and Chile fortification of flour with FA is required by law to reduce the risk of NTDs. Already in 1992 the U.S. Public Health Service recommended that all women of childbearing age consume 400 µg FA daily to prevent the first occurrence of NTDs (32). Older people with reduced folate intake are in need of supplementation to achieve the RDA. Therefore, particularly pregnant women and older adults benefit from the mandated FA fortification programs. However, there is evidence that high intake of FA is associated with adverse effects.

1.2.2.1 Folate fortification – benefit and risk

Fortification is the process of increasing the level of nutrients, which are normally present within a food vehicle (e.g. grain flour). In 1996, the U.S. became the first country to mandate fortification of all enriched cereal grain products with FA (fortification level of wheat flour = 140 µg/100 g) (76). Canada (fortification level of wheat flour = 150 µg/100 g) followed closely, then Costa Rica (180 µg/100 g), and Chile (220 µg/100 g). Up to date, more than 50 countries apply mandatory flour fortification programs but Germany is not among them (33). Supplemented or fortified FA is metabolized by the activity of DHFR, which has low affinity for FA. Therefore, the reduction of FA to DHF is very slow.

Clinical relevance

FA fortification policies resulted in a reduction of prevalence rate of NTDs and an increase of FA intake and blood folate concentrations (53;144). In the U.S. the National Health and Nutrition Examination Survey (NHANES) study series observed in adult non-supplement users a mean serum folate concentration of 11.4 nmol/L before (NHANES III, n = 9,430) and 26.9 nmol/L after (NHANES 1999 – 2000, n = 1,978) FA fortification (57), the mean RBC folate level increased from 375 nmol/L before (n = 9,438) to 590 nmol/L after (n = 2,007) fortification. In the Framingham Offspring Study cohort, non-supplemented participants showed a reduced incidence of abnormally low plasma folate (< 7 nmol/L) from 22% to < 1.7% and a reduced incidence of high tHcy concentrations from 18.7 – 9.8% after the fortification (112).

The safe upper limits for both folate intake and blood folate concentrations are still unknown (204). Apart from the beneficial effect of FA supplementation and fortification, in the past years suspicion arose about potential adverse effects of consumption of FA. The main targets for FA fortification are women of childbearing age. Therefore, question emerged if it is justifiable to expose the entire population to mandatorily fortified foods (173). Folate-dependent enzymes have different specificities for free FA than for naturally folates. Especially the FR develops an extremely tight binding with FA, which could interfere with the transport of other folates. High intake of FA might mask vitamin B_{12} deficiency, especially in older adults because of the high prevalence of vitamin B_{12} malabsorption (198). High plasma folate levels were associated with an increased risk of developing cardiovascular diseases (23), premenopausal (139) and postmenopausal (213) breast cancer, and colorectal cancer (228). In addition, high folate status promotes the progression of already existing preneoplasms (43). As 37.5% of the individuals > 50 years have colorectal adenomas, fortification with FA might pose a great risk (137).

1.2.2.2 Unmetabolized folic acid

High oral doses of FA from supplements or fortified foods have been shown to bypass the normal folate absorption mechanisms, which results in the presence of unmetabolized FA in serum (103). Unmetabolized FA poses risks in the diagnosis of vitamin B_{12} deficiency and the efficiency of chemotherapy based on antifolates, which limits the folate availability to tumor cells (204). In addition, there is scarce information dealing with the effect of long-term exposure to FA. One study measured lower natural killer cell cytotoxicity with increased FA concentration in plasma (> 3 nmol/L) in women > 60 years (225).

In the Framingham Offspring Cohort study unmetabolized FA in non-vitamin users before (55.0%, n = 705) and after (74.7%, n = 355) fortification, as well as in B-vitamins users (72.5%, n = 398 before fortification vs. 80.7%, n = 245 after fortification) was found (117). After

fortification, 19.1% of the non-vitamin users and 24.3% of the vitamin users had high plasma FA levels ≥ 1.35 nmol/L, with a positive association of FA intake and FA plasma concentration (117). Obeid *et al.* found in a randomized, double-blind, placebo-controlled supplementation study (n = 74 older adults, median age = 82 years, 63 women) that 19% of the participants had detectable amounts of unmetabolized FA (> 0.2 nmol/L) in plasma at baseline (168). After 3 weeks of supplementation with high doses of B-vitamins (5 mg FA, 2 mg cyanocobalamin, and 40 mg vitamin B_6) the median FA concentration increased from 0.08 nmol/L to 15.3 nmol/L. This was associated with higher concentrations of THF and 5-methylTHF and lower tHcy levels, suggesting the effective reduction and conversion of FA into active folate forms.

As FA supplementation is important during the pregnancy, pregnant women and their children are at risk of unmetabolized FA in the circulation. Sweeney *et al.* found unmetabolized FA in 17 of 20 placental cord blood samples, as well as in 18 of the corresponding pregnant women at delivery (217). Obeid *et al.* reported in a study with 87 pregnant women unmetabolized FA in 43.6% of the subjects and in 55% of umbilical cord serum samples (n = 29) (170). In contrast to 5-methylTHF and THF, concentrations of FA were not higher in cord than in maternal blood, suggesting that FA is not accumulated in the fetus.

1.2.3 Polymorphisms in the MTHFR gene

Folate metabolism and the interconnecting Met metabolism require the contribution of a number of enzymes and vitamin cofactors. Effects of gene-gene and gene-nutrient interactions have been reported for serum folate and serum tHcy concentrations (250). Several genetic polymorphisms for instance hundreds of single nucleotide polymorphisms (SNPs) have been identified. Some are demonstrated or suspected to be associated with HHCY or may play a role in altering the DNA methylation reactions.

The most common mutation in the *MTHFR* gene is a C → T substitution in exon 4 at bp 677. This causes a substitution of valine for alanine and results in a thermolabile variant of the enzyme activity at 37°C (79). The heterozygous *CT* genotype is associated with a 30% reduction and the homozygous *TT* genotype with a 50 – 70% reduction in the enzyme activity, which decreases the conversion of 5,10-methyleneTHF to 5-methylTHF (79;211). The *TT* variant is present in about 10 – 20% while the heterozygous genotype amounts approximately 40% of the population. As MTHFR is the sole producer of 5-methylTHF, polymorphisms may have a number of deleterious effects on the methylation cycle. Decreased 5-methylTHF and increased formylTHF in RBCs have been reported in *TT* individuals (12). The *TT* mutation causes elevated tHcy levels and leads to alterations in the intracellular folate distribution, especially in the absence of adequate folate

availability (79). Differences in tHcy concentrations between individuals with *CC* and *TT* genotype have been shown to increase as the folate concentrations decrease and may only be significant at low folate concentrations. At low folate concentrations, deficiencies of vitamin B_{12} have also been linked to elevated tHcy concentrations in *TT* individuals (250). In addition, some studies associate polymorphisms of *MTHFR C677T* with increased risks for cardiovascular disease (128), NTDs (174), as well as malignant tumors susceptibility (127).

The second common polymorphism in the *MTHFR* gene is the A → C substitution at bp 1,298. This SNP results in a glutamate to alanine substitution and to a 50 – 60% decreased enzyme activity but without significant differences in tHcy or folate plasma levels (227). The prevalence of this polymorphism ranges in Europe from 6 – 11% (39). Individuals with *CC* genotypes have only 60 – 70% of the *MTHFR 1298* activity compared to the *AA* genotype (243). The majority of studies concluded that the *MTHFR A1298C* polymorphism is not an independent risk factor for NTDs (227) or other birth defects such as congenital heart defects (232) or cardiovascular disease (85). The *MTHFR C677T* and the *A1298C* genotypes are in linkage disequilibrium. The *677T* and the *1298C* alleles are rarely found in the *cis* configuration.

1.3 Quantitative folate determination

The accurate measurement of folate forms is essential for monitoring the folate intake and the evaluation of the folate status. Therefore serum and plasma, as well as whole blood (WB) folates are determined. The quantification of folate forms from various sample material is challenging. First methods, including the microbiological and the protein-binding assay only determine the total folate (TFOL) of the sample and are unable to distinguish between the different folate forms. To this date, even chromatographic methods can separate and quantify some but not all folate vitamers. There are several difficulties of developing an appropriate quantification method. First, the number of possible folates (oxidation states and number of glutamate residues) is quite large. Second, the matrix of the sample material (biological samples or foods) is complex. Third, reduced folates are unstable and tend to undergo interconversions. Forth, folates are bound to binding proteins. In biological samples folates from serum and plasma are easier to determine because they consist exclusively of monoglutamates. In addition, the maintenance of polyglutamate chains of WB folate results in complex method outputs. Therefore, the folylpolyglutamates are often deconjugated to their monoglutamate forms by either endogenous or added conjugase. Prior this step, WB folates must first be extracted from the cells by hemolysis. There is high variability of WB folate forms depending on the incubation time, temperature, and pH during hemolysis (179). As a result, hemolysis can be incomplete, or folates can be trapped in the hemoglobin molecule (248).

1.3.1 Folate stability

Reduced folate forms are known to be sensitive to heat, pH, oxidation, pressure, and ultraviolet light (115). Cleavage occurs at the C-9-N-10-bond of the molecule. The major degradation products are *p*-aminobenzoyl-*L*-glutamic acid (*p*ABG) and pteridine fragments; both are no longer coenzymes available for one-carbon metabolism (63). THF, DHF, and 10-formylTHF are especially unstable *in vitro* and readily undergo oxidative degradation.

In addition to degradation, reduced folates easily undergo enzymatic and non-enzymatic interconversions, which are pH-dependent. Especially 5-formylTHF, 10-formylTHF, and 5,10-methenylTHF demonstrate complex interconversion reactions under different pH conditions. De Brouwer *et al.* summarized the pH and heat stability of individual folate forms in *in vitro* experiments (50) (**Figure 7**). FA and 5-methylTHF are stable at pH 2 – 10. 5-FormylTHF is relatively stable at 37°C and pH 3 – 10 but becomes instable under acidic conditions (pH < 3) during heating. 10-FormylTHF can be oxidized to 10-formyl FA. 5,10-MethenylTHF is instable at pH values between 3 – 9. 5,10-MethyleneTHF loses under acidic conditions its one-carbon unit and converts into THF and formaldehyde. Under basic conditions and in the presence of formaldehyde, THF can convert back to 5,10-methyleneTHF. THF is relatively stable in acetic solution (pH < 5), whereas DHF is relatively stable at pH > 8. DHF is instable under all pH conditions after heating. Under low pH conditions, THF can *in vitro* be oxidized to DHF and FA. In order to stabilize the reduced folates in *in vitro* experiments, it is decisive to add adequate antioxidants such as ascorbic acid, *β*-mercaptoethanol, or dithiothreitol (DTT).

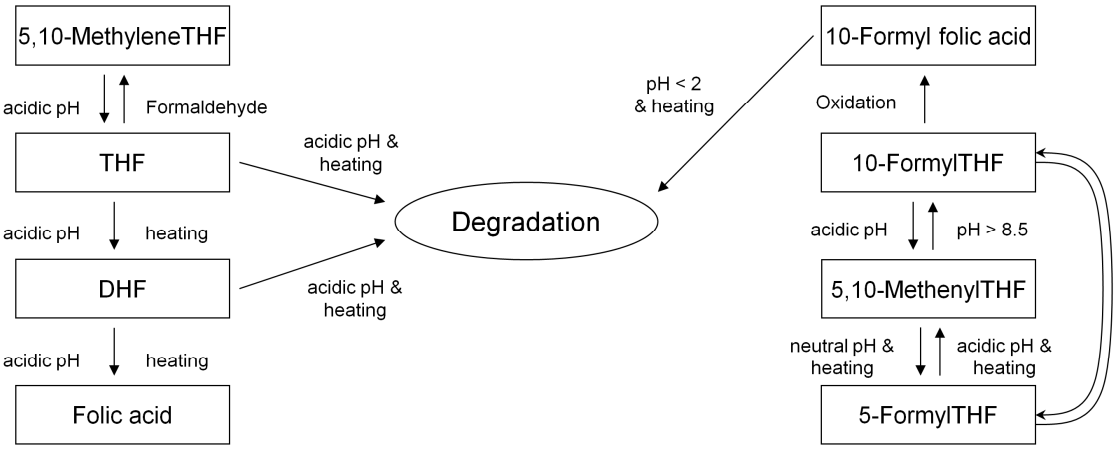

Figure 7: Stability of aqueous folic acid and folate solutions at different pH and temperature. Shown are the degradation and interconversion reactions of the folate coenzymes. Adapted from de Brouwer *et al.* (50).

1.3.2 Quantification methods

Folates in biological samples were analyzed over 50 years. First, the microbiological assay was introduced. This assay is based on the fact that a specific microorganism (*Lactobacillus rhamnosus*, formerly known as *L. casei*) grows proportionally to the available folate in the sample and is used for the determination of TFOL in serum, WB, and dried blood spots. *L. rhamnosus* is specific to biologically active folate with similar response to a variety of naturally occurring folate derivatives and does not respond to pteroic acid, breakdown products (e.g. *p*ABA), and folate stereoisomers. Next, the protein-binding assay was introduced. One example is the automated chemiluminescent immunoassay on the ADVIA Centaur platform (ADVIA Centaur XP System, Bayer Diagnostics, Leverkusen, Germany). This method consists of a competitive assay (FOL assay) using FBPs. This method depends on the release of the folate from its binding proteins. Prior measurement, samples are treated with DTT to release the folates from endogenous BPs. The released serum folate competes against acridinium ester-labeled FA for a limited amount of biotin-labeled FBP. The biotin-labeled FBP binds to avidin, which is covalently bound to paramagnetic particles in the solid phase. The amount of serum TFOL in the sample correlates inversely with the obtained signal.

Methods used for quantifying TFOL show large disagreements (158) and are unable to detect various forms of the vitamins. In the 1970s and 1980s, first chromatographic methods have been developed. These methods can distinguish between the different folate forms. Numerous HPLC (11;146) and gas chromatography (GC) (188;201) methods have been described for the detection of folate forms in serum and WB. Lately, liquid chromatography coupled with mass spectrometry (LC-MS) methods and liquid chromatography tandem mass spectrometry (LC-MS/MS) methods have been developed to quantify folate monoglutamates in biological fluids and foods (155;178;241). LC-MS/MS methods provide three levels of selectively: the chromatographic separation of the analytes, the mass-to-charge (*m/z*) ratio of ionized precursor molecules, and the transition of precursor to fragment ion. The functionality of LC-MS/MS, especially of ultra performance LC-MS/MS (UPLC-MS/MS), is explained in more detail in **Chapter 3.4.1**.

Modern LC-MS/MS methods for the determination of biological samples use more or less complex sample extraction and clean-up procedures from protein precipitation to solid phase extraction (SPE) with or without concentration of the analytes. They are capable to quantify several folate forms in the nanomolar range. The LC-MS/MS methods use a variety of monoglutamate or polyglutamate folate standards. In addition, stable isotope-labeled internal standards for most of the monoglutamate forms are available. The proper preparation and storage of the folate standards and internal standards are critical for the accurate measurement. The standard materials and samples should be kept frozen (≤ -70°C). The handling of the standards

and samples should be done under subdued light. Stock solutions and buffers should be prepared with degassed (N_2 or He) water to minimize the oxidation. The concentrations of stock solutions should be verified by spectrophotometry. Adequate amounts of antioxidants (10 g/L ascorbic acid and/or β-mercaptoethanol) are necessary in stock solutions to protect reduced folates from oxidation. Aliquots of lower concentrations should be prepared every month and once thawed they should be discarded after use.

2 Study aims

Folate deficiency is common in older adults, because of the low intake of the vitamin and compromised absorption or bioavailability. In addition, deficiencies of vitamin B_{12} and B_6 are also common in the elderly, suggesting that the folate metabolism might be sensitive to the deficiencies in other enzyme cofactors participating in folate cycle. Folate deficiency is a known risk factor for several age-related diseases such as stroke, dementia, cancer, and coronary vascular disease (91). In contrast, few studies support that increased folate and FA intake can be harmful (23;43;228). Folate has essential roles during cell growth and differentiation in the *de novo* synthesis of purines and thymidylates. Moreover, folates in the form of 5-methylTHF facilitate SAM synthesis, which is an important methyl donor for maintaining the methylation of cytosine.

Folate intake from natural sources is often below the RDA (74), suggesting that folate deficiency can be common even in a wealthy society. Folate fortification of grain products has been initiated in the U.S. in 1998 with the aim of reducing the incidence and recurrence of NTDs by ensuring a daily intake of folate of 400 µg for all women of child bearing age. Despite that, there has been some doubt about the application of folate fortification and exposure of the entire population to an increased intake of FA (173). Nevertheless, there have been no metabolic studies confirming the fate of supplemented FA in the cell. Furthermore, little is known about the metabolism of FA within the cell and the distribution of folate forms that promote the DNA synthesis. Moreover, it has not been tested whether subjects with polymorphisms in the *MTHFR* gene might show a different folate forms distribution after folate or B-vitamins supplementation. Because elderly people are at risk of developing cancer and other age-related diseases this population group is of special interest for the folate research.

Study aims

The aims of this study are:

The development of sensitive methods for the quantification of metabolites of folate and Met cycle using UPLC-MS/MS

- The development of a method for the quantification of folate forms in serum und WB samples.
- The development of a method for the quantification of methylation markers SAH and SAM.
- The development of a method for the quantification of alternative methyl donor betaine as well as choline and DMG.
- The determination of reference ranges for the new methods in healthy subjects.

The investigation of the folate forms distribution before and after short-term and long-term supplementation with B-vitamins in older adults in relation to the *MTHFR C677T* polymorphism

- The determination of the folate forms distribution in serum and WB before and after the short-term and long-term supplementation with B-vitamins at different doses.
- The determination of the influence of gender, age, and vitamin B_{12} status on the concentration and the distribution of folate forms.
- The determination of the influence of co-supplementation with vitamins B_6 and B_{12} on the folate forms distribution.
- The incidence of the *MTHFR C677T* polymorphism and the effects on the folate forms distribution in serum und WB before and after supplementation with B-vitamins.

3 Materials and methods

3.1 Study design

All studies were approved by the local ethics commission of the Saarland University Hospital and all participants signed informed consent documents. The studies were conducted at the Saarland University Hospital, Department of Clinical Chemistry and Laboratory Medicine – Central Laboratory, Homburg, Germany in collaboration with the rehabilitation center in St. Ingbert, Germany. Patient information sheets including consent form, a randomization sheet, and a questionnaire for telephone calls are summarized in **Appendix G**. Randomizations were performed at the Department of Medical Biometry, Epidemiology, and Medical Informatics of the Saarland University Hospital, Homburg, Germany. The trial (study I) was registered at clinicaltrials.gov as NCT01105351.

Study I: Short-term supplementation folic acid vs. folic acid plus vitamin B_6 and B_{12}

The aim of the study was to investigate the effect of low doses of oral FA supplementation with and without the co-supplementation of vitamin B_6 and vitamin B_{12} given over a period of several weeks on the folate forms and the polymorphisms in folate catabolizing enzymes (MTHFR) in elderly people (> 50 years). The study included two arms (T1: FA or T2: FA co-supplemented with vitamins B_6 and B_{12}) and was double-blind. Following approved supplements were applied: Folverlan 0.4 mg tablets (400 µg FA) and Medyn tablets (200 µg FA, 8 mg vitamin B_6, and 10 µg vitamin B_{12}) for the co-supplementation arm. The co-supplementation arm received additionally half a Folverlan tablet. Blood samples were collected under fasting conditions at baseline and at the end of the study (see **Figure 8**). The recruitment and the randomization of the volunteers were performed during August 2009 and June 2010. Inclusion criteria were: older adult Germans (> 50 years), performing several weeks of treatment in the rehabilitation center in St. Ingbert. Exclusion criteria were: renal dysfunction, recent stroke or coronary event within the last 3 months, current cancer, antifolate treatment, ileum resection, existing B-vitamins supplementation, and megaloblastic anemia. Termination criteria were: indication for a high-dose vitamin B, poor physical condition (e.g. heart attack, stroke), or surgical procedures during the study.

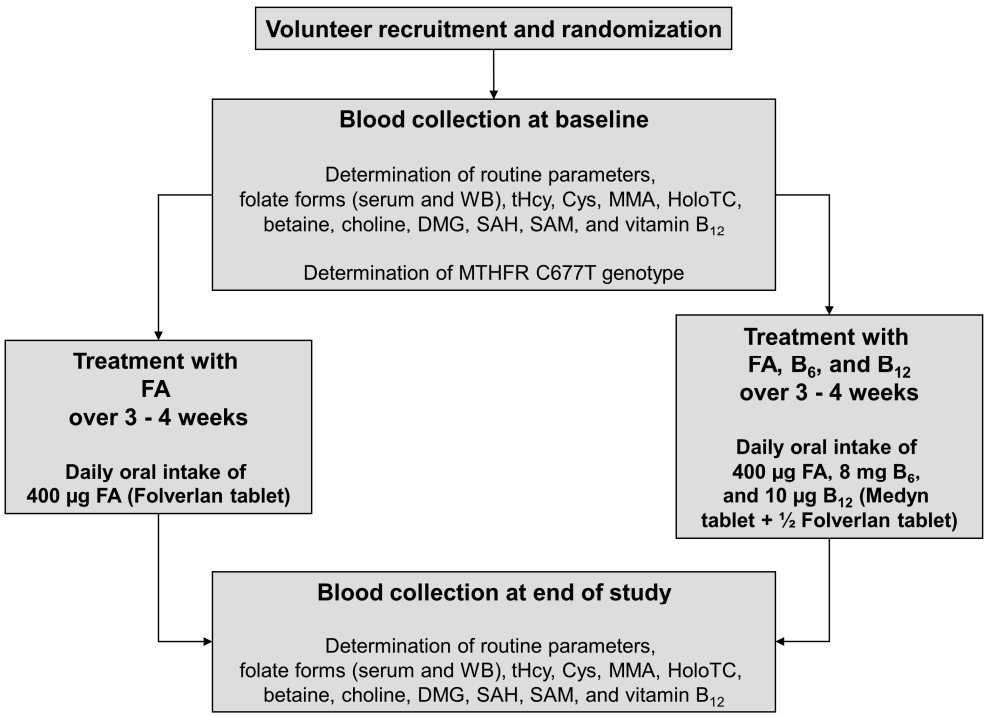

Figure 8: Study plan of the short-term supplementation study.

Study II: Long-term supplementation with folic acid, vitamin B_6, vitamin B_{12}, vitamin D, and calcium vs. vitamin D and calcium

Aim of the study was the supplementation of elderly subjects (> 50 years) with calcium and vitamin D in both study arms in addition to B-vitamins (FA, vitamin B_{12}, and vitamin B_6) over a period of 1 year in a randomized, and double-blind study (see **Figure 9**). Study participants' daily supplement intake contained 3 capsules: 1 green in the morning (group A: 500 µg FA, 50 mg vitamin B_6, 500 µg vitamin B_{12}, and 160 IU vitamin D, or group B: only 160 IU vitamin D), 1 white (228 mg calcium) at lunch, and 1 brown (228 mg calcium and 1,040 IU vitamin D) in the evening. The regular intake of the supplements was verified by telephone calls at intervals of 2 – 3 months. Blood was collected under fasting conditions at baseline, after 6 months, and at the end of the study (12 months). The recruitment and the randomization of the volunteers were performed during August 2009 and June 2010. Inclusion criteria were: older adult Germans (> 50 years). Exclusion criteria were: renal dysfunction, recent stroke or coronary event within the last 3 months, current cancer, antifolate treatment, ileum resection, existing B-vitamins supplementation, and megaloblastic anemia. Termination criteria were: indication for a high-dose vitamin B, poor physical condition (e.g. heart attack, stroke), or surgical procedures during the study.

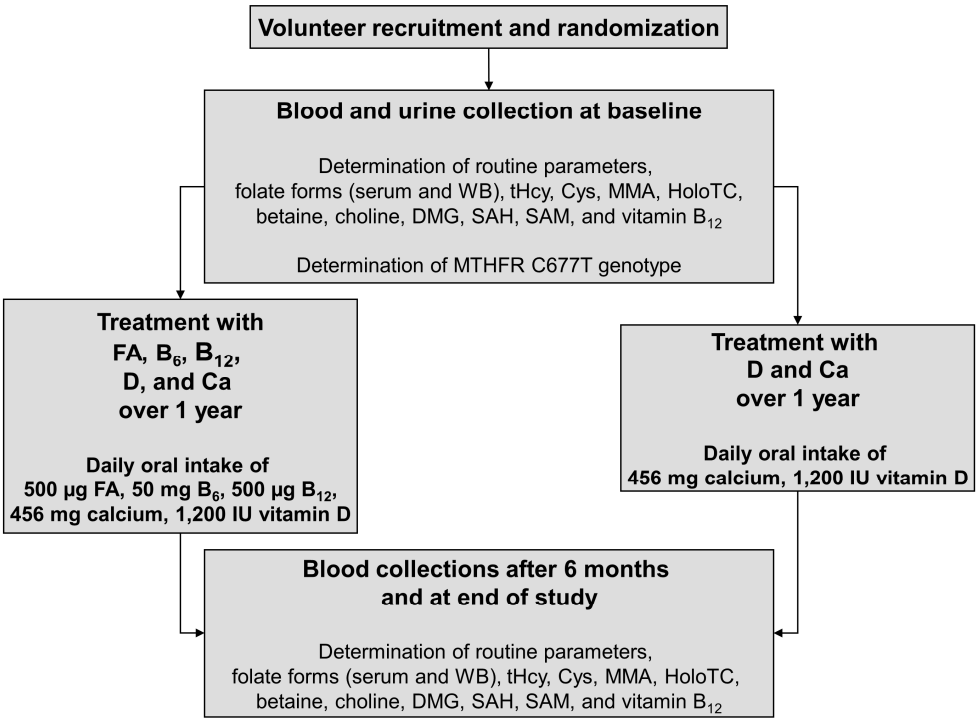

Figure 9: Study plan of the long-term supplementation study.

3.2 Sample collection

Blood samples from fasting subjects were collected by venous puncture either in vacutainer tubes containing an anticoagulant (EDTA (ethylenediaminetetraacetate), citrate, or lithium heparin) or in tubes without anticoagulant. For WB folate quantification, EDTA WB samples were immediately aliquoted and stored at -70°C until analysis. Serum samples were allowed to clot for 30 minutes at room temperature and were centrifuged at 2,000 x g and 4°C for 10 minutes. Plasma samples were centrifuged within 30 minutes at 2,000 x g and 4°C for 10 minutes. Serum and plasma were immediately separated, divided into multiple aliquots, and processed. For the SAH and SAM measurement, SAM has to be immediately stabilized by acidifying the sample to avoid the degradation to SAH. For this purpose, 500 µL EDTA plasma was acidified with 50 µL 1 N acetic acid and mixed thoroughly. For the tHcy determination, EDTA plasma has to be rapidly separated from erythrocytes, as a time and temperature-dependent release of Hcy has been reported (2;226). Sample aliquots were stored at -70°C until analysis. For quality control in all UPLC-MS/MS methods, pooled samples of serum, EDTA plasma, and EDTA WB were prepared.

3.3 Materials

3.3.1 Equipment, chemicals, and reagents

A list of the chemicals and reagents used, as well as their description, ordering number, and companies is provided in **Appendix A**. An overview of the equipment used is summarized in **Appendix B**.

General equipment

- Aurius CE2041 Spectrophotometer (CECIL Instruments Ltd., Cambridge, UK)
- Biohit m10 (0.5 – 10 µL), m200 (20 – 200 µL), m1000 (100 – 1000 µL) pipette (Biohit Deutschland GmbH, Rosbach v. d. Höhe, Germany)
- Eppendorf centrifuge 5810 R, A-4-62 Rotor (Eppendorf AG, Hamburg, Germany)
- Eppendorf concentrator 5301 (Eppendorf AG)
- Hettich Mikro 20 centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany)
- RM5-40 Horizontal Mixer (Bennett Scientific Ltd., Newton Abbot, UK)
- Schott Intruments Lab 870 pH meter and N 6000 A electrode (SI Analytics GmbH, Mainz, Germany)
- Thermo Scientific Haake Open-Bath Circulators C10-W19 (Thermo Fischer Scientific, Waltham, USA)
- VARIOMAG Monotherm Heatable Magnetic Stirrer (VARIOMAG-USA, Daytona Beach, USA)
- Waters Acquity UPLC coupled to a MicroMass Quattro Premier XE (Waters Corporation, Milford, USA)

General chemicals

- acetic acid (glacial; >99.99%; Sigma Aldrich, Munich, Germany)
- acetonitrile (ULC/MS grade; Biosolve, Valkenswaard, The Netherlands)
- ammonium acetate (ULC/MS grade; Biosolve)
- ammonia solution analaR NORMAPUR (25%; VWR International GmbH, Darmstadt, Germany)
- formic acid (ULC/MS grade; Biosolve)
- methanol (ULC/MS grade; Biosolve)
- water (18.2 MΩ; Milli-Q water purification system; Millipore, Molsheim, France)

3.4 Methods

3.4.1 Ultra-performance liquid chromatography tandem mass spectrometry

Chromatography was defined in 1993 by the International Union of Pure and Applied Chemistry (IUPAC) as follows (66):

"Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction."

HPLC uses a liquid mobile phase, which is delivered at the desired flow rate under high pressure (up to 400 bar), and a stationary phase in form of a separation column. The chromatographic separation occurs by the interaction of the compounds to different extents with the mobile and stationary phase, leading to different retention times (t_R) for each analyte from sample introduction to detection. In general, HPLC requires a mobile phase in which the target analytes are soluble. Two basic elution modes are used: a constant composition of mobile phase is termed isocratic elution. In the second mode (gradient elution) the mobile phase changes over a period of time. Gradient curve profiles are shown in (**Figure 10**).

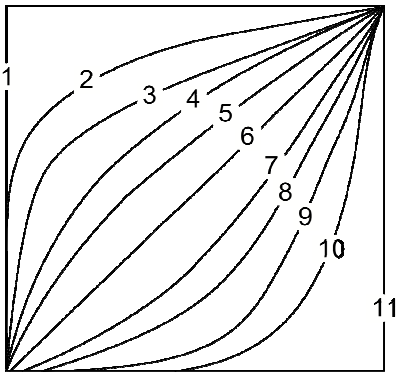

Figure 10: Gradient curve profiles. Curve no. 1: immediately goes to specified conditions; curve no. 2 – 5: convex; curve no. 6: linear gradient; curve no. 7 – 10: concave; curve no. 11: maintains start conditions until next step. Adapted from: MassLynx NT Inlet Control Guide, Version 4.0, Waters Part No. 7150000399, 2002.

Although the HPLC technique is frequently used in analytical chemistry and biochemistry, it has its limitations such as low efficiency and poor sensitivity. Using smaller particles enhances the column efficiency because it provides more theoretical plates per unit length. In 2004, UPLC

technology was introduced by Waters Corporation, which enables improved results in resolution, speed, and sensitivity.

The coupling of a LC system to a mass spectrometer (**Figure 11**), which is known as LC-MS leads to a more sensitive and far more specific detection of the analytes. It provides the molecular weight and thus reduces the number of possible structures for the analyte. Additional advantages of LC-MS are the identification of components in unresolved chromatographic peaks and the possibility to analyze compounds that lack a chromophore (e.g. choline). For mass spectrometric analysis the molecules are ionized and later on sorted and identified according to the *m/z* ratios. Common ionization methods in LC-MS include chemical ionization, electron ionization, electrospray (ESI) and atmospheric-pressure chemical ionization (APCI). In the ESI mode, a liquid including the dissolved analyte is passed through a metallic capillary at atmospheric pressure and maintained at high voltage between probe tip and sampling cone – a spray is created (6). Depending on the voltage polarity, highly charged droplets are created at the tip of the capillary, which are desolvated on their way to a counter electrode. The size of the droplets reduces while the density of charges at the droplet surface increases – until the repulsion forces between the charges are too high and the droplet explodes. Eventually, analyte ions are created, which pass into the source of the mass spectrometer for separation.

The quadrupole mass spectrometer consists of two pairs of parallel metallic rods; one set is at a positive electrical potential, the other one at a negative potential. Constant direct current (DC) and radio frequency (RF) voltages are both applied on each set (209). Only the ions of a given *m/z* ratio will resonate and can be detected for a given amplitude of the RF and DC voltages while other ions hit the rods (7;242) (**Figure 12**).

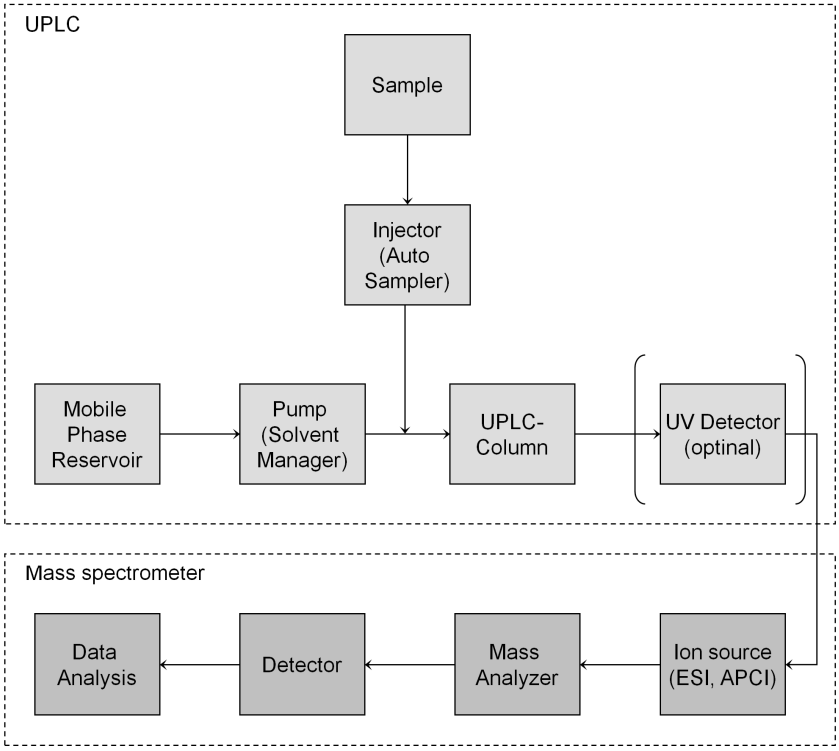

Figure 11: Schematic representation of an UPLC system coupled to a mass spectrometer. The sample is injected via injector device into the UPLC system and separated by the UPLC column. The mobile phase is pumped at high pressure through the column. After separation of the analytes, the sample is ionized in the ion source via ESI or APCI. Identification occurs by means of the mass-to-charge (*m/z*) ratio. Using a triple quadrupole, a specific analyte can be structurally investigated by fragmentation using a collision gas, creating precursor and product ions.

MS/MS covers a number of methods in which one stage of mass spectrometry is used for the isolation of an ion. The second stage is used to investigate the relationship of this ion with others from which it may have been generated or which it may have generated on decomposition. The most widely used MS/MS instrument is the triple quadrupole. It consists of three sets of quadrupoles in series. MS 1 and MS 2 are used as mass analyzers, whereas the second set of quadrupoles is used as a collision cell where the fragmentation and focusing of ions is carried out (**Figure 12**).

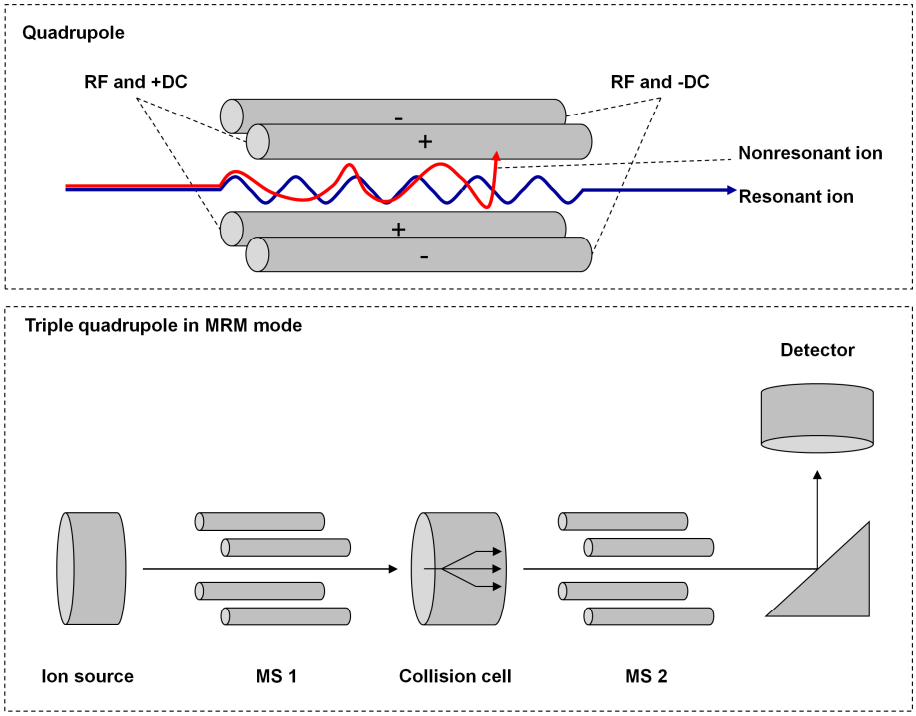

Figure 12: Assembly and functionality of a single quadrupole and a triple quadrupole in multiple reaction monitoring mode of a mass spectrometer. The quadrupole consists of four parallel metallic rods; one pair is at a positive electrical potential, the other one at a negative potential. Constant DC and RF voltages are applied. Ions of a given *m/z* ratio will resonate and can be detected for a given amplitude of the RF and DC voltages, while nonresonant ions hit the rods. The triple quadrupole consists of three sets of quadrupoles (MS 1, collision cell, and MS 2) in series. In MRM mode, a selected precursor-ion is isolated in MS 1, fragmented in the collision cell, and a selected product-ion is detected in MS 2.

Collision-induced dissociation is a mechanism by which molecular ions are fragmented in the gas phase by collision with neutral gas molecules (argon, helium, or nitrogen). Using MS/MS a large number of experiments can be carried out: the product-ion scan, the precursor-ion scan, the constant-neutral-loss scan, and the multiple reaction monitoring (MRM) (**Table 5**). MRM is a highly selective MS/MS mode, typically used for quantification of known analytes in complex samples. In MRM mode, a selected precursor-ion is isolated in MS 1, fragmented in the collision cell, and a selected product-ion is detected in MS 2 (**Figure 12**).

Table 5: Selected MS/MS techniques and operation modes of mass analyzers MS 1 and MS 2 in mass spectrometry.

MS/MS technique	MS 1	MS 2
Product-ion scan	static (selected precursor-ion)	full scan
Precursor-ion scan	full scan	static (selected product-ion)
Constant-neutral-loss scan	full scan (synchronized with MS 2)	full scan (synchronized with MS 1)
Multiple reaction monitoring	static (selected precursor-ion)	static (selected product-ion)

The quantification of analytes using LC-MS/MS involves the comparison of the intensity of the analyte signal in a sample with that obtained from standards containing known amounts of the analyte. The internal standard is added as early as possible in the same concentration, correcting the possible loss of analyte during the sample preparation or the sample inlet. In addition, in biological samples the internal standard corrects for the so called matrix effect, which influences the measurement by enhancing or suppressing the signal intensities of the analytes. Internal standards generally match the analyte of interest as closely as possible but not completely. Stable isotope labeled analogues of the analytes contain unusual isotopes (e.g. replacing hydrogen with deuterium) in their chemical composition. Since the stable isotopes and the analytes have different masses they can be distinguished from each other without difficulty by MS. For all UPLC-MS/MS methods, the data acquisition was performed by MassLynx V4.1 and the QuanLynx software. Calculations and statistics are presented in **Chapter 3.5**.

3.4.1.1 Quantification of folate forms

A sensitive and reliable method for the determination of key folate forms (5-methylTHF, 5-formylTHF, 5,10-methenylTHF, THF, and FA) in serum using UPLC-MS/MS technique was developed by Kirsch *et al.* (126). The method enables the quantification of the folate forms either in serum or plasma. A modified method for the quantification of 5-methylTHF and non-methylTHF (sum of formylTHF, 5,10-methenylTHF, 5,10-methyleneTHF, THF, DHF, and FA) in WB was recently described by Kirsch *et al.* (123). Both methods use the same liquid chromatography and mass spectrometry conditions but differ in the sample preparation step. As WB folate has to be first extracted from the cells and deconjugated into monoglutamates, the sample preparation is more complex and time consuming.

