Rheinische Friedrich-Wilhelms-Universität Bonn
Institut für Ernährungs- und Lebensmittelwissenschaften (IEL)
Fachgebiet Humanernährung
Leiter: Prof. Dr. Peter Stehle

Folate, vitamin B_6, and vitamin B_{12} status in European adolescents – associations with homocysteine as well as communicable and non-communicable factors

INAUGURAL-DISSERTATION
zur Erlangung des Grades

Doktor der Ernährungs- und Haushaltswissenschaft
(Dr.oec.troph.)

der
Hohen Landwirtschaftlichen Fakultät
der
Rheinischen Friedrich-Wilhelms-Universität zu Bonn

vorgelegt am 1.10.2009
von
Jasmin Al-Tahan

aus Dinslaken

Referent:	Prof. Dr. Peter Stehle
Korreferenten:	Prof. Dr. Marcela Gonzalez-Gross
	PD Dr. Reinhild Prinz-Langenohl
Tag der mündlichen Prüfung:	14. Juni 2010
Erscheinungsjahr:	2010

Meinen Eltern

Zusammenfassung

Die Adoleszenz beinhaltet wichtige psychologische, soziale und physiologische Veränderungen. Abgesehen vom ersten Lebensjahr ist der Energie- und Nährstoffbedarf am höchsten in der Adoleszenz. Um physiologisches Wachstum in dieser kritischen Zeit zu gewährleisten, ist eine adäquate Aufnahme von Folat, Vitamin B_6 und Vitamin B_{12} zwingend erforderlich. Diese B-Vitamine sind eng mit Homozystein, einem Risikofaktor für Herz-Kreislauf-Krankheiten verbunden. Bei Jugendlichen wurde ein steigender Verzehr von energiedichten, aber nährstoffarmen Lebensmitteln beobachtet. Gleichzeitig werden nachteilige Faktoren wie z.B. ein bewegungsarmer Lebensstil übernommen. Für Jugendliche existieren bislang keine Referenzwerte für die oben genannten B-Vitamine und Homozystein, die im Blut gemessen werden. Daher ist eine Bewertung kaum möglich. Voraussetzung für die Entwicklung von verlässlichen Referenzwerten ist erstens die Beschreibung von aktuellen Daten und zweitens die Aufklärung, inwiefern diese Substrate mit möglichen beeinflussenden Faktoren zusammenhängen. Die untersuchten Daten der vorliegenden Arbeit stammen aus der HELENA Studie (“Healthy Lifestyle in Europe by Nutrition in Adolescence”). Insgesamt untersuchte die vorliegende Arbeit verschiedene Assoziationen und stellte potentielle Referenzdaten bereit, die als Ausgangspunkt für die weitere Entwicklung von verlässlichen Referenzwerten dienen könnten.

Die Assoziationen zwischen dem Folat-, Vitamin B_6- und Vitamin B_{12}-Status sowie Homozysteinkonzentrationen und ausgewählten nicht-kommunizierbaren Faktoren wie Geschlecht, Alter, sexuelle Reife und dem Polymorphismus der Methylentetrahydrofolate Reduktase 677C/T und kommunizierbaren Faktoren wie Körperzusammensetzung, Gebrauch von Supplementen und Rauchverhalten wurden analysiert. Dabei zeigte sich, dass die Cobalamin- und Homozysteinkonzentrationen nach Geschlecht variierten. Ein höheres Alter war mit höheren Homozysteinkonzentrationen und vorangeschrittenere sexuelle Reife war mit niedrigeren Folat-, Erythrozytenfolat- und Holo-Transcobalaminkonzentrationen assoziiert. Analog zum Alter stieg Homozystein proportional mit dem sexuellen Reifegrad. Der Polymorphismus der Methylentetrahydrofolate Reduktase 677C/T war mit Folat-, Erythrozytenfolat-, Cobalamin-, Holo-Transcobalamin und Homozysteinkonzentrationen assoziiert. Normalgewichtige zeigten höhere Folat- und Cobalaminkonzentrationen als

übergewichtige Jugendliche. Nutzer von Supplementen wiesen höhere Folat-, Erythrozytenfolat- und Cobalaminkonzentrationen und niedrigere Homozysteinkonzentrationen auf als Nichtnutzer. Rauchen war mit niedrigeren Folat-, Erythrozytenfolat-, Cobalamin- und Pyridoxal-5-phosphatkonzentrationen assoziiert.

Des Weiteren wurde der Zusammenhang zwischen Homozystein und den kommunizierbaren Faktoren körperliche Aktivität, kardiovaskuläre Fitness und Fettleibigkeit untersucht. Wenige Studien evaluierten dieses Thema und diese konzentrierten sich meist auf Erwachsene. Studien mit Jugendlichen zeigten uneinheitliche Ergebnisse. In dieser Arbeit war Homozystein weder bei männlichen, noch bei weiblichen Teilnehmern signifikant mit körperlicher Aktivität, kardiovaskulärer Fitness oder Fettleibigkeit assoziiert.

Basierend auf Beobachtungen von Homozystinurie-Patienten, die in jungen Jahren unter anderem an Osteoporose und Frakturen litten, wurde die Hypothese aufgestellt, dass der Folat- und Vitamin B_{12}-Status, sowie Homozysteinkonzentrationen mit der Knochenmineraldichte assoziiert sein könnten. Bei Jugendlichen standen unterschiedliche Werte des Knochenmineralgehalts oder der -dichte nicht mit dem Folat-, Vitamin B_6- und Vitamin B_{12}-Status, sowie Homozysteinkonzentrationen in Verbindung.

Summary

Adolescence implies important psychological, social, and physiological changes. Apart from the first year of life, both energy and nutrient requirements are greatest during adolescence. To ensure physiological growth during this critical period, an adequate intake of folate, vitamin B_6, and vitamin B_{12} is mandatory. These B-vitamins are closely connected with homocysteine a well-known risk factor for cardiovascular diseases. In adolescents, a change towards an increasing consumption of energy dense but nutrient poor food is observed. Simultaneously, detrimental factors e.g. a sedentary lifestyle are implemented. Blood B-vitamin and homocysteine reference values for adolescents do not exist. Therefore, the assessment of B-vitamin and homocysteine concentrations is not yet possible, but still needed. A prerequisite for the development of reference values is to record the present situation and to elucidate the influencing factors of B-vitamin status and homocysteine concentrations. Underlying data were taken from the HELENA study ("Healthy Lifestyle in Europe by Nutrition in Adolescence"). Overall, the present work elucidated several associations and provided potential reference data that might be a starting point for the further development of sound reference values.

The relation of folate, vitamin B_6, vitamin B_{12} status and homocysteine levels with selected non-communicable factors gender, age, sexual maturity, and the methylenetetrahydrofolate reductase 677C/T as well as selected communicable factors body composition, supplement use, and smoking were investigated. Cobalamin and homocysteine levels varied between genders. Higher age was related with increased homocysteine concentrations. Sexual maturity was inversely associated with folate, red blood cell folate, and holo-transcobalamin concentrations. Analogue to age, homocysteine concentrations were positively associated with sexual maturity. The methylenetetrahydrofolate reductase 677C/T polymorphism was associated with folate, red blood cell folate, cobalamin, holo-transcobalamin, and homocysteine concentrations. Normal weight adolescents displayed higher folate and cobalamin concentrations than overweight adolescents. Supplement users had higher folate, red blood cell folate, and cobalamin concentrations compared with non-users and homocysteine concentrations were lower in supplement users than in non-users. Smoking was

associated with lower folate, red blood cell folate, cobalamin, and pyridoxal-5-phosphate concentrations.

Furthermore, homocysteine and its association with the communicable factors physical activity, cardiovascular fitness, and fatness was examined. Few studies have targeted this relationship sofar. Subjects were mostly adults and studies on adolescents display inconsistent results. In the present study homocysteine concentrations were not significantly associated with physical activity, cardiovascular fitness, or fatness after controlling for potential confounders, neither in males nor females.

Based on observing patients with homocystinuria suffering also from premature osteoporosis and fractures, it is hypothesised that also folate, vitamin B_6, and vitamin B_{12} status as well as homocysteine levels are associated with bone mineral density. However, in adolescents variations in bone mineral content and -density could not be explained by folate, vitamin B_6, vitamin B_{12} status, and homocysteine levels.

Contents

List of abbreviations and acronyms

a.m.	Ante meridiem
ALSPAC	Avon Longitudinal Study of Parents and Children
ANOVA	Analysis of variance
ASO	Allele-specific oligos
AVENA	Alimentación y Valoración del Estado Nutricional de los Adolescentes
BA	Bone area
BEFO	Behavioural and food choice study
BIA	Bioelectrical impedance analysis
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
CATCH	Child and Adolescent Trial for Cardiovascular Health
CBS	Cystathionin-ß-synthase
CDC	Centre for Disease Control and Prevention
COMS	Cross-over multi-centre study
CSS	Cross-sectional study
CV	Coefficient of variation
CVF	Cardiovascular fitness
DNA	Desoxyribonucleic acid
dPN	Deoxypyridoxin
DRI	Dietary reference intake
DTT	Dithiothreitol
DXA	Dual energy X-ray absorptiometer
e.g.	Exempli gratia
EALT	Erythrocyte alanine aminotransferase
EAR	Estimated average requirements
EAST	Erythrocyte aspartate aminotransferase
EDTA	Ethylene diamine tetraacetic acid
EU	European Union
EYHS	European Youth Heart Study
f	Females
FAO	Food and Agriculture Organization

FBP	Folate binding protein
HELENA	Healthy Lifestyle in Europe by Nutrition in Adolescence
HHS	Hordaland Homocysteine Study
HIF	Hog intrinsic factor
HPLC	High performance liquid chromatography
KiGGS	German Health Interview and Examination Survey for Children and Adolescents
LBM	Lean body mass
LSEI	Lifestyle education intervention
LSO	Locus-specific oligo
m	Males
METs	Metabolic equivalents
MMA	Methylmalonic acid
MRC	Medical Research Council
MS	Methionine synthase
MTHFR	Methylenetetrahydrofolate reductase
MVPA	Moderate or vigorous physical activity
NDNS	National Dietary and Nutritional Survey
NHANES	National Health And Nutrition Examination Survey
n.s.	Not significant
P	Percentile
p.m.	Post meridiem
PA	Physical activity
PCA	Perchloric acid
PCR	Polymerase chain reaction
PLP	Pyridoxal-5-phosphate
RBC folate	Red blood cell folate
rpm	Rounds per minute
SAH	S-adenosyl-homocysteine
SAM	S-adenosyl-methionine
sd	Standard deviation
SNP	Single nucleotide polymorphism
SPSS	Statistical Package for Social Sciences
THF	Tetrahydrofolate
TMB	Tetramethylbenzidine

UK	United Kingdom
US	United States
VO_2max	Maximal oxygen consumption
WHO	World Health Organization
WP	Work Packages
y	Years

1 General introduction

Adolescence is defined as the period between childhood and adulthood. The period of gradual transition that normally begins with signs of puberty, implies important psychological, social, and physiological changes. The transition may either be pursued by chronological age or by stages of sexual maturation. For instance, the term adolescent was defined by the World Health Organization (WHO) as a person aged 10-19 years [1]. Further sex organs and the reproductive system mature and growth spurt takes place. In terms of development, maturity, and lifestyle, adolescents are an inhomogeneous group. Even for a given place and age, there is a great diversity depending on personal and environmental factors. Therefore, adolescence may be divided into three developmental stages: early adolescence (10/13-14/15 years), mid-adolescence (14/15-17 years), late adolescence (17-21 years, but variable) [1].

Adolescence is a period of rapid growth: up to 45% of skeletal growth takes place and 15 to 25% of adult height is achieved during adolescence [2]. During the growth spurt of adolescence, up to 37% of total bone mass may be accumulated [3]. Height and weight gain can be used as an indicator of this high demand in nutrient requirement. Based on a selection of German studies from 1985-1999 including in total >34,000 children and adolescents aged 0-18 years and 10,378 adolescents aged 12-17.9 years, Kromeyer-Hauschild et al. developed age-dependent percentile curves for height, weight, and body mass index (BMI) [4]. Focussing on the age group 12.5-17.5 years used in the present work, the median for height increased by 24.9 cm (12.5-17.5 years: 155.2-180.1 cm) in males and by 11.1 cm (156.7-167.8 cm) in females. The median for weight increased by 25.3 kg (44.2-69.5 kg) in males and by 13.0 kg (45.6-58.6 kg) in females. These data are in accordance with results from the German Health Interview and Examination Survey for Children and Adolescents (KiGGS) [5] and the Centre for Disease Control and Prevention (CDC) growth charts from the United States (US) [6]. Nutrition influences growth and development throughout infancy, childhood, and adolescence. Apart from the first year of life, both energy and nutrient requirements are greatest during adolescence [7, 8].

To ensure physiological growth during this critical period in general an adequate intake of macro- and micronutrients and in particular an adequate intake of folate, vitamin B_6, and vitamin B_{12} is mandatory. These three vitamins belong to a group of eight water soluble

vitamins which will be referred to as B-vitamins. Major physiological functions of B-vitamins in human metabolism are their role as precursor of co-enzymes. Since B-vitamins play a decisive role in and are closely connected with the methionine cycle, the intermediate homocysteine, which is in contrast to B-vitamins not obtained from diet, will also be considered.

The term folate comprises all biologically active metabolites. The chemical structure of nutritive folates is a pteridine ring attached to a *p*-aminobenzoate and a polyglutamyl chain. Though, the length may vary from one glutamyl moiety up to a multiple. Synthetic folic acid occurs solely with an attached monoglutamyl moiety instead of a polyglutamyl chain. Due to its high stability and quantitative absorption (in pure substance >90%) folic acid is utilised for food fortification and vitamin supplements. Both folate and folic acid are used to build derivatives like tetrahydrofolate (THF) bound to one-carbon units with different oxidation states: 10-formyl-THF, 5-formyl-THF, 5,10-methenyl-THF, 5-formimino-THF, 5,10-methylene-THF, and 5-methyl-THF. The latter derivative is the predominant folate in human metabolism and the form of transport in plasma. As shown in Figure 1 (page 16) some of these derivatives function as one-carbon group donators within nucleic acid synthesis (10-formyl-THF and 5,10-methylene-THF) and within remethylation of homocysteine to methionine (5-methyl-THF). Methionine as S-adenosyl-methionine (SAM) regulates the formation of 5-methyl-THF from 5,10-methylene-THF through inhibition of the vitamin B_2-dependent enzyme methylenetetrahydrofolate reductase (MTHFR) and is also involved in the choline synthesis, a basic module for the synthesis of acetylcholine and lecithin. Subsequently, folate engages indirectly with the synthesis of neurotransmitters, cell membranes, and DNA methylation. In case of folate deficiency, the disturbed nucleic acid synthesis may lead to neural tube defects in newborns and megaloblastic anemia in later life which is morphologically identical to pernicious anemia caused by vitamin B_{12} deficiency due to lacking intrinsic factor (functional folate deficiency). Biochemical markers for folate status in human metabolism are plasma folate and red blood cell folate (RBC folate) concentrations. Plasma folate immediately changes after the intake of folates or folic acid. By contrast, RBC folate concentration changes slowly as folate is incorporated and accumulated only during erythropoiesis, the development of red blood cells having a lifetime of approximately 120 days. Therefore, RBC folate is often used as a long-term indicator for folate status [9, 10].

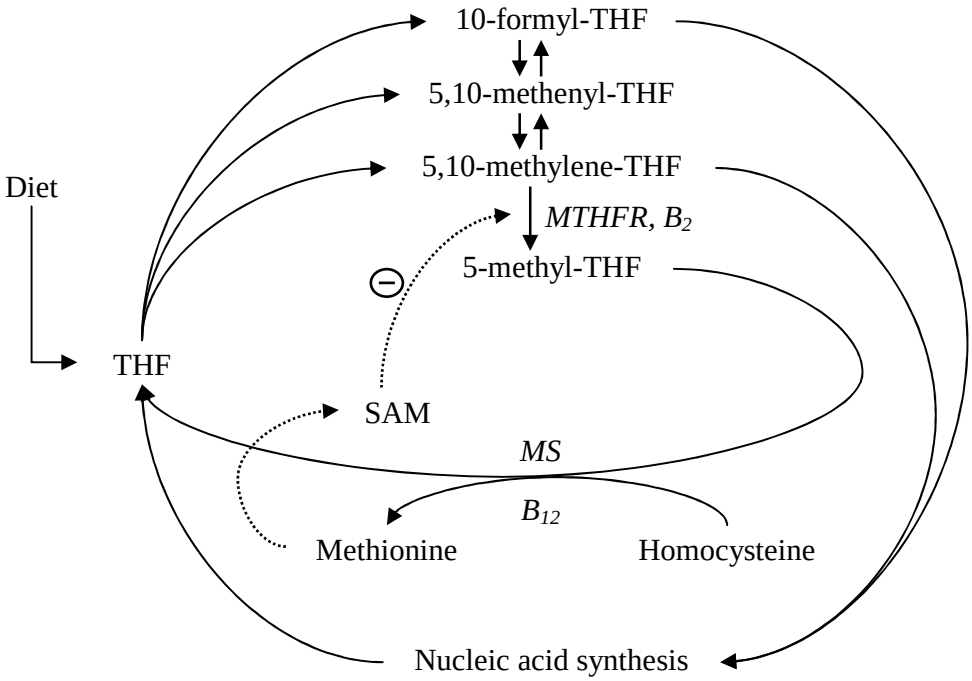


Figure 1: Simplified folate metabolism [modified from Bässler et al. (2002)]

THF = tetrahydrofolate, MTHFR = methylenetetrahydrofolate reductase, MS = methionine synthase, B_{12} = vitamin B_{12}, SAM = S-adenosyl-methionine

Vitamin B_6 summarises a group of six related compounds. The chemical structure is based on pyridine with varying substitutes at position four and their respective 5-phosphates: an alcohol substitute ($-CH_2OH$) leads to pyridoxine, aldehyde (-CHO) to pyridoxal, and an amino substitute ($-CH_2NH_2$) to pyridoxamine. The active co-enzyme form is pyridoxal-5-phosphate (PLP) but all derivatives can be interchanged and lead into PLP. Metabolic functions of PLP as a co-enzyme are numerous [11] including the metabolism of neurotransmitters [12], amino acids, fats, glycogen, hormones [13, 14], and immune functions [15]. Together with cystathionin-ß-synthase (CBS) and γ-cystathionase PLP describes an alternative pathway for the degradation of homocysteine to cysteine. Symptoms for vitamin B_6 deficiency are numerous e.g. seborrhoeic eczema, atrophic glossitis, somnolence, confusion, as well as neuropathy. An isolated vitamin B_6 deficiency is uncommon and often occurs in association with other vitamin deficiencies of the B complex. Biochemical markers for vitamin B_6 status are PLP concentration in plasma, erythrocytes, or wholeblood, urinary pyridoxic acid concentration, enzyme activities of erythrocyte alanine aminotransferase (EALT) and erythrocyte aspartate aminotransferase (EAST), and the measurement of tryptophan

catabolites. A review of established indicators of vitamin B_6 status suggests that plasma PLP is probably the best single indicator because it appears to reflect tissue stores [9, 10, 16].