For method comparison purposes, serum TFOL was determined using ADVIA Centaur XP System. With the assay, 150 µL of serum folate can be determined. The assay had a measurement range of 0.79 (= LOD) – 54.36 nmol/L, a mean recovery after dilution of serum samples of

101.0%, and a mean recovery after spiking of 97.2%. The inter- and intraassay CVs for serum samples of four different concentrations (3.85, 12.00, 22.67, and 33.86 nmol/L) were ≤ 7.93%. The reference range for TFOL with this method was > 5.38 ng/mL (> 12.19 nmol/L)

Solid-phase extraction column

- Oasis MAX (1 ccm/30 mg and 3 ccm/60 mg; Waters Corporation, Milford, USA)

Standards and internal standards

- (6S)-5-CH_3-H_4PteGlu-Na_2 (= 5-methylTHF)
- (6S)-5-CHO-H_4PteGlu-Na_2 (= 5-formylTHF)
- (6R)-10-CHO-H_4PteGlu-Na_2 (= 10-formylTHF)
- (6R)-5,10-CH^+-H_4PteGlu-Cl x HCl (= 5,10-methenylTHF)
- (6R)-5,10-CH_2-H_4PteGlu-Na_2 (= 5,10-methyleneTHF)
- (6S)-H_4PteGlu-Na_2 (= THF)
- 7,8-H_2PteGlu (= DHF)
- PteGlu-Na_2 (= FA)
- (6S)-5-CH_3-H_4Pte[$^{13}C_5$]Glu, Ca-salt (= [$^{13}C_5$]-5-methylTHF)
- (6S)-5-CHO-H_4Pte[$^{13}C_5$]Glu, Ca-salt (= [$^{13}C_5$]-5-formylTHF)
- (6R)-5,10-CH^+-H_4Pte[$^{13}C_5$]Glu (= [$^{13}C_5$]-5,10-methenylTHF) made from [$^{13}C_5$]-5-formylTHF by acidification with 10% formic acid and incubation at 4°C for 1 d
- (6S)-H_4Pte[$^{13}C_5$]Glu (= [$^{13}C_5$]-THF)
- Pte[$^{13}C_5$]Glu, free acid form (= [$^{13}C_5$]-FA; Merck Eprova AG, Schaffhausen, Switzerland)

Additional chemicals and equipment

- activated charcoal Darco, powder, ~ 100 mesh particle size (Sigma Aldrich)
- *L(+)*-ascorbic acid (puriss. 99.7%, Riedel-de-Haën (Sigma Aldrich))
- *L*-cysteine (≥ 99.5%; Fluka (Sigma Aldrich))
- Triton X-100 (for electrophoresis; Sigma Aldrich)
- 5 mL glass tubes (5-SV – EPA Screw Top Vials; Chromacol, Herts, UK)
- Maximum recovery glass vials (Waters Corporation)

Preparation of stock solutions, calibrators, and quality control samples

To avoid the degradation of reduced folates, it is decisive to avoid the exposure to light, high temperatures, and oxygen. Stock solutions of standards and internal standards were therefore quickly prepared under subdued light with N_2 degassed buffers containing antioxidants. Stock solutions I (440 µmol/L) were prepared according to Pfeiffer *et al.* in 20 mmol/L of di-ammonium hydrogen phosphate, pH 7.2 (178). The concentrations were verified by using an Aurius CE2041 spectrophotometer and the determination of the transmittance (T) of 1:20- and 1:50-diluted samples (19;178) (see **Table 1** for wavelengths and molar extinction coefficients). The concentration of stock solution I was calculated by following equations:

$$A = -log\ T \qquad (1)$$

$$A_\lambda = \varepsilon\ c\ l \qquad (2)$$

The absorbance (A) is defined as the logarithm (base 10) of the reciprocal of the transmittance (**Equation 1**). The Lambert Beer's law states, that the absorbance (A_λ) is proportional to the concentration (c) for a given substance dissolved in a given solute and measured at a given wavelength (**Equation 2**) (107). For avoiding the degradation of the reduced folates, ascorbic acid (10 g/L) and *L*-cysteine (1 g/L) were added to all solutions except to that of FA after photometric quantification. Stock solutions II (220 µmol/L) were prepared according to the determined concentration of stock solution I with 10 g/L ascorbic acid (reduced folates) or water (FA). Aliquots of stock solution II were stored at -70°C for no longer than one year. Working standard stock solutions III (22 µmol/L) were prepared in 1 g/L aqueous acetic acid solution (reduced folates) or water (FA). An internal standard mix from five internal standards was prepared containing 0.4 µmol/L of [$^{13}C_5$]-5-methylTHF, 0.2 µmol/L of [$^{13}C_5$]-5-formylTHF, 0.4 µmol/L of [$^{13}C_5$]-5,10-methenylTHF, 0.4 µmol/L of [$^{13}C_5$]-THF, and 0.6 µmol/L of [$^{13}C_5$]-FA. Aliquots were stored at -70°C for no longer than one month and discarded after use.

Calibrators were prepared from stock solution III in 1 g/L ascorbic acid solution and were included in each batch of samples. Concentrations of calibrators were 0, 0.2, 0.5, 2, 10, 50, and 100 nmol/L for all folate forms. Control samples at two different concentrations (0.6 and 160 nmol/L of each analyte) were prepared from stock solution III in 1 g/L ascorbic acid solution and were included in each batch of samples. One serum pool sample or WB pool sample was included in each batch of samples as quality control.

Sample preparation serum folate

Sample extraction and cleanup was performed with Oasis MAX SPE columns. These columns combine a mixed-mode anion exchange and reversed-phase sorbent and have high selectivity for acidic compounds. Folates are weak acids and are negatively charged at pH > 7. Ion-exchange works best with a positive charged stationary phase of the SPE column (anion exchange). The aqueous and basic sample is loaded onto the columns. For elution, a stronger solvent (methanol or acetonitrile) has to be used under acidic conditions (pH < 3).

Serum samples were thawed at 4°C in the dark. 250 µL of the sample and 500 µL of the calibrator or the quality control sample were incubated with 700 µL or 450 µL ammonium acetate buffer (200 mmol/L, pH 10), respectively. 50 µL internal standard solution mix was added (total volume = 1 mL). Samples were vortexed and incubated in the dark at room temperature for 15 – 30 minutes, to ensure the release of the folates from the binding proteins and the equilibration of the labeled internal standard and the unlabeled endogenous folate. The SPE columns were preconditioned with 2 x 1 mL methanol following 1 mL 200 mmol/L of ammonium acetate buffer (pH 10), containing 10 g/L ascorbic acid. The prepared samples were loaded onto the column and impurities were removed by washing the columns with 1 mL 5% aqueous NH_4OH following 1 mL methanol. The elution of the folates was performed by 6 x 250 µL elution solution (methanol containing 1% formic acid). The eluates (1,500 µL) were transferred into glass vials (Maximum Recovery vials) and taken to dryness in an Eppendorf Concentrator 5301 at 45°C. Dried samples were dissolved in 100 µL H_2O/methanol (60:40, v/v), containing 0.1% formic acid and 1g/l ascorbic acid. Concentrated eluates were immediately measured.

Sample preparation whole blood folate

Depending on the standards and internal standards, the developed UPLC-MS/MS method is only capable of quantification of monoglutamate folates. Therefore, folate polyglutamates have to be first deconjugated into their monoglutamate forms. WB hemolysates were prepared as follows: 200 µL EDTA WB was slowly dropped into 5 mL glass vials containing 2 mL of a 10 g/L ascorbic acid solution (pH 4.0, containing 0.2% Triton X-100), representing a 1:11-dilution. 73.3 µL of internal standard mix was added and samples were incubated for 1 h at 37°C for hemolysis and deconjugation of the polyglutamates by endogenous plasma folate conjugases. The hemolysates were processed using SPE or frozen at -70°C until analysis.

Hemolysates were centrifuged for 5 minutes at 4,000 x g at 4°C. 1,550 µL of the supernatant containing 1,500 µL hemolysate and 50 µL internal standard mix was added to 1,450 µL of a 200 mmol/L ammonium acetate buffer, pH 10 in 5 mL glass vials. Samples were incubated for 20 minutes for equilibration. For SPE conditioning, 2 x 3 mL methanol and 3 mL 200 mmol/L ammonium acetate buffer, pH 10 was used. Wash steps were performed with 2 x 3 mL 5% aqueous NH_4OH and 2 x 3 mL methanol. After resuspension, the concentrated samples were centrifuged in the Eppendorf Concentrator and supernatant was transferred to glass vials; samples were measured immediately.

UPLC-MS/MS conditions

Sample measurement was performed by using an UPLC-MS/MS system. Separating and analysis conditions for the folate assay were summarized in **Table 6**. Information on gradient curve profiles is shown in (**Figure 10**).

Table 6: UPLC-MS/MS conditions for the folate assay.

UPLC conditions	
UPLC column	Acquity UPLC HSS T3 column (50 mm x 2.1 mm (i.d.) 1.8 µm particle size; Waters Corporation)
UPLC pre-column	Acquity BEH C_{18} VanGuard pre-column (5 mm x 2.1 mm (i.d.); 1.7 µm particle size; Waters Corporation)
In-line filter	0.2 µm in-line filter (Waters Corporation)
Mobile phase A	Aqueous acetic acid (glacial), pH 2.636
Mobile phase B	Methanol
Column temperature	30°C
Sample temperature	4°C
Gradient	0.0 minutes, 10% B 0.4 minutes, 25% B (convex curve 2) 0.6 minutes, 45% B (convex curve 2) 0.8 minutes, 85% B (linear gradient) 1.0 minutes, 85% B (concave curve 11) 1.1 minutes, 10% B (linear gradient)
Run time	2.5 minutes
Flow rate	0.5 mL/minutes
Injection volume	10 µL
Mass spectrometer conditions	
Modus	ESI^+
Source temperature	110°C
Desolvation gas	N_2
Cone gas	N_2
Collision gas	Ar

Cone and collision energy voltages, dwell times, MRM mass transitions, and t_Rs of the folates and the corresponding internal standards in ESI^+ are summarized in **Table 7**.

Table 7: Multiple reaction monitoring in ESI$^+$ of folate compounds and internal standards.

Variable	Cone voltage [V]	Collision energy [eV]	Precursor ion (m/z)	Product ion (m/z)	Dwell time [s]	Retention time [min]
Folic acid	22	16	442.08	295.13	0.04	1.03
THF	25	22	446.31	299.28	0.04	0.67
5,10-MethenylTHF	55	27	456.12	412.12	0.04	0.70
5-MethylTHF	23	19	460.29	313.26	0.04	0.69
5-FormylTHF	27	20	474.27	327.24	0.08	0.97
[$^{13}C_5$]-Folic acid	22	16	447.08	295.13	0.04	1.00
[$^{13}C_5$]-THF	25	22	451.33	298.88	0.04	0.67
[$^{13}C_5$]- 5,10-MethenylTHF	55	27	461.12	416.12	0.04	0.70
[$^{13}C_5$]-5-MethylTHF	23	19	465.29	313.26	0.04	0.69
[$^{13}C_5$]-5-FormylTHF	25	20	479.27	327.24	0.04	0.96

Studies on the stability of folate forms *in vitro* and during sample preparation

Due to the known instabilities and interconversions of reduced folates, several preanalytic conditions should be considered (36;50;175). The stabilities of the folate coenzymes at 4°C over 24 h with and without ascorbic acid at different pHs were studied. For this purpose, solutions of each folate compound (100 nmol/L) were prepared in water, in 1 g/L aqueous solution of ascorbic acid without further pH adjustment (pH ~ 3.4), in 1 g/L aqueous solution of ascorbic acid at pH 2.6, and 1 g/L aqueous solution of ascorbic acid at pH 7.0. The folate forms were then measured at start (time 0), 1, 5, and 24 h. The stabilities of the folate forms in serum, and WB were determined using serum pool and WB pool samples, respectively.

Linearity and sensitivity

The linearity over the physiological range was tested for the folate forms between 0.2 – 200 nmol/L for each analyte. Limits of detection (LOD) and limits of quantification (LOQ) for serum and WB folate validation were calculated in a two-step approach. The first step is the determination of the instrumental detection limit (IDL) and instrumental quantification limit (IQL), which are defined as the smallest amount of an analyte that can be reliably detected or quantified from the background on an instrument. IDL and IQL were estimated by calculating the root mean square error (RMSE; **Equation 7**) of five 5-point calibration curves containing 0, 0.2, 0.5, 2, 5, and 10 nmol/L of each folate form. The next step is the calculation of the method detection limit (MDL) and method quantification limit (MQL) (**Equation 9** and **10**), which are defined as the smallest amount of an analyte that can be reliably detected or quantified from the background for a particular matrix. MDL (= LOD) and MQL (=LOQ) were determined in ten aliquots of folate-free serum pool and WB hemolysate samples using the $t_{99}s_{LLMV}$ method. To generate folate-free serum and WB hemolysate, serum pool (1:1, v/v with 1 g/L ascorbic acid

solution) and hemolysate of WB pool were treated with activated charcoal (5 mg/mL for serum and 20 mg/mL for hemolysates) for 40 minutes at ambient temperature under constant shaking. Samples were centrifuged for 10 minutes at 4,000 x g. Folate-free serum and WB hemolysate were spiked to obtain folate concentrations in the range of 1 – 5 times the IDL.

Precision and recovery

The precision of the method was assessed by quantifying folate in serum pool, in WB pool, and in quality control samples. The intraassay CV was determined by the measurement of 10 aliquots of the samples within one run, the interassay CV was assessed over a period of 10 days. Recovery experiments were performed by spiking serum and WB hemolysate samples with quality control samples at two different levels over a period of three days.

Evaluation of the relative matrix effect in whole blood

The relative matrix (CV% of mean slope) effects were determined by calculating the standard line slopes of 5 different lots (149) of WB samples spiked after the SPE with 0, 0.2, 0.5, 2, 10, 50, 100, and 200 nmol/L of each analyte and 50 µL of internal standard mix.

Method comparison

The sum of folates concentrations from 70 serum samples were measured by either the ADVIA Centaur System (TFOL) or the newly developed UPLC-MS/MS method. The agreement between methods for serum samples (UPLC-MS/MS method vs. immunological method performed by ADVIA Centaur) was assessed by Bland-Altman difference plots (20).

Studies on concentrations of folate forms and reference ranges in healthy individuals

The data from a subset of 32 apparently healthy non-vitamin users (8 males, age range: 17 – 55 years from the medical staff of the Saarland University Hospital) was used for studying the normal range of folate forms in serum. The samples were collected in the course of a medical checkup in February 2009. For the determination of the normal folate form ranges in WB hemolysate, data from a subset of study I and II participants (42 subjects (8 males) who were non-supplemented and from 35 subjects (11 males) supplemented with 500 µg FA, 50 mg vitamin B_6, and 500 µg vitamin B_{12} /day for 6 months) was used.

3.4.1.2 Quantification of S-adenosyl homocysteine and S-adenosyl methionine

For the simultaneous quantification of SAH and SAM, a sensitive UPLC-MS/MS method has been developed (125). The method comprises a phenylboronic acid-containing SPE procedure. The preparation of stock solutions, calibrators, and quality control samples, as well as the method validation were described in detail by Kirsch *et al.* (125) and can be found in **Appendix C**.

Solid-phase extraction column

- Varian Bond Elut PBA columns (Varian Inc., Palo Alto, USA)

Standards and internal standards

- *S*-(5'-adenosyl)-*L*-homocysteine, crystalline (= SAH; Sigma Aldrich)
- *S*-(5′-adenosyl)-*L*-methionine *p*-toluenesulfonate salt, from yeast (= SAM; Sigma Aldrich)
- *S*-adenosyl-*L*-methionine-d_3-tetra(*p*-toluenesulfonate) salt (= [$^2H_3$]-SAM; CDN Isotopes, Quebec, Canada)
- *S*-(5'-adenosyl)-*L*-homocysteine (= [$^{13}C_5$]-SAH; Henkjan Gellekink group, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands)

In brief, the method was linear over the ranges of 25 – 200 nmol/L for SAM and of 6 – 48 nmol/L for SAH. The coefficients of linear correlation were $R^2 > 0.999$ for both SAM and SAH. The LOD was 0.2 nmol/L for SAM and 0.3 nmol/L for SAH. Intraassay CVs in 12 plasma pool samples were 3.3% for SAM and 3.9% for SAH. Interassay CVs in 15 plasma pool samples were 7.9% for SAM and 8.6% for SAH. The mean (SD) recoveries were 101.7 (4.1)% for SAH and 100.0 (4.6)% for SAM.

SAH and SAM concentrations and reference ranges in healthy individuals

The data from 31 apparently healthy individuals (6 males, age range: 20 – 55 years) from the medical staff of the Saarland University Hospital was used for the study of normal range of SAH and SAM. The samples were collected in the course of a medical checkup in February 2009. The obtained reference ranges are presented in **Table 8**.

Table 8: SAH and SAM reference ranges in healthy subjects (124).

Variable	
Age range, years	20 – 55
SAH, nmol/L	14.6 (5.5)
SAM, nmol/L	94 (12)
SAM/SAH ratio	7.0 (1.8)

The data are means (SD) if not stated otherwise.

3.4.1.3 Quantification of betaine, choline, and dimethylglycine

For the simultaneous quantification of betaine, choline, and DMG in plasma, a sensitive hydrophilic interaction chromatography (HILIC) UPLC-MS/MS method has been developed (124). The method comprises a protein precipitation using acetonitrile. The preparation of stock solutions, calibrators, and quality control samples, as well as the method validation were described in detail by Kirsch *et al.* (124) and can be found in **Appendix D**.

Standards and internal standards

- betaine anhydrous (Sigma Aldrich)
- choline chloride (Sigma Aldrich)
- *N,N,*-dimethylglycine (= DMG; Sigma Aldrich)
- *N,N,N*-trimethyl-d_9-glycine hydrochloride (= d_9-betaine; Isotec, Sigma Aldrich)
- choline chloride-trimethyl-d_9 (=d_9-choline; Isotec)
- *N,N*-dimethyl-d_6-glycine HCl (= d_6-DMG; CDN Isotopes)

The assay was linear over 0.2 – 150 µmol/L for each analyte. The coefficients of linear regression (n = 5) were: $R^2 > 0.999$ for all analytes. The LOD was 0.18 µmol/L for betaine, 0.13 µmol/L for choline, and 0.13 nmol/L for DMG. Intraassay CVs in 10 plasma pool samples were 2.0% for betaine, 2.4% for choline, and 7.0% for DMG. Interassay CVs in 10 plasma pool samples were 5.8% for betaine, 8.4% for choline, and 9.6% for DMG. The mean recoveries in the plasma pool samples were: 93.0% for betaine, 100.4% for choline, and 102.5% for DMG.

Choline metabolite concentrations and reference ranges in healthy individuals

Concentrations of betaine, choline, and DMG in plasma samples from 44 older fasting adults (subset of study I and II, > 50 years; 24 males) were used for studying the normal range of betaine, choline, and DMG. The obtained reference ranges are presented in **Table 9**.

Table 9: Betaine, choline, and DMG reference ranges in fasting older subjects (124).

Variable	Females	Males	p
n	20	24	-
Age, years	59 (50 – 73)	66 (57 – 75)	0.062
Betaine, µmol/L	29.0 (18.7 – 37.5)	29.7 (25.2 – 46.8)	0.150
Choline, µmol/L	9.0 (7.0 – 11.7)	9.2 (7.43 – 12.4)	0.465
DMG, µmol/L	2.1 (1.5 – 3.7)	3.1 (2.0 – 4.3)	**0.001**

The data are medians (10^{th} – 90^{th} percentiles). P values are according to the Mann-Whitney-U test.

3.4.2 Gas chromatography tandem mass spectrometry

GC is used for separating and analyzing of compounds that can be vaporized. GC is in principle similar to LC. In LC a mobile phase is used while in GC an inert carrier gas (e.g. He, Ar, or N_2) is utilized. The stationary phase consists of a column, which is 1.5 to several meters in length. The packing of the column contains solid support material that is coated with a solid or liquid stationary phase. The separation of the analytes is carried out by adsorption of the molecules to the stationary phase, creating a specific t_R. Prior GC separation, many analytes must be derivatized. Derivatization reduces the polarity and increases the volatility, the stability, and the detectability of some compounds. After passing the ionization chamber, the vaporized analytes are mostly positively charged. Due to instabilities of the created ions, the molecules are fragmented, creating a characteristic fragmentation pattern.

3.4.2.1 Quantification of homocysteine, cystathionine, and methylmalonic acid

The quantification of tHcy, Cys, and MMA was performed by a GC-MS method, modified by the previously described protocol of Stabler *et al.* (210). Cys and tHcy were simultaneously determined in serum or EDTA plasma. The GC-MS system consisted of a HP GC System, 6890 Series, G 1530 with a HS 5973 Mass Selective Detector, G 1099AX and a HP-5ms GC column (cross-linked 5% PH ME siloxane; 30 m x 0.25 mm x 0.25 µm; Agilent Technologies, Waldbronn, Germany).

Solid-phase extraction column

- poly-prep chromatography columns, 0.8 x 4 cm
- anionic resin: AG MP-1, 100 – 200 mesh, chloride form (Bio-RAD)

Internal standards

- methyl-d_3-malonic acid (= d_3-MMA; CDN Isotopes)
- *DL*-homocystine-3,3,3',3',4,4,4',4'-d_8 (= d_8-Hcy; Cambridge Isotope Laboratories Inc., Andover, USA)
- *DL*-(2-amino-2-carboxyethyl)-homocysteine-3,3,4,4-d_4 (= d_4-Cys; CDN Isotopes)

Additional chemicals and equipment

- acetic acid, glacial EMPROVE (Merck Chemicals)
- dithiothreitol (AppliChem GmbH, Darmstadt, Germany)
- hydrochloric acid (Merck Chemicals)
- *N*-methyl-*N*-tert-butyldimethylsilyltrifluoro-acetamid (MBDSTFA; Machery and Nagel, Düren, Germany)
- sodium hydroxide (Merck Chemicals)
- 5 mL glass tubes (5-SV – EPA Screw Top Vials; Chromacol)
- Panasonic NN-5256 microwave (Panasonic Deutschland GmbH, Hamburg, Germany)

Column preparation

Equal volumes of anionic resin and 1 N HCl were incubated for 30 minutes at ambient temperature. The HCl was removed; an equal volume methanol was added and incubated for 30 minutes. The methanol was removed and the resin was left to dry for 1 – 2 days at 50°C. Poly-prep columns were packed with ~100 mg anionic resin and 1 mL methanol.

Methylmalonic acid assay

In 5 mL glass tubes, 1 mL water, 400 µL sample, and 50 µL d_3-MMA (1.635 nmol/L) were added. Methanol was removed from the poly-prep columns and columns were conditioned with 3.3 mL water. Preparated samples were loaded using glass Pasteur pipettes. The columns were washed with 3 mL water and 3 x 3 mL of 0.01 N acetic acid/methanol solution. The samples were eluted using 1.1 mL elution solution (4 N acetic acid/1 N HCl (9:1, v/v)).

Total homocysteine and cystathionine assay

In 5 mL glass tubes, 1 mL water, 250 µL sample, 15 µL d_8-Hcy (392 µmol/L), 20 µL d_4-Cys (413.36 pmol/50 µL), and 30 µL DTT (10 mg/mL in 1 N NaOH; freshly prepared) were added.

DTT is necessary for releasing the protein bound Hcy. After vortexing, the samples were incubated at 45°C for 35 minutes for the reduction of disulfides of homocystine to Hcy. The methanol was removed from the poly-prep columns and the columns were conditioned with 1 mL methanol and 3.3 mL water. Preparated samples were loaded using glass Pasteur pipettes. The columns were washed with 3 x 3 mL water and 3 mL methanol. The samples were eluted in a glass vial using 1.1 mL elution solution (0.4 N acetic acid in methanol).

Derivatization of the analytes

Eluted samples from both assays were taken to dryness for a minimum of 3 h at 60°C in an Eppendorf concentrator. Dried samples were derivatized (silylated) with 20 µL acetonitrile and 10 µL MBDSTFA. After vortexing, the samples were derivatized in the microwave at 440 Watts for 5 minutes. The samples were vortexed and analyzed by GC-MS. For both assays, serum pool samples were included at each batch of samples. The reference ranges for the analytes determined by this method were: tHcy < 12 µmol/L, Cys < 301 nmol/L, and MMA ≤ 271 nmol/L.

3.4.3 Quantification of vitamin B_{12} and holotranscobalamin II

The quantification of vitamin B_{12} (cyanocobalamin) was performed using a chemiluminescent immunoassay performed on the ADVIA Centaur XP System platform. The commercially available VB12 assay operates similar to the folate assay. The difference is the competition of released serum or plasma vitamin B_{12} against acridinium ester-labeled vitamin B_{12} for a limited amount of intrinsic factor. With the assay vitamin B_{12} can be determined from 100 µL serum or plasma. The assay has a measurement range of 33 (= LOD) – 1,476 pmol/L, a mean recovery after dilution of serum samples of 97.3%, and a mean recovery after spiking of 101.8%. The intraassay CVs of serum samples of four different concentrations (131.89, 152.89, 449.19, and 991.51 pmol/L) were ≤ 5.0%, whereas the interassay CVs were ≤ 9.2%. The reference range for vitamin B_{12} determined by this method was 156 – 672 nmol/L.

In serum, vitamin B_{12} is bound to two proteins: transcobalamin (TC) and haptocorrin (HC). The complex of cobalamin:TC is also called HoloTC or active B_{12}, whereas the complex of cobalamin:HC, which consists of 70 – 90% of the vitamin B_{12} in serum, is metabolically inert. The determination of HoloTC was performed using a micro particle enzyme immunoassay (AxSYM Active-B_{12}; Axis-Shield, Oslo, Norway), which was performed on an AxSYM platform (Abbott Diagnostics, Vienna, Austria). The AxSYM Active-B_{12} assay is based on a monoclonal mouse antibody specific for human HoloTC, which is bound to magnetic micro spheres, and a

monoclonal antibody specific for TC, which exists as conjugate of alkaline phosphatase. Serum HoloTC binds to the anti-HoloTC antibody. Further, the antigen-antibody complex is irreversibly bound to a glass fiber matrix. In the next step, the conjugate of anti-TC:alkaline phosphatase is added and binds to the antigen-antibody complex. After a washing step, the substrate 4-methylumbelliferyl-phosphate is added. The alkaline phosphatase conjugate catalyses the separation of a phosphate group from the substrate – the fluorescent 4-methylumbelliferon is generated. The fluorescent product is detected by the AxSYM system at 448 nm wavelength. With the assay HoloTC can be determined from 173 µL serum samples. The assay has a measurement range of 0 (LOD ≤ 1) – 128 pmol/L and a mean recovery of 109.3%. The interassay CVs of serum samples at different concentrations (22.8 and 48.2 pmol/L) were ≤ 8.5%. The reference range for HoloTC determined by this method was ≥ 35 pmol/L.

3.4.4 MTHFR C677T polymorphism determination

Genomic DNA was isolated from EDTA blood samples using either the automated TECAN Te-MagS magnetic bead separation module (TECAN Group Ltd., Männedorf, Switzerland) or manually with a QIAamp DNA blood mini kit (Qiagen, Hilden, Germany). The *MTHFR 677 CT* SNP was assessed by polymerase chain reaction (PCR) of genomic DNA and pyrosequencing (186;233). PCR conditions were: 13 µL genomic DNA (30 µg/mL), 1.5 µL forward-primer (10 pmol/µL; Invitrogen GmbH, Karlsruhe, Germany), 1.5 µL reverse-primer (10 pmol/µL; Invitrogen), 5.0 µL PCR reaction buffer (with $MgCl_2$, 10 x conc.; Roche Diagnostics GmbH, Mannheim, Germany), 5.0 µL dNTP mix (2.5 mmol/L each dNTP; 5 Prime GmbH, Hamburg, Germany), 1.5 µL *Taq* DNA polymerase (1 U/µL; Roche) in 50 µL of H_2O (Eppendorf AG). The thermal cycling in an Eppendorf Mastercycler ep gradient S (Eppendorf AG) was: 2 minutes at 94°C, 36 cycles of 94°C for 30 s, 59°C for 45 s, and 72°C for 30 s, with a final step at 72°C for 10 minutes. 30 µL of the biotinylated PCR product was immobilized with 3 µL streptavidin sepharose high performance beads (GE Healthcare, Freiburg, Germany), 37 µL binding buffer, and 10 µL H_2O. The samples were pyrosequences using a PSQ 96MA instrument (Biotage AB, Uppsala, Sweden). The immobilization, washing, denaturation, and primer annealing steps with 40 µL of sequencing-primer (100 pmol/µL; Invitrogen) were performed. Sequencing and analysis of the single-stranded biotinylated PCR product was performed with a PSQ 96MA instrument and SNP analysis software. The primer sequences were summarized in **Appendix E**.

3.4.5 Determination of routine parameters

The blood count was determined from EDTA WB either by the SYSMEX SF 3000 or the SYSMEX XE 5000 platform (SYSMEX Germany GmbH, Norderstedt, Germany). From lithium-heparin plasma following parameters were determined using the automated Roche/Hitachi 917a system (Roche Diagnostics GmbH): creatinine, alanine aminotransferase (ALAT), C-reactive protein (CRP), cholesterol, glucose, triglycerides, and high density lipoprotein (HDL) cholesterol. Reference ranges for the routine parameters are summarized in **Appendix F**.

3.5 Calculations and statistics

Calculations

For all UPLC-MS/MS assays, calibrators were used for the construction of a standard curve by plotting the response (y = area analyte/area internal standard) against the corresponding concentrations (c) of the calibrators. The slope (m) and the intercept (i) of the standard curve are used for calculating the concentration of the unknown sample:

$$Concentration\ of\ \ the\ unknown\ analyte = \frac{y - i}{m} \qquad (3)$$

WB folate was determined using following equation:

$$Concentration\ WB\ \ folate = \frac{Measured\ \ concentration \times dilution\ \ factor \times 100}{Hematocrit} \qquad (4)$$

, whereas the dilution factor for WB folate was 3.667 (1:11-dilution of 1,500 µL WB hemolysate, in relation to 500 µL sample volume of the calibrator):

Recovery [%] was calculated as:

$$Recovery\ [\%] = \frac{Measured\ \ concentration}{Expected\ \ concentration + concentration\ added} \times 100 \qquad (5)$$

Calculations and statistics

RMSE was calculated as:

$$RMSE = \left[\frac{\sum_{j=1}^{n} E_j^2}{n-2} \right]^{\frac{1}{2}} \quad (6)$$

, whereas n is the number of standards and E is the error associated with each measurement.

IDL and IQL were calculated as:

$$IDL = \frac{3 \times RMSE}{m} \quad (7)$$

$$IQL = \frac{10 \times RMSE}{m} \quad (8)$$

, whereas m is the slope of the calibration curve.

MDL and MQL were determined by the $t_{99}s_{LLMV}$ method, using the following equations:

$$MDL = t_{99(n-1)} \times SD \quad (9)$$

$$MQL = 3 \times MDL \quad (10)$$

, whereas $t_{99\ (n\ -\ 1)}$ is the one-tailed t-statistic for n - 1 observations at the 99% confidence level ($t_{99\ (n\ -1)}$ = 2.821 for 10 aliquots or 9 degrees of freedom) and SD is the standard deviation.

Quantification of tHcy, Cys, and MMA was carried out using following equations:

$$Concentration\ tHcy = \frac{Area\ sample \times concentration\ internal\ standard}{Area\ internal\ standard} \times 39.2 \quad (11)$$

$$Concentration\ Cys = \frac{Area\ sample \times concentration\ internal\ standard}{Area\ internal\ standard} \times 1{,}000 \quad (12)$$

$$Concentration\ MMA = \frac{Area\ sample \times concentration\ internal\ standard}{Area\ internal\ standard} \times 4{,}087.5 \quad (13)$$

Statistics

Statistical analyses were performed using SPSS (Statistical Package for the Social Sciences, version 19.0). The correlation analyses were performed by using the Spearman-Rho test. For folate assay, the agreement between methods (UPLC-MS/MS method vs. immunological method performed by ADVIA Centaur) was assessed by a Bland-Altman difference plot (20). Results are shown as medians (10^{th} – 90^{th} percentiles) or means (SD). Oneway ANOVA and the Tamhane-T test were used for testing possible differences in the means of continuous variables between several groups. The differences in continuous variables between two independent groups were tested by the Mann-Whitney-U test and those in categorical variables by the Chi square test. Bottom and top of box plots represent the 25^{th} – 75^{th} percentiles; a horizontal band indicates the median. Whisker ends represent the minimum and maximum values that are not outliers. All tests used were 2-sided and p values < 0.05 were considered to be statistically significant.

4 Results

4.1 Development of sensitive methods for the quantification of folate forms using UPLC-MS/MS

Chromatography and mass spectrometry

The quantification methods of folate forms in serum and WB using stable-isotope dilution UPLC-MS/MS were recently published by Kirsch *et al.* (123;126). Ideal MRM conditions were obtained in the positive electrospray ionization mode. Typical *m/z* transitions of the folate forms, as well as the MS/MS-conditions for serum and WB are shown in **Table 7**.

The mass loss from precursor to product ion can be explained by the neutral loss of the glutamic acid residue from the protonated molecule to produce the major product ions $[M + H^+ - 147]$ for unlabeled and $[M + H^+ - 152]$ for $[^{13}C_5]$-labeled compounds. The analytes 5,10-methenylTHF, 10-formylTHF, and DHF produced only small amounts of this ion. For 5,10-methenylTHF a non-specific fragment loss of CO_2 $[M + H^+ - 44]$ occurred, whereas 10-formylTHF mostly converted to 5,10-methenylTHF (*m/z* 456). DHF showed mainly a cleavage between the pteridine and the *p*ABA moiety $[M + H^+ - 266]$, which was confirmed by the work of other groups (206). The collision-induced product ion spectra are summarized in **Figure 13**.

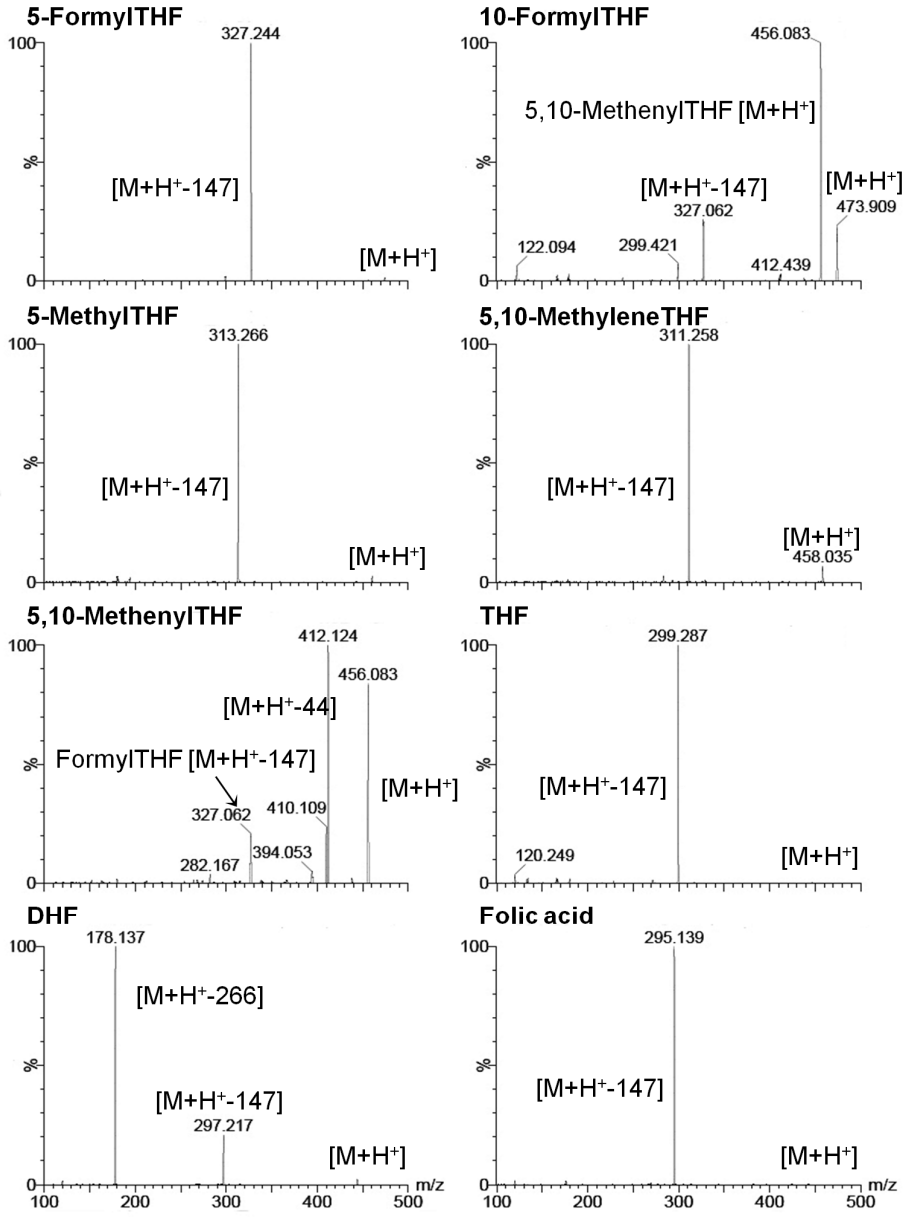

Figure 13: Collision-induced product ion spectra of the individual protonated folate forms. Spectra were produced in positive electrospray ionization mode of the mass spectrometer and were scaled to 100% on the basis of the most abundant product ion.

The MRM chromatograms from serum and WB are shown in **Figure 14**. In serum and WB, 5-methylTHF, [$^{13}C_5$]-5-methylTHF, 5,10-methenylTHF, [$^{13}C_5$]-5,10-methenylTHF, coeluted at t_Rs between 0.64 and 0.70 minutes. [$^{13}C_5$]-THF and THF coeluted in serum at 0.64 and 0.69 minutes but were not detected in WB hemolysates, possibly due to interconversions or degradation. 5-FormylTHF and FA, including their [$^{13}C_5$]-labeled compounds, coeluted between 0.93 and 1.01 minutes.

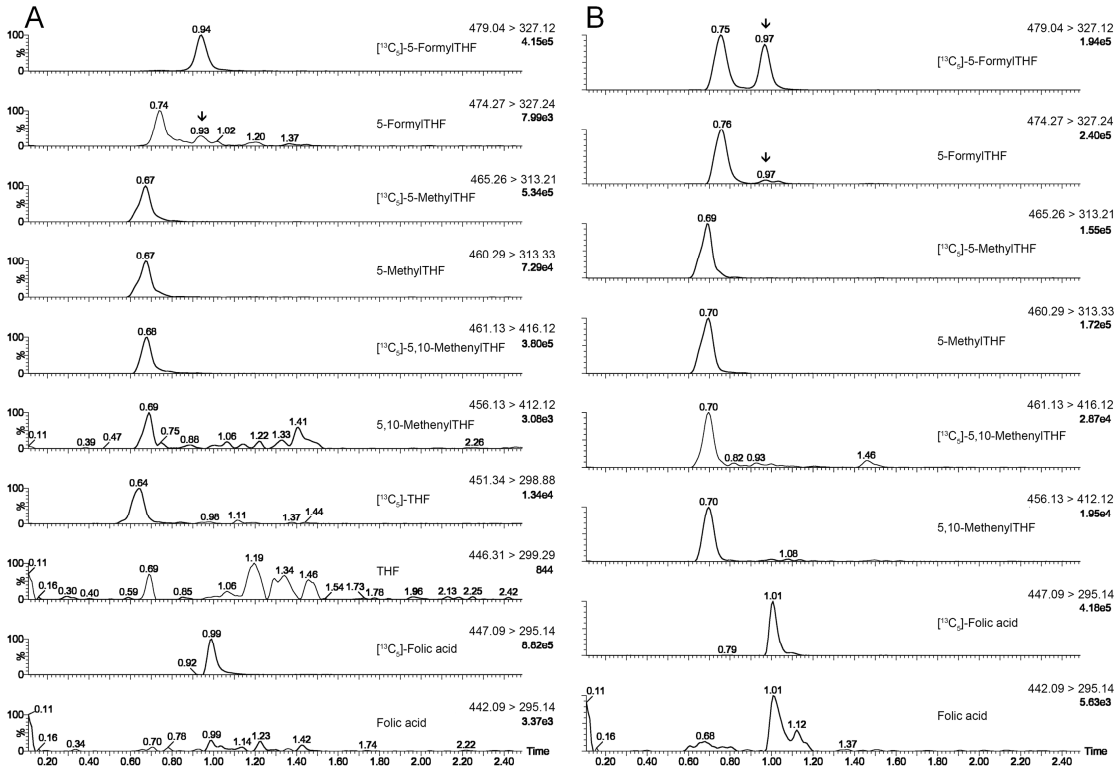

Figure 14: MRM-traces of folate forms in (A) a serum and (B) a whole blood sample. The serum sample concentrations (multiplied by the dilution factor) were 6.44 (12.89) nmol/L for 5-methylTHF, 0.08 (0.16) nmol/L for 5-formylTHF, 0.96 (1.93) nmol/L for THF, 0.03 (0.07) nmol/L for 5,10-methenylTHF, and 0.06 (0.11) nmol/L for FA. In the WB sample, measured concentrations (multiplied by the dilution factor and the hematocrit) of the folates were 61.0 (545) nmol/L for 5-methylTHF, 3.19 (28.5) nmol/L for 5-formylTHF, 28.3 (253) nmol/L for 5,10-methenylTHF, and 3.89 (34.8) nmol/L for FA. *m/z* transitions and peak intensities are shown in the upper right. The arrows indicate the correct peaks.

An additional peak emerged at ~ 0.75 minutes for 5-formylTHF and [$^{13}C_5$]-5-formylTHF. The additional peaks were excluded from the data analysis. 5-MethylTHF showed the highest peak intensity in serum and WB. Smaller but reproducible peaks were found for reduced folate forms, whereas 5,10-methenylTHF, THF, and FA were barely quantifiable in serum samples that have been collected from non-supplemented subjects. Due to low concentrations, 5-formylTHF was barely quantifiable in WB samples. THF was not detectable in WB samples.

Studies on the stability of folate forms *in vitro* and during sample preparation

The stability of aqueous folate solutions at 4°C over 24 hours with and without ascorbic acid at different pH was tested. Folate solutions (100 nmol/L) were prepared in water, in 1 g/L aqueous solution of ascorbic acid without pH adjustment (pH ~ 3.4), in 1 g/L aqueous solution of ascorbic acid (pH 2.6), and in 1 g/L aqueous solution of ascorbic acid (pH 7.0). The folate forms were measured at start, 1, 5, and 24 hours. The results are shown in **Table 10** and **Figure 15**.

Table 10: Interconversions of folate compounds after 24 h of incubation at 4°C (126).

Variable	**Main interconversion products**	**Main interconversion product of initial concentration in H_2O [%]**			
Antioxidant added		**-**	**ascorbic acid**	**ascorbic acid**	**ascorbic acid**
pH		**-**	**3.4**	**2.6**	**7.0**
Folic acid	none	-	-	-	-
5-MethylTHF	none	-	-	-	-
DHF	folic acid	15.0	16.7	16.2	12.7
5,10-MethenylTHF	5-formylTHF	4.8	4.7	4.3	3.4
5-FormylTHF	5,10-methenylTHF	1.2	24.1	51.2	1.4
	10-formylTHF	4.1	3.6	2.9	4.7
10-FormylTHF[a]	5,10-methenylTHF	75.2	102.0	104.1	70.6
	5-formylTHF	3.6	3.6	3.3	2.7
5,10-MethyleneTHF[b]	THF	0.1	34.2	28.1	45.9
	5,10-methenylTHF	2.0	1.3	2.2	1.6
THF	DHF	4.4	1.2	1.5	4.2
	folic acid	2.8	0.9	1.0	1.1
	5,10-methyleneTHF	0.1	0.2	0.3	2.7

Interconversions below the LOD were not included. The results are the percentages of the main interconversion products of the initial concentration at start time.
[a]: immediate interconversion to 5,10-methenylTHF at pH ≤ 7.0
[b]: immediate interconversion to THF and formaldehyde at pH ≤ 7.0

5-MethylTHF and FA were stable over 24 h at 4°C under all conditions with no evidence for interconversion to other forms (**Table 10**). DHF showed degradation at acidic conditions and interconversion to FA. 5-FormylTHF, 10-formylTHF, and 5,10-methenylTHF are known to undergo complex interconversion reactions under acidic conditions (50). We demonstrated that 5-formylTHF slowly converted under acidic conditions into 5,10-methenylTHF. In addition, small amounts of 10-formylTHF were detected. Due to acidic conditions during the sample preparation and the measurement, we expect that 5-formylTHF in serum samples might undergo interconversion thus causing some of the 5-formylTHF to be detected as 5,10-methenylTHF. 5,10-MethenylTHF was stable at acidic conditions and showed a minor interconversion to 5-formylTHF. 10-FormylTHF and 5,10-methyleneTHF were unstable under acidic and neutral conditions and were below the LOD of the assay.

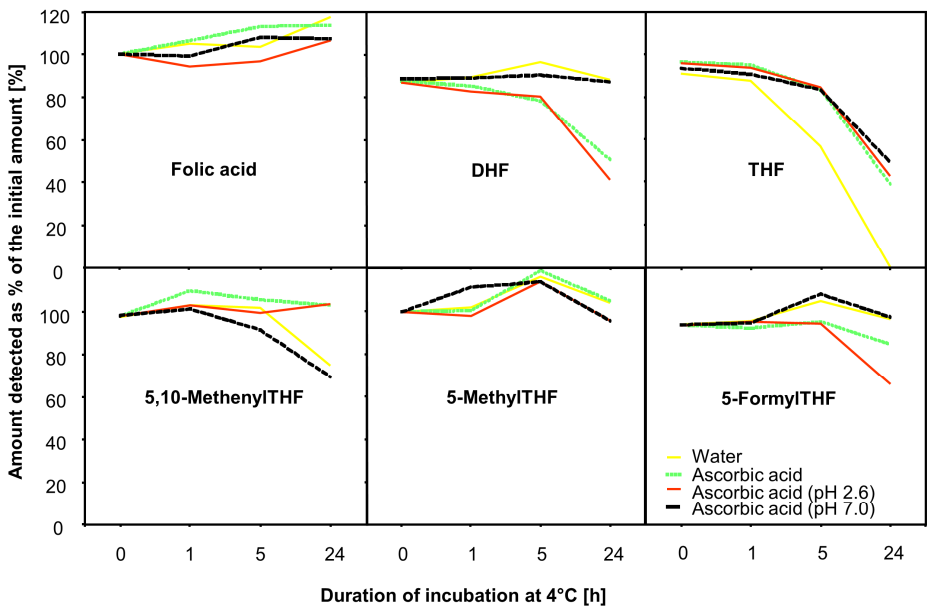

Figure 15: Stability of folate compounds (126). The amount of folate compounds are shown as percentage of the initial concentration (100 nmol/L) in H_2O, ascorbic acid solution (1 g/L), ascorbic acid solution pH 2.6, and ascorbic acid solution pH 7.0. The samples were incubated at 4°C for 24 h and measured immediately (start time), after 1, 5, and 24 h. 10-FormylTHF and 5,10-methyleneTHF were unstable under the selected conditions and were therefore not possible to detect.

10-FormylTHF immediately converted into 5,10-methenylTHF and small amounts of 5-formylTHF. Moreover, 5,10-methyleneTHF completely converted to THF and formaldehyde, with a slight interconversion to 5,10-methenylTHF. This unusual nonenzymatic oxidation reaction could not be prevented by the addition of the antioxidant ascorbic acid or flushing the H_2O with N_2 prior experiments. Impurities of the standards used could be an explanation. THF showed complete degradation within 24 h of incubation at 4°C in H_2O without antioxidant. By addition of ascorbic acid, this process could be strongly decelerated (**Figure 15**). Apart from the degradation, THF showed minor interconversions into FA and DHF. Interestingly, under pH 7.0 conditions small amounts of 5,10-methyleneTHF could be detected. This seems unusual due to the lack of formaldehyde as one-carbon donor.

In serum and WB, THF degraded to FA in a dose-dependent manner during the sample preparation (**Figure 16**). To exclude an enzymatic conversion of the folate forms, serum pool samples were inactivated by heating for 2 minutes at 100°C. THF was added at concentrations of 5 and 20 nmol/L to the serum pool samples, the heat inactivated serum pool samples (supernatant after 10 minutes at 10,000 rpm), and the WB pool hemolysate samples. The folate forms were measured after incubation for 15 minutes at ambient temperature in the dark. An increase of FA in serum by a mean of 0.9 nmol/L (after adding 5 nmol/L of THF) and 3.6 nmol/L (after adding 20 nmol/L of THF) was observed (**Figure 16**).

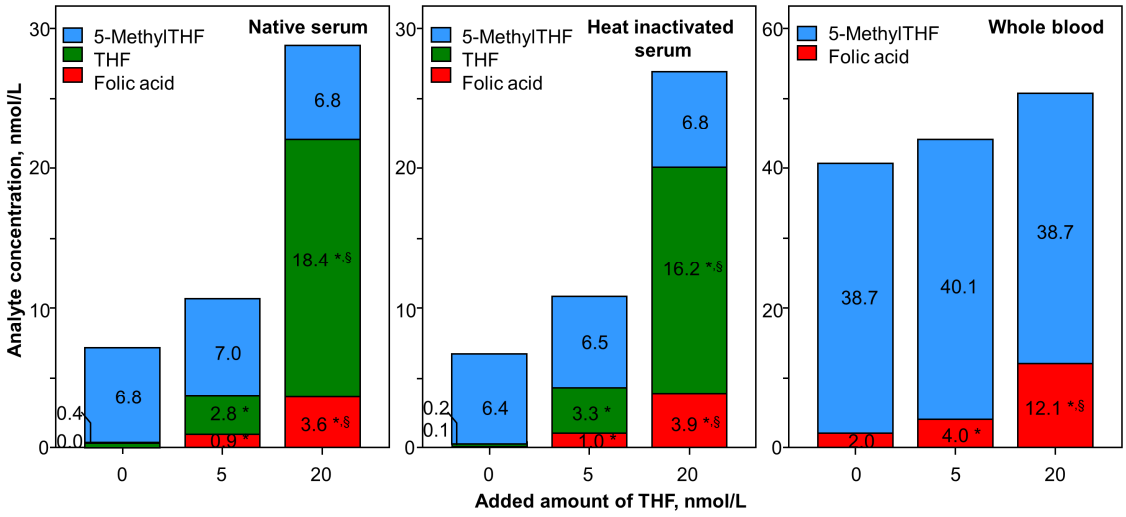

Figure 16: Median concentrations of folate forms in serum, in heat inactivated serum, and in whole blood pool hemolysates after spiking with tetrahydrofolate (123). THF was partly converted to FA in a dose-dependent manner. FA concentrations were 3 – 4 times higher in WB pool samples than in serum. The asterisks (*) indicate significant differences ($p < 0.05$) comparing the baseline concentration with the 5 nmol/L THF levels. The section signs (§) indicate significant differences ($p < 0.05$) comparing the 5 nmol/L THF concentration with the 20 nmol/L THF levels. P values were calculated using the Tamhane-T test.

The results were similar in the heat inactivated serum pool samples (0.9 and 3.8 nmol/L of FA after incubation with 5 and 20 nmol/L of THF, respectively), suggesting a non-enzymatic interconversion in the serum. The oxidation of THF to FA was ~ 2 – 3fold higher in WB hemolysates than in serum (mean 2.0 and 10.1 nmol/L increase in FA after spiking with 5 and 20 nmol/L of THF, respectively). This could be due to a higher proportion of ammonium acetate buffer (pH 10) or the longer time required for the SPE (more wash steps with larger volume). Possible oxidation and degradation products of THF are summarized in **Figure 17**.

We were able to quantify 5-methylTHF, 5-formylTHF, 5,10-methenylTHF, THF, and FA in serum. Due to interconversions of the folate forms, the measured THF concentrations were the sum of concentrations of THF and 5,10-methyleneTHF, whereas 5,10-methenylTHF concentrations represent the sum of 5,10-methenylTHF and 10-formylTHF. In WB we summarized the data as 5-methylTHF and non-methylTHF (sum of formylTHF, 5,10-methenylTHF, 5,10-methyleneTHF, THF, DHF, and FA).

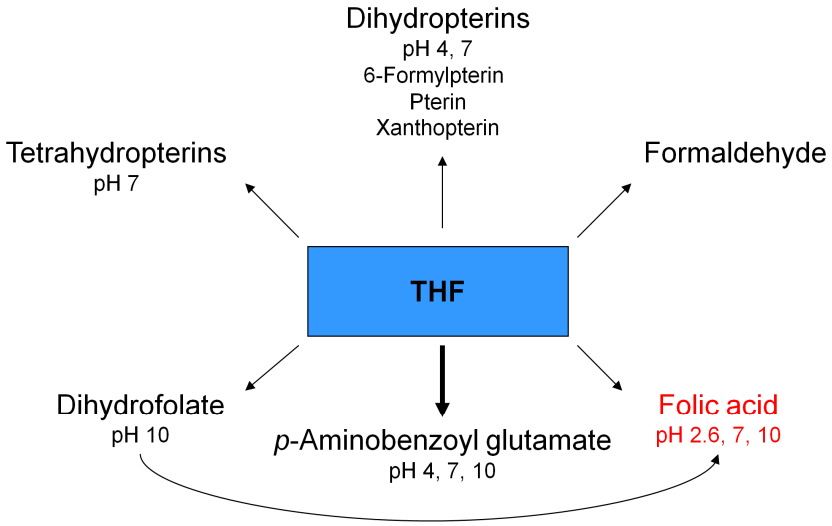

Figure 17: Possible ways of *in vitro* conversion of tetrahydrofolate and resulting compounds (123). Based on earlier reports (37;183) and own observations (126).

Linearity and sensitivity

Calibration curves were linear over the ranges of 0.2 – 200 nmol/L for all folate forms (**Figure 18**). The mean coefficients of linear regression for 5 independent experiments were $R^2 \geq 0.9999$ for all folate forms.

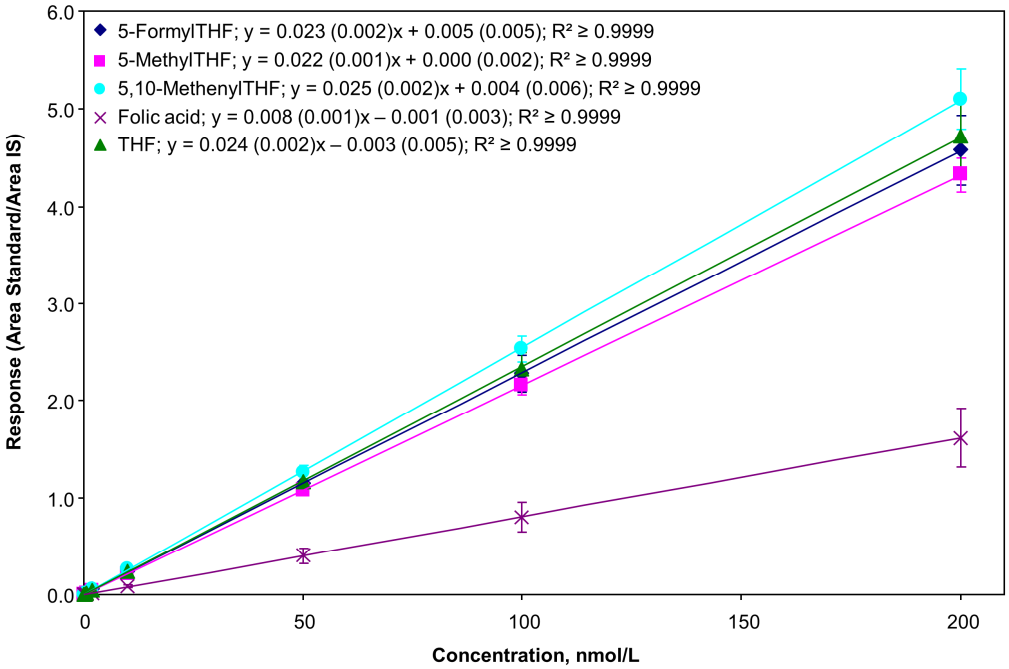

Figure 18: UPLC-MS/MS calibration curves for folate forms. Linear equations from 5 independent experiments are presented as mean (SD).

The mean (SD) IDLs were 0.23 (0.07) nmol/L for 5-methylTHF, 0.24 (0.11) nmol/L for 5-formylTHF, 0.26 (0.17) nmol/L for 5,10-methenylTHF, 0.76 (0.45) nmol/L for THF, and 0.14 (0.09) nmol/L for FA. The mean (SD) concentrations of the analytes in folate-free serum pool and WB pool hemolysates and the LODs were presented in **Table 11**. Corresponding LOQs in serum were 0.84 nmol/L for 5-methylTHF, 0.69 nmol/L for 5-formylTHF, 1.00 nmol/L for 5,10-methenylTHF, 2.01 nmol/L for THF, and 0.64 nmol/L for FA and in WB were 0.50 nmol/L for 5-methylTHF, 0.36 nmol/L for 5-formylTHF, 1.19 nmol/L for 5,10-methenylTHF, and 0.45 nmol/L for FA.

Table 11: Limit of detection and recovery of folate forms.

Variable	LOD[a]		Recovery[b]			
	Measured	LOD	Endogenous	Spike	Measured in spiked samples	% Recovery (SD)
Analyte concentrations in serum						
5-MethylTHF	1.44 (0.10)	0.28	6.9 (0.3)	40.0	46.9 (1.7)	99.5 (7.8)
				5.00	11.7 (0.4)	97.3 (2.3)
5-FormylTHF	1.06 (0.08)	0.23	0.12 (0.06)	4.00	4.15 (0.60)	98.6 (9.2)
				0.50	0.67 (0.10)	105.9 (9.7)
5,10-MethenylTHF[c]	1.29 (0.12)	0.33	0.02 (0.02)	4.00	4.46 (0.23)	112.4 (8.7)
				0.50	0.54 (0.06)	110.1 (14.2)
THF[d]	0.79 (0.24)	0.67	0.38 (0.20)	4.00	3.88 (1.28)	73.9 (25.1)
				0.50	0.73 (0.56)	82.0 (43.5)
Folic acid[e]	1.35 (0.24)	0.21	0.06 (0.01)	4.00	3.67 (0.04)	92.2 (5.7)
				0.50	0.56 (0.03)	104.3 (10.8)
Analyte concentrations in whole blood						
5-MethylTHF	1.52 (0.06)	0.17	45.2 (0.46)	20.0	64.3 (0.37)	98.4 (0.4)
				6.25	51.1 (1.55)	99.1 (2.3)
5-FormylTHF	1.81 (0.04)	0.12	1.41 (1.31)	20.0	22.0 (1.04)	101.6 (8.1)
				6.25	7.6 (0.71)	97.1 (6.9)
5,10-MethenylTHF[c]	2.07 (0.14)	0.40	4.76 (1.51)	20.0	24.9 (2.13)	100.9 (6.7)
				6.25	10.8 (1.73)	99.6 (6.0)
Folic acid[e]	1.34 (0.05)	0.15	0.76 (0.52)	20.0	20.9 (1.48)	99.7 (3.9)
				6.25	7.3 (0.71)	102.7 (4.1)

The data are means (SD), the concentration are nmol/L if not stated otherwise.
[a]: For the LOD determination, serum pool samples and hemolysates from WB pool samples (n = 10) were treated with activated charcoal for 40 minutes at ambient temperature prior experiment until they reached concentrations of 1 – 5 times the instrumental detection limit. Shown analyte concentrations have not been multiplied by the dilution factor and divided by the hematocrit.
[b]: Mean of 4 independent experiments for serum and n = 3 for WB. Experiments were performed using 3 individually prepared samples, each. Shown concentrations have not been multiplied by dilution factor and divided by hematocrit.
[c]: Sum of 5,10-methenylTHF and 10-formylTHF.
[d]: Sum of THF and 5,10-methyleneTHF.
[e]: Sum of FA, DHF, and partly oxidized THF.

Precision and recovery

The precision of the method was assessed by quantifying the folate forms in an in-house prepared serum and WB pool. The intraassay and the interassay CVs are shown in **Table 12**. In serum pool, the concentrations of FA, THF, and 5,10-methenylTHF were below the LOQs. 5-MethylTHF had an interassay CV of 2.8% in serum samples and 7.4% in WB samples. The interassay CVs for the quality control samples (0.6 and 160 nmol/L of each folate form) for 10 days were between 1.9% (5-formylTHF in 160 nmol/L quality control sample) and 11.2% (THF in 0.6 nmol/L of quality control sample) (123).

Table 12: Precision of folate forms in serum pool and whole blood pool samples.

Variable	Serum		Whole blood	
	Mean (SD) [nmol/L]	CV [%]	Mean (SD) [nmol/L]	CV [%]
Intraassay (n = 10)				
5-MethylTHF	12.6 (0.3)	2.0	399 (13.7)	3.4
5-FormylTHF	0.17 (0.01)	7.2	-	-
5,10-MethenylTHF[a]	< LOQ[e]	-	-	-
THF[b]	< LOQ[e]	-	-	-
Folic acid[c]	< LOQ[e]	-	-	-
Non-methylTHF[d]	-	-	65.5 (4.5)	6.9
Interassay (n = 10)				
5-MethylTHF	13.5 (0.4)	2.8	405 (29.9)	7.4
5-FormylTHF	< LOQ[e]	-	-	-
5,10-MethenylTHF[a]	< LOQ[e]	-	-	-
THF[b]	< LOQ[e]	-	-	-
Folic acid[c]	< LOQ[e]	-	-	-
Non-methylTHF[d]	-	-	64.2 (9.9)	15.4

Folates in WB have been multiplied by the dilution factor and divided by the hematocrit.
[a]: Sum of 5,10-methenylTHF and 10-formylTHF.
[b]: Sum of THF and 5,10-methyleneTHF.
[c]: Sum of FA, DHF, and partly oxidized THF.
[d]: Sum of formylTHF, 5,10-methenylTHF, 5,10-methyleneTHF, THF, DHF, and FA.
[e]: Between LOD and LOQ.

Recovery experiments were performed by spiking serum samples and WB pool hemolysates with two different levels over a period of three days (**Table 11**). Mean recoveries were 82.3% (THF), 98.2% (FA and 5-methylTHF), 102.3% (5-formylTHF), and 110.8% (5,10-methenylTHF) in serum samples and 98.8% (5-methylTHF), 99.3% (5-formylTHF), 100.3% (5,10-methenylTHF), and 101.2% (FA) in WB pool samples.

Evaluation of the relative matrix effect in whole blood

The relative matrix effect was 2.2% for 5-methylTHF, 2.5% for 5-formylTHF, 2.8% for 5,10-methenylTHF, and 4.0% for FA. The addition of the stable isotope-labeled analytes effectively eliminated the relative matrix effect in WB hemolysates (relative matrix effect $\leq$ 4.0%) (123).

Method comparison

The sum of folate forms in 70 serum samples was assessed by UPLC-MS/MS and by ADVIA Centaur (TFOL). Concentrations of the sum of folates measured by UPLC-MS/MS correlated strongly with those measured by the ADVIA Centaur ($R = 0.939$; $p < 0.001$). The mean (SD)

sum of folates concentration in serum samples was 18.5 (12.1) nmol/L for the UPLC-MS/MS method and the TFOL was 23.4 (13.7) nmol/L for the ADVIA Centaur.

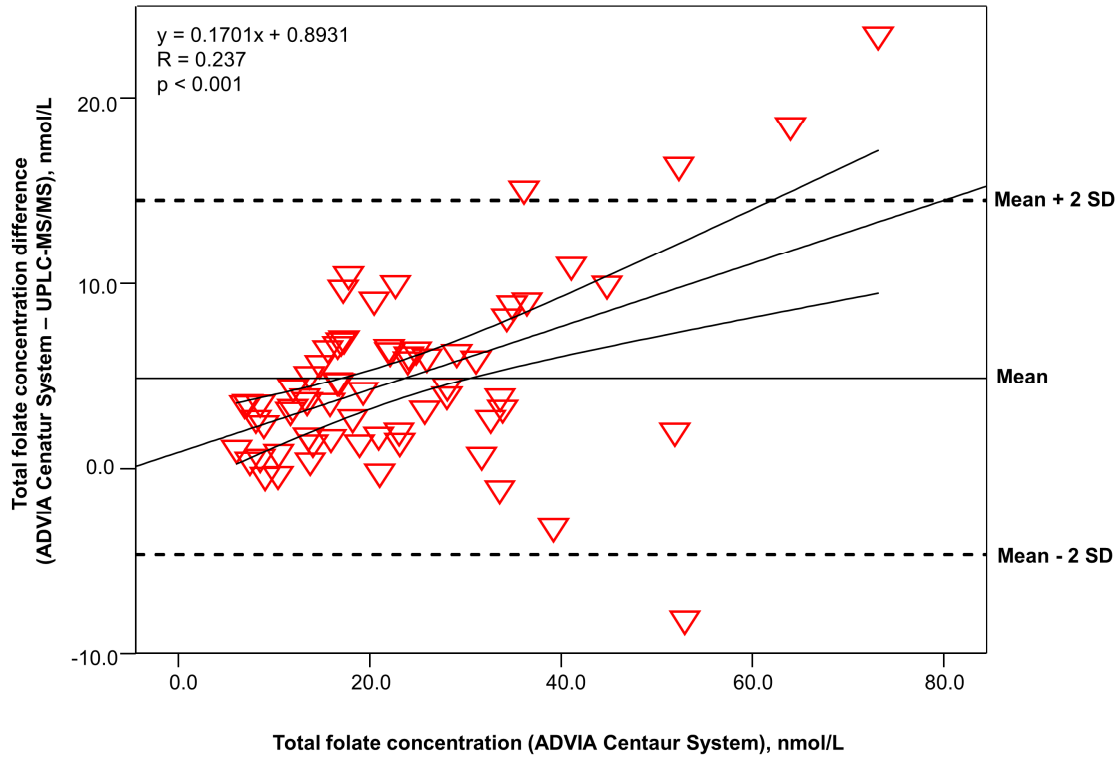

Figure 19: Bland-Altman difference plot between total folate concentrations of n = 70 serum samples obtained by immunological assay (ADVIA Centaur System) and UPLC-MS/MS (126). The *solid line* represents the mean difference between the two methods. The *dashed lines* represent the 95% limits of agreement of the differences between the two methods (mean difference ± 2 SD).

The sum of the folate forms measured by the UPLC-MS/MS method were generally lower than the TFOL measured by ADVIA Centaur. The Bland-Altman difference plot indicated that the difference between the two methods positively correlated to the TFOL measured by the immunoassay (**Figure 19**). At folate concentrations below 35 nmol/L, the two methods showed a mean (SD) difference of 4.9 (4.8) nmol/L.

Studies on concentrations of folate forms and reference ranges in healthy individuals

The concentrations of folate forms were measured in serum samples from 32 apparently healthy non-vitamin users (non-fasting conditions), in the WB of 42 non-supplemented fasting subjects, and in the WB of 35 supplemented fasting subjects (500 µg FA, 50 mg vitamin B_6, and 500 µg vitamin B_{12} /d for 6 months) (**Table 13**). 5-MethylTHF and its respective polyglutamates were the predominant folate forms in serum and WB. In serum, the median concentrations of 5-formylTHF, 5,10-methenylTHF, and FA were above the LOD but could not reach the LOQ.

5-MethylTHF and the non-methyl folate forms in WB were higher in supplemented than in non-supplemented subjects.

Our results regarding serum folate concentrations are in line with similar reports (**Table 13**). Wang *et al.* reported mean 5-methylTHF concentrations of 14.6 nmol/L, 4.35 nmol/L of 5-formylTHF, and 1.39 nmol/L of FA (241). The serum 5-methylTHF concentrations reported by Obeid *et al.* (168) were lower than those reported by other groups presumably due to the higher age of the participants. They detected higher levels of 5-formylTHF and FA, but no THF and 5,10-methenylTHF.

Folate concentrations from countries with FA fortification programs or supplemented subjects were higher in WB folate content than in non-vitamin users (**Table 13**). The WB 5-methylTHF concentration in our assay of 42 adults without FA fortification and supplementation is similar to that obtained by Smulders *et al.* (n = 109 adults from The Netherlands). However, we found higher non-methylTHF concentration. Fazili *et al.* reported 50% lower folate concentration, measured in 75 WB samples obtained from an European blood bank (71). Our results on 5-methylTHF after supplementation of 35 adults with B-vitamins (500 µg FA, 50 mg vitamin B_6, and 500 µg vitamin B_{12}) for 6 months are in the range of that obtained in fortified countries (104;216). The non-methylTHF concentrations in our study were higher than those reported by Summers *et al.* (216).

Table 13: Folate concentrations in serum, plasma, and whole blood samples determined by LC-MS/MS (123).

Reference	Material	Subjects	Supplementation	5-MethylTHF [nmol/L]	Non-methylTHF forms [nmol/L]
Countries without mandatory folic acid fortification					
Kirsch *et al.* (123;126)[a]	Serum WB WB	32 adults (8 males) 42 adults (8 males) 35 adults (11 males)	no no n = 35[g]	15.8 (5.6 – 26.7) 576 (264 – 886) 1,206 (841 – 2,067)	2.07 (< LOD – 4.05) THF 73.6 (52.5 – 120)[f] 155 (97.3 – 252)[f]
Obeid *et al.* (168)[a]	Serum	37 older adults (71 – 88 years)	no	5.6 (2.4 – 19.1)	3.4 (1.3 – 16.0) THF 0.08 (0.00 – 0.85) folic acid
Wang *et al.* (241)	Serum	50 pregnant women	no data available	14.58 (9.06)	4.35 (2.60) 5-formylTHF 1.39 (2.93) folic acid
Fazili *et al.* (71)	WB	75 from an European blood bank	no data available	207 (30.2 – 462)[b]	29.2 (13.1 – 68.9)[b] 5-formylTHF 9.65 (0 – 167) 5,10-methenylTHF 0 (0 – 23.5) THF
Smulders *et al.* (207)	WB	109 adults (52 males)	no	427 (92.5 – 1,089)[b]	4.1 (0 – 786)[b,f]
Countries with mandatory folic acid fortification (collected after introduction of folic acid fortification programs)					
Summers *et al.* (216)	Plasma WB	21 Caucasian women	n = 15[c]	50.2 (22.5)[d] 1,122 (279)[d]	no data available 37.5 (3.2)[d] THF
Fazili *et al.* (71)	WB	96 from an U.S. blood bank	no data available	304 (94.7 – 703)[b]	41.4 (22.7 – 93.9)[b] 5-formylTHF 10.1 (0 – 212) 5,10-methenylTHF 0 (0 – 142) THF

The data are medians (10^{th} – 90^{th} percentiles), [b]: medians (range), [d]: means (SD), or [e]: mean concentrations.
[a]: UPLC-MS/MS.
[c]: Supplements included multivitamins, B-vitamins, and FA.
[f]: Sum of formylTHF, 5,10-methenylTHF, 5,10-methyleneTHF, THF, DHF, and FA.
[g]: 500 µg FA, 50 mg vitamin B_6, 500 µg vitamin B_{12} /d.

4.2 Folate forms distribution before and after short-term and long-term supplementation with B-vitamins in older adults in relation to the MTHFR C677T polymorphism

For examination of the folate concentration and the distribution of the key folate forms in serum and in WB, as well as of related metabolites in older adults we combined the baseline values of the two studies (short-term supplementation and long-term supplementation study). Parts of the results were reported earlier (122). Information concerning the study population and the baseline characteristics of the two clinical studies are described in the following paragraphs.

Short-term supplementation with folic acid vs. folic acid plus vitamin B_6 and B_{12}

An overview of the number of study participants of the short-term co-supplementation study, as well was their group assignment over the duration of the study are presented in **Table 14**. Seventyseven subjects were randomized to receive the vitamin supplements (group T1 (FA) and group T2 (FA, B_6, B_{12})) out of which 54 participants completed the study. Ten (18.5%) of the 54 participants were males. The median (10^{th} – 90^{th} percentiles) duration of the supplement intake was 23.5 (15.0 – 34.5) days for group T1 and 26.0 (14.0 – 33.0) days for group T2.

Table 14: Overview of number and group assignment of study participants (short-term supplementation study).

	Group T1[a] FA	Group T2[b] FA, B_6, B_{12}	Total	Comment
Study participants randomized	40	37	77	
Blood collection at baseline	40	37	77	
Blood collection at study end	30	24	54	23 persons withdrew or were excluded due to termination criteria

[a]: 400 µg FA /d.
[b]: 400 µg FA, 8 mg vitamin B_6, 10 µg vitamin B_{12} /d.

The baseline study characteristics of the study participants who completed the study are presented in **Table 15**. We could find no significant differences between the two groups at baseline. The median (10^{th} – 90^{th} percentiles) plasma glucose concentration was above the reference range (≤ 100 mg/dL) in both groups.

Table 15: Baseline study characteristic (short-term supplementation study).

Variable	Group T1[a] FA n = 30	Group T2[b] FA, B_6, B_{12} n = 24	p value
Females, n (%)	26 (86.7%)	18 (75.0%)	-
Study duration, d	23.5 (15.0-34.5)	26.0 (14.0-33.0)	0.930
Age, y	81 (72 – 88)	84 (73 – 92)	0.106
Hemoglobin, g/dL	11.5 (9.7 – 13.7)	11.4 (9.2 – 13.8)	0.859
Hematocrit, %	35.2 (29.3 – 40.2)	34.6 (29.4 – 42.2)	0.579
Creatinine, µmol/L	70.7 (53.0 – 121.1)	70.7 (44.2 – 109.6)	0.745
Glucose, mg/dL	126 (88 – 216)	114 (84 – 236)	0.311
ALAT, U/L	22.0 (10.3 – 38.7)	22.0 (9.8 – 39.8)	0.936
CRP, mg/L	1.27 (0.27 – 4.49)	1.73 (0.20 – 4.38)	0.368
Total cholesterol, mg/dL	197 (138 – 263)	189 (135 – 270)	0.795
HDL cholesterol, mg/dL	45.1 (33.4 – 75.1)	47.0 (31.9 – 79.7)	0.975
Triglycerides, mg/dL	155 (73 – 229)	161 (70 – 297)	0.893

The data are medians (10^{th} – 90^{th} percentiles). P values are according to the Mann-Whitney-U test.
[a]: 400 µg FA /d.
[b]: 400 µg FA, 8 mg vitamin B_6, 10 µg vitamin B_{12} /d.

Long-term supplementation with calcium, vitamin D, folic acid, vitamin B_6, and vitamin B_{12} vs. calcium and vitamin D

An overview of the study participants of the long-term B-vitamins supplementation study, as well was their group assignment over the duration of the study is presented in **Table 16**. From a total sum of 111 randomized subjects, 96 were selected to take the vitamin supplements (group A (B, D, Ca) or group B (D, Ca)) out of which 65 participants completed the study (study duration: 12 months).

Table 16: Overview of number and group assignment of study participants (long-term supplementation study).

	Group A[a] B, D, Ca	Group B[b] D, Ca	Total	Comment
Study participants randomized	59	52	111	14 persons withdrew/were excluded: 10 on account of premedication, 4 on account of personal reasons
Blood collection at baseline	50	46	96	
Study participants after 6 months	37	34	71	25 persons withdrew/were excluded: 10 on account of personal reasons, 8 on account of adverse reactions such as gastric disorders and mood swings, 6 on account of termination criteria such as surgery, severe illness, infarction or intake of medication/ supplements, 1 deceased
Blood collection after 6 months	35	31	66	from 5 persons no blood was collected
Blood collection after 12 months	34	31	65	6 persons withdrew on account of personal reasons

[a]: 500 µg FA, 500 µg vitamin B_{12}, 50 mg vitamin B_6, 456 mg calcium, and 1,200 IU vitamin D /d.
[b]: 456 mg calcium and 1,200 IU vitamin D /d.

The baseline study characteristics of all participants (intention-to-treat) were presented in **Table 17**. The study included 39 males (40.6%) who started the intervention. We could find no significant differences between the two groups at baseline. The median (10^{th} – 90^{th} percentiles) plasma glucose concentrations were above the reference range (≤ 100 mg/dL) in both groups. In addition, the median total cholesterol concentration in group A was elevated (reference range: ≤ 200 mg/dL).

Table 17: Baseline study characteristic (long-term supplementation study).

Variable	Group A[a] B, D, Ca	n	Group B[b] D, Ca	n	p value
Females, n (%)	35 (70 %)	50	22 (48%)	46	-
Age, y	68 (54 – 83)	50	71 (58 – 86)	46	0.089
Hemoglobin, g/dL	14.4 (12.6 – 16.1)	39	14.2 (12.4 – 15.4)	39	0.121
Hematocrit, %	42.5 (39.3 – 47.5)	39	41.9 (36.9 – 45.2)	39	0.079
Creatinine, µmol/L	70.7 (53.0 – 113.2)	41	79.6 (53.0 – 114.0)	40	0.867
Glucose, mg/dL	104 (94 – 125)	41	106 (88 – 137)	40	0.590
ALAT, U/L	22.0 (14.2 – 44.4)	41	23.5 (14.1 – 40.5)	40	0.917
CRP, mg/L	1.50 (0.60 – 5.28)	41	1.05 (0.60 – 4.78)	40	0.211
Total cholesterol, mg/dL	208 (149 – 254)	41	200 (142 – 261)	40	0.688
HDL cholesterol, mg/dL	56 (37 – 84)	41	55 (33 – 94)	40	0.561
Triglycerides, mg/dL	101 (60 – 165)	41	116 (64 – 281)	40	0.279

The data are medians (10^{th} – 90^{th} percentiles). P values are according to the Mann-Whitney-U test.
[a]: 500 µg FA, 500 µg vitamin B_{12}, 50 mg vitamin B_6, 456 mg calcium, and 1,200 IU vitamin D /d.
[b]: 456 mg calcium and 1,200 IU vitamin D /d.

4.2.1 Folate forms distribution in older adults and the effect of B-vitamins supplementation

The combined study included 146 non-supplemented and non-fortified older adults (median (10^{th} – 90^{th} percentiles) age = 74 (58 – 87) years, 50 males). The concentrations of the folate forms and of related metabolites are presented in **Table 18**. The median serum concentration of 5-methylTHF was in the range found in our earlier study on older adults (168). 5-MethylTHF was the predominant folate form in serum and WB and constituted 87.5% (70.2 – 96.0%) of the sum of folates in serum. 5-MethylTHF and non-methylTHF in WB were in the normal range. The minor folate forms often did not reach the LOD. In serum, 5-formylTHF concentrations ≥ 0.10 nmol/L were detected in 122 (83.6%) subjects, THF ≥ 0.91 nmol/L in 88 (60.3%) subjects, and 5,10-methenylTHF ≥ 0.16 nmol/L in 70 (47.9%) subjects. Unmetabolized FA (≥ 0.21 nmol/L) was detected in the serum of 17 (11.6%) of the subjects.

Table 18: Folate forms in serum and whole blood (n = 146) (122).

Variable	
S sum of folates, nmol/L	11.9 (4.7 – 36.9)
S 5-methylTHF	10.0 (3.4 – 35.3)
S 5-formylTHF, nmol/L	0.14 (< LOD – 0.51)
S 5,10-methenylTHF, nmol/L[a]	0.08 (< LOD – 0.25)
S THF, nmol/L[b]	1.03 (< LOD – 3.47)
S 5-methylTHF/THF ratio	9.0 (2.8 – 40.9)
WB sum of folates, nmol/L	562 (325 – 1,013)
WB 5-methylTHF, nmol/L	495 (257 – 893)
WB non-methylTHF, nmol/L[c]	72.7 (47.9 – 126.2)

The data are medians (10^{th} – 90^{th} percentiles) unless otherwise specified.
[a]: Sum of 5,10-methenylTHF and 10-formylTHF.
[b]: Sum of THF and 5,10-methyleneTHF.
[c]: Sum of 5-formylTHF, 10-formylTHF, 5,10-methenylTHF, 5,10-methyleneTHF, THF, DHF, and FA.

Short-term supplementation folic acid vs. folic acid plus vitamin B_6 and B_{12}

We tested the effect of short-term FA supplementation with and without the combination of vitamin B_6 and B_{12} on the folate forms distribution in serum and WB. The folate forms in serum and WB samples of the study participants who completed the study and had WB and serum available from baseline and after the supplementation are summarized in **Table 19**. 5-MethylTHF was the predominant folate form in serum (as monoglutamate) and WB (as polyglutamates) at baseline and after supplementation in both study arms (**Table 19**). At baseline, median (10^{th} – 90^{th} percentiles) 5-methylTHF serum concentrations in both groups were lower than in younger and non-fasting adults (**Table 13**).

5-MethylTHF in serum was significantly higher after supplementation with FA in group T1 (~ 2.2fold, $p = 0.022$) and group T2 (~ 2.2fold, $p < 0.001$) (**Figure 20**). All other folate forms did not differ significantly comparing the baseline levels and the concentrations after the supplementation. After the supplementation, we found no significant changes of serum 5-methylTHF concentrations comparing group T1 and group T2 ($p = 0.338$). Compared to group T2, unmetabolized FA was significantly higher in group T1 after the supplementation. Folate forms in serum showed no significant differences between both groups at baseline and after supplementation for 3 – 4 weeks.