Vitamin B_{12} is a collective name for biologically active cobalamins in human metabolism. The basic chemical structure is a porphyrin-like compound, consisting of four pyrrol rings with a central cobalt atom. Depending on the moiety at the sixth ligand of the cobalt atom the porphyrin-like compound becomes: cyanocobalamin (CN^-), hydroxocobalamin (OH^-), aquocobalamin (H_2O), nitrocobalamin (NO_2), methylcobalamin (CH_3), or adenosylcobalamin (5-desoxyadenosyl). However, the two last-mentioned derivatives represent the most important metabolites. In human metabolism cobalamin functions as cofactor in two enzymes, methylcobalamin in the methionine synthase (MS) and adenosylcobalamin in the methylmalonyl-CoA mutase. Within folate metabolism, MS catalyses the remethylation of homocysteine to methionine (Figure 1, page 16) and within uneven fatty acid degradation methylmalonyl-CoA mutase catalyses the conversion of methylmalonyl-CoA to succinyl-CoA. A lack of vitamin B_{12} in the remethylation reaction of homocysteine to methionine induces reduced SAM concentrations. Due to a negative feed-back mechanism between SAM and MTHFR, the synthesis of 5-methyl-THF is increased and because of the irreversible reaction of 5,10-methylene-THF to 5-methyl-THF finally results in a functional folate deficiency (Figure 1, page 16). A lack of vitamin B_{12} also inhibits the folate independent methylmalonyl-CoA mutase and methylmalonic acid (MMA) accumulates. Along with lacking SAM an excess of MMA may cause degeneration of the spinal cord, called funicular myelosis. Subsequently, an adequate vitamin B_{12} status due to its role in DNA- and central nervous system synthesis is highly important at all ages. For transport and storage specific cobalamin binding proteins are necessary. These can be subdivided into extracellular proteins (intrinsic factor, holo-transcobalamin, and haptocorrin), membrane-bound proteins (intrinsic factor and holo-transcobalamin receptors), and intracellular proteins (MS and methylmalonyl-CoA mutase) [9, 10]. Biochemical markers for vitamin B_{12} status are cobalamin concentration in serum or plasma, holo-transcobalamin concentration, and MMA concentration. The measurement of serum or plasma cobalamin concentration is simple and practicable in studies with large sample sizes but has limited sensitivity and specificity for diagnostic use [17, 18]. Holo-transcobalamin is the vitamin B_{12} fraction that is available for cellular uptake and is supposed to be an early marker for changes in vitamin B_{12} metabolism [19]. MMA concentration is a very specific marker for vitamin B_{12} deficiency but cost-intensive [20].

The sulphur-containing amino acid homocysteine is biosynthesised from the essential amino acid methionine via SAM and S-adenosyl-homocysteine (SAH). Homocysteine degradation can be separated into the remethylation and transsulfuration pathway as depicted in Figure 2 (page 19). Remethylation requires a methyl group either from 5-methyl-THF or betaine to form methionine from homocysteine. The pathway with 5-methyl-THF occurs in all tissues via the vitamin B_{12}-dependent enzyme MS whereas the pathway with betaine is confined to the liver and takes place vitamin B_{12}-independent. The transsulfuration pathway converts homocysteine to cysteine through the intermediate cystathionine. Homocysteine condenses with serine to form cystathionine in a reaction catalysed by the enzyme CBS. Then, cystathionine is hydrolysed by γ-cystathionase to form cysteine and α-ketobutyrate. Both reactions are vitamin B_6-dependent [21]. In plasma, homocysteine appears mainly bound to protein but appears also in free disulfide or thiol forms. All forms are measured and referred to as plasma total homocysteine. However, within this work the term homocysteine is used for the sum of all homocysteine derivates.

Homocystinuria was first reported in 1962 in two sisters during the screening of mentally retarded children in Northern Ireland for abnormal amino acid excretion [22]. At the same time but independently, homocystine was identified in the urine of a retarded child in the US [23]. Major clinical manifestations involve the eyes as well as the central nervous, skeletal, and vascular systems. Based on the observation of arteriosclerosis in subjects with homocystinuria the homocysteine theory of arteriosclerosis was developed during the period 1970-1975 [24]. Ever since homocysteine research was focused mostly on adults. However, to what extend homocysteine is associated with physiological development in apparently healthy adolescents was neglected at large.

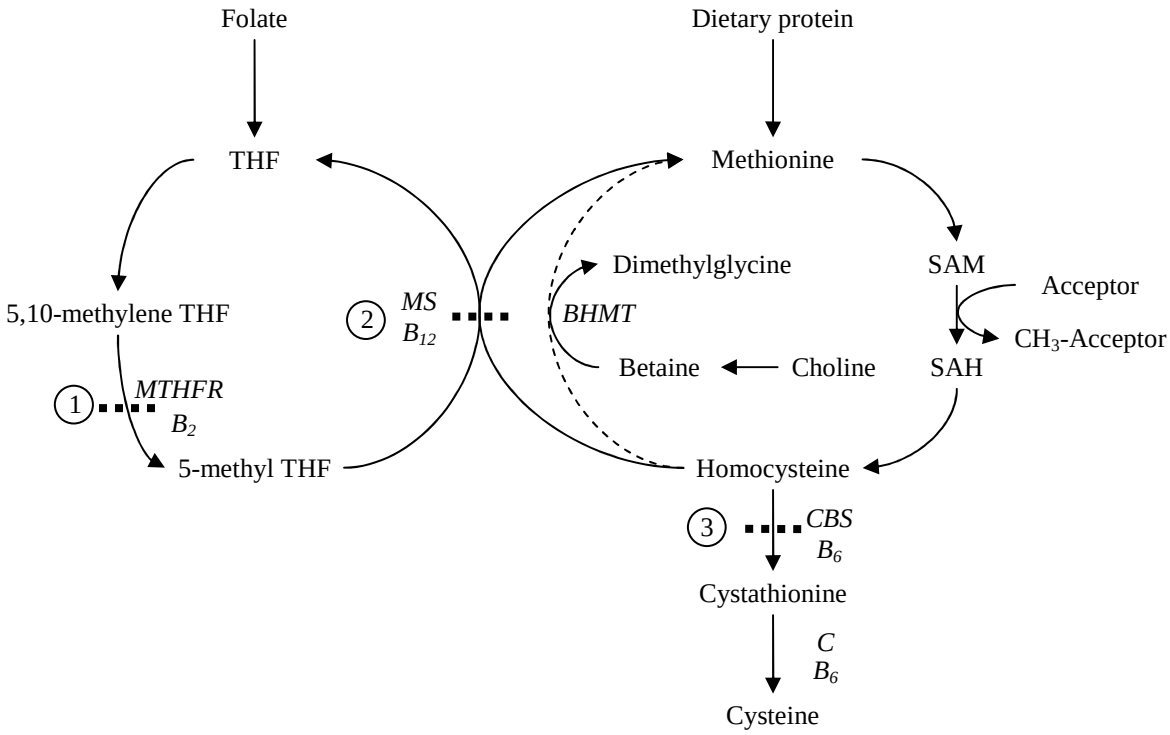


Figure 2: Simplified methionine cycle (modified from Heil et al. [25]). Important enzymatic disorders can lead to elevated homocysteine concentrations as indicated: 1) MTHFR, 2) MS, and 3) CBS leading to classical homocystinuria.

THF = tetrahydrofolate, MTHFR = methylenetetrahydrofolate reductase, MS = methionine synthase, B_2 = vitamin B_2, B_6 = vitamin B_6, B_{12} = vitamin B_{12}, CBS = cystathionine ß-synthase, C = γ-cystathionase, BHMT = betaine homocysteine methyltransferase, SAM = S-adenosyl-methionine, SAH = S-adenosyl-homocysteine.

Due to rapid degradation, circulating concentrations of homocysteine are generally low in healthy population (<14µmol/L). On the one hand, homocysteine as a regular intermediate in the methionine cycle is necessary to produce one of the most important methyl group donors SAM. However, on the other hand, elevated homocysteine concentrations are seen as a biomarker of increased oxidative stress which is associated with an increased risk for endothelial damage [26] as well as for cardiovascular diseases [27]. Several cut-offs were set up and Stanger et al. proposed a linear dose-response relationship with a relative risk for cardiovascular events of 1.3-1.7 for a 5µmol/L increase in plasma homocysteine [27]. Moreover, unphysiologically high homocysteine concentrations may contribute to the development of dementia and Alzheimer's disease [28, 29], osteoporosis [30-32], and adverse pregnancy outcomes [33-35].

Major dietary sources of folate are fruits and vegetables, wholemeal products, and liver that functions as folate storage amongst others. Dietary sources of vitamin B_6 are ubiquitous whereas vitamin B_{12} is exclusively found in animal and fermented products. Usually sub-optimal folate intake is widespread. Vulnerable groups for a deficient folate status are subjects with increased requirements like pregnant women and adolescents. Deficient vitamin B_{12} status predominantly appears in vegetarians and vegans [36] or in subjects lacking intrinsic factor like the elderly. Vitamin B_6 status could be adversely affected by alcoholism, age-related gastrointestinal changes, and the use of commonly prescribed medications, such as oral contraceptives [37].