Table 19: Folate forms in serum and whole blood of study participants (short-term supplementation study).

Variable	Group T1[a] FA n = 30 [nmol/L]	Group T2[b] FA, B_6, B_{12} n = 24 [nmol/L]	p value
Baseline			
S 5-methylTHF	6.9 (3.2 – 20.1)	6.1 (3.0 – 15.3)	0.562
S 5-formylTHF	< LOD	0.05 (< LOD – 0.27)	0.502
S 5,10-methenylTHF[c]	0.01 (< LOD – 0.26)	0.00 (< LOD – 0.20)	0.646
S THF[d]	1.08 (< LOD – 3.52)	0.99 (< LOD – 2.84)	0.376
WB 5-methylTHF	570 (296 – 1,086)	589 (239 – 845)	0.981
WB non-methylTHF[f]	69.4 (50.9 – 145.5)	81.0 (52.4 – 120.6)	0.644
After supplementation			
S 5-methylTHF	15.1 (7.0 – 24.9)	13.5 (7.6 – 24.9)	0.338
S 5-formylTHF	0.08 (< LOD – 0.32)	0.10 (< LOD – 0.24)	0.378
S 5,10-methenylTHF[c]	0.08 (< LOD – 0.26)	0.00 (< LOD – 0.31)	0.224
S THF[d]	1.74 (< LOD – 3.32)	1.64 (< LOD – 2.82)	0.377
S folic acid[e]	0.53 (< LOD – 1.56)	0.17 (< LOD – 0.51)	**0.001**
WB 5-methylTHF	731 (521 – 1,299)	689 (362 – 943)	0.295
WB non-methylTHF[f]	92.8 (64.1 – 165.9)	90.0 (55.4 – 161.6)	0.903

The data are medians (10^{th} – 90^{th} percentiles). P values are according to the Mann-Whitney-U test.
[a]: 400 µg FA /d.
[b]: 400 µg FA, 8 mg vitamin B_6, 10 µg vitamin B_{12} /d.
[c]: Sum of 5,10-methenylTHF and 10-formylTHF.
[d]: Sum of THF and 5,10-methyleneTHF.
[e]: Sum of FA, DHF, and partly oxidized THF.
[f]: Sum of 5-formylTHF, 10-formylTHF, 5,10-methenylTHF, 5,10-methyleneTHF, THF, DHF, and FA.

Comparing the treatment arm T1 with T2, we could find no significant differences of the baseline concentrations and after the supplementation of FA with and without co-supplementation with vitamins B_6 and B_{12} in the WB folate forms. After the supplementation, WB 5-methylTHF ($p < 0.001$ for both T1 and T2) and WB non-methylTHF concentrations (1.3fold, $p = 0.003$ for T1 and 1.1fold, $p = 0.014$ for T2) were significantly higher in both groups.

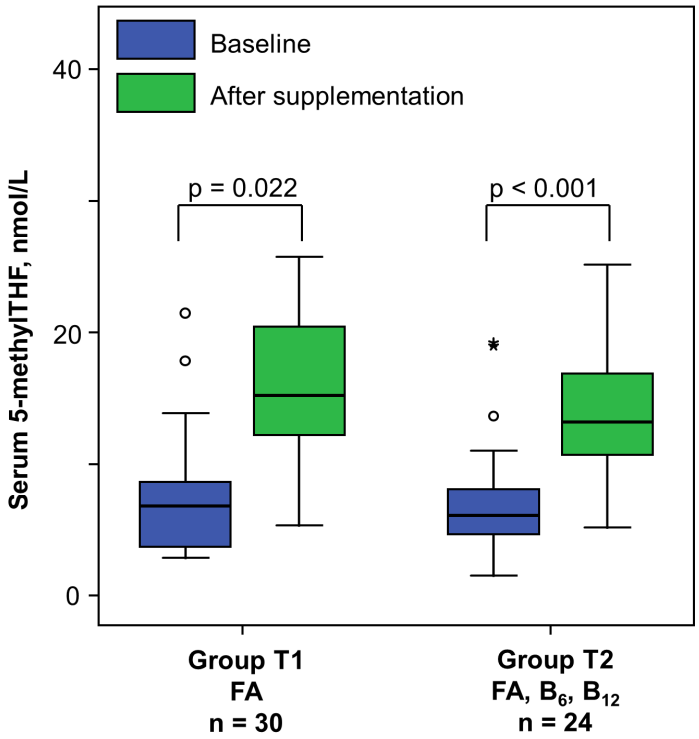

Figure 20: 5-MethylTHF concentrations in serum at baseline and after 3 – 4 weeks of supplementation (short-term supplementation study). After the supplementation group T1 and T2 showed significantly higher 5-methylTHF concentrations compared to the baseline. No significant changes were observed for 5-methylTHF concentrations comparing both groups**.**

Long-term supplementation with calcium, vitamin D, folic acid, vitamin B_6, and vitamin B_{12} vs. calcium and vitamin D

We tested the effect of long-term (12 months) B-vitamins supplementation on the folate forms distribution in serum and WB. The folate forms in serum and WB samples of the study participants, who completed the study and had WB and serum available from baseline, after 6 months, and after 12 months (n = 59) are summarized in **Table 20**. 5-MethylTHF, monoglutamates and polyglutamates, respectively, were the predominant folate form, in serum and WB at baseline and after the supplementation in both study arms (**Table 20**).

At baseline, the median ($10^{th} – 90^{th}$ percentiles) 5-methylTHF serum concentrations were comparable to the concentrations of younger and non-fasting adults (15.8 (5.6 – 26.7) nmol/L) shown in **Table 13**. Folate forms showed no significant differences between the two groups. After 6 months of supplementation, group A had significantly higher serum concentrations of 5-methylTHF (~ 2.5fold), 5,10-methenylTHF (~ 6fold), THF (~ 2fold), and FA (~ 1.5fold) compared to group B, which received no B-vitamins supplementation.

Table 20: Folate forms in serum and whole blood of study participants who completed the study (long-term supplementation study).

Variable	Group A[a] B, D, Ca n = 31 [nmol/L]	Group B[b] D, Ca n = 28 [nmol/L]	p value
Baseline			
S 5-methylTHF	15.4 (6.3 – 36.3)	19.4 (4.4 – 47.2)	0.616
S 5-formylTHF	0.19 (< LOD – 0.70)	0.26 (< LOD – 0.70)	0.649
S 5,10-methenylTHF[c]	0.13 (< LOD – 0.27)	0.12 (< LOD – 0.32)	0.613
S THF[d]	1.04 (< LOD – 3.96)	1.64 (< LOD – 5.59)	0.395
WB 5-methylTHF[f]	424 (219 – 882)	438 (251 – 902)	0.999
WB non-methylTHF[f,g]	69 (48 – 127)	68 (40 – 149)	0.930
After 6 months of supplementation			
S 5-methylTHF	46.8 (28.5 – 75.4)	17.0 (6.8 – 34.4)	**<0.001**
S 5-formylTHF	0.31 (0.18 – 0.65)	0.38 (0.20 – 0.72)	0.098
S 5,10-methenylTHF[c]	0.44 (0.18 – 6.45)	0.07 (< LOD – 0.26)	**<0.001**
S THF[d]	2.28 (0.98 – 8.88)	1.01 (< LOD – 4.29)	**0.001**
S folic acid[e]	0.12 (< LOD – 0.41)	0.08 (< LOD – 0.21)	**0.040**
WB 5-methylTHF[f]	1,278 (971 – 2,159)	534 (301 – 1,080)	**<0.001**
WB non-methylTHF[f,g]	157 (76 – 269)	74 (31 – 144)	**<0.001**
After 12 months of supplementation			
S 5-methylTHF	44.7 (17.6 – 70.9)	14.2 (5.8 – 35.7)	**<0.001**
S 5-formylTHF	0.21 (< LOD – 0.30)	0.18 (< LOD – 0.45)	0.808
S 5,10-methenylTHF[c]	0.06 (< LOD – 0.20)	0.08 (< LOD – 0.29)	0.460
S THF[d]	2.22 (< LOD – 5.74)	1.11 (< LOD – 3.46)	**0.004**
S folic acid[e]	0.39 (< LOD – 4.58)	0.06 (< LOD – 0.23)	**<0.001**
WB 5-methylTHF[f]	1,234 (694 – 2,030)	476 (242 – 836)	**<0.001**
WB non-methylTHF[f,g]	120 (94 – 241)	62 (43 – 124)	**<0.001**

The data are medians (10^{th} – 90^{th} percentiles). P values are according to the Mann-Whitney-U test.
[a]: 500 µg FA, 500 µg vitamin B_{12}, 50 mg vitamin B_6, 456 mg calcium, and 1,200 IU vitamin D /d.
[b]: 456 mg calcium and 1,200 IU vitamin D /d.
[c]: Sum of 5,10-methenylTHF and 10-formylTHF.
[d]: Sum of THF and 5,10-methyleneTHF.
[e]: Sum of FA, DHF, and partly oxidized THF.
[f]: WB folate forms were available for n = 19 subjects in group A and n = 22 subjects in group B.
[g]: Sum of 5-formylTHF, 10-formylTHF, 5,10-methenylTHF, 5,10-methyleneTHF, THF, DHF, and FA.

In group A, 5-methylTHF concentrations were comparable after 6 and 12 months and significantly higher compared to the baseline (~ 3fold higher, $p < 0.001$). In group B, the median 5-methylTHF concentrations in serum were highest at baseline and lower after 6 months and after 12 months. In group A, the THF concentrations in serum were significantly higher after 6 months ($p = 0.002$) and after 12 months ($p = 0.009$), as well as FA ($p = 0.009$ after 6 months and $p < 0.001$ after 12 months). Serum 5,10-methenylTHF was significantly higher after 6 months in group A. In group B, serum FA was significantly higher after 6 months compared to the baseline concentrations ($p = 0.016$).

Comparing group A and B, the median (10^{th} – 90^{th} percentiles) 5-methylTHF and non-methylTHF concentrations in WB were not significantly different at baseline, but after 6 months (~ 2.4fold higher 5-methylTHF and ~ 2.1fold higher non-methylTHF) and 12 months

of supplementation (~ 2.6fold higher 5-methylTHF and ~ 1.9fold higher non-methylTHF). After supplementation for 6 and 12 months, the WB 5-methylTHF and non-methylTHF concentrations were significantly higher (~ 2.5fold higher, $p < 0.001$) in group A compared to the baseline.

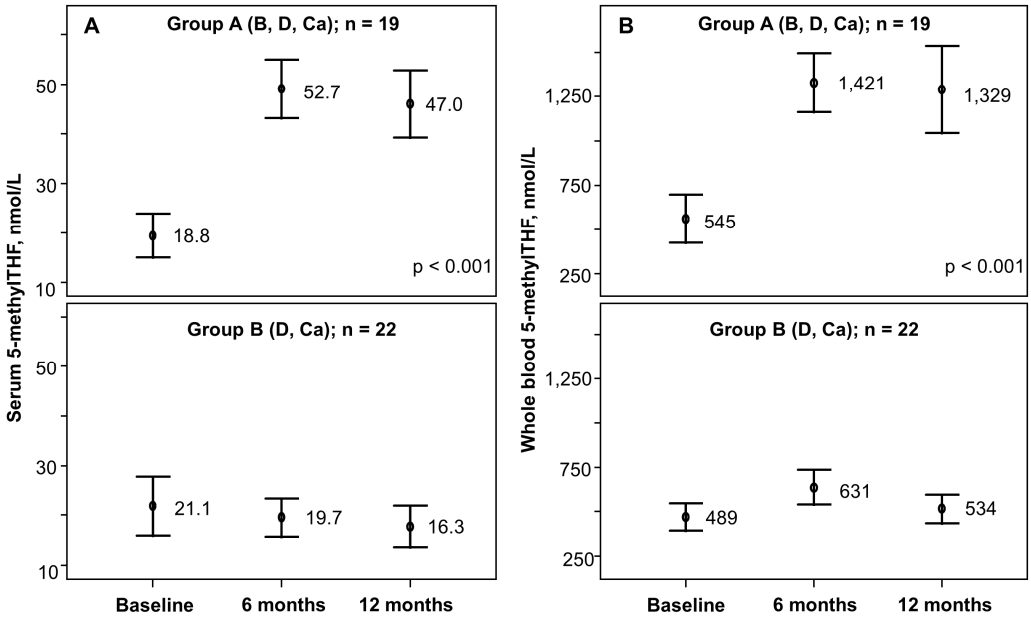

Figure 21: 5-MethylTHF concentrations in (A) serum and (B) whole blood at baseline, after 6 months, and after 12 months of supplementation (long-term supplementation study). After supplementation for 6 and 12 months with B-vitamins, group A showed significantly higher 5-methylTHF concentrations ($p < 0.001$) compared to the baseline in serum and WB. No significant changes were observed for 5-methylTHF concentrations in group B. Mean concentrations are stated in the figure. P values are according to the Mann-Whitney-U test.

The mean 5-methylTHF concentrations in serum and WB at baseline, after 6 and 12 months in both study arms are shown in **Figure 21**. Group A (n = 19 subjects) showed significantly higher mean 5-methylTHF concentrations in serum and WB after 6 and 12 months of supplementation ($p < 0.001$). No significant changes were observed in group B (n = 22 subjects). The correlation of the 5-methylTHF concentrations in serum and in WB for group A and B at baseline and after 6 and 12 months of supplementation are shown in **Figure 22**. The serum and WB 5-methylTHF concentrations of all study participants positively correlated at baseline ($R = 0.595$; $p < 0.001$) (**Figure 22**). After 6 months of supplementation, the 5-methyTHF serum and WB concentrations in group A were higher compared to group B, but were significantly correlated ($R = 0.458$; $p = 0.017$). After 12 months, the correlation of 5 methylTHF in serum and WB in group A became non significant. This indicates that a steady-state and the saturation of the folate storages in RBCs by supplements (500 µg FA/d) were reached between 6 – 12 months.

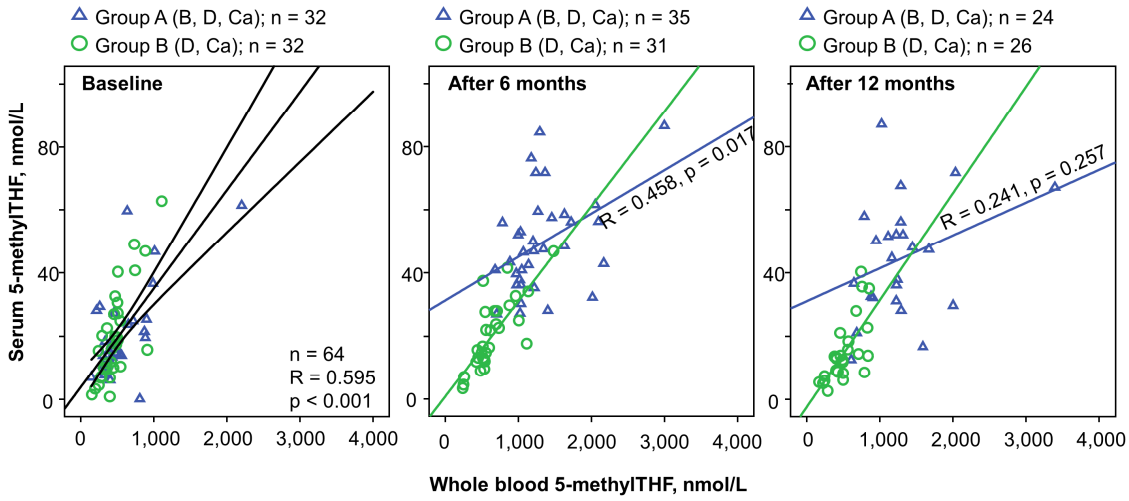

Figure 22: The correlation of 5-methylTHF in serum and whole blood according to the Spearman test (long-term supplementation study). Serum and WB 5 methylTHF concentrations were significantly correlated in the combined groups A and B at baseline, after 6 months for groups A and B, and after 12 months for group B. After 12 months of supplementation in group A, the correlation of 5-methylTHF in serum and WB became non significant, indication a steady-state of the RBC folate.

The correlations of the folate forms in 146 non-supplemented and non-fortified older adults are summarized in **Table 21**. At baseline (n = 93) and after 6 months of supplementation in group A (n = 35), serum 5-methylTHF correlated positively with its demethylated product THF. After 12 months of supplementation, the correlation was not significant anymore ($p = 0.073$). In addition, 5-methylTHF in serum correlated with WB 5-methylTHF at baseline ($p < 0.001$) and after 6 months of supplementation ($p = 0.017$), but not after 12 months of supplementation ($p = 0.257$). At baseline, serum 5-formylTHF correlated positively with 5,10-methenylTHF and THF in serum, but not after 6 and 12 months of supplementation. Serum 5-methylTHF positively correlated with serum 5,10-methenylTHF after 6 months of supplementation, but not at baseline and after 12 months. Furthermore, serum FA significantly correlated with its reduced product THF in serum ($R = 0.451$, $p = 0.007$) after 6 months of supplementation.

Table 21: Correlations of folate forms at baseline (combined study) and after 6 and 12 months of supplementation in group A (long-term supplementation study).

Variable	S 5,10-methenyl THF	S THF	WB 5-methyl THF	WB non-methyl THF[b]
Baseline (all participants)				
S 5-methylTHF		R = 0.379 p < 0.001 n = 93	R = 0.595 p < 0.001 n = 64	R = 0.501 p < 0.001 n = 64
S 5-formylTHF	R = 0.242 p = 0.019 n = 93	R = 0.248 p = 0.016 n = 93		
S THF				R = 0.283 p = 0.023 n = 64
S folic acid				R = 0.264 p = 0.035 n = 64
WB 5-methylTHF				R = 0.813 p < 0.001 n = 64
After 6 months of supplementation (group A)[a]				
S 5-methylTHF	R = 0.402 p = 0.01 n = 35	R = 0.362 p = 0.033 n = 35	R = 0.458 p = 0.017 n = 35	
S folic acid		R = 0.451 p = 0.007 n = 35		
WB 5-methylTHF				R = 0.458 p = 0.006 n = 35
After 12 months of supplementation (group A)[a]				
S folic acid				R = 0.496 p = 0.014 n = 24
WB 5-methylTHF				R = 0.662 p < 0.001 n = 24

The correlation analysis was performed using the Spearman-Rho test. Only significant correlations are shown.
[a]: 500 µg FA, 500 µg vitamin B_{12}, 50 mg vitamin B_6, 456 mg calcium, and 1,200 IU vitamin D /d.
[b]: Sum of 5-formylTHF, 10-formylTHF, 5,10-methenylTHF, 5,10-methyleneTHF, THF, DHF, and FA.

WB non-methylTHF correlated positively at baseline with 5-methylTHF ($p < 0.001$), THF ($p = 0.023$) in serum, and with 5-methylTHF in WB ($p < 0.001$). After 6 and 12 months of supplementation, WB non-methylTHF correlated with WB 5-methylTHF. We could find significant correlations with WB non-methylTHF and serum FA at baseline ($p = 0.035$) and after 12 months ($p = 0.014$), but not after 6 months of supplementation.

4.2.2 The influence of age and gender on the folate forms distribution

We tested the folate forms distribution in relation to the age in non-supplemented and non-fortified older adults. The folate form and metabolite concentrations according to the age quartiles are presented in **Table 22**. We found significant differences in concentrations of the sum of folates ($p = 0.003$), as well as that of 5-methylTHF ($p = 0.001$) and its percentage ($p = 0.001$) between the age quartiles. The youngest group (median age: 60 years) showed significantly higher sum of folate forms and 5-methylTHF in serum and lower tHcy compared to the oldest group (median age: 87 years). No significant differences were found in the concentrations of the other folate forms and FA between the age quartiles. Subjects in the lowest age quartile had significantly higher median 5-methylTHF proportion (% of the sum of folates) in comparison to that in the 3rd and 4th age quartile (**Figure 23**). The reason for this finding is unknown. In contrast to folate form concentrations in serum, we found no significant age-related changes in WB (**Table 22**).

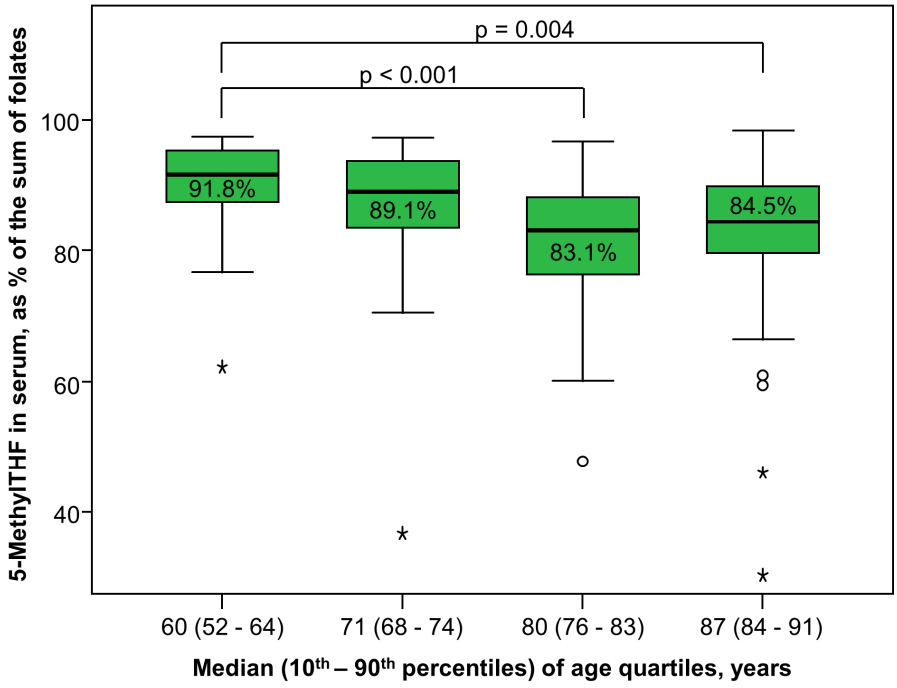

Figure 23: Serum 5-methylTHF as percentage of the sum of folates in relation to the age in 146 older subjects. Participants were divided into quartiles according to their age. Significantly lower 5-methylTHF contents (% of the sum of folates) were obtained in the 3rd and the 4th age quartile.

Table 22: Concentrations of folate forms and metabolites in relation to the age of the combined study (n = 146) (122).

Variable	Quartile of age				p
	1 (lowest)	2	3	4 (highest)	
Number	38	36	35	37	-
Age, years	60 (52 – 64)	71 (68 – 74)	80 (76 – 83)	87 (84 – 91)	-
S sum of folates, nmol/L	18.2 (8.6 – 51.7)	14.9 (6.4 – 38.9)	8.9 (4.5 – 22.3)	7.8 (3.3 – 17.7)	**0.003**
S 5-methylTHF, nmol/L	16.5 (7.6 – 49.1)	13.7 (4.0 – 36.3)	7.1 (3.3 – 19.3)	6.3 (2.6 – 16.1)	**0.001**
S 5-formylTHF, nmol/L	0.18 (< LOD – 0.50)	0.23 (< LOD – 0.65)	0.08 (< LOD – 0.47)	0.08 (< LOD – 0.67)	0.189
S 5-formylTHF ≥ LOD, n (%)	30 (78.9%)	31 (86.1%)	30 (85.7%)	31 (83.8%)	-
S 5,10-methenylTHF ≥ LOD, n (%)[a]	17 (44.7%)	15 (41.7%)	21 (60.0%)	17 (45.9%)	-
S THF, nmol/L[b]	1.14 (< LOD – 3.12)	1.06 (< LOD – 3.60)	1.27 (< LOD – 3.86)	0.96 (< LOD – 3.02)	0.851
S THF ≥ LOD, n (%)[b]	23 (60.5%)	19 (52.8%)	21 (60.0%)	25 (75.7%)	-
S Folic acid ≥ LOD, n (%)[c]	4 (10.5%)	3 (8.3%)	7 (20.0%)	3 (8.1%)	-
WB sum of folates, nmol/L[d]	545 (281 – 1,093)	487 (278 – 827)	679 (409 – 1,064)	590 (291 – 1,062)	0.118
WB 5-methylTHF, nmol/L[d]	475 (247 – 980)	421 (240 – 749)	572 (356 – 960)	519 (243 – 936)	0.106
WB non-methylTHF, nmol/L[d,e]	75.3 (36.2 – 131.7)	65.8 (48.8 – 107.3)	73.0 (52.5 – 169.4)	77.2 (41.6 – 125.7)	0.415
Creatinine, µmol/L	70.7 (53.0 – 106.1)	70.7 (32.7 – 126.4)	79.6 (26.5 – 123.8)	70.7 (47.7 – 144.9)	0.718
tHcy, µmol/L	11.9 (8.0 – 15.1)	12.6 (9.0 – 23.2)	18.4 (11.4 – 39.6)	17.5 (10.2 – 37.3)	**<0.001**
Cys, nmol/L	176 (111 – 472)	268 (150 – 647)	385 (165 – 1,148)	312 (172 – 1,018)	0.313
MMA, nmol/L	209 (120 – 300)	202 (115 – 501)	277 (139 – 511)	242 (138 – 472)	0.141
HoloTC, pmol/L	50 (26 – 66)	48 (31 – 88)	47 (26 – 128)	61 (28 – 128)	0.093
Vitamin B_{12}, pmol/L	306 (189 – 436)	280 (184 – 436)	265 (136 – 641)	266 (165 – 529)	0.459
SAH, nmol/L	17.6 (10.9 – 28.5)	19.4 (10.7 – 36.9)	27.9 (13.8 – 55.2)	26.9 (18.2 – 45.9)	**<0.001**
SAM, nmol/L	117 (88 – 181)	122 (90 – 159)	132 (99 – 206)	140 (106 – 185)	**0.013**
SAM/SAH ratio	7.2 (5.6 – 9.0)	6.2 (4.0 – 9.0)	4.8 (3.5 – 8.6)	5.0 (3.5 – 6.2)	**<0.001**
Betaine, µmol/L	34.1 (25.6 – 43.9)	30.7 (20.6 – 46.7)	27.3 (16.9 – 43.0)	31.5 (19.8 – 44.1)	**0.034**
Choline, µmol/L	8.7 (6.6 – 12.5)	9.2 (5.8 – 14.7)	10.5 (6.5 – 14.3)	11.3 (7.9 – 15.4)	**0.016**
DMG, µmol/L	2.8 (2.0 – 3.9)	3.3 (1.7 – 6.5)	5.5 (2.5 – 11.0)	5.1 (4.0 – 8.6)	**<0.001**

The data are medians (10^{th} – 90^{th} percentiles) unless otherwise specified. P values are according to the ANOVA test.

[a]: Sum of 5,10-methenylTHF and 10-formylTHF.

[b]: Sum of THF and 5,10-methyleneTHF.

[c]: Sum of FA, DHF, and partly oxidized THF.

[d]: WB folate forms were available from 30 subjects in quartile 1, 27 subjects in quartile 2, 28 subjects in quartile 3, and 22 subjects in quartile 4.

[e]: Sum of 5-formylTHF, 10-formylTHF, 5,10-methenylTHF, 5,10-methyleneTHF, THF, DHF, and FA.

The creatinine concentrations were in the normal ranges in all age groups, indicating a normal glomerular filtration rate and a normal renal function. SAH and SAM concentrations, as well as the resulting SAM/SAH ratio depended on the age of the subjects. Lowest SAH and SAM concentrations were found in the youngest group (median: 60 years), which corresponded with the highest SAM/SAH ratio. The SAM/SAH ratio was significantly lower in older subjects, indicating a reduced methylation capacity.

In addition, betaine, choline, and DMG concentrations were age-related. Highest choline and DMG concentrations were found in older subjects (3rd and 4th quartile). Lowest betaine plasma concentrations were found in the 3rd age quartile. These findings are in agreement with the elevated tHcy concentrations found at higher age. Due to low serum folate concentrations, betaine acts as alternative methyl donor for the methylation of Hcy, resulting in elevated DMG concentrations. In addition, we found a tendency for higher holoTC, MMA, and Cys concentrations with higher age. The elevated metabolite concentrations can not be explained by a reduced renal function, as the median creatinine concentrations were all in the normal ranges.

Table 23: Folate forms distribution (nmol/L) in older subjects according to gender (n = 146).

Variable	Males (n = 50)	Females (n = 96)	p
Serum			
Sum of folates	13.2 (5.0 – 35.9)	9.6 (4.6 – 38.9)	0.303
5-MethylTHF	12.2 (3.7 – 32.1)	8.3 (3.3 – 37.1)	0.246
5-FormylTHF	0.21 (< LOD – 0.52)	0.12 (< LOD – 0.51)	0.056
5,10-MethenylTHF[a]	0.11 (< LOD – 0.24)	0.08 (< LOD – 0.28)	0.329
THF[b]	1.12 (< LOD – 3.66)	1.01 (< LOD – 3.13)	0.940
Whole blood[c]			
Sum of folates	531 (331 – 1,011)	573 (296 – 1,031)	0.422
5-MethylTHF	454 (288 – 889)	505 (253 – 922)	0.520
Non-methylTHF[d]	67.0 (49.3 – 127.3)	74.8 (47.5 – 125.4)	0.399

The data are medians (10th – 90th percentiles). P values are according to the Mann-Whitney-U test.
[a]: Sum of 5,10-methenylTHF and 10-formylTHF.
[b]: Sum of THF and 5,10-methyleneTHF.
[c]: WB folate forms were available from 35 males and 72 females.
[d]: Sum of 5-formylTHF, 10-formylTHF, 5,10-methenylTHF, 5,10-methyleneTHF, THF, DHF, and FA.

We tested the study population of differences in the folate forms distribution according to the gender (**Table 23**). Folate forms did not differ significantly between males and females. Only a tendency (p = 0.056) was found for serum 5-formylTHF, where the median concentrations were higher in males than in females.

4.2.3 Unmetabolized folic acid and the effect of co-supplementation on the folate forms distribution

Seventeen (11.6%) of the non-supplemented subjects had detectable concentrations of unmetabolized FA in the serum (**Table 18**). We compared older adults with serum FA < 0.21 nmol/L (LOD = 0.21 nmol/L) to those with serum FA ≥ 0.21 nmol/L. Serum sum of folate forms and 5-methylTHF concentrations were higher in the group with detectable FA. However, we found no significant differences in the median 5-methylTHF or the sum of folates concentrations (**Table 24**). In addition, all other serum folate forms did not differ significantly.

Table 24: Serum folate forms distribution according to unmetabolized folic acid (n = 146).

Variable	Folic acid < 0.21 nmol/L	Folic acid ≥ 0.21 nmol/L	p
Number (%)	129 (88.4%)	17 (11.6%)	-
S sum of folates, nmol/L	11.6 (4.7 – 36.1)	13.6 (5.6 – 61.7)	0.276
S 5-methylTHF, nmol/L	9.5 (3.4 – 32.4)	12.7 (3.6 – 56.9)	0.357
S 5-formylTHF, nmol/L	0.14 (< LOD – 0.53)	0.06 (< LOD – 0.45)	0.177
S 5,10-methenylTHF, nmol/L[a]	0.08 (< LOD – 0.24)	0.08 (< LOD – 0.39)	0.827
S THF, nmol/L[b]	1.03 (< LOD – 3.04)	0.94 (< LOD – 5.94)	0.966

The data are medians (10^{th} – 90^{th} percentiles). P values are according to the Mann-Whitney-U test.
[a]: Sum of 5,10-methenylTHF and 10-formylTHF.
[b]: Sum of THF and 5,10-methyleneTHF.

Short-term supplementation folic acid vs. folic acid plus vitamin B_6 and B_{12}

The short-term co-supplementation with the vitamins B_6 and B_{12} had no effect on the concentrations of serum folate forms other than FA (**Table 19**). For further studying the effect of co-supplementation on the detected unmetabolized FA after supplementation, we divided the two study arms into two groups each: the 1^{st} with no detectable amounts of FA (< 0.21 nmol/L) and the 2^{nd} with detectable FA ≥ 0.21 nmol/L. The results are shown in **Figure 24**.

At baseline, 7 of the 30 subjects (23.3%) of group T1 and 2 of the 24 subjects (8.3%) of group T2 had detectable amounts of FA in serum. After the supplementation, 23 of the 30 subjects (76.7%) of group T1 had detectable amounts of unmetabolized FA in the serum. In comparison, in the group T2 with co-supplementation of vitamins B_6 and B_{12} only 9 of the 24 subjects (37.5%) had detectable FA ≥ 0.21 nmol/L in the serum. This might be related to a higher turnover of the supplemented FA in the presence of the vitamins B_6 and B_{12}.

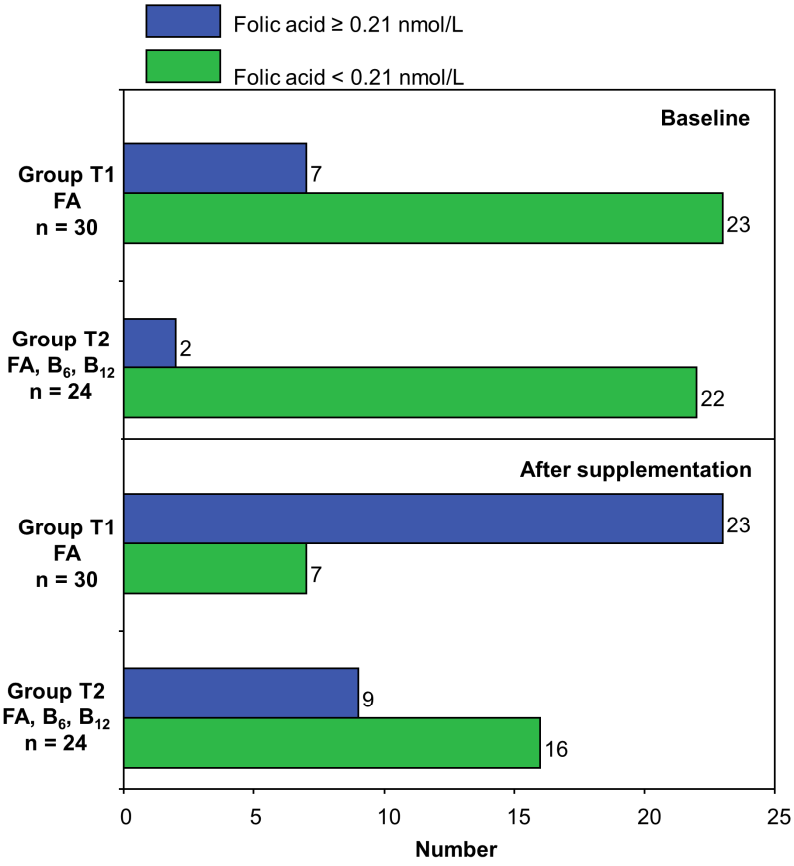

Figure 24: Unmetabolized folic acid at baseline and after 3 – 4 weeks of supplementation (short-term supplementation study). After the supplementation, 23 of 30 subjects of group T1 had detectable amounts of unmetabolized FA in the serum. In comparison, in the group with co-supplementation of vitamins B_6 and B_{12} (T2) only 9 of the 24 subjects had detectable FA ≥ 0.21 nmol/L in the serum.

4.2.4 The influence of the vitamin B_{12} status on the folate forms distribution

We tested the folate forms distribution in serum and WB, as well as the metabolite concentration in relation to the MMA and tHcy concentrations (n = 35) (93). Subjects with MMA > 271 nmol/L and tHcy > 12.0 µmol/L were likely to be vitamin B_{12} or vitamin B_{12} and folate deficient. The results are summarized in **Table 25**. Elevated tHcy and MMA concentrations were significantly correlated with higher age and higher creatinine concentration compared to normal metabolites. The concentrations of HoloTC, total vitamin B_{12}, 5-methylTHF, and sum of folates were significantly lower in the group with elevated tHcy and MMA. In addition, elevated MMA and tHcy were associated with a lower 5-methylTHF proportion (% of sum of folates) and a higher 5-formylTHF concentrations compared to the group with normal MMA and tHcy. The ratio of 5-methylTHF/THF was also lower in subjects with elevated MMA and tHcy concentrations compared to those with normal metabolites. Folates in WB did not differ significantly.

Table 25: Folate forms and metabolites according to the vitamin B_{12} status (122).

Variable	MMA ≤ 271 nmol/L and tHcy ≤ 12.0 µmol/L	MMA > 271 nmol/L and tHcy > 12.0 µmol/L	p
Number	35	38	-
Age, years	66 (56 – 84)	81 (62 – 87)	**<0.001**
Creatinine, µmol/L	61.9 (40.7 – 88.4)	79.6 (53.0 – 125.5)	**0.007**
S sum of folates, nmol/L	25.4 (7.2 – 50.9)	10.1 (4.4 – 28.2)	**<0.001**
S 5-methylTHF, nmol/L	23.7 (6.2 – 47.7)	10.2 (2.8 – 26.3)	**<0.001**
S 5-methylTHF, % of sum of folates	92.6% (78.1 – 97.1%)	83.2% (58.1 – 91.2%)	**<0.001**
S THF, nmol/L[a]	1.10 (< LOD – 2.94)	1.49 (< LOD – 4.29)	0.194
S THF ≥ LOD, n (%)[a]	22 (62.9%)	28 (73.9%)	0.450[e]
S 5-formylTHF, nmol/L	0.20 (< LOD – 0.45)	0.33 (< LOD – 0.91)	**0.014**
S 5-formylTHF ≥ LOD, n (%)	29 (82.9%)	33 (86.8%)	0.748[e]
S 5-methylTHF/THF ratio	14.77 (4.14 – 58.75)	6.67 (1.60 – 27.74)	**<0.001**
WB sum of folates, nmol/L	541 (297 – 940)	640 (289 – 1,163)	0.383
WB 5-methylTHF, nmol/L[b]	455 (258 – 836)	533 (239 – 1,061)	0.456
WB non-methylTHF, nmol/L[b, c]	75.7 (42.8 – 118.3)	72.1 (60.4 – 189.0)	0.702
tHcy, µmol/L	9.2 (7.1 – 11.8)	18.8 (13.6 – 42.3)	**<0.001**
Cys, nmol/L	190 (104 – 385)	445 (167 – 1,314)	**<0.001**
MMA, nmol/L	174 (106 – 243)	374 (284 – 623)	**<0.001**
HoloTC, pmol/L[d]	54 (39 – 110)	39 (25 – 103)	**0.002**
Vitamin B_{12}, pmol/L[d]	322 (195 – 498)	221 (132 – 434)	**0.002**
SAH, nmol/L	16.0 (10.9 – 25.4)	24.0 (14.7 – 63.5)	**<0.001**
SAM, nmol/L	113 (89 – 155)	131 (98 – 209)	**0.001**
SAM/SAH ratio	7.0 (4.9 – 9.0)	5.1 (3.4 – 8.4)	**<0.001**
Betaine, µmol/L	35.0 (24.2 – 48.5)	27.0 (18.3 – 41.5)	**0.001**
Choline, µmol/L	9.4 (6.2 – 12.6)	11.4 (7.2 – 16.7)	**0.007**
DMG, µmol/L	3.0 (2.0 – 6.0)	4.9 (2.4 – 11.4)	**0.002**

The data are medians (10^{th} – 90^{th} percentiles) unless otherwise specified. P values are according to the Mann-Whitney-U test unless otherwise specified.

[a]: Sum of THF and 5,10-methyleneTHF.

[b]: WB folate forms were available from 23 subjects in the group with normal vitamin B_{12} status and 24 subjects in the group with low vitamin B_{12} status.

[c]: Sum of 5-formylTHF, 10-formylTHF, 5,10-methenylTHF, 5,10-methyleneTHF, THF, DHF, and FA.

[d]: HoloTC and vitamin B_{12} concentrations were available from 32 subjects in the group with normal vitamin B_{12} status and 33 subjects in the group with low vitamin B_{12} status.

[e]: P values according to the Chi square test for categorical variables.

Subjects with elevated MMA and tHcy had higher concentrations of SAH, SAM, choline, and DMG but a lower SAM/SAH ratio and plasma betaine concentrations. This suggests that betaine acts as alternative methyl donor – the action of BHMT is vitamin B_{12} independent. As a consequence, Hcy is methylated to Met and more DMG is produced (median 3.0 vs. 4.9 µmol/L of DMG) in the group with elevated MMA and tHcy. In addition, at high tHcy concentration SAH concentrations were also high (median 16.0 vs. 24.0 nmol/L of SAH; $p < 0.001$). Elevated SAM concentrations promote the transsulfuration of Hcy to Cys by the action of the CBS (median 190 vs. 445 nmol/L of Cys).