In adolescents, however, a decreasing consumption of nutrient dense food e.g. fruits and vegetables towards an increasing consumption of energy dense but nutrient poor food can be observed [38, 39]. Smoking, alcohol consumption and the use of oral contraceptives begin during adolescence [40] and possibly other detrimental factors like a sedentary lifestyle are implemented [41, 42]. Thus, during adolescence B-vitamin intake may deteriorate due to an altered eating behaviour. B-vitamin and homocysteine concentrations could be affected by changing lifestyle factors. In the case of obesity, which may be a consequence of lacking physical activity (PA), longitudinal studies have shown that these behaviours to some extend might track into adulthood [43]; associations between BMI in adolescence and mortality in adulthood have been reported [44]. Whether an inadequate B-vitamin intake during childhood or adolescence influences morbidity in later life can only be answered by longitudinal studies whereof at present only one is available. The British population-based birth cohort study National Survey of Health and Development implemented by the Medical Research Council (MRC) intended to correlate dietary intake data of six B-vitamins at age 4, 36, 43 and 53 years with psychological distress at 53 years, however, without finding statistical significant associations [45].

The assessment of B-vitamin intake requires reliable reference values. However, up to date adolescent dietary reference values for B-vitamins e.g. as published by Germany, Austria, and Switzerland (D-A-CH) [46] or by the US (dietary reference intakes (DRI)) [9] are extrapolated from adult reference values and might not account for special needs during growth [47]. The underlying methodology of extrapolation could not be discerned from D-A-CH reference values whereas in the DRI the methodology is described in detail [9]. The estimated average requirements (EAR) are extrapolated by weighing for body weight. Data on body composition from the Third National Health and Nutrition Examination Survey

(NHANES) and data on proportional increase in protein requirements for growth from Food and Agriculture Organization (FAO) and WHO from 1985 form the basis of the following extrapolation:

$$EAR_{child} = EAR_{adult}\ F$$

$$\text{where } F = (\text{weight}_{child} / \text{weight}_{adult})^{0.75}\ (1 + \text{growth factor})$$

Furthermore, dietary reference values implicate uncertainties like bioavailability and nutrient loss due to varying methods of food preparation, which is allowed for by safety margins, as well as the disregard of fortified food items. Accordingly, the direct measurement of B-vitamin and homocysteine concentrations in several specimens reflects the situation more precisely. However, blood B-vitamin and homocysteine reference values for adolescents do not exist. So the assessment of B-vitamin and homocysteine concentrations based on sound reference values is not possible. A prerequisite for the development of reference values is to record the present situation and elucidate the influencing factors of B-vitamin status and homocysteine concentrations.

The factors that may be associated with B-vitamin status and homocysteine levels can be divided into non-communicable and communicable factors. The non-communicable factors age and gender are commonly examined in relation with B-vitamin status and homocysteine levels. However, particularly in adolescents the investigation of sexual maturity would be more appropriate because maturation tends to proceed independently of chronological age, so that chronological age is not a good indicator of sexual maturity [48, 49]. Genetics represent another non-communicable factor. The well known single nucleotide polymorphism (SNP) of the MTHFR at nucleotide position 677C/T, which results in decreased enzyme activity in the CT- and TT-genotype, is the main genetic determinant of homocysteine levels [50]. Communicable factors such as fatness, vitamin or mineral supplement use, smoking habits, PA, and cardiovascular fitness (CVF) may alter B-vitamin and homocysteine concentrations. With respect to BMI and B-vitamins, an association with vitamin B_{12} was found in an Israeli adolescent cohort [51]. Several studies observed higher homocysteine concentrations in adolescents with a higher BMI [52-55]. Regarding supplement use in adolescents, ambiguous results were reported. De Laet et al. did not observe any difference in B-vitamin status between supplement and non-supplement users [56] whereas Osganian et al. discovered significantly higher B-vitamin concentrations and lower homocysteine concentrations in supplement users than in non-supplement users [53]. Smoking seems to be associated with

homocysteine as published by several authors [53, 57, 58]. Additionally, in a Spanish study [58] lower folate concentrations in adolescent smokers compared with non-smokers were found. Physical activity and fitness was mostly surveyed in adults [59-63]. However, inconsistent results exist relating to the possible association between PA and CVF and homocysteine concentrations in childhood and adolescence [64, 65].

During the adolescent growth spurt also skeletal growth related demands are elevated [2, 3]. Based on recent studies on elderly it is hypothesised that also folate and vitamin B_{12} status as well as homocysteine concentrations are associated with bone mineral density (BMD) [66]. These hypotheses were motivated by observations in patients with homocystinuria who also suffer from premature osteoporosis and fractures [67]. Independent of age, in vitro studies support the hypothesis that homocysteine deteriorates the collagen cross-linking in bone [68]. Low concentrations of cobalamin have been associated with suppressed osteoblast activity [69] and low concentrations of folate, PLP, and cobalamin have been related with stimulated osteoclast activity [70]. It remains unclear whether BMD is associated with B-vitamin status and homocysteine concentrations in apparently healthy adolescents [71, 72].

Outlines of this thesis

This thesis deals with the present B-vitamin status in adolescents as well as non-communicable and communicable factors that might be associated with B-vitamin status and homocysteine levels. The following research questions will be addressed:

- To study the current folate, vitamin B_6, vitamin B_{12} status, and homocysteine blood concentration in European adolescents
- To analyse selected non-communicable and communicable factors associated with B-vitamin status and homocysteine concentrations in adolescents
- To discuss potential reference data for folate, vitamin B_6, vitamin B_{12}, and homocysteine concentrations in European adolescents

(Chapter 3)

- To study if there is an association between the communicable factors physical activity, CVF, and/ or fatness and homocysteine concentrations in adolescents

(Chapter 4)

- To analyse if B-vitamin status and homocysteine concentrations are related to bone parameters in adolescents

(Chapter 5)

References

1. Nutrition in adolescence – Issues and Challenges for the Health Sector: World Health Organization, 2005.

2. Rees JM, Christine MT. Nutritional influences on physical growth and behavior in adolescence. In: G. A, ed. Biology of adolescent behaviour and development. California: Sage Publications, 1989.

3. Key JD, Key LL, Jr. Calcium needs of adolescents. Curr Opin Pediatr 1994;6:379-382.

4. Kromeyer-Hauschild K, Wabitsch M, Kunze D, Geller F, Geiß HC, Hesse V, von Hippel A, Jaeger U, Johnsen D, Korte W, Menner K, Müller G, Müller JM, Niemann-Pilatus A, Remer T, Schaefer F, Wittchen H-U, Zabransky S, Zellner K, Ziegler A, Hebebrand J. Perzentile für den Body-mass-index für das Kindes- und Jugendalter unter Heranziehung verschiedener deutscher Stichproben. Monatsschr Kinderheilkd 2001;149:807-818.

5. Stolzenberg H, Kahl H, Bergmann KE. [Body measurements of children and adolescents in Germany. Results of the German Health Interview and Examination Survey for Children and Adolescents (KiGGS)]. Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz 2007;50:659-669.

6. Kuczmarski RJ, Ogden CL, Grummer-Strawn LM, Flegal KM, Guo SS, Wei R, Mei Z, Curtin LR, Roche AF, Johnson CL. CDC growth charts: United States. Adv Data 2000:1-27.

7. Lifshitz F, Tarim O, Smith MM. Nutrition in adolescence. Endocrinol Metab Clin North Am 1993;22:673-683.

8. Koletzko B, Aggett PJ, Bindels JG, Bung P, Ferre P, Gil A, Lentze MJ, Roberfroid M, Strobel S. Growth, development and differentiation: a functional food science approach. Br J Nutr 1998;80 Suppl 1:S5-45.

9. Dietary Reference Intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin and choline/ a report of the Standing Comittee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, Other B Vitamins, and Choline and Subcommittee on Upper Levels of Nutrients, Food and Nutrition Board. Institute of Medicine. Washington DC: National Academy Press, 1998.

10. Bässler K-H, Golly I, Loew D, Pietrzik K. Vitamin-Lexikon. 3. ed. München Jena: Urban & Fischer, 2002.

11. Spinneker A, Sola R, Lemmen V, Castillo MJ, Pietrzik K, Gonzalez-Gross M. Vitamin B6 status, deficiency and its consequences--an overview. Nutr Hosp 2007;22:7-24.

12. Clayton PT. B6-responsive disorders: a model of vitamin dependency. J Inherit Metab Dis 2006;29:317-326.

13. Coursin DB. Present status of vitamin B6 metabolism. Am J Clin Nutr 1961;9:304-314.

14. Bender DA. Novel functions of vitamin B6. Proc Nutr Soc 1994;53:625-630.

15. Rall LC, Meydani SN. Vitamin B6 and immune competence. Nutr Rev 1993;51:217-225.

16. Lui A, Lumeng L, Aronoff GR, Li TK. Relationship between body store of vitamin B6 and plasma pyridoxal-P clearance: metabolic balance studies in humans. J Lab Clin Med 1985;106:491-497.

17. Herrmann W, Obeid R, Schorr H, Geisel J. Functional vitamin B12 deficiency and determination of holotranscobalamin in populations at risk. Clin Chem Lab Med 2003;41:1478-1488.

18. Klee GG. Cobalamin and folate evaluation: measurement of methylmalonic acid and homocysteine vs vitamin B12 and folate. Clin Chem 2000;46:1277-1283.

19. Nexo E, Hvas AM, Bleie O, Refsum H, Fedosov SN, Vollset SE, Schneede J, Nordrehaug JE, Ueland PM, Nygard OK. Holo-transcobalamin is an early marker of changes in cobalamin homeostasis. A randomized placebo-controlled study. Clin Chem 2002;48:1768-1771.

20. Stabler SP, Lindenbaum J, Allen RH. The use of homocysteine and other metabolites in the specific diagnosis of vitamin B-12 deficiency. J Nutr 1996;126:1266S-1272S.