4.2.5 The interaction between the MTHFR C677T polymorphism and folate forms and the effect of supplementation

Folate forms distributions from 32 non-fasting, middle-aged subjects (median age: 33 years) according to the *MTHFR C677T* genotypes are shown in (**Table 26)** (126). The incidence for the *CC* genotype was 62.5% and 37.5% for the *CT* and the *TT* genotype. Serum sum of folates concentrations were significantly different between carriers and non carriers of the mutated *MTHFR 677* T allele (p = 0.036). Compared to the *CC* genotypes, carriers of the T allele showed significantly lower concentrations of 5-methylTHF (p = 0.043), and THF (p = 0.004).

Table 26: Serum folate forms distribution in 32 serum samples from middle-aged non-fasting subjects according to the *MTHFR C677T* genotype (126).

Variable	*MTHFR C677T* genotype		p
	CC **(n = 20)**	***CT + TT*** **(n = 12)**	
Age, years	40 (20 – 51)	32 (19 – 54)	-
tHcy, µmol/L	10.3 (7.1 – 16.9)	10.0 (7.3 – 37.5)	0.967
S sum of folates, nmol/L	22.2 (8.0 – 30.6)	12.2 (6.0 – 29.9)	**0.036**
S 5-methylTHF, nmol/L	18.5 (6.6 – 27.2)	10.7 (4.7 – 26.3)	**0.043**
S THF, nmol/L[a]	2.77 (1.18 – 4.43)	1.09 (< LOD – 3.50)	**0.004**

The data are medians (10^{th} – 90^{th} percentiles). P values are according to the Mann-Whitney-U test.
[a]: Sum of THF and 5,10-methyleneTHF

In addition, we tested the folate forms distribution, as well as the vitamin and metabolite concentrations according to the *MTHFR C677T* genotype in elderly subjects. The incidence for the CC genotype was 40.8%, 48.3% for the CT genotype, and 10.8% for the TT genotype. In contrast to the study on non-fasting subjects, we found no significant differences in serum and WB sum of folates and folate forms according to the *MTHFR C677T* genotype (**Table 27**). However, we could find a lower folate status in the older subjects. The lower THF concentrations indicate a reduced methyl transfer from 5-methylTHF which might be related to a folate trap caused by a low vitamin B_{12} status. The low folate status and a possible folate trap both may overlay the effect of the *MTHFR C677T* polymorphism on the folate forms distribution in this population.

Table 27: Concentrations of folate forms and metabolites in relation to the *MTHFR C677T* genotype in 120 older non-supplemented subjects of the combined study (122).

Variable	*MTHFR C677T* genotype			p
	CC	*CT*	*TT*	(*CC* vs. *TT*)
Number (%)	49 (40.8%)	58 (48.3%)	13 (10.8%)	-
Age, years	76 (56 – 88)	73 (58 – 87)	78 (57 – 92)	0.508
S sum of folates, nmol/L	10.8 (4.7 – 49.8)	13.6 (5.3 – 37.0)	13.0 (4.9 – 48.1)	0.306
S 5-methylTHF, nmol/L	14.7 (4.5 – 55.6)	14.2 (4.5 – 33.8)	13.9 (3.3 – 44.3)	0.949
S 5-methylTHF, % of sum of folates	89.1 (72.9 – 96.1)	87.6 (69.6 – 96.7)	91.4 (54.8 – 96.1)	0.425
S THF, nmol/L[a]	1.27 (< LOD – 4.29)	1.31 (< LOD – 3.69)	0.94 (< LOD – 3.31)	0.384
S 5-formylTHF, nmol/L	0.20 (< LOD – 0.70)	0.24 (< LOD – 0.70)	0.40 (< LOD – 0.98)	0.131
WB sum of folates , nmol/L	567 (304 – 1,013)	561 (328 – 1,079)	454 (199 – 978)	0.472
WB 5-methylTHF, nmol/L	494 (255 – 895)	497 (285 – 950)	396 (159 – 849)	0.368
WB non-methylTHF, nmol/L[b]	71.4 (48.1 – 121.4)	72.7 (48.1 – 127.0)	64.1 (39.1 – 238.3)	0.897
tHcy, µmol/L	14.1 (8.7 – 29.6)	14.0 (9.2 – 24.8)	14.2 (7.2 – 54.4)	0.815
Cys, nmol/L	287 (138 – 1,057)	271 (113 – 728)	385 (133 – 2,011)	0.713
MMA, nmol/L	199 (123 – 390)	235 (145 – 462)	238 (126 – 540)	0.367
HoloTC, pmol/L	49 (25 – 108)	49 (31 – 123)	54 (29 – 81)	0.886
Vitamin B_{12}, pmol/L	259 (163 – 448)	302 (162 – 503)	265 (164 – 370)	0.977
SAH, nmol/L	24.7 (12.3 – 61.1)	19.5 (12.4 – 38.9)	24.0 (11.2 – 57.6)	0.061
SAM, nmol/L	132 (96 – 193)	122 (91 – 182)	129 (95 – 228)	0.403
SAM/SAH ratio	5.5 (3.4 – 8.1)	6.1 (4.0 – 8.9)	4.9 (3.9 – 9.0)	0.124
Betaine, µmol/L	30.9 (19.8 – 42.9)	31.8 (19.9 – 46.8)	28.6 (17.2 – 41.5)	0.430
Choline, µmol/L	10.0 (5.9 – 13.4)	10.2 (6.3 – 14.9)	10.1 (6.4 – 16.4)	0.676
DMG, µmol/L	4.4 (2.2 – 9.9)	3.8 (2.1 – 7.8)	4.6 (2.8 – 8.1)	0.128

The data are median (10^{th} – 90^{th} percentiles) unless otherwise specified. P values are according to the ANOVA test.

[a]: Sum of THF and 5,10-methyleneTHF.

[b]: Sum of 5-formylTHF, 10-formylTHF, 5,10-methenylTHF, 5,10-methyleneTHF, THF, DHF, and FA.

Short-term supplementation folic acid vs. folic acid plus vitamin B_6 and B_{12}

The mean (SD) concentrations of folate forms in serum and WB, as well as of the related metabolites after 3 – 4 weeks of supplementation according to the treatment arm and the *MTHFR C677T* genotype are summarized in **Table 28**. The *CT* + *TT* genotype of group T1 consisted of 11 subjects with the *CT* and 2 subjects with the *TT* genotype. In group T2 the *CT* + *TT* genotype consisted of 13 subjects with the *CT* and 1 subject with the *TT* genotype.

In group T2 we observed a significantly higher 5-methylTHF proportion (% of sum of folates) in subjects with the *MTHFR 677 CT* + *TT* genotypes ($p = 0.032$). A tendency in group T2 was found for lower SAH and SAM concentrations, as well as for higher plasma choline concentrations in subjects with the *CT* + *TT* genotype, but this was not significant. In addition, we observed higher choline concentrations in group T1 in T allele carriers. All other parameters did not differ significantly between the genotypes.

Table 28: Concentrations of folate forms and metabolites in relation to the *MTHFR C677T* genotype in 54 older subjects after 3 – 4 weeks of supplementation (short-term supplementation study).

Variable	Group T1[a] FA			Group T2[b] FA, B_6, B_{12}		
	MTHFR C677T genotype					
	CC	***CT + TT***	**p**	***CC***	***CT + TT***	**p**
Number	17	13	-	10	14	-
Age, years	81 (5)	81 (7)	0.952	84 (6)	83 (7)	0.809
S sum of folates, nmol/L	18.9 (5.1)	22.3 (14.9)	0.392	14.9 (5.4)	17.7 (6.6)	0.261
S 5-methylTHF, nmol/L	16.2 (4.5)	19.0 (13.7)	0.438	12.7 (5.0)	15.9 (6.1)	0.161
S 5-methylTHF, % of sum of folates	86.0 (2.8)	84.1 (7.5)	0.476	84.2 (7.5)	90.0 (5.0)	**0.032**
S THF, nmol/L[c]	1.86 (1.34)	1.96 (0.89)	0.584	1.77 (0.96)	1.42 (0.82)	0.402
S 5-formylTHF, nmol/L	0.12 (0.13)	0.11 (0.14)	0.760	0.10 (0.10)	0.12 (0.10)	0.429
S folic acid, nmol/L[d]	0.61 (0.40)	1.10 (2.10)	0.865	0.27 (0.39)	0.17 (0.17)	0.621
WB sum of folates, nmol/L	921 (252)	947 (522)	0.878	738 (244)	827 (248)	0.429
WB 5-methylTHF, nmol/L	821 (229)	838 (457)	0.906	639 (212)	730 (225)	0.370
WB 5-methylTHF, % of sum of folates	89.0 (2.8)	88.4 (3.1)	0.632	86.6 (1.9)	87.8 (4.8)	0.494
WB non-methylTHF, nmol/L[e]	100.3 (35.2)	108.3 (73.0)	0.730	98.6 (37.5)	97.3 (37.6)	0.937
tHcy, µmol/L	20.1 (7.2)	20.6 (6.8)	0.706	16.1 (6.0)	14.7 (3.7)	0.999
Cys, nmol/L	625 (548)	496 (334)	0.818	388 (305)	299 (156)	0.578
MMA, nmol/L	346 (203)	341 (161)	0.802	275 (134)	239 (73)	0.753
SAH, nmol/L	29.8 (10.6)	34.5 (17.7)	0.615	41.1 (26.6)	29.3 (17.3)	0.121
SAM, nmol/L	153 (31)	148 (39)	0.530	149 (20)	130 (40)	0.057
SAM/SAH ratio	5.5 (1.4)	4.9 (1.9)	0.137	4.8 (2.9)	5.6 (2.4)	0.198
Betaine, µmol/L	29.0 (9.9)	32.4 (10.5)	0.368	36.9 (10.2)	40.2 (26.6)	0.639
Choline, µmol/L	9.7 (2.7)	12.2 (3.6)	0.069	12.3 (3.2)	18.3 (20.9)	0.219
DMG, µmol/L	5.9 (2.2)	6.2 (2.2)	0.818	6.0 (2.0)	5.5 (1.5)	0.682

The data are means (SD). P values are according to the ANOVA test.
[a]: 400 µg FA/ d.
[b]: 400 µg FA, 8 mg vitamin B_6, 10 µg vitamin B_{12}/d.
[c]: Sum of THF and 5,10-methyleneTHF.
[d]: Sum of FA, DHF, and partly oxidized THF.
[e]: Sum of 5-formylTHF, 10-formylTHF, 5,10-methenylTHF, 5,10-methyleneTHF, THF, DHF, and FA.

Long-term supplementation with calcium, vitamin D, folic acid, vitamin B_6, and vitamin B_{12} vs. calcium and vitamin D

The mean (SD) concentrations of the folate forms in serum and WB, as well as of the related metabolites after 12 months of supplementation according to the treatment arm and the *MTHFR C677T* genotype from 60 older adults are summarized in **Table 29**. The *CT + TT* genotype of group A consisted of 18 subjects with the *CT* and 4 subjects with the *TT* genotype. In group B the *CT + TT* genotype consisted of 16 subjects with the *CT* and 3 subjects with the *TT* genotype.

In group A we observed significantly higher concentrations of serum THF in subjects with the *CC* genotype (3.75 vs. 2.14 nmol/L; p = 0.038). The THF concentrations in serum were higher after the supplementation with B-vitamins. This indicates that the methyl trap of 5-methylTHF was annulled which led to an undisturbed methyl transfer and THF formation – the effect of the *MTHFR C677T* polymorphism on the folate forms distribution is not overlayed anymore. In addition to the higher THF concentrations in serum, we found higher sum of folate forms and 5-methylTHF levels in serum and WB and higher plasma betaine and choline concentrations in subjects with the *CC* genotype, but these were not significant. In group B we found a significantly higher WB 5-methylTHF proportion (% of sum of folates; p = 0.017) and plasma betaine concentrations (p = 0.050) in subjects with the *CC* genotype. Participants with the *MTHFR 677 CT + TT* genotype were older (p = 0.054) and had lower sum of folate forms and 5-methylTHF in serum and plasma, but these differences were not significant. We found higher tHcy, Cys, and SAH concentrations and lower betaine and choline concentrations in T-allele carriers, but again these were not significant.

The interaction between the MTHFR C677T polymorphism and folate forms and the effect of supplementation

Table 29: Concentrations of folate forms and metabolites in relation to the *MTHFR C677T* genotype in 60 older subjects after 12 months of supplementation (long-term supplementation study).

Variable	Group A[a] Vit B, D, Ca			Group B[b] Vit D, Ca		
	MTHFR C677T genotype					
	CC	***CT + TT***	**p**	***CC***	***CT + TT***	**p**
Number	10	22	-	9	19	-
Age, years	64 (11)	64 (9)	0.996	65 (8)	71 (10)	0.054
S sum of folates , nmol/L	57.7 (20.7)	47.0 (18.0)	0.148	23.2 (11.5)	17.8 (10.6)	0.229
S 5-methylTHF, nmol/L	52.1 (18.3)	43.5 (18.0)	0.221	21.0 (11.1)	16.1 (10.5)	0.272
S 5-methylTHF, % of sum of folates	90.7 (4.6)	91.7 (6.0)	0.647	88.4 (8.6)	87.9 (10.5)	0.912
S THF, nmol/L[c]	3.75 (3.02)	2.14 (1.24)	**0.038**	1.80 (1.19)	1.29 (1.19)	0.301
S 5-formylTHF, nmol/L	0.20 (0.10)	0.21 (0.13)	0.824	0.22 (0.16)	0.19 (0.13)	0.678
S folic acid, nmol/L[d]	1.49 (2.51)	1.07 (1.88)	0.600	0.12 (0.08)	0.07 (0.12)	0.303
WB sum of folates , nmol/L	1,612 (1,088)	1,343 (444)	0.408	641 (244)	590 (225)	0.633
WB 5-methylTHF, nmol/L	1,445 (984)	1,213 (415)	0.435	578 (227)	512 (198)	0.489
WB 5-methylTHF, % of sum of folates	89.3 (2.0)	90.0 (2.5)	0.564	89.7 (2.4)	86.5 (2.8)	**0.017**
WB non-methylTHF, nmol/L[e]	167.1 (107.1)	129.5 (43.2)	0.243	62.5 (21.9)	77.7 (30.6)	0.249
tHcy, µmol/L	9.1 (2.5)	9.9 (5.8)	0.673	12.4 (3.2)	21.1 (22.1)	0.254
Cys, nmol/L	162 (78)	223 (151)	0.241	213 (95)	513 (679)	0.202
MMA, nmol/L	211 (96)	230 (87)	0.593	278 (151)	266 (131)	0.831
SAH, nmol/L	18.9 (8.5)	28.0 (29.7)	0.430	17.9 (4.5)	24.2 (15.8)	0.254
SAM, nmol/L	134 (41)	131 (57)	0.895	112 (16)	117 (41)	0.761
SAM/SAH ratio	7.6 (2.0)	6.6 (2.5)	0.269	6.7 (1.9)	5.7 (2.2)	0.259
Betaine, µmol/L	38.5 (8.0)	34.2 (7.2)	0.142	40.7 (8.0)	31.2 (12.8)	**0.050**
Choline, µmol/L	13.4 (3.8)	11.8 (2.9)	0.210	13.4 (3.8)	11.0 (3.1)	0.094
DMG, µmol/L	3.0 (0.5)	3.9 (3.2)	0.385	4.2 (1.4)	3.5 (0.8)	0.094

The data are means (SD). P values are according to the ANOVA test.
[a]: 500 µg FA, 500 µg vitamin B_{12}, 50 mg vitamin B_6, 456 mg Ca, and 1,200 IU vitamin D /d.
[b]: 456 mg calcium and 1,200 IU vitamin D /d.
[c]: Sum of THF and 5,10-methyleneTHF.
[d]: Sum of FA, DHF, and partly oxidized THF.
[e]: Sum of 5-formylTHF, 10-formylTHF, 5,10-methenylTHF, 5,10-methyleneTHF, THF, DHF, and FA.

4.3 The effect of short-term and long-term B-vitamins supplementation on vitamins and methionine cycle related metabolites in older adults

Short-term supplementation folic acid vs. folic acid plus vitamin B_6 and B_{12}

The baseline concentrations and the concentrations after 3 – 4 weeks of supplementation of vitamins and Met cycle related metabolites are summarized in **Table 30**. Shown are the results of all study participants. At baseline, the plasma betaine, the total vitamin B_{12}, and the HoloTC concentrations were significantly higher in group T2. All other vitamins and metabolites did not differ significantly between both study arms. In comparison to the group T1, tHcy and Cys concentrations were significantly lower and choline concentrations were significantly higher in group T2 after the co-supplementation with the vitamins B_6 and B_{12}.

After the supplementation, the median tHcy concentrations were significantly higher in group T1, while choline concentrations were significantly lower in group T1. The median ($10^{th} - 90^{th}$ percentiles) tHcy baseline concentration was elevated in both groups (moderate HHCY) (**Table 30**). After supplementation, tHcy was significantly lower ($p < 0.001$) comparing the concentrations at baseline and after supplementation in group T2 (co-supplementation with vitamins B_6 and B_{12}). Oral 400 µg/d FA led to significantly lower tHcy concentrations (~ 6% lower). The absolute reduction was ~ 1.2 nmol/L. The group receiving 400 µg/d FA in combination with the vitamins B_6 and B_{12} (T2) had significantly lowered the tHcy concentrations after the treatment (~ 4% lower; ~ 0.5 nmol/L). The median ($10^{th} - 90^{th}$ percentiles) tHcy concentrations were elevated in both groups (moderate HHCY) even after the supplementation. The supplementation with 400 µg FA daily for 3 – 4 weeks was not sufficient to effectively lower the tHcy concentration.

Table 30: Vitamins and metabolites of all study participants (short-term supplementation study).

Variable	Group T1[a] FA n = 30	Group T2[b] FA, B_6, B_{12} n = 24	p value
Baseline			
tHcy, µmol/L	19.2 (13.6 – 39.6)	16.2 (10.7 – 27.8)	0.174
Cys, nmol/L	398 (167 – 1,049)	304 (198 – 1,356)	0.971
MMA, nmol/L	265 (156 – 483)	198 (120 – 460)	0.057
Vitamin B_{12}, pmol/L	210 (137 – 500)	342 (203 – 608)	**0.020**
HoloTC, pmol/L	49 (25 – 111)	66 (37 – 128)	**0.034**
SAH, nmol/L	30.2 (14.8 – 52.2)	26.6 (16.2 – 76.8)	0.910
SAM, nmol/L	147 (94 – 205)	138 (108 – 190)	0.986
SAH/SAM ratio	5.0 (3.6 – 6.6)	4.9 (2.2 – 8.4)	0.899
Betaine, µmol/L	25.5 (16.3 – 41.4)	31.2 (22.0 – 56.7)	**0.013**
Choline, µmol/L	11.1 (5.5 – 16.6)	11.4 (7.6 – 15.2)	0.531
DMG, µmol/L	6.1 (3.7 – 10.1)	5.7 (4.0 – 14.9)	0.807
After supplementation			
tHcy, µmol/L	18.0 (12.8 – 31.7)	15.6 (9.4 – 22.3)	**0.003**
Cys, nmol/L	383 (191 – 1,201)	271 (149 – 588)	**0.012**
MMA, nmol/L	307 (156 – 701)	239 (141 – 390)	0.085
SAH, nmol/L	30.1 (13.8 – 46.8)	27.0 (13.4 – 70.0)	0.801
SAM, nmol/L	151 (100 – 189)	139 (102 – 181)	0.158
SAH/SAM ratio	4.8 (3.6 – 8.0)	4.9 (2.2 – 9.3)	0.851
Betaine, µmol/L	29.8 (16.8 – 43.6)	34.6 (24.4 – 54.8)	0.076
Choline, µmol/L	10.2 (6.2 – 15.7)	13.0 (8.6 – 17.6)	**0.023**
DMG, µmol/L	5.4 (3.8 – 9.9)	5.0 (4.0 – 8.7)	0.542

The data are medians (10^{th} – 90^{th} percentiles). P values are according to the Mann-Whitney-U test.
[a]: 400 µg FA /d.
[b]: 400 µg FA, 8 mg vitamin B_6, 10 µg vitamin B_{12} /d.

Long-term supplementation with calcium, vitamin D, folic acid, vitamin B_6, and vitamin B_{12} vs. calcium and vitamin D

Vitamins and metabolites at baseline and after 6 and 12 months of supplementation are summarized in **Table 31**. Shown are the results of all study participants. At baseline, vitamins and metabolites showed no significant differences between both study arms. In group A, SAM concentrations were significantly higher after 6 months compared to the baseline ($p = 0.026$). In addition, the median SAM concentrations were significantly higher after 6 months of supplementation when comparing group A with group B ($p = 0.029$) (**Table 31**). No significant differences were found for median SAH concentrations.

After 6 and 12 months of supplementation, the median tHcy and Cys concentrations were significantly lower in group A. The median tHcy concentration in group A was significantly lowered (~ 30%, $p < 0.001$) after 6 and 12 months from moderate HHCY (median 12.9 µmol/L of tHcy at baseline) to normal tHcy concentrations (median 8.9 µmol/L of tHcy after 6 and

12 months), whereas the moderate HHCY remained in group B. Compared to the short-term supplementation, the supplementation with B-vitamins over several months efficiently normalized the tHcy concentrations.

Table 31: Vitamins and metabolites of all study participants (long-term supplementation study).

Variable	Group A[a] B, D, Ca	n	Group B[b] D, Ca	n	p value
Baseline					
tHcy, µmol/L	12.9 (8.7 – 20.4)	46	13.0 (8.6 – 21.3)	45	0.968
Cys, nmol/L	232 (120 – 651)	46	225 (109 – 513)	45	0.470
Vitamin B_{12}, pmol/L	280 (176 – 495)	41	288 (190 – 425)	36	0.752
MMA, nmol/L	212 (142 – 402)	47	212 (126 – 388)	45	0.916
HoloTC, pmol/L	53 (35 – 79)	41	47 (26 – 85)	36	0.288
SAH, nmol/L	17.6 (11.9 – 29.2)	46	18.3 (10.8 – 33.1)	44	0.508
SAM, nmol/L	121 (88 – 167)	46	116 (95 – 178)	44	0.821
Betaine, µmol/L	33.2 (22.5 – 42.7)	46	32.0 (22.0 – 48.9)	45	0.994
Choline, µmol/L	8.9 (6.3 – 12.8)	46	8.9 (6.0 – 12.6)	45	0.592
DMG, µmol/L	2.8 (1.8 – 4.8)	46	3.0 (2.1 – 5.2)	45	0.298
After 6 months of supplementation					
tHcy, µmol/L	8.9 (7.1 – 14.1)	35	13.1 (9.4 – 20.9)	31	**<0.001**
Cys, nmol/L	157 (104 – 438)	35	218 (112 – 487)	31	**0.002**
Vitamin B_{12}, pmol/L	405 (277 – 848)	21	260 (197 – 436)	19	**<0.001**
MMA, nmol/L	275 (209 – 391)	35	285 (162 – 514)	30	0.869
HoloTC, pmol/L	150 (73 – 150)	21	77 (35 – 126)	20	**<0.001**
SAH, nmol/L	16.5 (10.6 – 34.1)	35	17.3 (8.6 – 29.9)	31	0.662
SAM, nmol/L	126 (98 – 188)	35	115 (91 – 151)	31	**0.029**
Betaine, µmol/L	36.3 (25.2 – 56.3)	35	33.7 (21.7 – 49.0)	31	0.289
Choline, µmol/L	10.4 (7.2 – 14.5)	35	9.3 (7.0 – 12.7)	31	0.199
DMG, µmol/L	2.5 (1.5 – 4.3)	35	3.0 (1.8 – 4.6)	31	0.066
After 12 months of supplementation					
tHcy, µmol/L	9.1 (6.0 – 14.1)	33	14.9 (8.7 – 28.5)	31	**<0.001**
Cys, nmol/L	152 (92 – 352)	33	283 (106 – 1,236)	31	**0.005**
Vitamin B_{12}, pmol/L	459 (316 – 862)	34	261 (169 – 360)	31	**<0.001**
MMA, nmol/L	212 (146 – 303)	34	237 (143 – 478)	31	0.203
SAH, nmol/L	18.4 (11.4 – 41.9)	34	18.9 (11.3 – 41.2)	31	0.646
SAM, nmol/L	120 (92 – 196)	34	120 (79 – 159)	31	0.250
Betaine, µmol/L	36.9 (25.4 – 46.6)	34	32.8 (20.4 – 49.6)	31	0.351
Choline, µmol/L	12.0 (7. 8 – 16.3)	34	11.1 (8.3 – 18.0)	31	0.490
DMG, µmol/L	3.0 (2.2 – 4.5)	34	3.8 (2.4 – 5.8)	31	**0.033**

The data are medians (10^{th} – 90^{th} percentiles). P values are according to the Mann-Whitney-U test.
[a]: 500 µg FA, 500 µg vitamin B_{12}, 50 mg vitamin B_6, 456 mg calcium, and 1,200 IU vitamin D /d.
[b]: 456 mg calcium and 1,200 IU vitamin D /d.

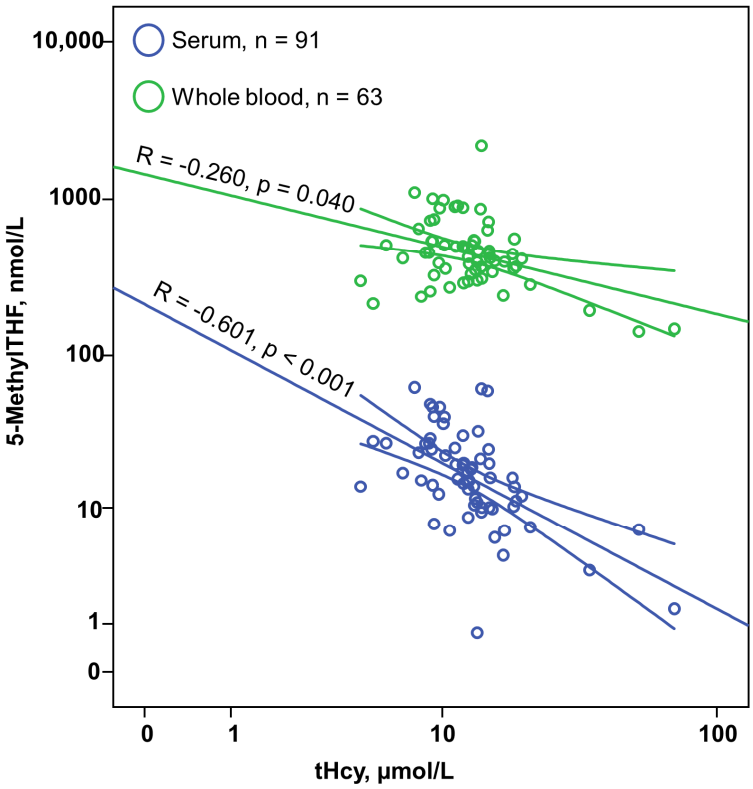

Figure 25: Correlation of baseline 5-methylTHF concentrations in serum and whole blood with the total homocysteine concentration (long-term supplementation study). At baseline, serum and WB 5-methylTHF concentrations were significantly correlated with tHcy concentrations. 5-MethylTHF in serum showed a stronger negative correlation with tHcy than WB 5-methylTHF.

5-MethylTHF correlated negatively with tHcy in serum ($R = -0.601$, $p < 0.001$) and in WB ($R = -0.260$, $p = 0.040$) (**Figure 25**) at baseline. The stronger correlation of tHcy with serum 5-methylTHF indicates that serum 5-methylTHF is a better determinant of tHcy as WB 5-methylTHF.

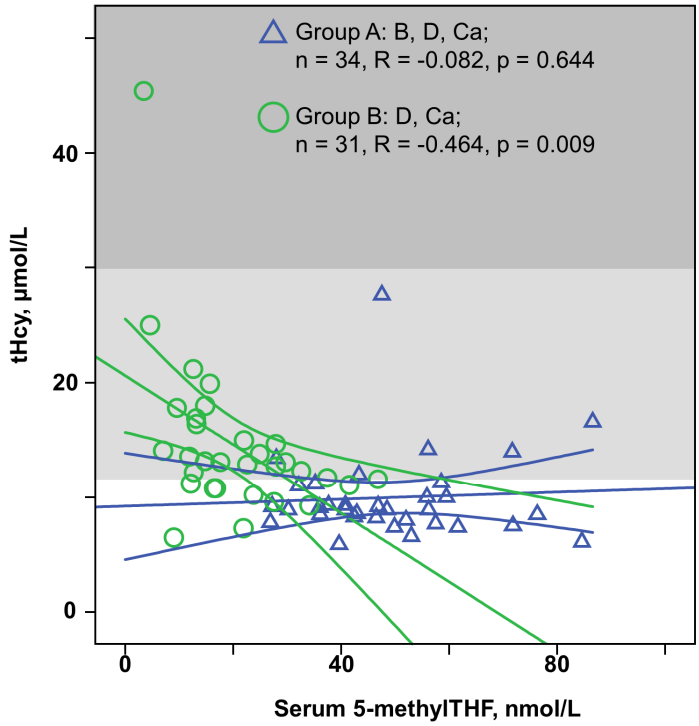

Figure 26: 5-MethylTHF concentrations in serum according to the tHcy concentration of group A and group after 6 months of supplementation (long-term supplementation study). After 6 months of B-vitamins supplementation in group A, no significant correlations were found between 5-methylTHF and tHcy. In group B, 5-methylTHF concentrations were significantly and negatively correlated with tHcy. The dark grey area intermediate HHCY (≤ 30 µmol/L); the light grey area indicates moderate HHCY (12 – 30 µmol/L).

After the long-term supplementation for 6 months, group B showed a significant correlation of serum 5-methylTHF with tHcy while in group A no correlation was found in group A (**Figure 26**). The increased intake of FA and the therefore increased serum 5-methylTHF and reduced tHcy levels might interfere with each other, which led to the loss of the correlation.

5 Discussion

Folate and other B-vitamins deficiencies are common in older adults, due to a lower vitamin intake, bioavailability, or absorption. Folate deficiency is a risk factor for various age-related diseases including cardiovascular diseases, stroke, and cancer (91;194;214). Little is known about the metabolism of folate within the cell and the distribution of folate forms in serum and WB. FA, the synthetic vitamin, is often used as an oral supplement (8). To date, there are only few metabolic studies confirming the fate of supplemented FA. Insufficient concentrations of folates, vitamin B_6, and vitamin B_{12} are related to an elevated tHcy concentration, which is an independent risk factor for pathologic conditions. Although the underlying pathogenic mechanisms are still poorly understood, it has been recently suggested that an increase of SAH, resulting in an altered SAM/SAH ratio, might play a role in developing age-related vascular and neurodegenerative diseases (31;91;142). The metabolisms of choline and folate are interrelated and both participate in Hcy methylation and delivering of SAM. Alterations in choline metabolism, including betaine and DMG, have also been associated with tHcy accumulation and age-related disorders such as cognitive dysfunction and dementia (193). It has been barely tested whether subjects with polymorphisms in the *MTHFR 677* gene might show different folate forms distribution after B-vitamins supplementation compared to carriers of other genotypes.

We developed sensitive UPLC-MS/MS methods for the quantification of key folate forms in serum and WB, SAM and SAH in acidified EDTA plasma, and betaine, choline, and DMG in EDTA plasma. We determined the fasting folate forms distribution in serum and WB, as well as the concentrations of related metabolites in non-supplemented and non-fortified older adults. We tested the hypothesis if gender, age, vitamin B_{12} status, unmetabolized FA, or the *MTHFR C677T* genotypes have an influence on the distribution of the folate forms in serum and WB. We performed two B-vitamins supplementation studies with short-term (3 – 4 weeks FA with and without co-supplementation with vitamin B_6 and B_{12}) and long-term (12 months with calcium, and vitamin D and with and without FA and vitamins B_6 and B_{12}) supplementation. We analyzed the concentrations of folate forms, vitamin B_{12} markers, tHcy, and related metabolites before and after the supplementation with B-vitamins. Furthermore, we tested the hypothesis if the co-supplementation with the vitamins B_6 and B_{12} influences the folate forms distribution.

5.1 Development of sensitive methods for the quantification of folate forms using UPLC-MS/MS

The reliable quantification of folate forms in serum and WB is difficult due to rapid interconversions and instabilities of non-methyl folate forms. We previously described the quantification methods of folate forms in serum and WB using stable-isotope dilution UPLC-MS/MS (123;126). The method enables the specific and sensitive measurement of 5-methylTHF, 5-formylTHF, 5,10-methenylTHF (sum of 5,10-methenylTHF and 10-formylTHF), THF (sum of THF and 5,10-methyleneTHF), and FA in serum, and 5-methylTHF and non-methylTHF (sum of 5-formylTHF, 10-formylTHF, 5,10-methyleneTHF, 5,10-methenylTHF, DHF, THF, and FA) in WB hemolysates.

The short time required for the sample preparation (serum: 40 samples in 120 minutes, WB: 40 samples in 200 minutes) and measurement (2.5 minutes/sample) enables folate quantifications in large scale clinical studies. Compared to our method, earlier methods for the WB folate quantification have a longer incubation time (68;70;71;159;206) and a longer run time per sample (between 45 and 6 minutes) (70;104;159;206). The folate assay shows a linearity over a broad range (0.2 – 200 nmol/L for each folate form), which enables the measurement of serum and WB hemolysates from supplemented subjects without dilution of the samples. The high sensitivity (LODs between 0.21 – 0.67 nmol/L in serum, 0.12 – 0.40 nmol/L in WB hemolysates) of our assay enables the accurate measurement of serum and WB folate in the low or deficient ranges. Compared to other methods, the described method for the folate forms determination in WB has a greater sensitivity (expressed as LOQ) for the minor forms 5-formylTHF (0.36 nmol/L by our method vs. 4 nmol/L by Smith *et al.* method) and FA (0.45 nmol/L by our method vs. 2 nmol/L by Smith *et al.* method) (206), which led to more precise determinations of non-methyl folates. Recoveries for folate forms in serum and WB hemolysates were in the range of 73.9 – 112.4%. The interassay CV for 5-methylTHF in serum was < 2.8% and < 7.4% in WB samples. Higher CVs for non-methyl folates can be explained by low concentrations of those compounds.

Comparing the new UPLC-MS/MS method with the established immunological assay (ADVIA Centaur), a strong correlation was observed. However, the correlation was lower in the upper range of serum folate (> 35 nmol/L). Preliminary results suggested that the higher the THF fraction, the bigger the difference between the two methods was. The immunological assay might not equally detect 5-methylTHF and other forms of folate. Another explanation might be that the UPLC-MS/MS method uses the same analytes for preparing the calibration curve, whereas the immunological assay uses 5-methylTHF for quantifying the TFOL.

In contrast to 5-methylTHF, the quantification of 5-formylTHF, 10-formylTHF, 5,10-methyleneTHF, 5,10-methenylTHF, DHF, THF, and FA has a relatively low accuracy in serum and WB hemolysates. Moreover, the interconversion of the folate forms especially the oxidation of THF to FA during the sample processing could not be completely avoided by the addition of antioxidants. Therefore, in WB we considered the sum of these compounds as non-methylTHF. Similar observations have been reported by other groups (36;50;175). Smith *et al.* summarized their results into 5-methylTHF, non-methylTHF (sum of THF, 5,10-methyleneTHF, 5,10-methenylTHF, 5-formylTHF, and 10-formylTHF), and unsubstituted (partly) oxidized folates (sum of FA and DHF) (206). This was not the case in our method, since we were able to demonstrate the partial oxidation of THF into FA during sample preparation. The FA formation was more pronounced in WB pool hemolysates than in serum pool samples. The oxidation into FA could explain why we were not able to detect THF and its internal standard in WB samples. This could be due to a higher proportion of ammonium acetate buffer (pH 10) or the longer time required for the sample processing (more wash steps with larger volume). Our study showed that free FA can be artificially generated from THF and DHF during sample processing in serum and WB. This might lead to misinterpretation of studies looking at FA concentrations in subjects with slightly higher THF.

In accordance with previous studies (178), we confirmed that 5-methylTHF is the predominant folate form in serum (as monoglutamate) (126) and WB (as polyglutamates) (123). In serum, we detected less FA compared to a study by Pfeiffer *et al.* (178). Nevertheless, this might be explained by differences in the population tested since the study by Pfeiffer *et al.* was conducted on an U.S. population after the introduction of folate fortification programs and the subjects in our study were from Germany, a country without mandatory folate fortification (178). Pfeiffer *et al.* found a mean sum of folates concentration in serum of 35.5 nmol/L (178). Samples with a sum of folates < 50 nmol/L contained 93.3% 5-methylTHF, 4.4% 5-formylTHF, and 2.3% FA. In comparison, in our study we found 87.2% 5-methylTHF, 1.1% 5-formylTHF, and 0.6% FA in serum samples of 32 subjects (126). However, we additionally found 11.4% THF and 0.2% 5,10-methenylTHF in the serum. Differences might be related to the population tested and the use of vitamin supplements or fortified foods. The concentrations of WB folate obtained by our methods (123) are comparable to data from countries not applying fortification with FA. The median concentration of WB 5-methylTHF in 48 non supplemented subjects from our study is similar to that reported in subjects from the Netherlands (n = 109 adults) (207). However, the concentration of the non-methylTHF was ~ 20times higher in our study, which could be related to differences in sample processing. The sample extraction in the method by Smith *et al.* is accomplished by affinity chromatography. Due to different affinities of the folate binding proteins for the reduced folates, the folate forms distribution might be altered. Moreover, the

LOQs in our study were below that reported by Smith *et al.*, which might also explain the higher non-methylTHF concentration. Compared to our results, Fazili *et al.* reported lower folate concentrations in 75 WB samples obtained from an European blood bank (207 vs. 445 nmol/L of 5-methylTHF and 38.9 vs. 71.2 nmol/L of non-methylTHF) (71). In addition, the same group reported lower WB sum of folates in earlier studies (68) compared to other research groups (104;207;216). This might be related to differences in sample preparation, sample storage, or in the tested population. Concentrations of WB 5-methylTHF in subjects receiving FA (500 µg/d) for 6 months are in the range of that observed in countries applying mandatory fortification with FA (104;216). The non-methylTHF concentrations in our supplemented subjects were ~ 5times higher than that reported by studies on subjects consuming fortified staple foods (104;216).

Despite the good correlation of the UPLC-MS/MS method and the immunoassay, the results for the quantification of the folates may vary greatly in individuals due to the non-specific detection of the folate forms via the immunological methods. In addition, the UPLC-MS/MS method provides more information than the TFOL of the sample. 5-MethylTHF in serum negatively correlated stronger with tHcy than the sum of folates, indicating that 5-methylTHF in serum is a better determinant of tHcy. In addition, (UP)LC-MS/MS methods can monitor the metabolization of FA or occurrence of unmetabolized FA after fortification or supplementation. Furthermore, by determining the 5-methylTHF/THF ratio the remethylation of Hcy by means of the MS gene can be monitored which adds information of a possible methyl trap of the vitamin and a resulting secondary folate deficiency. This is especially of interest in populations with a low vitamin B_{12} status, such as the elderly, pregnant women, or vegetarians.

In conclusion, we developed a sensitive, precise, and reliable method for the quantification of 5-methylTHF, 5-formylTHF, 5,10-methenylTHF, THF, and FA in serum, and 5-methylTHF and non-methylTHF in WB samples. The method can be used in large-scale clinical studies. We observed a conversion of THF into FA during sample preparation. This could not be prevented by the addition of several antioxidants. Therefore we highly recommend a fast sample preparation and measurement to minimize the effect.

5.2 Folate forms distribution before and after short-term and long-term supplementation with B-vitamins in older adults in relation to the MTHFR C677T polymorphism

Seventy seven participants were recruited for the short-term supplementation study and 96 subjects were recruited for the long-term supplementation study. A total of 54 (short-term supplementation study) and 65 subjects (long-term supplementation study), respectively, completed the studies. We could find no significant changes in the baseline study characteristic of both studies. In order to investigate the folate forms distribution in serum and WB of non-supplemented and non-fortified older adults (median age: 74 years) according to gender, age, vitamin B_{12} concentrations, unmetabolized FA, or the *MTHFR C677T* genotype we combined the baseline concentrations of the vitamins and metabolites of the two studies (n = 146). Parts of the results were published previously (122).

5.2.1 Folate forms distribution in older adults and the effect of B-vitamins supplementation

The median concentrations of the serum sum of folates were comparable to those found in our earlier investigations (168), but were approximately 50% lower than in populations regularly consuming products fortified with FA (69). As expected, 5-methylTHF was the predominant folate form in serum, which is consistent with earlier results (126;168). However, compared to our earlier study (168), we found higher 5-methylTHF (combined study: 10.0 vs. 6.5 nmol/L) and lower THF (combined study: 1.0 vs. 5.5 nmol/L) concentrations with a comparable serum sum of folates concentration (combined study: 11.9 vs. 11.7 nmol/L). The reasons for the higher 5-methylTHF and the lower THF are unknown. It might be related to the lower age of the subjects (combined study: 74 vs. 81 years).