21. Selhub J. Homocysteine metabolism. Annu Rev Nutr 1999;19:217-246.

22. Carson NA, Neill DW. Metabolic abnormalities detected in a survey of mentally backward individuals in Northern Ireland. Arch Dis Child 1962;37:505-513.

23. Gerritsen T, Vaughn JG, Waisman HA. The identification of homocystine in the urine. Biochem Biophys Res Commun 1962;9:493-496.

24. McCully KS. Hyperhomocysteinemia and arteriosclerosis: historical perspectives. Clin Chem Lab Med 2005;43:980-986.

25. Heil SG, Lievers KJ, Boers GH, Verhoef P, den Heijer M, Trijbels FJ, Blom HJ. Betaine-homocysteine methyltransferase (BHMT): genomic sequencing and relevance to hyperhomocysteinemia and vascular disease in humans. Mol Genet Metab 2000;71:511-519.

26. Papatheodorou L, Weiss N. Vascular oxidant stress and inflammation in hyperhomocysteinemia. Antioxid Redox Signal 2007;9:1941-1958.

27. Stanger O, Herrmann W, Pietrzik K, Fowler B, Geisel J, Dierkes J, Weger M. Clinical use and rational management of homocysteine, folic acid, and B vitamins in cardiovascular and thrombotic diseases. Z Kardiol 2004;93:439-453.

28. Haan MN, Miller JW, Aiello AE, Whitmer RA, Jagust WJ, Mungas DM, Allen LH, Green R. Homocysteine, B vitamins, and the incidence of dementia and cognitive impairment: results from the Sacramento Area Latino Study on Aging. Am J Clin Nutr 2007;85:511-517.

29. Seshadri S, Beiser A, Selhub J, Jacques PF, Rosenberg IH, D'Agostino RB, Wilson PW, Wolf PA. Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. N Engl J Med 2002;346:476-483.

30. McLean RR, Jacques PF, Selhub J, Tucker KL, Samelson EJ, Broe KE, Hannan MT, Cupples LA, Kiel DP. Homocysteine as a predictive factor for hip fracture in older persons. N Engl J Med 2004;350:2042-2049.

31. Gjesdal CG, Vollset SE, Ueland PM, Refsum H, Meyer HE, Tell GS. Plasma homocysteine, folate, and vitamin B 12 and the risk of hip fracture: the hordaland homocysteine study. J Bone Miner Res 2007;22:747-756.

32. van Meurs JB, Dhonukshe-Rutten RA, Pluijm SM, van der Klift M, de Jonge R, Lindemans J, de Groot LC, Hofman A, Witteman JC, van Leeuwen JP, Breteler MM, Lips P, Pols HA, Uitterlinden AG. Homocysteine levels and the risk of osteoporotic fracture. N Engl J Med 2004;350:2033-2041.

33. Little J, Gilmour M, Mossey PA, Fitzpatrick D, Cardy A, Clayton-Smith J, Hill A, Duthie SJ, Fryer AE, Molloy AM, Scott JM. Folate and clefts of the lip and palate--a U.K.-based case-control study: Part II: Biochemical and genetic analysis. Cleft Palate Craniofac J 2008;45:428-438.

34. Knott L, Hartridge T, Brown NL, Mansell JP, Sandy JR. Homocysteine oxidation and apoptosis: a potential cause of cleft palate. In Vitro Cell Dev Biol Anim 2003;39:98-105.

35. Vollset SE, Refsum H, Irgens LM, Emblem BM, Tverdal A, Gjessing HK, Monsen AL, Ueland PM. Plasma total homocysteine, pregnancy complications, and adverse pregnancy outcomes: the Hordaland Homocysteine study. Am J Clin Nutr 2000;71:962-968.

36. Elmadfa I, Singer I. Vitamin B-12 and homocysteine status among vegetarians: a global perspective. Am J Clin Nutr 2009;89:1693S–1698S.

37. Morris MS, Picciano MF, Jacques PF, Selhub J. Plasma pyridoxal 5'-phosphate in the US population: the National Health and Nutrition Examination Survey, 2003-2004. Am J Clin Nutr 2008;87:1446-1454.

38. Klepp KI, Perez-Rodrigo C, De Bourdeaudhuij I, Due PP, Elmadfa I, Haraldsdottir J, Konig J, Sjostrom M, Thorsdottir I, Vaz de Almeida MD, Yngve A, Brug J. Promoting fruit and vegetable consumption among European schoolchildren: rationale, conceptualization and design of the pro children project. Ann Nutr Metab 2005;49:212-220.

39. Libuda L, Alexy U, Buyken AE, Sichert-Hellert W, Stehle P, Kersting M. Consumption of sugar-sweetened beverages and its association with nutrient intakes and diet quality in German children and adolescents. Br J Nutr 2009;101:1549-1557.

40. Currie C, Gabhain S, Godeau E, Smith R, Curie D, Pickett W, Richter M, Morgan A. Inequalities in young people's health. HBSC international report from the 2005/2006 survey. Copenhagen: World Health Organization, Regional Office for Europe, 2008.

41. Kahn JA, Huang B, Gillman MW, Field AE, Austin SB, Colditz GA, Frazier AL. Patterns and determinants of physical activity in U.S. adolescents. J Adolesc Health 2008;42:369-377.

42. van Mechelen W, Twisk JW, Post GB, Snel J, Kemper HC. Physical activity of young people: the Amsterdam Longitudinal Growth and Health Study. Med Sci Sports Exerc 2000;32:1610-1616.

43. Yang X, Telama R, Leskinen E, Mansikkaniemi K, Viikari J, Raitakari OT. Testing a model of physical activity and obesity tracking from youth to adulthood: the cardiovascular risk in young Finns study. Int J Obes (Lond) 2007;31:521-527.

44. Engeland A, Bjorge T, Sogaard AJ, Tverdal A. Body mass index in adolescence in relation to total mortality: 32-year follow-up of 227,000 Norwegian boys and girls. Am J Epidemiol 2003;157:517-523.

45. Mishra GD, McNaughton SA, O'Connell MA, Prynne CJ, Kuh D. Intake of B vitamins in childhood and adult life in relation to psychological distress among women in a British birth cohort. Public Health Nutr 2009;12:166-174.

46. DACH, Deutsche Gesellschaft für Ernährung ÖGfE, Schweizerische Gesellschaft für Ernährungsforschung, Schweizerische Vereinigung für Ernährung, eds. Referenzwerte für die Nährstoffzufuhr. 1. ed. Frankfurt am Main: Umschau/ Braus, 2001.

47. Lambert J, Agostoni C, Elmadfa I, Hulshof K, Krause E, Livingstone B, Socha P, Pannemans D, Samartin S. Dietary intake and nutritional status of children and adolescents in Europe. Br J Nutr 2004;92 Suppl 2:S147-211.

48. Sizonenko PC. Physiology of puberty. J Endocrinol Invest 1989;12:59-63.

49. Beunen GP, Rogol AD, Malina RM. Indicators of biological maturation and secular changes in biological maturation. Food Nutr Bull 2006;27:S244-256.

50. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJ, den Heijer M, Kluijtmans LA, van den Heuvel LP, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10:111-113.

51. Pinhas-Hamiel O, Doron-Panush N, Reichman B, Nitzan-Kaluski D, Shalitin S, Geva-Lerner L. Obese children and adolescents: a risk group for low vitamin B12 concentration. Arch Pediatr Adolesc Med 2006;160:933-936.

52. Huemer M, Vonblon K, Fodinger M, Krumpholz R, Hubmann M, Ulmer H, Simma B. Total homocysteine, folate, and cobalamin, and their relation to genetic polymorphisms, lifestyle and body mass index in healthy children and adolescents. Pediatr Res 2006;60:764-769.

53. Osganian SK, Stampfer MJ, Spiegelman D, Rimm E, Cutler JA, Feldman HA, Montgomery DH, Webber LS, Lytle LA, Bausserman L, Nader PR. Distribution of and factors associated with serum homocysteine levels in children: Child and Adolescent Trial for Cardiovascular Health. JAMA 1999;281:1189-1196.

54. Narin F, Atabek ME, Karakukcu M, Narin N, Kurtoglu S, Gumus H, Coksevim B, Erez R. The association of plasma homocysteine levels with serum leptin and apolipoprotein B levels in childhood obesity. Ann Saudi Med 2005;25:209-214.

55. Shen M-H, Chu N-F, Wu D-M, Chang J-B. Plasma homocysteine, folate and vitamin B12 levels among school children in Taiwan: The Taipei Children Heart Study. Clinical Biochemistry 2002;35:495-498.

56. De Laet C, Wautrecht JC, Brasseur D, Dramaix M, Boeynaems JM, Decuyper J, Kahn A. Plasma homocysteine concentration in a Belgian school-age population. Am J Clin Nutr 1999;69:968-972.

57. Bates CJ, Mansoor MA, Gregory J, Pentiev K, Prentice A. Correlates of plasma homocysteine, cysteine and cysteinyl-glycine in respondents in the British National Diet and Nutrition Survey of young people aged 4-18 years, and a comparison with the survey of people aged 65 years and over. Br J Nutr 2002;87:71-79.

58. Al-Tahan J, Sola R, Ruiz JR, Breidenassel C, Garcia-Fuentes M, Moreno L, Castillo MJ, Pietrzik K, Gonzalez-Gross M. Methylenetetrahydrofolate reductase 677CT polymorphism and cobalamin, folate, and homocysteine status in Spanish adolescents. Ann Nutr Metab 2008;52:315-321.

59. Woolf K, Manore MM. B-vitamins and exercise: does exercise alter requirements? Int J Sport Nutr Exerc Metab 2006;16:453-484.

60. Joubert LM, Manore MM. The Role of Physical Activity Level and B-Vitamin Status on Blood Homocysteine Levels. Med Sci Sports Exerc 2008.

61. Chrysohoou C, Panagiotakos DB, Pitsavos C, Zeimbekis A, Zampelas A, Papademetriou L, Masoura C, Stefanadis C. The associations between smoking, physical activity, dietary habits and plasma homocysteine levels in cardiovascular disease-free people: the 'ATTICA' study. Vasc Med 2004;9:117-123.

62. Kuo HK, Yen CJ, Bean JF. Levels of homocysteine are inversely associated with cardiovascular fitness in women, but not in men: data from the National Health and Nutrition Examination Survey 1999-2002. J Intern Med 2005;258:328-335.

63. Unt E, Zilmer K, Magi A, Kullisaar T, Kairane C, Zilmer M. Homocysteine status in former top-level male athletes: possible effect of physical activity and physical fitness. Scand J Med Sci Sports 2008;18:360-366.

64. Ruiz JR, Rizzo NS, Hurtig-Wennlof A, Ortega FB, Warnberg J, Sjostrom M. Relations of total physical activity and intensity to fitness and fatness in children: the European Youth Heart Study. Am J Clin Nutr 2006;84:299-303.

65. Ruiz JR, Sola R, Gonzalez-Gross M, Ortega FB, Vicente-Rodriguez G, Garcia-Fuentes M, Gutierrez A, Sjostrom M, Pietrzik K, Castillo MJ. Cardiovascular fitness is negatively associated with homocysteine levels in female adolescents. Arch Pediatr Adolesc Med 2007;161:166-171.

66. McLean RR, Jacques PF, Selhub J, Fredman L, Tucker KL, Samelson EJ, Kiel DP, Cupples LA, Hannan MT. Plasma B vitamins, homocysteine, and their relation with bone loss and hip fracture in elderly men and women. J Clin Endocrinol Metab 2008;93:2206-2212.

67. Brenton DP. Skeletal abnormalities in homocystinuria. Postgrad Med J 1977;53:488-496.

68. Kang AH, Trelstad RL. A collagen defect in homocystinuria. J Clin Invest 1973;52:2571-2578.

69. Carmel R, Lau KH, Baylink DJ, Saxena S, Singer FR. Cobalamin and osteoblast-specific proteins. N Engl J Med 1988;319:70-75.

70. Herrmann M, Schmidt J, Umanskaya N, Colaianni G, Al Marrawi F, Widmann T, Zallone A, Wildemann B, Herrmann W. Stimulation of osteoclast activity by low B-vitamin concentrations. Bone 2007;41:584-591.

71. Dhonukshe-Rutten RA, van Dusseldorp M, Schneede J, de Groot LC, van Staveren WA. Low bone mineral density and bone mineral content are associated with low cobalamin status in adolescents. Eur J Nutr 2005;44:341-347.

72. Steer C, Emmett P, Lewis S, Smith GD, Tobias J. The Methylenetetrahydrofolate Reductase (MTHFR) C677T Polymorphism is Associated with Spinal BMD in Nine-Year-Old Children. J Bone Miner Res 2008.

2 General methodology

2.1 The HELENA project – study design

The "Healthy Lifestyle in Europe by Nutrition in Adolescence" (acronym: HELENA; contract number: 007034) study is a European multi-centre study supported within the 6th framework program (FP6-2003-Food-2-A, FOOD-CT-2005-007034) under the coordination of Prof. Luis A. Moreno Aznar (Universidad de Zaragoza). In order to organise the work efficiently, 14 work packages (WP) were introduced dealing with specific topics and research fields. Accordingly, the HELENA project was split into four experimental parts: (I) a cross-over multi-centre study (COMS); (II) a lifestyle education intervention (LSEI); (III) a behavioural and food choice study (BEFO); (IV) a cross-sectional study (CSS). All work presented in this thesis was part of the CSS, specifically WP 9 (haematological, biochemical, and immunologic data). Data of WP 7 (PA and fitness), 8 (body composition), and 10 (genetics) were used, too.

2.1.1 The HELENA-cross-sectional study – study design and subject recruitment

Within the CSS, an adolescent group was assessed for food and nutrient intake, nutrition knowledge, PA and fitness, body composition, vitamin and mineral status, lipid and blood profile, and genetic variability. Therefore, methods were developed and harmonised in cooperation with all partners in order to obtain comparable data within the study centres. Subject recruitment was done by one research group or institute within the ten participating centres.

Subjects with an age range of 12.5-17.49 years were recruited in ten cities across Europe. Selection of cities was based on two criteria: regional distribution and presence of an active research group assuring sufficient expertise and resources to successfully perform epidemiological studies. Within the study, Stockholm (Sweden) represented Northern Europe, Athens and Heraklion (Greece), Rome (Italy), Saragossa (Spain) Southern Europe, Pécs (Hungary) Eastern Europe, Gent (Belgium), Lille (France) Western Europe, and Dortmund (Germany), Vienna (Austria) Central Europe. The sample size of 3,000 adolescents has been

estimated using BMI (confidence level of 95%, and a ±0.3 error). Each partner was asked to include 150 male and female subjects per protocol, respectively.

Reliable and objective data concerning age and gender were obtained by analysing complete school classes. On the city level, diversity of the sample with respect to cultural and socio-economic aspects was achieved by performing a random proportional distribution of all schools taking into account the site of the school (district/zone of the city) and the type of school (public or private). Blood sampling was performed in one third of the adolescents recruited (choice of entire classes representing the desired age of the subjects). The school and class random selection procedure, including the subset of classes for blood sampling, has been done centrally by one partner (Gent) for all study centres. In case a selected school denied its participation, a school with comparable characteristics from a list of substitutes was taken.

Individual exclusion criteria were not being able to speak the local language and simultaneous participation in another clinical trial. All protocols and informed consents for this study were reviewed and approved by an Ethics Review Committee in each country according to the Declaration of Helsinki and International Conferences on Harmonization for Good Clinical Practice. Quality control was assured throughout the whole project as described by Beghin et al. [1].

Prior to the start of the HELENA-CSS all methods were tested in a pilot study to assure an optimal sampling procedure and to optimise transport logistics and analytics. These results have been described and published elsewhere more in detail [2].

2.1.2 Medical examination, blood sampling, and anthropometry

Prior to the study day, participants were asked to abstain from eating and drinking after 8 p.m. At the study day, a medical doctor visited the school classes and asked all participants for medical history and acute diseases. A blood sampling questionnaire was used to assess fasting status, acute infections, allergies, smoking, vitamin and mineral supplements, and medication (Appendix). Maturity was assessed by means of Tanner stage [3]. Medical data and all information were recorded in a case report form for each participant (Appendix).

Blood sampling generally took place between 8-10 a.m. Approximately 30 mL of blood were collected from an antecubital vein in serum, heparin, and ethylene diamine tetraacetic acid (EDTA) monovettes® (Sarstedt AG & Co., Nümbrecht, Germany). Then, breakfast was offered to all participants.

After blood sampling, body weight was measured in kg using a standard beam balance (Seca, precision 100 g, range 0-150 kg). Height was measured in cm using a precision stadiometer (Seca, precision 0.2 cm, range 70-200 cm). BMI was calculated with the equation weight in kg divided by height in m squared (kg/m²). For bioelectrical impedance analysis (BIA) measurements, a classical tetrapolar technique was used. Standard instructions for BIA measurements were followed.

2.1.3 Sample pre-treatment and transport

The blood sampling procedure within the HELENA CSS has been described in detail by Gonzalez-Gross et al. [4]. Briefly, serum gel tubes were centrifuged at 3,500 rounds per minute (rpm) for 10 minutes within one hour after blood drawing for measuring holo-transcobalamin and creatinine. For the measurement of folate, cobalamin, and total homocysteine heparin gel tubes were immediately placed on ice and centrifuged at 3,500 rpm for 10 minutes within 30 minutes after blood drawing. For the RBC folate analysis and desoxyribonucleic acid (DNA) isolation EDTA wholeblood was collected. Within 24 hours the supernatant of heparin and the EDTA wholeblood were transported at a stable temperature of 4-7°C while serum was transported at room temperature to the central laboratory in Bonn. Then, EDTA wholeblood was diluted 1:5 with freshly prepared 0.1% ascorbic acid for cell lysis and incubated for 60 minutes in the dark. For the measurement of PLP, EDTA plasma was obtained by centrifugation at 3,500 rpm for 10 minutes. All samples were stored at -80°C until withdrawn for bunched analyses.

2.2 Material

The following tables summarise all materials used for the biochemical (folate, RBC folate, cobalamin, holo-transcobalamin, PLP, and homocysteine) and anthropometric measurements within the HELENA study. More precisely, Table 2.1 (page 32) displays the used laboratory materials and facilities, whereas Table 2.2 (page 33) presents the used chemicals. Table 2.3 (page 34) includes an overview of all machines used for this work.