Concentrations of WB folates were in the range reported in our earlier study (123) (**Table 13**). However, these data originate from a subset of the short-term and the long-term supplementation study. 5-MethylTHF in WB was comparable to that reported by Smulders *et al.* and approximately twice as high as published by Fazili *et al.* in non-supplemented and non-fortified adults (71;207). Non-methylTHF concentrations were twice as high as described by Fazili *et al.* and ~ 20fold higher than that reported by Smulders *et al.* (71;207). The WB 5-methylTHF concentrations were approximately 50% lower than in countries applying FA fortification programs (104;216).

The baseline serum 5-methylTHF concentrations of the short-term supplementation study were in the lower normal or deficiency range (**Table 13**) and comparable to that reported by Obeid *et al.* (168). The intake of 400 µg/d or 500 µg/d of FA led in a short time to a significant increase of the 5-methylTHF levels in serum (2.2 – 2.5fold) and in WB (1.1 – 2.1fold) (**Table 19**, **Table 20**). This led to a normalization of the 5-methylTHF concentration in the short-term supplementation study after 3 – 4 weeks and to higher 5-methylTHF levels after 6 months in group A of the long-term supplementation study. The increase in serum 5-methylTHF was lower than that reported by Obeid *et al.* after 3 weeks of B-vitamins supplementation (168). However, they used high doses of B-vitamins (5 mg FA, 1 mg vitamin B_{12}, and 40 mg vitamin B_6 /d), which can explain the lower 5-methylTHF (as well as THF and FA) levels in serum in our study. Van Oort *et al.* supplemented 43 older adults (50 – 75 years) with 400 µg/d FA for 4 and 12 weeks (229). After 4 weeks of supplementation, they found a mean (SD) increase of plasma sum of folates from 13.8 (5.3) nmol/L to 31.9 (15.3) nmol/L. This is twice as high as the concentrations we found in our study. Nevertheless, they showed a 2.3fold increase of the sum of folates, which is consistent with our findings. In a recent study Hursthouse *et al.* long-term supplemented women of childbearing age with placebo (n = 47), 140 µg/d (n = 49), and 400 µg/d (n = 48) FA (105). In the group receiving 400 µg/d FA the mean (SD) or mean (95% CI) plasma sum of folates concentrations were 19.3 (1.9) nmol/L at baseline, 37.3 (31.1 – 43.4) nmol/L after 29 weeks, and 39.5 (33.0 – 46.1) nmol/L after 40 weeks. Corresponding, the RBC sum of folates concentrations were 757 (1.6) nmol/L at baseline, 1,122 (978 – 1,265) nmol/L after 29 weeks, and 1,273 (1,110 – 1,437) nmol/L after 40 weeks (105). In our study, we found lower WB 5-methylTHF concentrations, and higher serum 5-methylTHF concentrations after 6 and 12 months of supplementation. These differences can be explained by the different study population and the higher FA dose in our study. The concentrations of WB non-methylTHF were significantly higher after the supplementation for 3 – 4 weeks (short-term supplementation study), 6 months, and 12 months (long-term supplementation study). All other folate forms did not differ significantly. Long-term supplementation with 500 µg/d of FA led to significantly higher serum concentrations of THF and FA after 6 and 12 months.

5-MethylTHF levels in serum and WB were correlated at baseline and after 6 months of supplementation but the correlation was lost after 12 months of supplementation. This suggests that a steady-state was reached and that the folate storages in the RBCs were saturated between 6 and 12 months of supplementation. This is in line with the findings of Pietrzik *et al.* who calculated the RBC folate steady-state conditions after daily supplementation of FA or 5-methylTHF in women of childbearing age (180). They state that a steady-state was not achieved after 24 weeks of supplementation with 400 µg/d FA. They state further, that a RBC folate steady-state should be reached after 5 $t_{1/2}$ (~ 40 weeks; $t_{1/2}$ of RBCs ~ 60 days) (180).

Comparing the groups T1 and T2 of the short-term supplementation study, no significant changes were found for 5-methylTHF (serum and WB), 5-formylTHF, 5,10-methenylTHF, THF, and WB non-methylTHF. This indicates that the co-supplementation of vitamin B_6 and B_{12} had no measureable effect on the mentioned folate forms. Only the serum FA concentrations were significantly higher in group T1. Comparing the group A (B-vitamins supplementation) with group B (no B-vitamins supplementation) of the long-term supplementation study, as expected we could find significantly higher levels of almost all folate forms in group A.

In serum, 5-methylTHF was positively correlated with its demethylated product THF (**Table 21**). Serum 5-formylTHF was positively correlated with 5,10-methenylTHF. The folate interconversion from 5-formylTHF to 5,10-methenylTHF can either be carried out enzymatically by the action of the MTHFS enzyme (**Figure 4**) or by changes in the pH value and heating (50), presumably during heating step in sample preparation (**Figure 7**). In addition, 5-formylTHF and THF were positively correlated in serum. The WB non-methylTHF was positively associated with the serum folate forms 5-methylTHF, THF, and FA and with the WB 5-methylTHF.

5.2.2 The influence of age and gender on the folate forms distribution

Our study confirms the inverse association between folate and age (lower sum of folates comparing the 1st and the 4th age quartile; $p = 0.003$) in serum (41;171) but not in WB ($p = 0.118$). Furthermore, age is also inversely related to serum concentrations of 5-methylTHF but not to the other folate forms. This might partly be explained by the renal function. The reason for a lower 5-methylTHF proportion (% of sum of folates) in serum at an older age is unknown. A lower intake of folate or a lower absorption might be related to a lower sum of folates at an older age; however, we did not assess the daily folate intake. Other possible explanations might be decreased enzyme activities at an older age, lower absorption of the 5-methylTHF in the intestine, or the relatively common vitamin B_{12} deficiency in older subjects that affects the turnover rate of 5-methylTHF (200). In a recent study, Zappacosta *et al.* found significant gender-dependent serum folate concentrations with a higher serum TFOL in 79 female Italian blood donors (mean: 10.3 vs. 11.9 nmol/L; $p < 0.05$) (251). These could be explained by differences in dietary folate intake, e.g. in form of fruit and vegetable consumption. Flynn *et al.* demonstrated, that the mean daily folate intake was higher in males than in females (288 µg/d vs. 241 µg/d) (74). In our study, we did not find gender-dependend changes in folate forms distribution in serum and WB.

The serum or plasma concentrations of tHcy, SAH, and SAM increase with higher age while the SAM/SAH ratio decreases (171). We could confirm these findings in our study. The

physiological decline in renal function might explain the increase in the tHcy levels in the elderly (92). Konstantinova *et al.* found higher betaine and choline concentrations in older (71 – 74 years) than in middle-aged (47 – 49 years) subjects (129). We could confirm the finding that choline was higher in older subjects (median age: 87 years) than in younger subjects (median age: 60 years). The DMG levels according to the age were similar to the tHcy concentrations, which were lowest in the youngest age group and highest in the 3rd age quartile (median age: 80 years). In combination with the finding that serum sum of folates and 5-methylTHF concentrations were significantly lower in older adults, this indicates that the Hcy remethylation increasingly occurred via the alternative betaine pathway. This is in line with our finding that the betaine concentrations were highest in the 1st age quartile and lowest in the 3rd age quartile, suggesting an increased depletion during the Hcy remethylation.

5.2.3 Unmetabolized folic acid and the effect of co-supplementation on the folate forms distribution

Increased FA intake promotes the appearance of unmetabolized FA in blood, which is suspected to mask the vitamin B_{12} deficiency and to interfere with antifolates. To date, several studies have questioned the role of unmetabolized FA in blood (117;170;225). In a recent study by Obeid *et al.*, the plasma folate forms concentrations of 74 older German subjects (median age: 82 years) were examined at baseline and after 3 weeks of administrating therapeutic doses of B-vitamins (5 mg FA, 40 mg vitamin B_6, and 2 mg vitamin B_{12}/d) or placebo (168). At baseline, 19% of the participants had detectable concentrations of unmetabolized FA in their plasma. In the present study, we could find serum FA concentrations > LOD in 17 (11.6%) of the participants. This was lower than that reported by Obeid *et al.* (168). However, the presence of unmetabolized FA was not associated with significant differences in concentrations of sum of folates or 5-methylTHF in serum, suggesting that unmetabolized FA in serum might be related to consuming fortified foods. Nevertheless, this does not account for the improvement of the individual folate status of the participants. Additionally, minor amounts of FA in serum might be generated during the sample preparation (45°C during sample drying step) from the oxidation of THF and DHF (183).

Unmetabolized FA showed no age-dependent changes in non-supplemented subjects. This might not exclude age-related differences in FA metabolism in case of FA supplementation. A higher turnover rate of folates in young people compared to older adults has been proposed earlier (52). To show this, the absorption and conversion of [$^2H_2$]-FA were studied in 12 middle-aged (≥ 50 years) and 12 young adults (< 30 years) before and after the supplementation of 400 µg FA or [6*R,S*]-5-methylTHF for 5 weeks (52). Middle-aged subjects showed lower absorption of [$^2H_2$]-FA compared to the younger subjects. In the group of the young adults, 400 µg FA caused

an increase, and [6*R,S*]-5-methylTHF caused a decrease in the absorption of [$^2H_2$]-FA. FA or [6*R,S*]-5-methylTHF caused no changes in the absorption of [$^2H_2$]-FA in the middle-aged group (52). Differences in the FA absorption according to age might be related to higher gastric pH, atrophic gastritis, ess turnover rate, or changes in folate enzyme activities in the elderly (52).

To our knowledge, this is the first study investigating the effect of FA supplementation and co-supplementation with vitamins B_6 and B_{12} on folate forms in serum and WB. The co-supplementation with the vitamins B_6 and B_{12} had no significant influence on the levels of folates other than FA in serum. We could find significantly higher serum FA concentrations in the group only receiving the FA supplement. Oral doses of FA from supplements or fortified foods > 200 µg have been shown to bypass the normal folate absorption mechanisms and result in the presence of unmetabolized FA in serum (103;116;119). The co-supplementation with the vitamins B_6 and B_{12} led to lower concentrations of unmetabolized FA in the serum. The division of the participants into two groups (FA < 0.21 nmol/L and FA ≥ 0.21 nmol/L) led to a more pronounced result. After supplementation, 76.7% of the subjects in group T1 (FA supplementation) but only 37.5% of the subjects in group T2 (co-supplementation with vitamin B_6 and B_{12}) had detectable amounts of serum FA. This suggests that the presence of the vitamins B_6 and B_{12} led to a higher turnover of the supplemented FA.

5.2.4 The influence of the vitamin B_{12} status on the folate forms distribution

Elevated tHcy and MMA levels, as markers of the B-vitamin status, are common in older adults, which were shown in our study. Elevated levels of tHcy and MMA are related to lower serum concentrations of active vitamin B_{12} (HoloTC) and 5-methylTHF. The concentrations of serum MMA and tHcy were directly related, indicating a decreased Hcy remethylation to methionine when vitamin B_{12} is limited. Interestingly, the content of the predominant folate form decreased with increasing MMA and tHcy levels (lower 5-methylTHF proportion). However, a lower 5-methylTHF proportion was also related to age, because subjects with elevated tHcy and MMA concentrations were older than those with normal metabolite levels. Low vitamin B_{12} is common in elderly subjects and is possibly related to a lower dietary vitamin B_{12} intake or vitamin B_{12} malabsorption. The lower HoloTC and 5-methylTHF concentrations in subjects with elevated concentrations of the metabolites might suggest a lower intake or absorption of folates and vitamin B_{12}. However, this does not explain the reduction of the 5-methylTHF proportion. Our results suggest that vitamin B_{12} might play a yet underestimated role in the absorption of 5-methylTHF or in the *in vivo* turn-over of folates, which was proposed earlier (167;222).

Similar to tHcy the Cys levels were significantly higher in the group with elevated metabolite concentrations and consequently vitamin B_{12} or folate and vitamin B_{12} deficiency. We found elevated SAH and SAM concentrations but a reduced SAM/SAH ratio in the group with higher tHcy levels (113). The elevated SAM concentrations in combination with elevated SAH (and tHcy) levels can be explained by the inhibitory function of the SAH. By the inhibition of the methyltransferase reaction by SAH, SAM can not be demethylated and is accumulated. The betaine levels were lower and the DMG levels were higher in subjects with vitamin B_{12} or folate and vitamin B_{12} deficiency. This indicates that betaine is used as an alternative methyl donor for the remethylation of Hcy generating Met and DMG.

5.2.5 The interaction between the MTHFR C677T polymorphism on the folate forms distribution and the effect of supplementation

The enzyme MTHFR converts 5,10-methyleneTHF to 5-methylTHF and thus makes this available for Hcy remethylation. The common *MTHFR C677T* polymorphism results in a thermolabile enzyme with less activity (79). Accordingly, higher folate levels can stabilize the enzyme and retain its activity (79). The polymorphism in *MTHFR C677T* is associated with lower levels of serum 5-methylTHF, serum THF (126), or 5-methylTHF in RBCs compared with the *CC* genotype (126;207). The *TT* variant has been reported to cause accumulation of formylated THF (12). In our earlier study with 32 healthy non-supplemented subjects, we confirmed that the *CC* subjects have higher serum sum of folates than the group of T allele carriers (126). Subjects with the *CT* and *TT* genotype who had less 5-methylTHF available for the tHcy methylation had also a lowered THF concentrations. This can be explained by a reduced remethylation rate and a consequently reduced formation of THF. Moreover, the serum 5-methylTHF levels available for the tHcy remethylation seemed to be sufficient thus C and T allele carriers had comparable concentrations of plasma tHcy. The tHcy increasing effect of the C to T polymorphism of the *MTHFR* gene is preferably seen in subjects having serum folate in the low normal to decreased concentration range.

However, in the present study of the 120 subjects with *MTHFR C677T* genotyping results available, no differences in the concentrations of folate forms or metabolites in the serum, as well as in the WB were found between the *CC* and *TT* genotypes (**Table 27**). The reasons for these findings are unknown but might be related to the fasting vs. the non-fasting conditions of the subjects in our earlier report. In addition, in the earlier study the subjects were younger (age range: 17 – 55 years) than in the present study (126). Furthermore, the older subjects had a lower folate status with a reduce methyl transfer rate and a possible trap of the folates as 5-methylTHF which both might overlay the effect of the gene polymorphism.

The interaction between the MTHFR C677T polymorphism on the folate forms distribution and the effect of supplementation

We had the *MTHFR C677T* genotyping results available of 54 older subjects in the short-term supplementation study and 60 older subjects in the long-term supplementation study who terminated the studies. After the supplementation for 3 – 4 weeks with FA with and without co-supplementation we could find a significantly lower serum 5-methylTHF content (as % of sum of folates) in the participants with the *CC* genotype in the study arm receiving the combined B-vitamins (**Table 28**). After the long-term supplementation with B-vitamins we found significantly higher concentrations of serum THF in the subjects with the *CC* genotype in comparison to the T allele carriers (**Table 29**). A tendency for higher sum of folates and 5-methylTHF in serum and WB was found in the subjects with the *CC* genotype. This is consistent with earlier work (47;126;207). The differences in the folate forms distribution should only be present in low folate status (71;78;128) but this was not the case in our study. In the group receiving no B-vitamins, we found a significantly higher WB 5-methylTHF proportion (as % of sum of folates) in the *MTHFR 677 CC* subjects. Moreover, we found significantly lower betaine concentrations in the subjects with the *CT* and the *TT* genotype. This was in line with findings earlier reported by Holm *et al.* (99).

5.3 The effect of short-term and long-term B-vitamins supplementation on vitamins and methionine cycle related metabolites in older adults

In the short-term co-supplementation study we found significantly lower total vitamin B_{12} and HoloTC baseline levels in the group T1. Unfortunately we did not quantify the total vitamin B_{12} and HoloTC levels after the supplementation but we can assume that the total vitamin B_{12} and the HoloTC levels were higher after the supplementation with vitamin B_{12} in the group T2. In addition, the betaine concentrations were higher at baseline ($p = 0.013$) and after the supplementation ($p = 0.076$) in the group T2. This might be related to a higher proportion of male participants in this group (13.3% males in group T1 and 25% males in group T2). This is in line with findings from Lever *et al.* and from Holm *et al.* who found significantly higher betaine concentrations in males (98;133), which may be related to the effect of sex steroids on plasma betaine (98). After the supplementation for 3 – 4 weeks, the Cys levels were significantly lower in the group T2, which is in agreement with the lower tHcy concentrations. The choline concentration was significantly higher in the group T2. This is in line with a recent supplementation trial on rats where B-vitamins supplemented rats showed higher choline concentrations than rats on a B-vitamins poor diet (230).

In the long-term B-vitamins supplementation study we found significantly lower tHcy and Cys concentration, as well as higher total vitamin B_{12} levels in the group receiving B-vitamins after

6 months and after 12 months. The HoloTC and SAM levels were higher in group A after 6 months of supplementation. Both findings are related to the higher folate and vitamin B_{12} status after the supplementation. Similar to the short-term supplementation study we found higher betaine concentrations in group A but the differences were not significant. However, the elevated betaine concentrations might be related to the B-vitamins intake (153). We found significantly higher DMG levels in group B only receiving calcium and vitamin D, which is presumably associated with the lower folate status in serum and WB in the participants in this group.

In both of our studies, the baseline tHcy concentrations were elevated. The Hcy metabolism is regulated by the nutritional status of folate, vitamin B_6, and vitamin B_{12}, whereas the folate status has the greatest impact on the plasma tHcy concentrations (195). It is a known fact that the supplementation of FA with and without other B-vitamins effectively reduces the tHcy levels and normalizes the HHCY in a dose-dependent manner (25-27;55). In a recent meta-analysis on 25 trials and 2,596 subjects (mean (SD) age: 52 (19) years) it has been shown that the supplementation of FA plus vitamin B_{12} has a better tHcy-lowering effect than FA alone (100). The mean (SD) treatment duration was 8 (6) weeks. The supplementation with 400 µg/d FA led to 20% lower tHcy concentrations; the addition of vitamin B_{12} was associated with 7% more reduction of the tHcy level. This led to an absolute reduction of ~ 3 – 4 µmol/L of tHcy in populations with a median tHcy level of ~ 10 – 12 µmol/L. However, in our study, short-term supplementation was associated with ~ 6% lower (group T1; 400 µg/d FA) and ~ 4% lower (group T1; 400 µg/d FA and vitamin B_6 and B_{12}) tHcy levels. This might be the case due to the shorter time of supplementation (3 – 4 vs. 8 weeks) and the lower number of participants. The median tHcy concentrations were significantly lower ($p = 0.003$) in the group receiving co-supplementation. This might either be because of the co-supplementation or the fact that the baseline tHcy concentrations were lower in the group T2 at the start of the study. Nevertheless, the supplementation with FA or B-vitamins for only 3 – 4 weeks did not efficiently normalize the tHcy levels.

The long-term supplementation with B-vitamins normalized the tHcy concentrations within 6 months (~ 30% or ~ 4 nmol/L lower tHcy). This is in line with earlier findings (100). It has to be mentioned that the baseline tHcy levels were lower than that in the short-term supplementation study (~ 13 nmol/L vs. ~ 18 nmol/L of tHcy). In the group receiving only calcium and vitamin D the moderate HHCY remained after 12 months of supplementation.

Previous findings from our group (126;168) and many other studies (60;110;231) have shown strong negative correlations of serum or plasma folate, particularly 5-methylTHF, and circulating tHcy levels. We could confirm this finding and add that the tHcy concentrations are negatively

correlated to WB 5-methylTHF (**Figure 25**). In an earlier study we demonstrated that tHcy correlates stronger with serum 5-methylTHF than with the sum of folates (126). Serum 5-methylTHF and tHcy were not correlated after 6 months of supplementation with B-vitamins (**Figure 26**). The increased intake of FA and the therefore increased serum 5-methylTHF and reduced tHcy levels might interfere with each other. The stronger correlation of tHcy with serum 5-methylTHF indicates that the serum 5-methylTHF is a better determinant of tHcy as WB 5-methylTHF.

6 Conclusion

We aimed at investigating the folate forms distribution in serum and WB, as well as that of related vitamins and metabolites at baseline and after the supplementation with B-vitamins in older German adults. Therefore we performed two randomized and double-blind studies. In the short-term supplementation study we supplemented the participants with daily 400 µg FA or 400 µg FA, 8 mg vitamin B_6, and 10 µg vitamin B_{12} for 3 – 4 weeks. In the long-term supplementation study we daily supplemented the participants with 500 µg FA, 500 µg vitamin B_{12}, 50 mg vitamin B_6, 456 mg calcium, and 1,200 IU vitamin D or 456 mg calcium, and 1,200 IU vitamin D for one yeat. The main novel findings of this study are:

- The development of UPLC-MS/MS methods for the quantification of folate forms and related metabolites of the Met cycle and the establishment of reference ranges.
- The baseline sum of folates and the 5-methylTHF concentrations in serum and the 5-methylTHF content (as % of sum of folates) in serum are age- but not gender-dependent.
- The vitamin B_{12} status influences the concentrations of folate forms in serum, as well as that of the related metabolites.
- Unmetabolized FA could be detected in the serum at baseline and after supplementation. The co-supplementation with vitamins B_6 and B_{12} led to lower amounts of unmetabolized FA in the serum, which might reflect a higher turnover of the vitamin.
- The long-term oral supplementation with B-vitamins led to a steady-state and a saturation of the RBC folate between 6 and 12 months.
- The *MTHFR C677T* genotype had a significant influence on the folate forms distribution, whereas the sum of folates, the 5-methylTHF, and the THF concentrations were significantly higher in subjects with the *CC* genotype in non-fasting middle-aged participants but not in fasting older adults. After short-term supplementation with the combined B-vitamins subjects with the *CC* genotype had significantly lower serum 5-methylTHF contents. Long-term supplementation with B-vitamins resulted in significantly higher serum THF concentrations in subjects with the *CC* genotype.

Compared to the immunoassay or other related methods that only determine the TFOL, the UPLC-MS/MS method provides more information concerning the folate forms distribution. We could demonstrate that 5-methylTHF in serum is a better determinant of tHcy than the sum of folates. By using (UP)LC-MS/MS methods the metabolization of FA or occurrence of unmetabolized FA after fortification or supplementation can be monitored, and by determining

the 5-methylTHF/THF ratio the remethylation of Hcy by means of the MS gene can be observed. This is especially of interest in populations with a low vitamin B_{12} status, such as the elderly, pregnant women, or vegetarians. We therefore strongly recommend the use of (UP)LC-MS/MS methods in clinical studies in the future.

7 References

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8 Appendix

Appendix A: Standards and chemicals

Chemical	Purity	Company	CAS number	Catalogue number
Folate monoglutamates: standards and internal standards				
(6S)-5-CHO-H_4PteGlu-Na_2 (= 5-formylTHF)	97.3%	Merck Eprova AG, Schaffhausen, Switzerland	641-41-8	
(6R)-10-CHO-H_4PteGlu-Na_2 (= 10-formylTHF)	95.9%	Merck Eprova AG, Schaffhausen, Switzerland	2800-34-2	
(6R)-5,10-CH^+-H_4PteGlu-Cl x HCl (= 5,10-methenylTHF)	96.0%	Merck Eprova AG, Schaffhausen, Switzerland	7444-29-3	
(6S)-5-CH_3-H_4PteGlu-Na_2 (= 5-methylTHF)	97.7%	Merck Eprova AG, Schaffhausen, Switzerland	76937-22-9	
(6R)-5,10-CH_2-H_4PteGlu-Na_2 (= 5,10-methyleneTHF)	91.4%	Merck Eprova AG, Schaffhausen, Switzerland	3432-99-3	
(6S)-H_4PteGlu-Na_2 (= THF)	94.0%	Merck Eprova AG, Schaffhausen, Switzerland	135-16-0	
7,8-H_2PteGlu (= DHF)	95.0%	Merck Eprova AG, Schaffhausen, Switzerland	4033-27-6	
PteGlu-Na_2 (= folic acid)	96.4%	Merck Eprova AG, Schaffhausen, Switzerland	9007-43-6	
(6S)-5-CHO-H_4Pte[$^{13}C_5$]Glu, Ca-salt (= [$^{13}C_5$]-5-formylTHF)	>99 atom % D	Merck Eprova AG, Schaffhausen, Switzerland		
(6S)-5-CH_3-H_4Pte[$^{13}C_5$]Glu, Ca-salt (= [$^{13}C_5$]-5-methylTHF)	>99 atom % D	Merck Eprova AG, Schaffhausen, Switzerland		
Pte[$^{13}C_5$]Glu, free acid form (= [$^{13}C_5$]-folic acid)	>99 atom % D	Merck Eprova AG, Schaffhausen, Switzerland		

Appendix A: Standards and chemicals (continued)

Chemical	Purity	Company	CAS number	Catalogue number
(6S)-H_4Pte[$^{13}C_5$]Glu, Ca-salt (= [$^{13}C_5$]-THF)	>99 atom % D	Merck Eprova AG, Schaffhausen, Switzerland		
SAH and SAM: standards and internal standards				
S-(5'-Adenosyl)-*L*-homocysteine, crystalline (= SAH)		Sigma Aldrich, Munich, Germany	979-92-0	A9384
S-(5′-Adenosyl)-*L*-methionine *p*-toluenesulfonate salt, from yeast (L-methionine enriched) (= SAM)	≥80% (HPLC)	Sigma Aldrich, Munich, Germany	17176-17-9	A2408
S-Adenosyl-*L*-methionine-d_3 (S-methyl-d_3) tetra(*p*-toluenesulfonate) salt (= [$^2H_3$]-SAM)	85% chemical purity; 99 atom % D	CDN Isotopes, Quebec, Canada	17176-17-9 (unlabeled compound)	D-4093
S-(5'-Adenosyl)-*L*-homocysteine (= [$^{13}C_5$]-SAH)		Henkjan Gellekink group, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands	979-92-0 (unlabeled compound)	
Choline related metabolites: standards and internal standards				
Betaine anhydrous	ultra; ≥ 99.0%	Sigma Aldrich, Munich, Germany	107-43-7	61962
Choline chloride	≥ 99%	Sigma Aldrich, Munich, Germany	67-48-1	C7017
N,N,-Dimethylglycine (= DMG)	99%	Sigma Aldrich, Munich, Germany	1118-68-9	D1156
N,N,N-Trimethyl-d_9-glycine hydrochloride (= d_9-betaine)	98 atom % D	Isotec, Sigma Aldrich, Munich, Germany		616656
Choline chloride-trimethyl-d_9 (=d_9-choline)	98 atom % D	Isotec, Sigma Aldrich, Munich, Germany	61037-86-3	492051
N,N-Dimethyl-d_6-glycine HCl (= d_6-DMG)	99 atom % D	CDN Isotopes, Quebec, Canada	347840-03-3	D-3509
Homocysteine, methylmalonic acid, cystathionine: internal standards				
DL-(2-Amino-2-carboxyethyl)-homocysteine-3,3,4,4-d_4 (= d_4-Cys)	98 atom % D	CDN Isotopes, Quebec, Canada	56-88-2	D-3349
DL-Homocystine-3,3,3',3',4,4,4',4'-d_8 (= d_8-Hcy)	98 atom % D	Cambridge Isotope Laboratories Inc., Andover, MA, USA	870-93-9	DLM-3619

Appendix A: Standards and chemicals (continued)

Chemical	Purity	Company	CAS number	Catalogue number
Methyl-d_3-malonic acid (= d_3-MMA)	99.7 atom % D	CDN Isotopes, Quebec, Canada	42522-59-8	D-2810
Other chemicals				
Acetic acid, glacial	99.99+%	Sigma Aldrich, Munich, Germany	64-19-7	338826
Acetonitrile	ULC/MS grade	Biosolve, Valkenswaard, The Netherlands	75-05-8	01204102
Activated charcoal Darco, powder, ~ 100 mesh particle size		Sigma Aldrich, Munich, Germany	7440-44-0	242276
Ammonia solution analaR NORMAPUR	25%	BDH Prolabo (VWR International GmbH, Darmstadt, Germany)	1336-21-6	1133.2500
Ammonium acetate	ULC/MS grade	Biosolve, Valkenswaard, The Netherlands	631-61-8	01244156
Ammonium formate	ULC/MS grade	Biosolve, Valkenswaard, The Netherlands	540-69-2	01984156
L(+)-Ascorbic acid	puriss; 99.7%	Riedel-de-Haën (Sigma Aldrich, Munich, Germany)	50-81-7	33034
L-Cysteine	≥ 99.5%	Fluka (Sigma Aldrich, Munich, Germany)	52-90-4	30089
Dithiothreitol (= DTT)	≥ 99.5%	AppliChem GmbH, Darmstadt, Germany	27565-41-9	A2948
Ethylenediaminetetraacetic acid disodium salt dihydrate (= EDTA)	99.6%	Sigma Aldrich, Munich, Germany	6381-92-6	E4884
Formic acid	ULC/MS grade; 99%	Biosolve, Valkenswaard, The Netherlands	64-18-6	06914131
Hydrochloric acid	fuming, pro analysi; 37%	Merck Chemicals, Darmstadt, Germany	7647-01-0	100317

Appendix A: Standards and chemicals (continued)

Chemical	Purity	Company	CAS number	Catalogue number
Methanol, absolute	ULC/MS grade	Biosolve, Valkenswaard, The Netherlands	67-56-1	13684102
N-Methyl-N-tert-butyldimethylsilyltrifluoro-acetamid (= MBDSTFA)		Machery and Nagel, Düren, Germany	77377-52-7	701440.201
β-Mercaptoethanol	cell culture tested	Sigma Aldrich, Munich, Germany	60-24-2	M7522
Sodium hydroxide, solid	pro analysi; ≥ 99%	Merck Chemicals, Darmstadt, Germany	1310-73-2	106495
Triton X-100	for electrophoresis	Sigma Aldrich, Munich, Germany	9002-93-1	T8532

Appendix B: Equipment

Equipment	Description	Company
Analytical balance	Sartorius CP224S-0CE Sartorius ME215P-0CE	Sartorius AG, Göttingen, Germany
Blood collection	Citrate: S-Monovette 5 mL 9NC, 92 x 11 mm EDTA: S-Monovette 2.7 mL K3E, 66 x 11 mm; S-Monovette 9 mL K3E, 92 x 16 mm Li-Hep: S-Monovette 4.7 mL LH-Gel, 75 x 15 mm Needle: Safety-Multifly 21G tube 200 mm Serum: S-Monovette 4.7 mL Z-Gel, 75 x 15 mm	Sarstedt, Nümbrecht, Germany
Centrifuge	Eppendorf centrifuge 5810 R, A-4-62 Rotor	Eppendorf AG, Hamburg, Germany
	Hettich Mikro 20	Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany
	Sigma 3K12,	Sigma Laborzentrifugen GmbH, Osterode, Germany
	Optima LE-80K Preparative Ultracentrifuge, Type 50.2 Ti Rotor	Beckman Coulter GmbH, Krefeld, Germany
Cooling	Ziegra Ice machine ZBE 70-35	Ziegra Eismaschinen GmbH, Isernhagen, Germany
	Heraeus HERA freeze HFU-Basic Series, -86°C	Thermo Fischer Scientific, Waltham, USA
Concentrator	Eppendorf concentrator 5301	Eppendorf AG, Hamburg, Germany
DNA isolation	QIAamp DNA blood mini kit	Qiagen GmbH, Hilden, Germany
	TECAN Te-MagS magnetic bead separation module	TECAN Group Ltd., Männedorf, Switzerland
Gases	ALPHAGAZ 1 Argon	AIR LIQUIDE Deutschland GmbH, Düsseldorf, Germany
	Cmc instruments NGM nitrogen-membrane-generator (LCMS)	Cmc instruments GmbH, Eschborn, Germany
Heating	Thermo Scientific Haake Open-Bath Circulators C10-W19	Thermo Fischer Scientific, Waltham, USA
HPLC system	Waters 2795 alliance HT	Waters Corporation, Milford, USA
Mass spectrometer	MicroMass Quattro Micro API (coupled to HPLC system) MicroMass Quattro Premier XE (coupled to UPLC system)	Waters Corporation, Milford, USA
Microwave	NN-5256, 900 watts	Panasonic Deutschland GmbH, Hamburg, Germany

Equipment	Description	Company
Mixer/Shaker/Vortex	IKA MS2 Minishaker	IKA – Werke GmbH & Co. KG, Staufen, Germany
	RM5-40 Horizontal Mixer	Bennett Scientific Ltd., Newton Abbot, UK
	VARIOMAG Monotherm Heatable Magnetic Stirrer	VARIOMAG-USA, Daytona Beach, USA
PCR	Eppendorf Mastercycler ep gradient S	Eppendorf AG, Hamburg, Germany
	PSQ 96MA instrument	Biotage AB, Uppsala, Sweden
pH meter	Schott Intruments Lab 870 pH meter (N 6000 A electrode)	SI Analytics GmbH, Mainz, Germany
Photometer	Aurius CE2041 Spectrophotometer	CECIL Instruments Ltd., Cambridge, UK
Pipetting	Biohit m10 (0.5-10 µL), m200 (20-200 µL), m1000 (100-1000 µL) pipette	Biohit Deutschland GmbH, Rosbach v. d. Höhe, Germany
	Combitips plus; 0.5, 1, 5, 10, 25 mL Multipette plus	Eppendorf AG, Hamburg, Germany
	Pipette tips 20 µL, 200 µL, 1000 µL Serological Pipette 10 mL, 25 mL	Sarstedt, Nümbrecht, Germany
Reaction tubes	PCR Tubes 0.2 mL, PCR clean	Eppendorf AG, Hamburg, Germany
	Microtube 1.5 mL Tube 12 mL, 105x16.8 mm, PS; Tube 15 ml, 120x17 mm, PP; Tube 50 mL, 114x28 mm, PP	Sarstedt, Nümbrecht, Germany
	OptiSeal Polyallomer Centrifuge Tubes 1 x 3 ¼ in. (26 x 77 mm), 29.9 mL	Beckman Coulter GmbH, Krefeld, Germany
Sample preparation	Oasis MAX (1 ccm/30 mg and 3 ccm/60 mg) columns	Waters Corporation, Milford, USA
	Varian Bond Elut PBA columns	Varian Inc., Palo Alto, USA
Ultrasonic cleaner	VWR USC600T	VWR International GmbH, Darmstadt, Germany
UPLC columns	Acquity UPLC HSS T3 column (50 mm x 2.1 mm (i.d.) 1.8 µm particle size) Acquity UPLC BEH C_{18} column (50 mm x 2.1 mm (i.d.); 1.7 µm particle size) Acquity UPLC BEH HILIC column (100 mm x 2.1 mm (i.d.); 1.7 µm particle size)	Waters Corporation, Milford, USA
UPLC in-line filter	0.2 µm in-line filter	Waters Corporation, Milford, USA

Equipment	Description	Company
UPLC precolumns	Acquity BEH C_{18} VanGuard pre-column (5 mm x 2.1 mm (i.d.); 1.7 µm particle size) Acquity HILIC VanGuard pre-column (5 mm x 2.1 mm (i.d.); 1.7 µm particle size)	Waters Corporation, Milford, USA
UPLC system	Waters Acquity UPLC	Waters Corporation, Milford, USA
Vials and glassware	5-SV – EPA Screw Top Vials, 5 mL glass tubes	Chromacol, Herts, UK
	11 mm Snap Cap with PTFE/Silicone septa	SUN-Sri (Thermo Fischer), Rockwood, USA
	12 x 32 Maximum Recovery glass vials including Snap Caps with locked-in, Pre-slit PTFE/Sil Septa	Waters Corporation, Milford, USA
	Snap/Crimp V-Vial 8002-SC-D/V15µ	Glastechnik Graefenroda GmbH, Gräfenroda, Germany
	Wheaton Sample Vial Clear, with Screw Thread, 19x65 mm, 12 mL, with Screw Caps	neoLab Migge Laborbedarf-Vertriebs GmbH, Heidelberg, Germany
	DURAN GL 45 glass bottles, 200 mL, 500 mL, 1000 mL	DURAN Group GmbH, Wertheim/Main, Germany
Water purification	Milli-Q Academic	Millipore, Molsheim, France

Appendix C: Quantification of S-adenosyl homocysteine and S-adenosyl methionine

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Simultaneous quantification of S-adenosyl methionine and S-adenosyl homocysteine in human plasma by stable-isotope dilution ultra performance liquid chromatography tandem mass spectrometry

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ABSTRACT

S-adenosyl methionine (SAM) is an important methyl group donor that is formed from methionine. S-adenosyl homocysteine (SAH) is formed after demethylation of SAM and represents a potent inhibitor of many methyltransferases. We developed an improved stable-isotope dilution ultra performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) method for the simultaneous quantification of SAM and SAH in biological samples. The method comprises a phenylboronic acid-containing solid-phase extraction procedure, serving for binding and clean-up of SAM and SAH. After extraction, samples were separated and detected using either a HPLC SymmetryShield RP_{18} or an Acquity UPLC BEH C_{18} column with a HPLC–MS/MS or an UPLC–MS/MS system. The best results were obtained by Acquity UPLC BEH C_{18} column. In plasma samples, the estimated intraassay coefficients of variation (CVs) for SAM and SAH were 3.3% and 3.9%, respectively, the interassay CVs were 10.1% for SAM and 8.3% for SAH. Mean recovery of SAM and SAH at two different concentrations was 100.0% for SAM and 101.7% for SAH. The quantification limits were 0.5 and 0.7 nmol/L for SAM and SAH, respectively. In 31 plasma samples, the mean concentrations (SD) were 85.5 (11.1) nmol/L for SAM and 13.3 (5.0) nmol/L for SAH with a SAM/SAH ratio of 7.0 (1.8). The new UPLC–MS/MS method showed very high sensitivity and selectivity for SAM and SAH, low CVs and fast sample preparation (40 samples in 60 min) and analysis time (3 min). This new assay can be used for large-scale clinical studies.

1. Introduction

Moderate hyperhomocysteinemia (HHCY) (tHcy > 12 μmol/L) is associated with several pathologic conditions, including cardiovascular and neurodegenerative diseases [1]. An increase in serum total homocysteine (tHcy) is frequently caused by vitamin B_{12}, vitamin B_6, and folate deficiency. Apart from oxidative stress another main pathomechanism in HHCY is hypomethylation which is poorly reflected by the tHcy plasma level.

S-adenosyl methionine (SAM) is the most prominent methyl group donor and formed from methionine by methionine adenosyltransferase (MAT) in the presence of ATP. It plays a decisive role in many transmethylation reactions of compounds such as DNA, RNA, proteins, lipids, myelin, and neurotransmitters. The demethylated product of SAM, S-adenosyl homocysteine (SAH), can further be hydrolyzed to homocysteine and adenosine in a reversible reaction catalyzed by SAH-hydrolase in a reaction that favored SAH formation in case of HHCY [2]. SAH is known as a potent competitive inhibitor for most cellular methyltransferases [3]. It can bind to the active site of the enzymes and thus inhibits the binding of SAM. An increase in SAH level or a decrease in SAM level will result in a decrease in the SAM/SAH ratio, which has been termed methylation index. Maintenance of the SAM production and cleavage of newly formed SAH are therefore important for regulation of methylation index [4]. Hypomethylation has been related to changed DNA methylation, gene expression or other methylation-dependent functional biological reactions [5–8]. In addition, studies have shown that SAH represents a stronger risk factor for cardiovascular disease than tHcy [9]. Therefore, measurement of plasma SAM and SAH is of great interest because they better reflect the methylation potential than tHcy [10].

Due to low plasma concentrations in the nanomolar range, accurate quantification of SAM and SAH requires sensitive equipments. Over the last years, several HPLC methods combined with ultra-

Abbreviations: HC, high control; HHCY, hyperhomocysteinemia; HPLC, high-performance liquid chromatography; LC, low control; LC–MS/MS, liquid chromatography with tandem mass spectrometry; MAT, methionine adenosyltransferase; MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometry; SAH, S-adenosyl homocysteine; SAM, S-adenosyl methionine; SPE, solid-phase extraction; tHcy, total homocysteine; UPLC, ultra performance liquid chromatography.

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violet detection for simultaneous measurement of SAM and SAH in biological samples have been introduced [11,12]. However, due to lack of sensitivity and poor reproducibility, these methods are not suitable for reliable quantification of SAM and SAH in plasma and for high throughput analyses. Liquid chromatography coupled with mass spectrometry (LC–MS) is characterized by high sensitivity and short retention time which enables the efficient and accurate assay of biological samples [13,14]. Nevertheless, sample preparation is often time consuming. Most recently, the technique of liquid chromatography tandem mass spectrometry (LC–MS/MS) has been introduced. Advantages of this method are high selectivity, sensitivity, and sample throughput by less time-consuming sample preparation [15–17]. In addition, the use of stable-isotope-labeled internal standards offers many advantages. It is generally accepted that these standards show better performance in quantitative LC–MS analysis because they behave like the target analyte during sample preparation and chromatography. They correct the matrix effect and variations associated with the analysis. However, precision, reproducibility, and sample throughput obtained by LC–MS/MS has not turned out satisfactory. Some methods are sensitive (LOQ of 2.0 nmol/L for SAM and 1.0 nmol/L for SAH) but need between 5 and 55 min analysis time per sample [14,16] or are fast (3 min analysis time per sample) but lack sensitivity (LOQ of 15.0 nmol/L for SAM and 5.0 nmol/L for SAH) [15]. We aimed at developing a high throughput method, which is sensitive enough for accurate determination of SAH and SAM concentrations in human plasma. The resulting methylation index serves as basis to investigate the relationships between methylation and biological disorders in large-scale clinical studies.

In this report, we describe a fast, stable-isotope dilution LC–MS/MS method for the simultaneous quantification of SAM and SAH in biological samples. The introduction of ultra performance liquid chromatography (UPLC) technique in combination with tandem mass spectrometry (UPLC–MS/MS) has further improved the quantification of SAM and SAH because of higher selectivity and sensitivity. This method can be used in large-scale clinical studies.

2. Materials and methods

2.1. Sample collection

Blood samples were collected from 31 apparently healthy participants (6 males) from the medical staff of the Saarland University Hospital (age range: 20–55 years) in a vacutainer tube containing EDTA. The samples were collected in the course of a medical checkup in February 2009. The study was approved by the local ethics commission.

The blood samples were immediately centrifuged at 2000 × *g* for 10 min at 4 °C, the plasma was separated. SAM is unstable in biological samples and can partially degrade into SAH. Therefore, strict pre-analytical conditions such as immediate centrifugation and acidification of the samples are strongly recommended. 1 mL of the EDTA plasma was directly acidified with 100 μL of 1 N acetic acid to prevent SAM degradation, mixed thoroughly, and then stored at −70 °C until analysis. In addition, a plasma pool was prepared in-house.

2.2. Standards and chemicals

SAH and SAM (Sigma–Aldrich) were used for the preparation of standard curves. $^{2}H_3$-SAM (CDN Isotopes, Quebec, Canada), and $^{13}C_5$-SAH (provided by Henkjan Gellekink group (Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands) were used as internal standards. Stock solutions were stable for at least 1 year. Chemicals used: acetic acid (glacial; 99.99+%; Sigma–Aldrich), ammonium acetate (ULC/MS grade; Biosolve, The Netherlands).

2.3. Sample preparation

Plasma samples were thawed and centrifuged at 2000 × *g* for 5 min at 4 °C. 500 μL of the supernatant was neutralized with 50 μL of 1 mol/L ammonia to a pH of 7.4–7.5. 25 μL internal standard solution mix (concentrations: 3.0 μmol/L $^{2}H_3$-SAM and 0.82 μmol/L $^{13}C_5$-SAH, leading to final concentrations of 195 nmol/L $^{2}H_3$-SAM and 64 nmol/L $^{13}C_5$-SAH) was added. Sample cleanup was performed with solid-phase extraction (SPE) columns containing phenylboronic acid (Varian Bond Elut PBA columns, Varian Inc., USA). Before loading the samples, the SPE columns were preconditioned with 1 mL of aqueous acetic acid (pH 2.636), then 5 × 1 mL of 20 mmol/L aqueous ammonium acetate (pH 7.4). Columns were centrifuged at 500 × *g* for 1 min at 4 °C after each washing step.

Each sample was then loaded on top of the cartridges and centrifuged at 250 × *g* (slow start) for 2 min at 4 °C. Water-soluble impurities were removed by washing the column with two 1-mL volumes of 20 mmol/L ammonium acetate, pH 7.4 (500 g, 1 min, 4 °C). Finally, SAM and SAH were eluted from the column using 3 × 350 μL of aqueous acetic acid (pH 2.636) with a centrifugation step every time at 250 × *g* for 2 min at 4 °C (slow start). The eluates were stored at −20 °C until analysis and were stable for at least 6 months.

2.4. Preparation of calibrators and quality controls

Calibrators were included in each batch of samples at concentrations of 0, 6, 12, 24, and 48 nmol/L for SAH and 0, 25, 50, 100, and 200 nmol/L for SAM in 20 mmol/L ammonium acetate buffer (pH 7.4). Quality controls at two different concentrations were added to each batch of samples. The high control (HC) contained 160 nM SAM and 32 nM SAH, the low control (LC) contained 40 nM SAM and 8 nM SAH in 20 mmol/L ammonium acetate buffer (pH 7.4). For recovery experiments, plasma samples were spiked with SAH and SAM. For this purpose, 250 μL plasma pool samples were added to 250 μL HC and LC, respectively. Prior sample loading, 50 μL of 20 mmol/L ammonium acetate buffer and 25 μL of internal standard solution (3.0 μmol/L for $^{2}H_3$-SAM and 0.82 μmol/L for $^{13}C_5$-SAH) were added. Calibrators and quality control samples were then processed as described above.

2.5. Calculations

Data acquisition was carried out by MassLynx V4.1 and QuanLynx software. The concentration of the analyte in plasma was calculated by interpolation of the observed analyte/internal standard peak-area ratio. Linear regression analysis (Microsoft Excel) was used to verify the linearity of the calibration curves. Resolution of the two peaks was calculated by the following equation: $R_s = 2[(t_R)_B - (t_R)_A]/w_{bA} + w_{bB}$ where R_s is the resolution, w_{bA} and w_{bB} are the widths at the base of peaks A and B, respectively; $(t_R)_B$ and $(t_R)_A$ are the retention times of the peaks. The spiked plasma concentration was calculated as: (endogenous concentration + concentration of quality control)/2. Recovery [%] was calculated as: (measured concentration/calculated concentration) × 100.

2.6. HPLC–MS/MS conditions

HPLC–MS/MS measurement was carried out using a Waters 2795 alliance HT, which was coupled to a MicroMass Quattro Micro API tandem mass spectrometer (Waters Corporation, Milford, MA, USA). The samples were separated on a SymmetryShield RP_{18} column [100 mm × 2.1 mm (i.d.); 3.5 μm particle size; Waters Corporation]. Solvent for HPLC was 100% aqueous acetic acid (glacial), pH 2.636. The sample injection volume was 20 μL. The column tem-

Table 1
MS–MS conditions (Quattro Premier XE) for multiple reaction monitoring in ESI⁺.

Compound	Cone voltage [V]	Collision energy [eV]	Parent ion (*m/z*)	Daughter ion (*m/z*)	Dwell time [s]
SAH	20.00	21.00	385.12	136.10	0.150
SAM	20.00	16.00	398.91	250.23	0.300
$^{13}C_5$-SAH	20.00	21.00	390.12	136.10	0.150
$^{2}H_3$-SAM	20.00	16.00	402.91	250.23	0.300

perature was 25 °C, the flow rate was 0.3 mL/min over a total run time of 3.0 min.

SAM and SAH, as well as their internal standards, were identified using a triple quadrupole mass spectrometer (data analysis software MassLynx V4.0 and QuanLynx), which was used in positive electrospray ionization mode. Nitrogen was used as cone gas, argon was used as collision gas. Desolvation gas flow was 700 L/h, and cone gas flow was maintained at 5 L/h. Desolvation and source temperature were 500 °C and 110 °C, respectively. Inter-scan and inter-channel delays were both set to 0.05 s. Cone voltage was 20.0 V for all compounds, collision energies for SAH and $^{13}C_5$-SAH were 16.0 eV and 20.0 eV for SAM and $^{2}H_3$-SAM, respectively. The *m/z* transitions were the same used for Quattro Premier XE tandem quadrupole mass spectrometer (Table 1).

2.7. UPLC–MS/MS conditions

UPLC–MS/MS analyses were performed using an Acquity Ultra Performance LC system, which was coupled to a MicroMass Quattro Premier XE tandem quadrupole mass spectrometer (Waters Corporation). Separation was achieved by using two different LC columns: a SymmetryShield RP_{18} column [100 mm × 2.1 mm (i.d.); 3.5 μm particle size], and an Acquity UPLC BEH C_{18} column [50 mm × 2.1 mm (i.d.); 1.7 μm particle size] with an Acquity BEH C_{18} VanGuard pre-column [5 mm × 2.1 mm (i.d.); 1.7 μm particle size] and a 0.2 μm in-line filter (Waters Corporation). The column temperature was maintained at 25 °C (HPLC compared to UPLC separation) or 30 °C, respectively, with a mobile phase flow rate of 0.35 mL/min. The mobile phase consisted of 100% aqueous acetic acid (glacial), pH 2.636. The total run time was 3.0 min. The sample volume injected was 10 μL.

MicroMass Quattro Premier XE mass spectrometer was operated using a positive electrospray ionization mode. The ionization parameters were: capillary voltage 0.9 kV; source temperature 110 °C; desolvation gas temperature 500 °C at a flow rate of 800 L/h (N_2); cone gas flow rate 20 L/h (N_2). Argon was used as collision gas at a flow rate of 0.35 mL/min. Multiple reaction monitoring (MRM) transitions as well as the individual cone voltages and collision energy voltages were summarized in Table 1. Inter-scan and inter-channel delays were set to 0.05 s.

3. Results

3.1. Chromatography and mass spectra

Optimal MRM conditions were obtained in the positive electrospray ionization mode, yielding the following transitions: SAM, *m/z* 398.91 → 250.23 (adenosine); $^{2}H_3$-SAM, *m/z* 402.91 → 250.23; SAH, *m/z* 385.12 → 136.10 (derived from adenosine backbone [15]); $^{13}C_5$-SAH, *m/z* 390.12 → 136.10 (Table 1).

Typical MRM chromatograms of a human plasma sample are shown in Fig. 1. Fig. 1a represents mass spectra obtained after separation via a HPLC column (SymmetryShield RP_{18}) on the UPLC system. Fig. 1b shows a chromatogram of an Acquity UPLC BEH C_{18} column. Column temperatures were set to 25 °C. As can be inferred from Fig. 1, the chromatograms of human plasma samples did not contain any interfering peaks. In addition, we tested the following UPLC-columns: an Acquity UPLC BEH C_8 column [100 mm × 2.1 mm

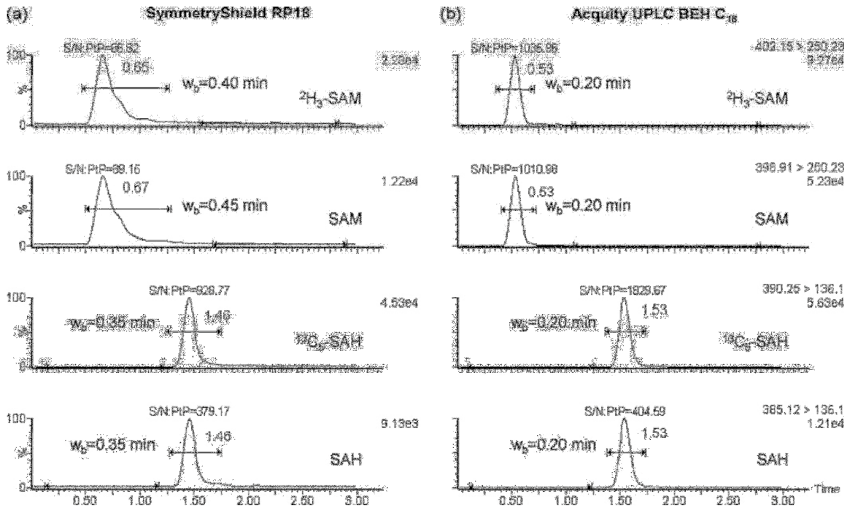

Fig. 1. Extracted MRM-traces of the target compounds in a plasma sample obtained from separation via UPLC–MS/MS (SymmetryShield RP_{18} (a) and Acquity UPLC BEH C_{18} column (b)). Flow rates are 0.3 mL/min, temperature was set to 25 °C. Signal-to-noise-ratio and retention times are shown above the peak maximum, peak intensity and *m/z* are shown in the upper right. Peak widths at base (w_b) are shown next to the peaks.

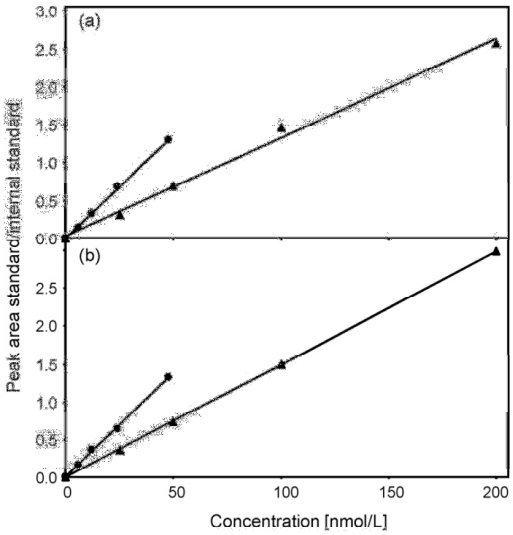

Fig. 2. UPLC–MS/MS calibration curves for SAH (●) and SAM (▲) with SymmetryShield RP_{18} column (a) and Acquity UPLC BEH C_{18} column (b). Calibration curves were linear over a range of 0–200 nmol/L SAM and 0–48 nmol/L SAH. The coefficients of linear regression for the SymmetryShield RP_{18} column are: $r^2 = 0.999$ ($y = 0.0273x - 0.0023$) for SAH and $r^2 = 0.994$ ($y = 0.013x + 0.0326$) for SAM. The coefficients of linear regression for the Acquity UPLC BEH C_{18} column are: $r^2 = 0.999$ ($y = 0.0274x + 0.0058$) for SAH and $r^2 = 0.999$ ($y = 0.0149x - 0.0041$) for SAM.

(i.d.); 1.7 μm particle size], an Acquity UPLC BEH Shield RP_{18} column [100 mm × 2.1 mm (i.d.); 1.7 μm particle size]; an Acquity UPLC HSS T3 column [50 mm × 2.1 mm (i.d.); 1.8 μm particle size] and an Acquity UPLC HSS C_{18} SB column [100 mm × 2.1 mm (i.d.); 1.8 μm particle size; Waters Corporation]. Due to longer retention times, inferior peak shape, or high back pressure in the UPLC system, no further experiments were carried out with these columns.

The retention times for SymmetryShield RP_{18} column (25 °C column temperature) were 0.65 min ($^2H_3$-SAM), 0.67 min (SAM), and 1.46 min (SAH and $^{13}C_5$-SAH). Retention times for the Acquity UPLC BEH C_{18} column were 0.53 min (SAM and $^2H_3$-SAM) and 1.53 min (SAH and $^{13}C_5$-SAH). Due to tailing effects during SymmetryShield RP_{18} separation, chromatographic peaks of SAM and SAH were not completely separated and peak widths at base (w_b) for SAM and SAH were 0.45 and 0.35 min, respectively. The resolution of the two peaks were $R_s = 1.975$. The mean signal-to-noise ratios (S/N) of 5 plasma samples for SAM were 357.5 and 269.1 for SAH. Using UPLC columns improved the separation as can be seen in Fig. 1b. Peak widths at base obtained by using an Acquity UPLC BEH C_{18} column were markedly lower than that obtained by HPLC–MS/MS (0.20 min for both SAM and SAH). The resolution of the two peaks were $R_s = 5.0$. By using an UPLC column we could improve the mean plasma ($n = 5$) signal-to-noise ratios for SAH to 661.8 and for SAM to 3914.0, which is 1.9- and 14.5-fold, respectively, higher than that obtained by a HPLC column. An increase in column temperature to 30 °C in the UPLC system led to shorter retention times of the compounds.

3.2. Linearity and sensitivity

The calibration curves were linear over the ranges 25–200 nmol/L for SAM and 6–48 nmol/L for SAH. The coefficients of linear correlation (r^2) for the used columns were $r^2 > 0.999$ for the calibration curves for both SAM and SAH (calculated with QuanLynx 4.1 software) and additionally verified using linear regression analysis (Microsoft Excel), as shown in Fig. 2.

The limits of detection (LOD) of the method, defined as signal-to-noise ratio ≥5, and the limits of quantification (LOQ), defined as signal-to-noise ratio ≥10, are presented in Table 2. LODs and LOQs for SAM and SAH were estimated by verifying the peak height of the analyte in comparison to the height of the noise in plasma pool samples. LOD and LOQ of SAH showed similar concentrations for both columns and LC–MS/MS systems, respectively. For SAM, best results were obtained by using an Acquity UPLC BEH C_{18} column. LOD was 0.2 nmol/L for SAM and 0.3 nmol/L for SAH. As can be seen in Table 2, an improvement in the sensitivity was achieved due to the application of the HPLC column to the UPLC system. Concentrations of LOD and LOQ could be reduced by 50%. Further improvement was obtained by using an Acquity UPLC BEH C_{18} column. In comparison with the SymmetryShield RP_{18} column, LOQ using an Acquity UPLC BEH C_{18} column could be reduced by approximately 7-fold.

3.3. Precision and recovery

The precision of the methods (HPLC–MS/MS and UPLC–MS/MS) was assessed by quantification of SAM and SAH in an in-house prepared plasma pool. Coefficients of variation (CV) for intraassay and interassay measurement are shown in Table 3. Intraassay CV was assessed by quantification of the compounds in twelve individual aliquots of the same plasma pool sample. SAH plasma pool concentrations, depending on column and method selection, ranged from 12.0 to 13.7 nmol/L, SAM concentrations from 77.9 to 81.8 nmol/L, yielding intraassay CVs between 3.9% and 13.5% (SAH), and between 3.3% and 6.2% (SAM). The transfer of the SymmetryShield RP_{18} column from HPLC system to the UPLC system improved the intraassay CV values. The lowest CVs were obtained by Acquity UPLC BEH C_{18} column with CVs of 3.9% (SAH) and 3.3% (SAM).

Interassay CV was assessed by the quantification of SAM and SAH in HC, LC and plasma pool samples over a period of seven days. Lowest CVs were obtained by Acquity UPLC BEH C_{18} column (see Table 3). In plasma pool samples, CVs of 8.3% (SAH) and 10.1% (SAM) were estimated.

Recovery experiments were performed by spiked plasma samples. Calculated concentrations, mean sample concentrations and recovery were illustrated in Table 3. Best recoveries were obtained by Acquity UPLC BEH C_{18} column with mean recoveries of 100.0% for SAM and 101.7% for SAH.

Table 2
Limit of detection and limit of quantification.

		HPLC–MS/MS	UPLC–MS/MS	
		SymmetryShield RP_{18}	SymmetryShield RP_{18}	Acquity UPLC BEH C_{18}
LOD [nmol/L]	SAH	0.2	0.4	0.2
	SAM	3.1	1.5	0.2
LOQ [nmol/L]	SAH	0.3	0.7	0.7
	SAM	6.3	3.6	0.5

LOD: limit of detection, LOQ: limit of quantification. $n = 12$ plasma pool samples were used.

Table 3
Summary of precision and recovery obtained by HPLC- and UPLC–MS/MS analysis (MRM mode) of SAH and SAM.

	Measured in	Intraassay ($n=12$)		Interassay ($n=7$)		Recovery ($n=2$)		
		Mean (SD) [nmol/L]	CV [%]	Mean (SD) [nmol/L]	CV [%]	Calculated in spiked plasma [nmol/L]	Mean in spiked plasma [nmol/L]	Mean recovery [%]
Acquity UPLC BEH C_{18} (UPLC–MS/MS)								
SAH	HC 32 nM			31.1 (1.8)	5.8			
	LC 8 nM			8.2 (0.7)	8.8			
	Plasma	12.9 (0.5)	3.9	13.7 (1.1)	8.3	10.0 and 22.0	10.2 and 23.0	101.7
SAM	HC 160 nM	81.8 (2.7)	3.3	162.5 (6.0)	3.7	95.0 and 155.0	92.9 and 159.5	100.0
	LC 40 nM			40.6 (3.6)	8.8			
	Plasma			85.9 (8.7)	10.1			
SymmetryShield RP_{18} (HPLC–MS/MS)								
SAH	HC 32 nM			30.5 (2.8)	9.2			
	LC 8 nM			8.0 (1.1)	13.4			
	Plasma	11.0 (0.9)	8.4	12.7 (1.5)	11.7	10.0 and 22.0	8.2 and 20.6	92.8
SAM	HC 160 nM	77.9 (2.8)	3.6	160.8 (9.5)	5.9	95.0 and 155.0	94.5 and 216.6	120.0
	LC 40 nM			40.7 (4.3)	10.5			
	Plasma			83.6 (7.8)	7.8			
SymmetryShield RP_{18} (UPLC–MS/MS)								
SAH	HC 32 nM			29.2 (2.9)	9.8			
	LC 8 nM			7.6 (1.0)	12.6			
	Plasma	12.0 (1.6)	13.9	14.4 (2.9)	20.2	10.0 and 22.0	10.2 and 23.0	103.1
SAM	HC 160 nM	79.5 (4.9)	6.2	157.3 (4.5)	2.8	95.0 and 155.0	91.2 and 159.5	99.5
	LC 40 nM			38.9 (2.9)	7.4			
	Plasma			88.5 (7.2)	8.1			

CV: coefficient of variation, HC: high control, LC: low control, n: number of experiments, SD: standard deviation.

3.4. Stability

Recent reports indicated that SAM in plasma may be unstable during storage at −20 °C and acidification is strongly recommended [14,15]. Therefore, the acidified plasma was stored at −70 °C with no apparent degradation of SAM over at least 1 year. According to Gellekink et al. and our own observations, samples are stable after SPE (at −20 °C) for at least 6 months due to the acidic elution (aqueous acetic acid, pH 2.636) of the compounds.

3.5. Normal plasma SAM and SAH concentrations

Normal SAM and SAH concentrations in plasma samples from 31 apparently healthy volunteers were measured. Mean (SD) SAM concentrations were 85.5 (11.1) nmol/L, mean (SD) SAH concentrations were 13.3 (5.0) nmol/L, which is presented in Table 4. The mean (SD) methylation index was 7.0 (1.8). The determined concentrations of SAM and SAH obtained by Acquity UPLC BEH C_{18} column are comparable to the results obtained by SymmetryShield RP_{18} with correlation coefficients (UPLC–MS/MS vs. HPLC–MS/MS method) of $r^2 = 0.96$ (SAH) and $r^2 = 0.83$ (SAM) (see Table 4).

In comparison with other studies, mean SAM plasma concentrations (85.5 nmol/L) measured by UPLC–MS/MS method are in between of those reported by Gellekink et al. (94.5 nmol/L) and Struys et al. (74.7 nmol/L) (see Table 4). SAM plasma concentrations reported by Stabler et al. (109 nmol/L (71–168 nmol/L, 95% CI)) were approximately 22% higher. SAH plasma concentrations (13.3 nmol/L) are comparable with that obtained by Gellekink et al. (12.3 nmol/L), 13% lower than that obtained by Stabler et al. (15 nmol/L (8–26 nmol/L, 95% confidence interval)) and approximately half of the concentration than that reported by Struys et al. (26.2 nmol/L).

4. Discussion

Mild to moderate hyperhomocysteinemia is a well-established risk factor for vascular and neurodegenerative diseases. Although the underlying pathogenic mechanisms are still poorly understood, it has been recently suggested that an increase in SAH, which results

Table 4
Comparison of SAM and SAH in samples of apparently healthy subjects.

n	Parameter	RT [min]	Mean (SD) [nmol/L]	Recovery [%]	LOQ [nmol/L]	Method	Reference
31	SAH	1.38	13.3 (5.0)	101.8	0.7	UPLC–MS/MS[a]	Present study
	SAM	0.53	85.5 (11.1)	100.0	0.5		
31	SAH	1.38	13.0 (4.8)	92.8	0.3	HPLC–MS/MS	Present study
	SAM	0.53	83.0 (13.0)	120.0	6.3		
26	SAH	2.88	12.3 (3.7)	96.8	1.0	HPLC–MS/MS	[14]
	SAM	2.42	94.5 (15.2)	94.5	2.0		
15	SAH	2.15	26.2 (6.1)	93.0	5.0	HPLC–MS/MS	[13]
	SAM	1.30	74.7 (14.5)	93.0	15.0		
48	SAH	10.27	15 (8–26)[b]	96.0	~5 pmol in serum sample	HPLC–MS	[15]
	SAM	10.37	109 (71–168)[b]	94.0			

LOQ: limit of quantification, RT: retention time.
[a] Acquity UPLC BEH C_{18} column.
[b] 95% confidence interval.

in an altered SAM/SAH ratio, might play a role [1,7,13]. To assess possible association of the methylation index with human disease states, it is necessary to develop sensitive methods for quantitative measurement of SAM and SAH in biological samples.

We present a sensitive and specific high-throughput method for the simultaneous measurement of SAM and SAH in plasma samples using stable-isotope dilution HPLC–MS/MS and UPLC–MS/MS with positive electrospray ionization. In this study we examined several UPLC columns and one HPLC column (SymmetryShield RP_{18}). The latter was used in both HPLC and UPLC systems. Best results in sensitivity and selectivity were obtained by the Acquity UPLC BEH C_{18} column. Through acidification of the plasma samples with acetic acid to a pH <5.0, as described by Gellekink et al. [16], degradation of SAM into SAH could be prevented. A degree of discrepancy in SAH concentrations obtained by different LC–MS methods is probably due to differences in sample preparation, which in some cases does not completely inhibit SAM to SAH transformation before measurement.

We measured SAM and SAH in 31 healthy volunteers. The determined concentrations of SAM and SAH obtained by Acquity UPLC BEH C_{18} column are comparable to the results obtained by SymmetryShield RP_{18}. In comparison with other studies, mean SAM plasma concentrations measured by UPLC–MS/MS method are in between of those reported by Struys et al. and Gellekink et al. SAM plasma concentrations reported by Stabler et al. were approximately 22% higher. SAH plasma concentrations are comparable with that obtained by Gellekink et al., 13% lower than that obtained by Stabler et al., and 50% lower than that reported by Struys et al. Lower SAM and higher SAH circulating plasma concentrations, especially reported by Struys et al., can be explained by partial SAM degradation because plasma samples were not immediately acidified. Due to similar sample collection and preparation, our results are mostly comparable with that reported by Gellekink et al.

Although several methods for quantification of SAM and SAH have been described, most of them have disadvantages, including time-consuming sample preparation and analysis, and low sensitivity or selectivity of the LC–MS/MS. Our method using UPLC–MS/MS overcame these difficulties. This new method requires 60 min sample preparation time for 40 samples and enables us the fast measurement of SAM and SAH within 3 min per sample with high precision (intraassay CVs <4%, interassay CVs ≤10%) and sensitivity (LOQ <0.7 nmol/L). Although the method was designed for the analysis of plasma samples, it can be applied to other biological materials, such as CSF and tissues [18]. A couple of general shortcomings have to be mentioned. Using stable-isotope labeled standards is expensive. In addition, isotope exchange between the standard and sample is possible, and interferences in biological systems can occur. One important disadvantage is that labeled standards are mostly not commercially available and have to be obtained either from other experts [16] or prepared by enzymatic synthesis [15].

In conclusion, we developed a fast, precise, and accurate method for the simultaneous quantification of SAM and SAH in biological samples, which can be used in large-scale clinical studies. The described method can contribute in investigating the biological relationships between methylation and certain diseases.

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Appendix D: Quantification of betaine, choline, and dimethylglycine

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Quantification of acetylcholine, choline, betaine, and dimethylglycine in human plasma and urine using stable-isotope dilution ultra performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

Disorders in choline metabolism are related to disease conditions. We developed a stable-isotope dilution ultra performance liquid chromatography–mass spectrometry (UPLC–MS/MS) method for the simultaneous quantification of acetylcholine (ACh), betaine, choline, and dimethylglycine (DMG). We used this method to measure concentrations of the analytes in plasma and urine in addition to other biological fluids after a protein precipitation by acetonitrile. The detection limits were between 0.35 nmol/L (for ACh in urine) and 0.34 μmol/L (for betaine in urine). ACh concentrations were not detectable in plasma. Intraassay and interassay coefficient of variation (CVs) were all <10.0% in biological fluids, except for DMG in cerebrospinal fluid (CV = 12.44%). Mean recoveries in urine pool samples were between 99.2% and 103.9%. The urinary excretion of betaine, choline, and DMG was low, with approximately 50.0% higher excretion of choline in females compared to males. Median urinary excretion of ACh were 3.44 and 3.92 μmol/mol creatinine in males and females, respectively ($p = 0.689$). Plasma betaine concentrations correlated significantly with urinary excretions of betaine ($r = 0.495$, $p = 0.027$) and choline ($r = 0.502$, $p = 0.024$) in females. Plasma choline concentrations correlated significantly with urinary excretion of ACh in males ($r = 0.419$, $p = 0.041$) and females ($r = 0.621$, $p = 0.003$). The new method for the simultaneous determination of ACh, betaine, choline, and DMG is sensitive, precise, and fast enough to be used in clinical investigations related to the methylation pathway.

1. Introduction

Choline is an essential micronutrient that is involved in several biochemical pathways. First, choline is a precursor of acetylcholine (ACh) that is formed by choline acetyltransferase (EC 2.3.1.6) [1]. Second, choline is utilized for synthesis of phosphatidylcholine and sphingomyelin thus having a functional role in cellular membrane composition and functions [1]. Third, the enzymatic oxidation of choline (mainly in the liver) delivers betaine, a methyl donor for the remethylation of homocysteine (Hcy) into methionine [2]. This reaction is catalyzed by the enzyme betaine:homocysteine methyltransferase (BHMT; EC 2.1.1.5) [3]. Betaine can be alternatively obtained from the diet [1] and it is also considered an important osmolyte. The product of betaine-demethylation is dimethylglycine (DMG) which can be further converted to sarcosine, and later on to glycine.

Deficiency of choline can cause developmental disorders, fetal brain damage, fatty liver [4], or muscle damage [5]. Moreover, deficiencies of choline, betaine, and folate cause Hcy accumulation, which is a risk factor for several diseases [6]. In addition, reduced plasma concentration of betaine has been related to lipid disorders [7], the metabolic syndrome [7], and diabetes [8]. The metabolisms of choline and folate are interrelated thus explaining the Hcy-lowering effect of betaine [9] and choline [10–12].

ACh is an important neurotransmitter and an endothelium-dependent vasodilator in the central and peripheral nervous systems [13]. In animal models, choline deficient diet caused renal necrosis [14,15] and hepatic dysfunction which could be partly related to phospholipid dysbalance or to low ACh production. Cholinesterase inhibitors that allow high concentrations of ACh for a longer time have been developed and tested for dementia treat-

Abbreviations: ACh, acetylcholine; AD, Alzheimer's disease; AF, amniotic fluid; BHMT, betaine:homocysteine methyltransferase; CSF, cerebrospinal fluid; DMG, dimethylglycine; Hcy, homocysteine; HILIC, hydrophilic interaction liquid chromatography; HPLC, high-performance liquid chromatography; IDL, instrumental detection limit; IQL, instrumental quantification limit; LC–MS/MS, liquid chromatography with tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MDL, method detection limit; MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometry; MQL, method quantification limit; RMSE, root mean square error; SAM, S-adenosyl methionine; tHcy, total homocysteine; TIC, total ion chromatogram; UPLC, ultra performance liquid chromatography.

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ment [16], and for protection against ischemic acute renal failure [17]. Recent data suggests that the activity of acetylcholinesterase is induced during ischemia reperfusion thus causing apoptosis in vivo [17] and probably connecting acute renal failure to ACh. Ye et al. have shown that inhibition of acetylcholinesterase lowered serum creatinine and urea nitrogen in animals subjected to ischemia reperfusion [17]. Therefore, ACh might be causally related to renal dysfunction.

The quantification of ACh, betaine, choline, and DMG in biological material is of interest. Choline is a small polar molecule that lacks a chromophore and thus cannot be detected by immunoassays. Chemiluminescent enzyme assays [18], as well as several UV-detection based high-performance liquid chromatography (HPLC) methods [19] have been described. Recently, liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods for plasma samples have been developed [20–22]. We used a hydrophilic interaction liquid chromatography (HILIC) which might offer an advantage, because the metabolites are weakly retained on reversed phase liquid chromatographic columns.

The physiological concentrations of ACh are in the nanomolar range and that of betaine, choline, and DMG are in the micromolar range. The ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) offers higher sensitivity and allows for all 4 metabolites to be measured in one run. We describe an improved, stable-isotope dilution UPLC–MS/MS HILIC method for the simultaneous quantification of ACh, betaine, choline, and DMG in several biological materials, including EDTA plasma, urine, amniotic fluid (AF), and cerebrospinal fluid (CSF). In addition, we utilized the new method for investigating concentrations of the metabolites in plasma and urine samples.

2. Materials and methods

2.1. Standards and chemicals

Acetylcholine iodide (Merck Chemicals, Darmstadt, Germany), betaine chloride, choline chloride, and *N,N*-dimethylglycine (Sigma–Aldrich, Munich, Germany) were used for preparation of standard solutions. d_9-Betaine chloride, d_9-choline chloride (Isotec, Sigma–Aldrich, Munich, Germany), d_9-acetylcholine chloride, and d_6-dimethylglycine HCl (CDN isotopes, Quebec, Canada) were used as internal standards. Other chemicals used were: ammonium formate, formic acid, acetonitrile (ULC/MS grade; Biosolve, Valkenswaard, The Netherlands), and ultrapure water (18.2 MΩ) from a Milli-Q water purification system (Millipore, Molsheim, France).

Stock solutions of ACh and d_9-ACh (each 1000 nmol/L) and betaine, d_9-betaine, choline, d_9-choline, DMG, and d_6-DMG (each 1000 μmol/L) were prepared in acetonitrile/H_2O (1:1; v/v). Aliquots of each stock solution were stored at −70 °C, used within 6 months, and thawed only once.

2.2. Subjects and sample collection

EDTA plasma samples from a total of 146 adults (34 males) were available. Serum samples were available from a subset of 74 females. The concentrations of choline and betaine were compared between serum and plasma samples from a subset of 74 females (mean age 35 years). Additionally, we investigated the effect of fasting and non-fasting conditions on the concentrations of the analytes. To this end, plasma samples from 28 subjects (mean age 52 years; 10 males) were available after overnight fasting and on the same day 4 h after lunch. Finally, we tested concentrations of ACh, betaine, choline, and DMG in plasma and urine samples from 44 older adults (>50 years; 24 males). For this purpose, fasting plasma and urine samples were collected on the same morning.

Blood was collected both in potassium EDTA-containing tubes and in tubes without anticoagulant. Blood samples were centrifuged within 30 min at 2000 × *g* and 4 °C for 10 min. The EDTA plasma and serum were then separated and immediately stored at -70 °C until analysis. Samples of cerebrospinal fluid (CSF) and amniotic fluid (AF) were collected for diagnostic purposes. Aliquots of these samples were immediately separated by centrifugation and stored at −70 °C. Pooled samples of EDTA plasma, CSF, AF, and urine were prepared, stored at −70 °C, and one aliquot was measured in each run. We used in-house prepared pools of EDTA plasma, CSF, AF, and urine for determination of detection and quantification limits of the assay. Pool of each biological material (3 mL) was dialyzed using a Spectra/Por Float-A-Lyzer CE (MWCO: 8,000 Dalton; Carl Roth GmbH, Karlsruhe, Germany) against 150 volumes of phosphate-buffered saline containing 4 mmol/L sodium EDTA (Sigma–Aldrich, Munich, Germany). Samples were collected for the determination of the detection and quantification limits when the concentrations of the analyte were in the ranges of 1–5 times of the instrumental detection limit (IDL). Urinary creatinine concentrations were determined by COBAS INTEGRA System (Roche Diagnostics, Mannheim, Germany). The study was approved by the local ethics committee and each participant signed an informed consent.

2.3. Sample preparation

Sample preparation was carried out on ice and vials were immediately sealed to avoid evaporation of acetonitrile at room temperature. Six calibrators of different concentrations were included in each batch of samples (Table 1). Quality control samples at two different concentrations (high and low) were included in each batch (Table 1). Calibrators and quality control samples were prepared from stock solutions in acetonitrile and processed as described below.

Plasma, serum, urine, CSF, and AF samples were thawed and centrifuged at 2000 × *g* for 5 min at 4 °C. The urine samples were diluted with H_2O (1:5) prior to analysis because of the high salt concentrations which can damage or shorten the life of the UPLC column and tubings. The clear sample, calibrator, and quality control (100 μL) were each added to a 1.5 mL tube containing 300 μL internal standard mix [5 nmol/L d_9-ACh and d_9-betaine, d_9-choline, d_6-DMG (each 10 μmol/L)] in acetonitrile. The addition of acetonitrile caused protein precipitation and the analytes were extracted by vigorous vortexing of the sample for 30 s. The samples were centrifuged for 5 min at 10,000 × *g* at room temperature; the super-

Table 1
Concentration of calibrators and quality control samples.

	ACh (nmol/L)	Betaine (μmol/L)	Choline (μmol/L)	DMG (μmol/L)
Calibration	0, 0.2, 0.4, 1, 5, 10, 40	0, 0.5, 1, 2.5, 12.5, 25, 100	0, 0.3, 0.6, 1.5, 7.5, 15, 60	0, 0.2, 0.4, 1, 5, 10, 40
Calibration for instrumental detection limit	0, 0.4, 0.8, 1.6, 3.2, 6.4	0, 0.2, 0.4, 0.8, 1.6, 3.2	0, 0.2, 0.4, 0.8, 1.6, 3.2	0, 0.2, 0.4, 0.8, 1.6, 3.2
Calibration for linearity range determination	0, 0.2, 0.4, 1, 10, 50, 150	0, 0.2, 0.4, 1, 10, 50, 150	0, 0.2, 0.4, 1, 10, 50, 150	0, 0.2, 0.4, 1, 10, 50, 150
High control	30	75	45	30
Low control	3	7.5	4.5	3

ACh: acetylcholine, DMG: dimethylglycine.

Table 2
Instrument parameters for LC–MS/MS analysis of ACh, betaine, choline, DMG, and their internal standards.

Analyte	Cone voltage (V)	Collision energy (eV)	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Retention time (min)
ACh	21.0	15.0	146.13	86.93	1.75
Betaine	31.0	17.0	118.08	59.22	3.20
Choline	34.0	16.0	104.08	60.05	2.19
DMG	21.0	12.0	104.08	58.18	3.00
d_9-ACh	22.0	14.0	154.92	86.86	1.75
d_9-betaine	26.0	18.0	127.15	68.16	3.20
d_9-choline	29.0	18.0	113.23	69.13	2.18
d_6-DMG	21.0	12.0	109.97	64.17	3.00

ACh: acetylcholine, DMG: dimethylglycine.

natant was transferred to glass vials. Sealed vials were either immediately measured or stored at −70 °C for no longer than one week.

2.4. UPLC–MS/MS conditions

UPLC–MS/MS analyses were carried out using an Acquity Ultra Performance LC system coupled to a MicroMass Quattro Premier XE tandem quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA). The samples were separated on an Acquity UPLC BEH HILIC column (100 mm × 2.1 mm (i.d.); 1.7 μm particle size) with an Acquity HILIC VanGuard pre-column (5 mm × 2.1 mm (i.d.); 1.7 μm particle size) and a 0.2 μm in-line filter (Waters Corporation). The column temperature was set to 30 °C. The flow rate was 0.6 mL/min. The solvents were 15 mmol/L ammonium formate (solvent A, pH 3.5) and acetonitrile (solvent B) with a gradient over the 6.5 min run time as follow: 0.0–3.5 min (12.5% A), 3.5–3.6 min (20% A), and 4.5–4.6 min (12.5% A). All gradient steps were linear. The sample injection volume was 1 μL.

The target analytes and their internal standards were identified by a triple quadrupole mass spectrometer (MicroMass Quattro Premier XE) using positive electrospray ionization mode. Source temperature was 150 °C, capillary voltage was 0.40 kV, desolvation gas (N_2) temperature was 350 °C at a flow rate of 700 L/h, and the cone gas (N_2) flow rate was 50 L/h. Collision gas (Ar) flow was maintained at 0.30 L/h. Inter-scan and inter-channel delay were set to 0.02 s, dwell times were 0.05 s. Individual cone and collision energy voltages, as well as multiple reaction monitoring (MRM) mass transitions are summarized in Table 2.