Table 2.1: List of used laboratory materials and facilities

Product	Specification	Manufacturer
Balance	861	Seca, Hamburg, Germany
Calliper		Holtain, Crosswell, United Kingdom (UK)
Centrifuge	Heraeus Megafuge 1.0R	Thermo-Electron. Corporation, Waltham, USA
Crushed ice		
Eppendorf pipettes	Eppendorf Research®, 10-100 µL, 100-1,000 µL	Eppendorf, Hamburg, Germany
Inelastic tape	200	Seca, Hamburg, Germany
Laboratory scale	1 2200 P	Soehnle, Nassau, Germany
Laboratory scale Research	R 160 P	Sartorius, Göttingen, Germany
Medical gloves	Flexam, Latex, REF 88471	Cardinalhealth, Illinois, USA
Monovette® serum-gel	01.1602.001	Sarstedt, Nümbrecht, Germany
Monovettes® K-EDTA	05.1167.001	Sarstedt, Nümbrecht, Germany
Monovettes® lithium-heparin	05.1553.001	Sarstedt, Nümbrecht, Germany
Multifly®	85.1638.005	Sarstedt, Nümbrecht, Germany
Multipette®plus	4981 000.019	Eppendorf, Hamburg, Germany
Pipette tips	For 10-100 µL, 100-1,000 µL pipettes	Eppendorf, Hamburg, Germany
Reaction tubes	Safe-lock tubes, 1.5mL	Eppendorf, Hamburg, Germany
Reaction tubes	Tubes with screw-cap, 1.5mL	Eppendorf, Hamburg, Germany
Stadiometer	225	Seca, Hamburg, Germany
Transportable centrifuge		Various
Vials		Macherey-Nagel, Düren, Germany
Vortex	Vortex-Genie®	Zimmermann Laborausrüster, Cologne, Germany

Table 2.2: List of used chemicals

Product	**Manufacturer**
Aqua bidest, H2O	
AxSYM® Active-B12 kit 1P43-20	AXIS-SHIELD Ltd., Dundee, Scotland, UK
Deoxypyridoxine, dPN	Sigma-Aldrich, St. Louis, MO, USA
Dipotassiumhydrogenphosphate, K_2HPO_4	Carl Roth, Karlsruhe, Germany
Immulite® 2000 Folic acid kit L2KFO2	DPC Biermann GmbH, Bad Nauheim, Germany
Immulite® 2000 Homocysteine kit L2KHO2	DPC Biermann GmbH, Bad Nauheim, Germany
Immulite® 2000 Vitamin B12 kit L2KVB2	DPC Biermann GmbH, Bad Nauheim, Germany
Perchloric acid (PCA) 70%	Carl Roth, Karlsruhe, Germany
Phosphoric acid, H_3PO_4, 70%	Carl Roth, Karlsruhe, Germany
Pyridoxal-5-phosphate, PLP	Sigma-Aldrich, St. Louis, MO, USA
Sodium hydroxide, NaOH	Carl Roth, Karlsruhe, Germany
Sodiumdisulfite, $Na_2S_2O_5$	Carl Roth, Karlsruhe, Germany

Table 2.3: List of used machines

Product	Manufacturer
Autosampler	Spark Holland Triathlon
AxSYM® Analyzer	Abbot Diagnostics S.A., Spain
Column oven S 4010	Column Thermo Controller
Dual energy X-ray absorptiometer (DXA)	
Immulite® 2000 Analyzer	DPC Biermann GmbH, Bad Nauheim, Germany
High performance liquid chromatography (HPLC) column	Synergi 4u hydro-reversed-phase 80A, 150*4.6mm, Phenomenex, Aschaffenburg, Germany
HPLC detector RF-551	Spectrofluorometer, Shimadzu, Duisburg, Germany
HPLC pump S 1100	Solvent delivery system, Sykam, Fürstenfeldbruck, Germany

2.3 Biochemical analyses

2.3.1 Clinical biochemistry and haematology

Routine parameters of clinical biochemistry were measured at the university hospital in Bonn. Creatinine was measured on the Dimension RxL clinical chemistry system (Dade Behring, Schwalbach, Germany) with enzymatic methods using the manufacturer's reagents and instructions. The proteins contained in the serum sample become immune complexes with specific antibodies. These complexes scatter a beam of light when passed through the sample. The intensity of the scattered light is proportional to the relevant protein concentration in the sample. The result is evaluated by comparison with a known standard concentration. Haematology including hematocrit and mean corpuscular volume (MCV) was done in situ.

2.3.2 Analyses of folate, red blood cell folate, pyridoxal-5-phosphate, cobalamin, holo-transcobalamin, and homocysteine

The biochemical analyses of folate, RBC folate, cobalamin, and homocysteine were done with the use of Immulite 2000 (DPC Biermann GmbH, Bad Nauheim, Germany). For the analysis of holo-transcobalamin, AxSYM® Active-B12 (AXIS-SHIELD Ltd., Dundee, Scotland, UK)

was used. Pyridoxal-5-phosphate was determined by a modified HPLC method developed by Kimura et al. [5].

Based on antigen capture technology, the Immulite 2000 performs a two-cycle on-board sample treatment of plasma or ascorbic acid treated wholeblood for measuring folate or RBC folate, respectively (Figure 2.1, page 36). The required volume was 50 µL plasma for folate and 100 µL wholeblood for RBC folate. For the preparation of RBC folate, cooled EDTA wholeblood was diluted 1:5 with freshly prepared 0.1% ascorbic acid for cell lysis and incubated for 60 minutes in the dark. Below, the principle of a competitive immunoassay is explained using the example of folate. During the first treatment cycle, folate was separated from endogenous binding proteins by addition of dithiothreitol (DTT) as denaturant and sodium hydroxide and potassium cyanide (NaOH/ KCN) for the scission of peptide bonds. The treated sample was transferred to a second reaction tube containing folate binding protein (FBP) and murine anti-FBP antibody-coated polystyrene beads that were used to enlarge the surface. During the second cycle, folate which had been released from endogenous binding proteins in the sample competed with a defined amount of ligand labelled folate for binding with FBP; FBP bound to the anti-FBP antibody-coated polystyrene beads. The beads were washed in order to remove unbound materials. The signal-generating molecule, alkaline phosphatase labelled anti-ligand, was added to bind unoccupied FBP sites. The unbound enzyme conjugate was removed by centrifugal wash. Chemiluminescent substrate was added and thus transformed to a fluorescent product in the presence of alkaline phosphatase. The substrate consisted of a phosphate ester of adamantly dioxetane in a 2-amino-2-methyl-1-propanol buffer with polyvinylbenzyltributylphosphonium chloride as enhancer. Folate was quantified by measuring the fluorescent product with the use of the microparticle enzyme immunoassay optical assembly. The intensity of the measured signal was inversely proportional to the amount of folate in the sample. RBC folate concentrations were calculated according to the following equation:

RBC folate = [(wholeblood folate * 100) – (plasmafolate * [100 - hematocrit])] / hematocrit.

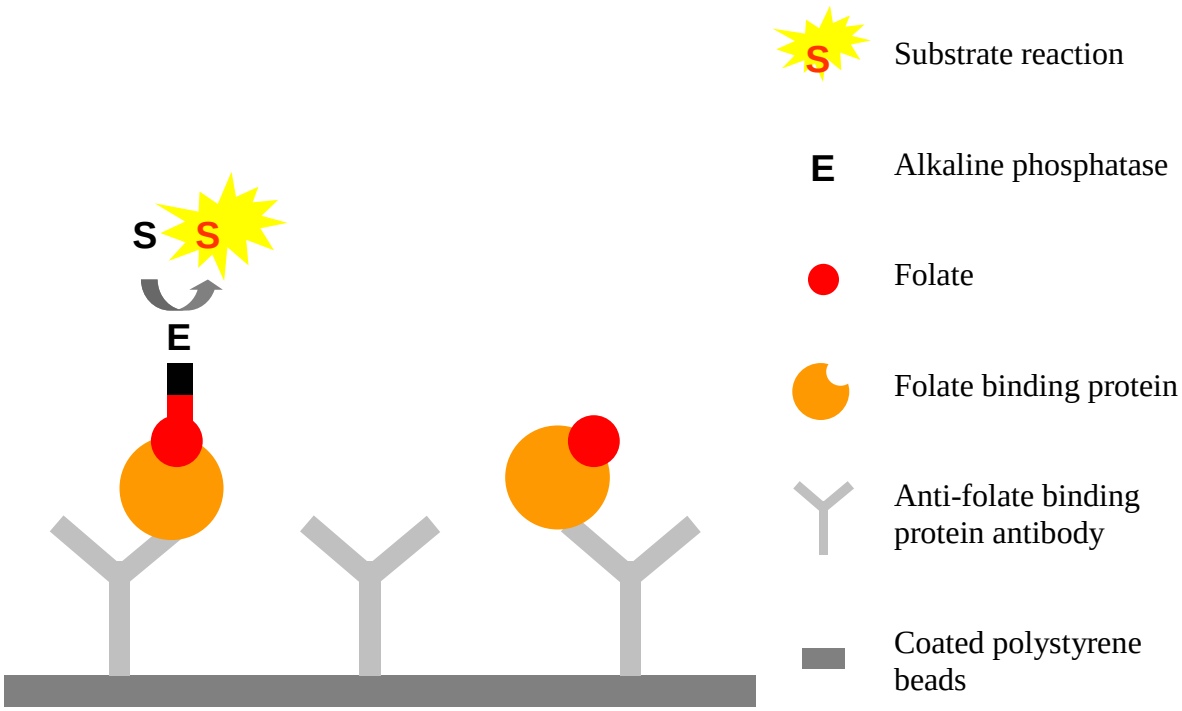


Figure 2.1: Competitive immunoassay principle using the example of folate

For the analysis of cobalamin 75 µL plasma was required. The first cycle of the cobalamin measurement was identical to the pretreatment of folate. In the second cycle, the sample was transferred to a reaction tube containing vitamin B_{12}-coated polystyrene beads and hog intrinsic factor (HIF). During the subsequent incubation of 30 minutes, the vitamin B_{12} released from endogenous binding proteins competed with immobilised vitamin B_{12} for binding with HIF. Alkaline phosphatase labelled anti-HIF was introduced which binds any HIF that is immobilised on the vitamin B_{12}-coated beads. Unbound enzyme conjugate was removed by centrifugal wash. Substrate was added and the procedure continued as previously described.