2.5. Data analyses

Data was acquired and processed using MassLynx V4.1 and QuanLynx software. Calibrators were used for the construction of a standard curve by plotting the response (y = area analyte/area internal standard) against the corresponding concentrations (c) of the calibrators. The slope (m) and the intercept (i) of the standard curve were used for calculating the concentration of the unknown sample (x) as $y = m \times x + i$.

Recovery (%) was calculated as: (Measured concentration/(Expected concentration + Concentration added)) × 100.

Root mean square error (RMSE) was calculated as follows:

$$\mathrm{RMSE} = \left[\frac{\sum_{j=1}^{n} E_j^2}{n-2}\right]^{\frac{1}{2}}$$

where n is the number of standards and E is the error associated with each measurement.

IDL was calculated as 3 × RMSE/m, and instrumental quantification limit (IQL) was calculated as 10 × RMSE/m. Method detection limit (MDL) was determined by $t_{99}s_{\mathrm{LLMV}}$ method, using the following equation:

$$\mathrm{MDL} = t_{99(n-1)} \times \mathrm{SD}$$

where $t_{99(n-1)}$ is the one-tailed t-statistic for $n-1$ observations at the 99% confidence level ($t_{99(n-1)}$ = 2.821 for 10 aliquots or 9 degrees of freedom) and SD is the standard deviation. Method quantification limit (MQL) = 3 × MDL.

Urinary excretion was calculated as the ratio of concentration of the analyte:creatinine concentration. Linear regression analysis was used to verify the linearity of the calibration curves. Results are shown as median (10–90th percentile) or mean (standard deviation; SD).

Paired t-test was used for comparison of sample material and fasting conditions on the analytes. Correlation analyses were performed by using Spearman-Rho test. Statistical analyses were performed using SPSS (Statistical Package for the Social Sciences, version 17.0).

3. Results

3.1. Chromatography and tandem mass spectrometry

Optimal MRM conditions for the quantification of the analytes and their stable-isotope labeled internal standards were obtained in the positive electrospray ionization mode of the Quattro Premier XE mass spectrometer. MRM chromatograms from quality control and urine pool samples are shown in Fig. 1. ACh and d_9-ACh coeluted at 1.79 and 1.78 min, choline and d_9-choline at 2.22 min, DMG and d_6-DMG at 3.03 and 3.00 min, and betaine and d_9-betaine at 3.14 and 3.16 min (Table 1). No peaks above the detection limit for ACh were found in plasma, serum, AF, and CSF samples. In urine, interfering peaks for ACh appeared approximately at 0.87 min, 2.56 min, 3.66 min, and 5.67 min. Because these peaks did not appear in calibration and quality control samples, they might be caused by sample matrix. Due to similar mass transitions, an additional choline peak (m/z 104.08 → 60.05) could be detected in the DMG trace (m/z 104.08 → 58.15) at 2.23 min. All interfering peaks were chromatographically separated from the compounds and were excluded from the data analyses. Compared to calibration and quality control samples, ACh showed minor ion suppression effects in pool samples, whereas there was a slight ion enhancement for choline, betaine, and DMG. Using stable-isotope labeled analogs of the analytes eliminated matrix effects.

3.2. Stability

Determining ACh concentration in biological samples requires the inhibition of the naturally occurring cholinesterases, for instance by using carbamates, Ca^+ chelators (e.g. EDTA), or organophosphate compounds, or by protein precipitation. EDTA plasma pool samples were measured immediately after collection and again after freezing over several months at −70 °C. Concentra-

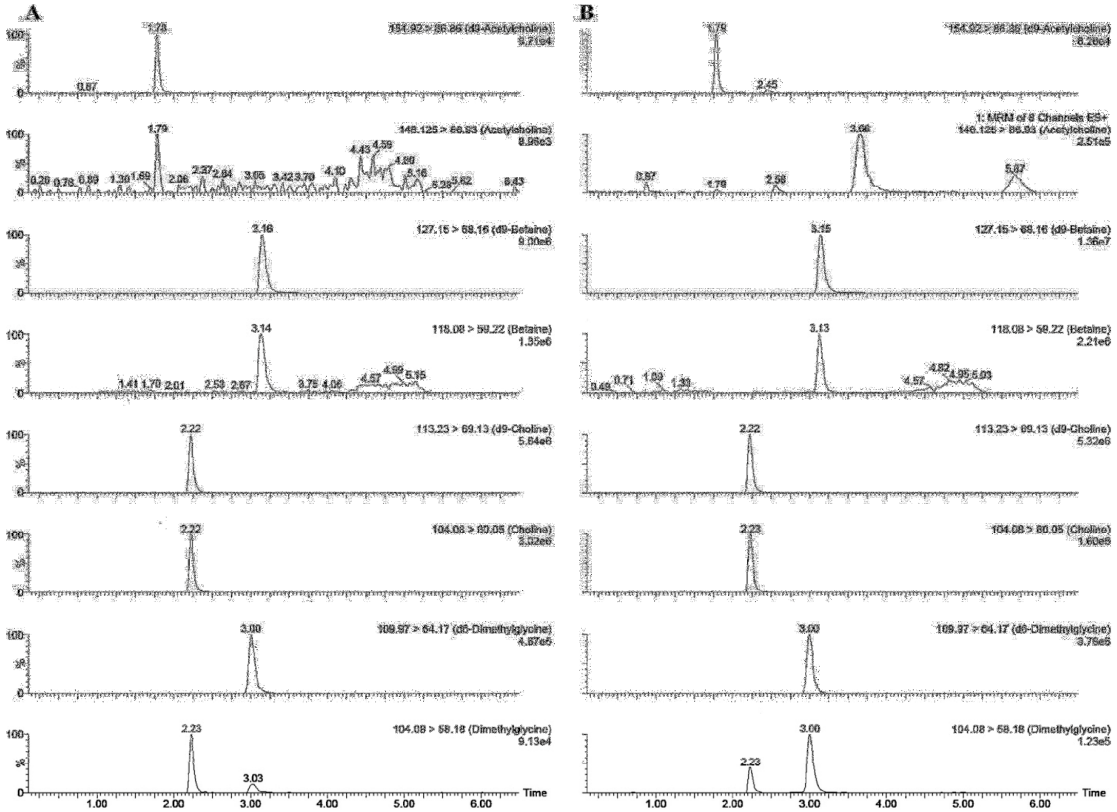

Fig. 1. MRM chromatograms of acetylcholine (ACh), betaine, choline, dimethylglycine (DMG), and their internal standards obtained by UPLC–MS/MS in (A) a quality control sample, containing 3 nmol/L ACh, 7.5 μmol/L betaine, 4.5 μmol/L choline, and 3 μmol/L DMG, and (B) one urine pool sample (1:5 diluted with H_2O) containing 3.62 nmol/L ACh, 7.63 μmol/L betaine, 2.45 μmol/L choline, and 3.03 μmol/L DMG. *m/z* transitions and peak intensities are shown in the upper right.

tions of betaine, choline, and DMG in frozen CSF, AF, and plasma samples were stable for at least 6 months. Concentrations of ACh, betaine, choline, and DMG were also stable in frozen urine pool for at least 6 months. No remarkable changes in concentrations of betaine, choline, or DMG were observed after at least three freeze/thaw cycles (data not shown).

3.3. Linearity and sensitivity

The assay was linear over the physiological ranges (Table 1). Concentrations of the standard solutions correlated strongly to the peak areas. The coefficients of linear regression for 5 independent experiments were: $r^2 > 0.999$ (mean (SD) linear equation $y = 0.0318$ (0.0028) $x + 0.0239$ (0.0075)) for ACh, $r^2 > 0.999$ ($y = 0.0189$ (0.0023) $x + 0.0030$ (0.0038)) for betaine, $r^2 > 0.999$ ($y = 0.1232$ (0.0084) $x + 0.0274$ (0.0065)) for choline, and $r^2 > 0.999$ ($y = 0.0095$ (0.0016) $x + 0.0035$ (0.0022)) for DMG (Supplemental Data Fig. 1).

The limits of detection (LOD) and quantification (LOQ) were calculated in a two-step approach, proposed by the U.S. Environmental Protection Agency. This consists of (1) the determination of the IDL and IQL (defined as the smallest amount of an analyte that can be reliably detected/quantified from the background on an instrument), and using these values to estimate MDL and MQL and (2) the calculation of the MDL and MQL (defined as the smallest amount of an analyte that can be reliably detected/quantified from the background for a particular matrix) using 10 aliquots of the dialyzed pool sample [25]. IDL and IQL were estimated by calculating the RMSE of five 5-point calibration curves (concentrations see Table 1) [24]. Mean (SD) IDL was 0.32 (0.24) nmol/L for ACh, 0.18 (0.08) μmol/L for betaine, 0.09 (0.02) μmol/L for choline, and 0.20 (0.12) μmol/L for DMG. MDLs were estimated by first preparing EDTA plasma, CSF, AF, and urine (1:5 diluted with H_2O) samples with concentrations between 1–5 times the IDL. In addition, 10 blank calibrators containing internal standard were prepared. Mean concentration of blank calibrators was subtracted from each sample concentration. Mean (SD) concentrations for plasma and urine samples and their LOD are presented in Table 3. LODs [mean (SD) concentrations] for AF were 0.25 μmol/L [0.61 (0.09) μmol/L] for betaine, 0.03 μmol/L [0.29 (0.01) μmol/L] for choline, and 0.07 μmol/L [0.42 (0.02) μmol/L] for DMG and LODs [mean (SD) concentrations] for CSF were 0.11 μmol/L [0.19 (0.04) μmol/L] for betaine, 0.04 μmol/L [0.33 (0.01) μmol/L] for choline, and 0.13 μmol/L [0.43 (0.05) μmol/L] for DMG.

3.4. Recovery and precision

Recovery experiments were performed by spiking the urine (1:5 diluted) or plasma pools with two different concentrations of the analytes in the physiological range (Table 3). Mean recoveries in the diluted urine pool samples were 102.6% for ACh, 103.9% for

Table 3
Limit of detection (LOD) and recovery of ACh, betaine, choline, and DMG.

Analyte	LOD		Recovery			
	Mean (SD)[a]	LOD	Measured mean (SD)	Added	Measured in spiked samples mean (SD)[a]	Recovery[a] (SD)(%)
Analyte concentrations in diluted urine pool[c]						
ACh (nmol/L)	0.56 (0.12)	0.35	3.97 (0.19)	20.0	23.58 (2.27)	98.4 (9.5)
				10.0	14.93 (0.46)	106.9 (3.3)
Betaine (μmol/L)	0.90 (0.12)	0.34	6.40 (0.36)	20.0	27.03 (1.11)	102.4 (4.2)
				10.0	17.29 (0.15)	105.4 (0.9)
Choline (μmol/L)	0.37 (0.06)	0.16	2.50 (0.10)	20.0	22.19 (0.18)	98.6 (0.8)
				10.0	12.48 (0.30)	99.8 (2.4)
DMG (μmol/L)	0.45 (0.04)	0.12	2.19 (0.15)	4.0	5.87 (0.38)	94.8 (6.1)
				2.0	4.52 (0.27)	107.9 (6.4)
Analyte concentrations in plasma pool						
Betaine (μmol/L)	0.16 (0.06)	0.18	32.55 (0.92)	20.0	49.72 (1.27)	94.6 (2.4)
				10.0	38.92 (1.81)	91.5 (4.3)
Choline (μmol/L)	0.20 (0.04)	0.13	12.59 (0.66)	20.0	33.19 (1.43)	101.8 (4.4)
				10.0	22.35 (0.94)	98.9 (4.2)
DMG (μmol/L)	0.40 (0.04)	0.13	3.83 (0.38)	4.0	8.08 (0.80)	103.2 (10.2)
				2.0	5.93 (0.66)	101.8 (11.3)

ACh: acetylcholine, DMG: dimethylglycine, SD: standard deviation. For LOD determination, pool samples were dialyzed prior experiment until they reached concentrations of 1–5 times the instrumental detection limit (IDL).
[a] $n = 10$ samples.
[b] Mean of 3 independent experiments.
[c] Urine samples were 1:5 diluted with H_2O prior experiments.

betaine, 99.2% for choline, and 101.4% for DMG. Mean recoveries in the plasma pool samples were: 93.0% for betaine, 100.4% for choline, and 102.5% for DMG.

Precision of the method was determined by quantification of ACh, betaine, choline, and DMG in the in-house prepared pools of EDTA plasma, CSF, AF, and urine, as well as quality control samples at two defined concentrations (low and high). Coefficients of variation (CVs) for different sample matrixes are presented in Table 4. Intraassay CVs ($n = 10$) ranged between 2.59–3.36% for ACh, 1.56–5.15% for betaine, 1.84–4.90% for choline, and 4.48–9.27% for DMG.

Interassay CVs for different biological samples ($n = 10$ each) ranged between 2.18–4.19% for ACh, 2.65–6.50% for betaine, 3.61–8.76% for choline, and 4.88–12.44% for DMG. The highest CVs for DMG in CSF pool of 12.44% were probably related to the low concentrations of this analyte there. A summary and a comparison with available methods are shown in Table 5.

3.5. ACh, betaine, choline, and DMG concentrations in blood and urine

Concentrations of choline-related metabolites were measured in plasma, serum, and urine samples. In 74 female subjects, plasma and serum concentrations of betaine were significantly correlated (Spearman correlation coefficient $r = 0.792$, $p < 0.001$) (AppendixB-Supplemental Data Fig. 2). The mean (SD) betaine concentration was 11.65 (3.06) μmol/L in plasma and 12.75 (3.36) μmol/L in serum ($p < 0.001$; paired t-test). Mean (SD) choline concentration in serum (13.54 (5.86) μmol/L) was significantly higher than the plasma concentration (6.68 (1.51) μmol/L) ($p < 0.001$), probably reflecting increased choline release in serum from phospholipids by means of cholinesterases. Because EDTA is a calcium chelator, the activity of cholinesterase might be inhibited in EDTA plasma.

The median (10–90th percentile) plasma betaine concentration of 28 subjects in fasting condition was 46.98 (30.95–63.44) μmol/L, which was not significantly different from that in non-fasting condition [42.67 (24.88–74.61) μmol/L ($p = 0.414$; paired t-test)]. We observed significantly higher plasma choline concentrations under non-fasting compared to fasting conditions [median 14.05 (10.61–18.90) μmol/L vs. 7.58 (4.42–9.78) μmol/L ($p < 0.001$; paired t-test)]. This might be related to enhanced homocysteine remethylation in the liver in non-fasting conditions. The fasting and non-fasting concentrations of choline were strongly correlated ($r = 0.779$, $p < 0.001$) (AppendixBSupplemental Data Fig. 3).

From 44 older adults (age > 50 years) the ranges of choline-related analytes as well as gender-related analyte concentrations were assessed (Table 6). The concentrations of betaine in plasma and those in urine showed wide inter-individual variations. Males showed significantly higher concentrations of plasma DMG (44.4%) than females which might be explained by a larger liver size in males compared to females. Females showed higher urinary excretion of choline (Table 6). In females, the concentration of plasma betaine correlated significantly to urinary excretion of betaine ($r = 0.495$, $p = 0.027$) and that of choline ($r = 0.502$, $p = 0.024$). This was not the case in male subjects (AppendixBSupplemental Data Table 1). In male subjects, a significant positive correlation of plasma DMG and urinary DMG excretion ($r = 0.418$, $p = 0.042$) was found. In addition, the concentration of plasma choline and urinary ACh:creatinine ratio showed a strong direct correlation in female subjects ($r = 0.621$, $p = 0.003$), which was lower in male subjects ($r = 0.419$, $p = 0.041$). Significant positive correlations between betaine and betaine excretions in urine, as well as choline excretion were observed in male and female subjects. Positive correlations between urinary betaine and DMG excretion were found in males ($r = 0.768$, $p < 0.001$) and females ($r = 0.543$, $p = 0.013$). In male subjects, significant correlations between urinary choline and betaine excretion, as well as DMG excretion were found. In the total group ($n = 44$) significant correlations of plasma betaine and DMG ($r = 0.380$, $p = 0.011$) were found. Plasma choline correlated significantly with urinary ACh excretion ($r = 0.455$, $p = 0.002$). Positive correlations between urinary betaine and choline excretion ($r = 0.587$, $p < 0.001$) and DMG excretion ($r = 0.700$, $p < 0.001$), as well as urinary choline and DMG excretion ($r = 0.514$, $p < 0.001$) were found.

4. Discussion

Choline is utilized for synthesis of ACh or phosphatidylcholine. Choline metabolism has been related to Hcy accumulation, fatty liver, renal and corneal disorders [1,6], or cognitive dysfunction

Table 4
Precision of ACh, betaine, choline, and DMG in quality control and pool samples ($n = 10$).

Analyte	High control		Low control		EDTA plasma pool		Urine pool		AF pool		CSF pool	
	Mean (SD)	CV (%)	Mean (SD)	CV (%)	Mean (SD)	CV (%)	Mean (SD)	CV (%)	Mean (SD)	CV (%)	Mean (SD)	CV (%)
Intraassay CV												
ACh (nmol/L)	30.61 (0.79)	2.59	3.22 (0.11)	3.34	ND	–	16.93 (0.57)	3.36	ND	–	ND	–
Betaine (μmol/L)	75.32 (1.17)	1.56	7.72 (0.16)	2.02	34.27 (0.69)	2.01	34.76 (0.82)	2.35	39.97 (2.06)	5.15	6.11 (0.15)	2.52
Choline (μmol/L)	44.22 (1.26)	2.86	4.60 (0.14)	3.04	14.02 (0.34)	2.42	12.23 (0.39)	3.23	26.28 (1.29)	4.90	4.65 (0.09)	1.84
DMG (μmol/L)	30.93 (1.39)	4.48	3.24 (0.17)	5.35	3.91 (0.27)	6.97	15.43 (1.17)	7.61	4.57 (0.42)	9.27	0.22 (0.01)	6.58
Interassay CV												
ACh (nmol/L)	30.21 (0.88)	2.90	3.08 (0.13)	4.19	ND	–	17.95 (0.39)	2.18	ND	–	ND	–
Betaine (μmol/L)	75.50 (2.00)	2.65	7.68 (0.26)	3.40	33.91 (1.95)	5.75	35.16 (1.70)	4.84	38.38 (2.09)	5.43	6.30 (0.41)	6.50
Choline (μmol/L)	45.44 (1.69)	3.72	4.65 (0.17)	3.61	13.77 (1.15)	8.37	13.62 (1.07)	7.85	25.00 (1.53)	6.11	4.89 (0.43)	8.76
DMG (μmol/L)	30.16 (1.57)	5.20	3.05 (0.15)	4.88	4.20 (0.40)	9.59	13.90 (1.17)	8.42	4.58 (0.40)	8.84	0.25 (0.03)	12.44

ACh: acetylcholine, AF: amniotic fluid, CSF: cerebrospinal fluid, CV: coefficient of variation, DMG: dimethylglycine, ND: not detected, SD: standard deviation.
High control samples contained 30 nmol/L ACh, 75 μmol/L betaine, 45 μmol/L choline, and 30 μmol/L DMG.
Low control samples contained 3 nmol/L ACh, 7.5 μmol/L betaine, 4.5 μmol/L choline, and 3 μmol/L DMG. Urine pool samples were diluted 1:5 with H_2O prior experiment; shown results have been multiplied by the dilution factor.

Table 5
Comparison of LC–MS/MS methods.

Analyte	Material	Run time; sample preparation	LOD (μmol/L)	Recovery	Intraassay CV in EDTA plasma	Linearity range	Reference
ACh Betaine Choline DMG	EDTA plasma Serum Urine AF CSF	6.5 min; Protein precipitation	ACh: 0.35 nmol/L Betaine: 0.11–0.34 Choline: 0.03–0.16 DMG: 0.07–0.13 (Various materials)	93.0–102.5% (EDTA plasma) 99.2–103.9% (urine)	Betaine: 2.01% Choline: 2.42% DMG: 6.97%	ACh: 0.2–150 nmol/L Betaine, choline, DMG: 0.2–150 μmol/L	Present study
Betaine Choline DMG	EDTA plasma Serum	6 min; Protein precipitation	Betaine: 0.3 Choline: 0.1 DMG: 0.2 (EDTA plasma)	90.2–104.5% (EDTA plasma[a])	Betaine: 2.1% Choline: 4.3% DMG: 4.9%	LOD–400 μmol/L	[20]
ACh Betaine Choline[b]	Plasma Tissues Foods	>30 min; Complex sample preparation	1–40 pmol (pure standards)	91.6–104% (rat liver)	ACh: 2.1% Betaine: 2.4% Choline: 8.5% (rat liver)	~10–200 nmol	[22]
Choline	EDTA plasma Whole blood	5 min; Protein precipitation	–	–	Choline: 4.7%	0.1–100 μmol/L	[21]

ACh: acetylcholine, AF: amniotic fluid, CSF: cerebrospinal fluid, CV: coefficient of variation, DMG: dimethylglycine, LOD: limit of detection.
[a] Mean of low and high concentration.
[b] In addition, following choline metabolites were analyzed: phosphocholine, glycerophosphocholine, cytidine diphosphocholine, phosphatidylcholine, and sphingomyelin.

Table 6
Median (10–90th percentiles) concentrations in fasting older subjects.

	Females	Males	p^a
n	20	24	
Age, years	59 (50–73)	66 (57–75)	0.062
EDTA plasma			
Betaine (μmol/L)	28.99 (18.67–37.49)	29.71 (25.21–46.78)	0.150
Choline (μmol/L)	8.96 (6.99–11.74)	9.19 (7.43–12.35)	0.465
DMG (μmol/L)	2.14 (1.47–3.65)	3.09 (1.97–4.26)	0.001
Urine			
ACh (μmol/mol creatinine)	3.92 (2.57–5.81)	3.44 (1.78–6.57)	0.689
Betaine (mmol/mol creatinine)	5.57 (3.28–11.42)	4.06 (2.40–25.30)	0.322
Choline (mmol/mol creatinine)	2.58 (1.14 4.04)	1.72 (1.01 4.70)	0.027
DMG (mmol/mol creatinine)	2.26 (1.30–4.65)	2.07 (0.57–7.02)	0.494

ACh: acetylcholine, DMG: dimethylglycine.
[a] *p*-values according to Mann–Whitney test.

[25]. Cholinesterase and inhibitors of this enzyme have been used to maintain concentrations of ACh in dementia [16]. Therefore, quantification of choline metabolites seems promising for epidemiological studies.

We described a new method for the simultaneous measurement of ACh, betaine, choline, and DMG in various biological fluids using stable-isotope dilution UPLC–MS/MS. The linearity over a broad concentration range, the short time for sample preparation (2 h/48 samples) and measurement (6.5 min/sample), and the small sample volume (100 μL) required make the method optimal for use in large-scale clinical studies. Coefficients of variation were <10% in intra- and interassay experiments. A higher CV for DMG assay in CSF (12.44%) is probably related to the low CSF concentrations of DMG (mean 0.25 μmol/L; LOQ = 0.26 μmol/L).

Our results on the performance of the betaine, choline, and DMG assay in plasma are comparable to that by Holm et al. (Table 5) [20]. However, our method also enables the measurement of ACh and it can be applied to different biological samples like urine, AF, and CSF. The sample preparation procedure and the assay time is more convenient than that of Koc et al. (Table 5) [22]. The method described by Yue et al. enables the measurement of only choline in plasma and whole blood [21].

In line with an earlier report [20] concentrations of betaine and choline were significantly different between serum and plasma. Moreover, the fasting concentrations of choline were lower than the non-fasting ones (AppendixBSupplemental Data Fig. 3) [20,26,27], but in contrast to an earlier study [20] plasma betaine concentrations did not differ according to fasting status. Different dietary intakes between the two populations might explain the effect of fasting status on betaine concentration [28,29]. Additionally, differences in choline metabolites according to gender are consistent with earlier studies [7,30–32]. The urine choline concentrations found in our study [median (10–90th) 22.41 (10.19–49.69) μmol/L] are in agreement with earlier results [33,34]. Urinary excretion of ACh correlated stronger to plasma choline in females than in males suggesting that plasma choline might be related to a higher production of ACh. The regulation (possibly hormonal) and the biological significance of these results have to be established.

In conclusion, we developed a fast, precise, and reliable method for the quantification of choline-related metabolites in various sample materials. Our method using UPLC–MS/MS provides high efficiency separation and enables for large-scale clinical studies. In comparison with other LC–MS/MS methods our method allows the simultaneous quantification of ACh, betaine, choline, and DMG.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.10.016.

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Appendix E: Primer for the MTHFR C677T genotype determination

Primer	Sequence	Company
MTHFR 677 CT forward-primer	TTG AGG CTG ACC TGA AGC AC	Invitrogen GmbH, Karlsruhe, Germany
MTHFR 677 CT reverse-primer	Biotin-5GTG ATG CCC ATG TCG GTG	Invitrogen GmbH, Karlsruhe, Germany
MTHFR 677 CT sequencing-primer	GGT GTC TGC GGG AG	Invitrogen GmbH, Karlsruhe, Germany

Appendix F: Reference ranges for laboratory parameters

Parameter	Sample material	Method of determination	Reference range
ALAT	Lithium-heparin plasma	IFCC (37°C)	males: 10 – 50 U/L females: 10 – 35 U/L
Blood count	EDTA whole blood	SYSMEX SF 3000 or XE 5000	males: Hb: 14.0 – 18.0 g/dL Hct: 41 – 53% females: Hb: 12.0 – 16.0 g/dL Hct: 36 – 46%
CRP	Lithium-heparin plasma	Immunoturbidimetric determination	< 5 mg/L
Cholesterol, HDL, triglycerides	Lithium-heparin plasma	Colorimetric enzyme assay	cholesterol: < 200 mg/dL HDL: males: 35 – 55 mg/dL females: 45 – 65 mg/dL triglycerides: < 200 mg/dL
Creatinine	Lithium-heparin plasma	Jaffé determination (kinetic colorimetric assay)	males: 0.7 – 1.2 mg/dL (61.9 – 106.1 µmol/L[a]) females: 0.5 – 0.9 mg/dL (44.2 – 79.6 µmol/L)
Glucose	Lithium-heparin plasma	UV test (hexokinase/G6P-DH)	60 – 100 mg/dL

[a]: Conversion factor creatinine: 88.4

Reference values were retrieved October 11, 2010 from: http://www.uniklinikum-saarland.de/facilities/departments-and-institutes/zentrallabor/referenzwerte-verfahrensliste/.

Conversion factors were retrieved October 11, 2010 from: http://jama.ama-assn.org/content/vol295/issue1/images/data/103/DC6/JAMA_auinst_si.dtl.

Appendix G: Study information sheets long-term supplementation study

Informationen für die Hausärztin bzw. den Hausarzt

KnoViB: Knochenstoffwechsel und Vitamin B

Sehr geehrte Frau Kollegin, sehr geehrter Herr Kollege,

Ihre Patientin bzw. Ihr Patient _____ nimmt an einer von der Ethikkommission genehmigten prospektiven, randomisierten, doppelblinden und placebokontrollierten Studie teil. Diese wird von der Universitätsklinik Homburg in Zusammenarbeit mit der Geriatrischen Rehaklinik St. Ingbert durchgeführt.

Ziel:

Es ist das Ziel, die in vitro beobachteten positiven Effekte von Vitamin B auf den Knochenstoffwechsel auch prospektiv in vivo bei älteren Menschen nachzuweisen. Dies könnte Auswirkungen auf die Ernährungsempfehlungen einer großen Bevölkerungsschicht haben.

Ablauf:

Jeder Patient erhält als Basistherapie Vitamin D und Calcium. Zusätzlich erhalten die Patienten entweder Vitamin B_6, Folsäure (=B_9) und Vitamin B_{12} oder Placebo. Die Verum- und Placebokapseln unterscheiden sich äußerlich nicht. Die Vitamin B – Dosen entsprechen denen, die zur Vorbeugung und Behandlung von Vitaminmangelerscheinungen empfohlen sind. Jeder Patient nimmt ein Jahr lang täglich morgens eine grüne, mittags eine weiße und abends eine braune Kapsel ein. Den Medikamentenvorrat erhalten die Patienten von uns. Zu Beginn der Untersuchung und am Ende wird von uns Blut und Urin abgenommen, um darin verschiedene Marker des Vitamin- und Knochenstoffwechsels zu messen. Wir werden in regelmäßigen Abständen telefonisch mit den Patienten oder den Angehörigen in Kontakt bleiben.

Abbruchkriterien:

Selbstverständlich hat jede medizinisch erforderliche Behandlung Vorrang vor der Studie. Deshalb ergeben sich folgende Abbruchkriterien:

Medikamente:	Corticosteroide, Methotrexat, spezifische osteologische Therapie, zusätzliche Therapie mit Vitamin B_{12}, Folsäure, Vitamin B_6, Vitamin D oder Calcium.
Akute Erkrankungen:	Herzinfarkt, Krebserkrankung, Apoplex oder ein operativer Eingriff führen ebenfalls zum Studienabbruch.

Ihre Aufgabe:

Außer den Abbruchkriterien brauchen Sie nichts zu beachten. Sollten diese eintreten, bitten wir um eine kurze Benachrichtigung.

Wenn Sie weitere Fragen haben oder Informationen wünschen, können Sie sich gerne mit uns in Verbindung setzen:
Frau Prof. Dr. Obeid: Tel. 06841 - 1630711, CA Dr. Eckert: s.o.

Vielen Dank

Mit freundlichen kollegialen Grüßen

Dr. Eckert

Das Kreiskrankenhaus St. Ingbert

Gesundheitspark 1
66386 St. Ingbert
Telefon: 0 68 94 – 108 451
Telefax: 0 68 94 – 108 452
E-mail: geriatrie@kkh-geriatrie-igb.de
Internet: www.kkh-geriatrie-igb.de

GERIATRISCHE REHAKLINIK

Zertifiziert nach DIN EN ISO 9001
Gem. Kr. nach § 4 Abs. 5 MB/KK

CA Dr. Rudolf Eckert

Ihr Ansprechpartner/-in:
Frau Ley
Unsere Zeichen: Eck /
Telefon: 0 68 94 / 108 - 451
Datum: August 2009

Wir behandeln ältere multimorbide Patienten, deren Selbständigkeit eingeschränkt ist:

fachärztlich:
- **Innere Medizin**
- **Physikalische und Rehabilitative Medizin**
- **Allgemeinmedizin**
- **konsiliarisch u. a.**
  Chirurgie
  Unfallchirurgie
  Kardiologie
  Neurologie

rehabilitativ:
- **Ergotherapie**
- **Logopädie**
- **Pflegedienst**
- **Physiotherapie**
- **Physikalische Therapie**
- **Psychologie**
- **Sozialdienst**

Geschäftsführer:
Wolfgang Steil
Dipl.-Kfm. Frank F. Banowitz

Aufsichtsratsvorsitzender:
Clemens Lindemann

Amtsgericht Saarbrücken:
HRB 32799

Patienteninformation und Einwilligungserklärung

Untersuchung möglicher Auswirkungen der Nahrungsergänzung mit Vitamin B_6, Folsäure (= Vitamin B_9) und Vitamin B_{12} auf den Knochenstoffwechsel zusätzlich zur Therapie mit Vitamin D und Kalzium

Sehr geehrte Patientin, sehr geehrter Patient,
wir möchten Sie bitten, an einer Studie der Universitätsklinik Homburg in Zusammenarbeit mit der Geriatrischen Rehaklinik St. Ingbert teilzunehmen, mit der die Auswirkungen von Vitaminpräparaten auf den Knochenstoffwechsel untersucht werden.

Gründe, Ablauf und Ziel der Untersuchung
Mit zunehmendem Alter wird der Knochen immer stärker entkalkt. Dies führt schließlich zu Osteoporose. Wir wollen untersuchen, ob die Vitamine B_6, Folsäure (= Vitamin B_9) und Vitamin B_{12}, die zusätzlich zu der üblichen Therapie mit Vitamin D und Kalzium eingenommen werden, eine positive Wirkung auf den Knochenaufbau haben.
Der Knochenstoffwechsel reagiert sehr langsam. Deshalb dauert die Therapie ein Jahr. Sie nehmen in dieser Zeit täglich morgens eine grüne, mittags eine weiße und abends eine braune Kapsel ein, die Vitamin D und Kalzium (mit schon nachgewiesener guter Wirkung auf den Knochen) und ggf. zusätzlich die oben genannten B-Vitamine ein. Zu Beginn der Untersuchung und nach einem Jahr wird der Urin untersucht und es wird Ihnen Blut abgenommen, um darin die Konzentrationen der Vitamine und die der Marker des Knochenstoffwechsels zu messen.
Die Einnahme der niedrig dosierten Vitaminpräparate ist unbedenklich, da die Dosen offiziell zur Behandlung und Vorbeugung von Vitaminmangelerscheinungen empfohlen sind und zuvor Gegenindikationen ausgeschlossen wurden. Sie werden alle 3 Monate telefonisch nach Ihrem Befinden gefragt und erhalten in diesem Intervall von uns kostenlos die nächsten Kapseln. Die Blutentnahme aus einer Armvene erfolgt mit den im Krankenhaus üblichen Nadeln. Die damit verbundenen Risiken wie z. B. Bluterguss oder Verletzung anderer Gewebe sind minimal.
Durch die Studie haben Sie praktisch keine zusätzlichen Belastungen. Sie sollten lediglich darauf achten, dass Sie keine anderen Vitamine der B – Gruppe und nicht zusätzlich Vitamin D und Kalzium einnehmen. Wenn die Einnahme von bestimmten Medikamenten, die den Knochenaufbau oder den Knochenabbau beeinflussen, während des Studienzeitraumes notwendig wird, ist die weitere Teilnahme an dieser Untersuchung nicht möglich. Ihr Hausarzt ist darüber informiert.
Diese Studie kann einen Beitrag zum effektiven und nebenwirkungsarmen Vorbeugen oder zur Therapie mit Vitaminen liefern, um der Knochenentkalkung entgegenzuwirken. Ihre Teilnahme hat damit Bedeutung für die Weiterentwicklung der medizinischen Therapie. Zusätzlich sparen Sie die Kosten der ohnehin sinnvollen Vitaminergänzung.

Einwilligungserklärung
Die Studienteilnahme ist freiwillig. Sie können jederzeit Ihre Einwilligung widerrufen und die in der Studie verwendete Blutprobe von der Untersuchung zurückziehen. Versicherungsschutz besteht im Rahmen einer allgemeinen Patientenversicherung der Geriatrischen Rehaklinik St. Ingbert.

Datenschutz
1) Ich erkläre mich damit einverstanden, dass die im Rahmen dieser klinischen Prüfung erhobene Daten, insbesondere Angaben über meine Gesundheit, in Papierform und auf elektronischen Datenträgern in der geriatrischen Rehaklinik St. Ingbert aufgezeichnet werden. Soweit erforderlich, dürfen die erhobenen Daten pseudoanonymisiert (verschlüsselt) weitergegeben werden.

2) Außerdem erkläre ich mich damit einverstanden, dass autorisierte und zur Verschwiegenheit verpflichtete Beauftragte die zuständigen inländischen und ausländischen Überwachungsbehörden in meine beim Prüfarzt vorhandenen personenbezogenen Daten, insbesondere meine Gesundheitsdaten, Einsicht nehmen, soweit dies für die Überprüfung der ordnungsgemäßen Durchführung der Studie notwendig ist. Für diese Maßnahme entbinde ich den Prüfarzt von der ärztlichen Schweigepflicht.

3) Die Einwilligung zur Erhebung und Verarbeitung meiner personenbezogenen Daten, insbesondere der Angaben über meine Gesundheit, ist unwiderruflich. Ich bin bereits darüber aufgeklärt worden, dass ich jederzeit die Teilnahme an der klinischen Prüfung beenden kann. Im Fall eines solchen Widerrufs meiner Einwilligung, an der Studie teilzunehmen, erkläre ich mich damit einverstanden, dass die bis zu diesem Zeitpunkt gespeicherten Daten ohne Namensnennung weiterhin verwendet werden dürfen, soweit dies erforderlich ist.

4) Ich erkläre mich damit einverstanden, dass meine Daten nach Beendigung oder Abbruch der Prüfung mindestens zehn Jahre aufbewahrt werden, wie es die Vorschriften über die klinische Prüfung von Arzneimitteln bestimmen. Danach werden meine personenbezogenen Daten gelöscht, soweit nicht gesetzliche, satzungsmäßige oder vertragliche Aufbewahrungsfristen entgegenstehen.

Einwilligungserklärung zu Studienteilnahme und Datenschutz

Mit Ihrer Unterschrift erklären Sie, dass Sie diese Probandeninformation gelesen und verstanden haben und freiwillig zur Teilnahme an der Untersuchung bereit sind.

Ferner stimmen Sie mit Ihrer Unterschrift der Weitergabe der während der Studie erhobenen Daten in anonymisierter Form an autorisierte Dritte zu.

Sie haben das Recht, jederzeit die Teilnahme an der Untersuchung ohne Nennung von Gründen zu widerrufen.

_____	_____	_____
Ort, Datum	**Name Patient**	**Unterschrift Patient**

_____
aufklärender Arzt

Beeinflussung des Knochenstoffwechsels durch Vitamin B (KnoViB)

Untersuchung möglicher Auswirkungen einer Vitamin B_6-, Folat- und Vitamin B_{12} - Supplementation zusätzlich zu Vitamin D und Calcium auf den Knochenstoffwechsel bei älteren Menschen (randomisiert, doppelblind)

Randomisierungsbogen

1 Im Zentrallabor UKS zu erledigen:

Sr.: Aktuelles Datum: ☐☐.☐☐.20☐☐

Sr.: Interne Patienten-Nr.: ☐☐☐

Sr.: Initialen Patient: Vorname, Name: ☐☐

Sr.: Geschlecht: ☐ männlich ☐ weiblich

Sr.: Geburtsdatum: ☐☐.☐☐.19☐☐

CA: Handzeichen Zentrallabor (Obeid): _____

Sek: Fax an Biometrie: 06841 – 16 22062
(Bei Rückfragen: Tel. 06841 – 16 22060)

2 In der Biometrie zu erledigen:

Zuordnung Therapiegruppe: ☐ A ☐ B

Patienten-Nr. in der Studie: ☐☐☐

Handzeichen Biometrie: _____

Fax an Zentrallabor: 06841 – 16 30703
(Bei Rückfragen: Tel. 06841 – 16 30711)

3 Therapiebeginn:

CA: Info an Station und Therapiebeginn: ☐☐.☐☐.20☐☐

CA: Handzeichen Zentrallabor (Obeid): _____

Untersuchung über die Beeinflussung des Knochenstoffwechsels durch Vitamin B (KnoViB)

Telefoncheckliste

(s. R. = siehe Bemerkungen auf der Rückseite diese Blattes)

Datum: _____

Guten Tag Frau/Herr _____!

Mein Name ist _____ von der Universitätsklinik Homburg. Sie / Ihre Angehörige nehmen an einer Untersuchung über den Einfluss verschiedener Vitamine auf den Knochenstoffwechsel teil. Die Untersuchung wurde in der geriatrischen Rehaklinik St. Ingbert begonnen. Jetzt bin ich für Sie zuständig. Haben Sie einen Moment Zeit zur Beantwortung einiger Fragen? (Wenn nein: Wann kann ich Sie wieder anrufen (Tag, Uhrzeit)?

1. Wie geht es Ihnen? ☐ s. R._____
2. Haben Sie außergewöhnliche gesundheitliche Beschwerden? ☐ s. R._____
3. Wurden Sie in letzter Zeit operiert? ☐ s. R. _____
4. Waren Sie in letzter Zeit im Krankenhaus? ☐ s. R._____
5. Nehmen Sie noch regelmäßig die Vitamintabletten ein? ☐ s. R._____
6. Wann nehmen Sie die Tabletten ein? ☐ s. R._____
7. Wie viele Vitamintabletten haben Sie noch? ☐ s. R._____
8. Wir würden Ihnen gerne den Bedarf für die nächsten 3 Monate per Post schicken! ☐ s. R.
   Haben Sie vom Arzt neue Medikamente oder Vitamine bekommen? ☐ s. R._____
9. Wenn ja, welche? ☐ s. R._____
10. Haben Sie Ihren Hausarzt gewechselt oder sind Sie noch bei Dr._____?
    ☐ s. R._____

Das sind schon alle meine Fragen. Vielen Dank für die Auskunft, Frau/Herr _____! Ich wünsche Ihnen (auch weiterhin) alles Gute und werde mich in 3 Monaten wieder telefonisch bei Ihnen melden. Sollten Sie noch weitere Fragen haben, können Sie mich gerne anrufen. Meine Telefonnummer ist _____.

Auf Wiederhören!