AxSYM Active-B12 is based on microparticle enzyme immunoassay technology. The required plasma volume was 75 µL. A reaction mixture is formed by combining diluted sample and microparticles coated with anti-holo-transcobalamin monoclonal antibodies in the sample well of the reaction vessel. Holo-transcobalamin antigen binds to the coated microparticles, forming antigen-antibody complexes on the microparticles. An aliquot of the reaction mixture is transferred to the matrix cell. The microparticles bind irreversibly to the glass fiber matrix. The matrix cell is washed to remove materials not bound to the

microparticles. The anti-transcobalamin antibody alkaline phosphatase conjugate is dispensed onto the matrix cell binding with the antigen-antibody complexes. The matrix cell is washed to remove conjugate not bound to the microparticles. The substrate, 4-methylumbelliferyl phosphate, is added to the matrix cell. The alkaline phosphatase-labelled conjugate catalyses the removal of a phosphate group from the substrate, yielding a fluorescent product measured by the microparticle enzyme immunoassay optical assembly.

For the measurement of homocysteine, the sample was pretreated with SAH hydrolase and DTT and incubated for 30 minutes during the first cycle. The required plasma volume was 15 µL. During the second cycle, the sample was transferred to another tube containing SAH coated polystyrene beads and alkaline phosphatase-labelled antibodies specific for SAH. The converted SAH from the sample pretreatment competed with immobilised SAH for binding alkaline phosphatase labelled anti-SAH antibodies. Unbound enzyme conjugate was removed by centrifugal wash. Substrate was added and the procedure continued as described for folate.

The HPLC method for the determination of PLP was described in detail by Kimura et al. [5]. We modified this method by adding the internal standard deoxypyridoxin (dPN) in order to compensate the loss of analyte during processing. The internal standard (20 µmol/L) was produced by dissolving 0.0001 g dPN in 5 mL 0.8 M PCA and then diluting 2 mL parent solution with 8 mL 0.8 M PCA. The sample (250 µL) was precipitated by adding 250 µL of the PCA containing internal standard. After centrifuging the sample for 10 minutes at 4,000 U and 10°C the supernatant (200 µL) was injected into a vial.

Intra- and interassay coefficients of variation (CV) are given in Table 2.4 (page 38). To avoid interassay variation, samples from each city were preferably measured by using a single kit. The analytical detection limit of folate, vitamin B_{12}, and homocysteine according to the manufacturer is shown in Table 2.5 (page 38). With respect to specificity the manufacturer observed a cross-reactivity of 0.9% between methotrexate and folate, 0.6% between S-adenosyl-L-methionine and homocysteine, 6.1% between cystathionine and homocysteine.

Table 2.4: Intra- and interassay coefficients of variation for folate, wholeblood folate, pyridoxal-5-phosphate, cobalamin, holo-transcobalamin, and homocysteine.

Coefficient of variation	intraassay	interassay
Folate	5.4%	8.1%
Wholeblood folate	10.7%	14.0%
PLP	1%	2%
Cobalamin	5.0%	12.7%
Holo-transcobalamin	5.1%	8.9%
Homocysteine	7.1%	10.7%

Table 2.5: Sensitivity of folate, cobalamin, holo-transcobalamin, and homocysteine.

	Sensitivity
Folate	≤ 0.8ng/mL (1.8 nmol/L)
Cobalamin	≤ 125 pg/mL (92 pmol/L)
Holo-transcobalamin	≤ 1 pmol/L
Homocysteine	≤ 0.5 µmol/L

2.3.3 Stability

To guarantee the stability of routine biochemistry analyses in fresh serum samples, three samples with high, mean and low baseline values were tested over different points in time during 24 hours after blood extraction. The results of the stability tests carried out of each biochemical parameter were measured at the central laboratory of the university hospital in Bonn. No changes in fresh serum samples were observed over a time span of 24 hours, either at high or low baseline levels [4].

To test the stability of folate and cobalamin, blood was collected from six volunteers (29±3years, five females), then immediately centrifuged and aliquoted. Plasma aliquots were stored at room temperature or under cooled conditions over 24 hours. All samples were repeatedly analysed over 24 hours at the laboratory in Bonn. Due to practical reasons, on cobalamin and folate, stability tests were performed directly after centrifugation and after 24

hours. No significant differences were observed during these 24 hours of storage at room temperature [4].

2.4 Genetic analyses

The DNA was extracted from white blood cells with the Puregene kit (QIAGEN, Courtaboeuf, France). The SNP MTHFR 677C/T was genotyped by Illumina (Eindhoven, Netherlands) with Golden Gate assay with 100% success rate. The genotype distribution of the polymorphism respects the Hardy-Weinberg equilibrium (P=0.17) in the sample.

In this paragraph, the principle of the Golden Gate assay is explained. The DNA sample used in this assay is activated for binding to paramagnetic particles. Assay oligonucleotides, hybridisation buffer, and paramagnetic particles are then combined with the activated DNA in the hybridisation step two. Three oligonucleotides are designed for each SNP locus. Two oligos are specific to each allele of the SNP site, called the allele-specific oligos (ASO). A third oligo that hybridises several bases downstream from the SNP site is the locus-specific oligo (LSO). All three oligonucleotide sequences contain regions of genomic complementarity and universal polymerase chain reaction (PCR) primer sites. The LSO also contains a unique address sequence targeting a particular bead type. During the primer hybridisation process, the assay oligonucleotides hybridise to the genomic DNA sample bound to paramagnetic particles. As hybridisation occurs prior to any amplification steps, no amplification bias can be introduced into the assay. Following hybridisation, several wash steps are performed to remove excess and mis-hybridised oligonucleotides. Extension of the appropriate ASO and ligation of the extended product to the LSO joins information about the genotype present at the SNP site to the address sequence on the LSO. These joined, full-length products provide a template for PCR using universal PCR primers P1, P2, and P3. Universal PCR primers P1 and P2 are Cy3- and Cy5-labeled. After downstream-processing, the single-stranded, dye-labeled DNAs are hybridised to their complement bead type through their unique address sequences. Hybridisation of the Golden Gate assay products onto the Array Matrix or BeadChip allows for the separation of the assay products in solution, onto a solid surface for individual SNP genotype readout. After hybridisation, the BeadArray Reader is used to analyse a fluorescence signal on the Sentrix Array Matrix or BeadChip, which is in turn analysed using software for automated genotype clustering and calling.

2.5 Evaluation and statistics

Corresponding to the development of gonads/breasts and pubic hair, adolescents were graduated into five categories of maturity, ranging from stage I prepubertal state (absence of development) and stage II the initial, overt development of each characteristic that marks the puberty onset. Stages III and IV mark the progress in maturation, stage V indicates the adult (mature) state. If the grade of maturity differed between both observations (gonads/breasts and pubic hair) the higher grade was chosen [3]. Regarding the point mutation in the MTHFR gene at nucleotide position 677, adolescents were identified as carriers of the CC-, CT- or TT-genotype. Furthermore, adolescents were classified into four BMI categories (thinness, normal weight, overweight, and obese) according to age and gender specific cut-offs developed by Cole et al. [6, 7]. Adolescents were classified into supplement users and non-supplement users (questionnaires). Smoking behaviour was categorised into daily smoking, smoking at least once a week but not every day, smoking less than once a week, and non-smokers. The number of cigarettes per day was not considered.

All data analyses were performed by using Statistical Package for Social Sciences (SPSS) version 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA). A weighing factor was introduced. Descriptive statistics are shown as mean ± standard deviation (sd) unless otherwise stated. We considered P-values <0.05 as statistically significant. A detailed description of statistic procedure is presented in each chapter.

References

1. Beghin L, Castera M, Manios Y, Gilbert CC, Kersting M, De Henauw S, Kafatos A, Gottrand F, Molnar D, Sjostrom M, Leclercq C, Widhalm K, Mesana MI, Moreno LA, Libersa C. Quality assurance of ethical issues and regulatory aspects relating to good clinical practices in the HELENA Cross-Sectional Study. Int J Obes (Lond) 2008;32 Suppl 5:S12-18.

2. De Henauw SG, F.; De Bourdeaudhuij, I.; Gonzalez-Gross, M.; Leclercq, C.; Kafatos, A.; Molnar, D.; Marcos, A.; Castillo, M. Dallongeville, J.; Gilbert, C.C.; Bergmann, P.; Widhalm, K.; Manios, Y.; Breidenassel, C.; Kersting, M.; Moreno, L.A.; on behalf of the HELENA Study Group. Nutritional status and lifestyles of adolescents from a public health perspective. The HELENA Project - Healthy Lifestyle in Europe by Nutrition in Adolescence. J Public Health 2007;15:187-197.

3. Tanner JM, Whitehouse RH. Clinical longitudinal standards for height, weight, height velocity and stages of puberty. Arch Dis Child 1976;51:170-179.

4. Gonzalez-Gross M, Breidenassel C, Gomez S, Ferrari M, Beghin L, Spinneker A, Diaz L, Maiani G, Demailly A, Al-Tahan J, Albers U, Warnberg J, Stoffel-Wagner B, Jiménez-Pavón D, Libersa C, Pietrzik K, Marcos A, Stehle P, Group obotHS. Sampling and processing of fresh blood samples within a European multicenter nutritional study: evaluation of biomarker stability during transport and storage. Int J Obes 2008;32:S66-S75.

5. Kimura M, Kanehira K, Yokoi K. Highly sensitive and simple liquid chromatographic determination in plasma of B6 vitamers, especially pyridoxal 5'-phosphate. J Chromatogr A 1996;722:296-301.

6. Cole TJ, Flegal KM, Nicholls D, Jackson AA. Body mass index cut offs to define thinness in children and adolescents: international survey. BMJ 2007;335:194.

7. Cole T, Bellizzi M, Flegal M, Dietz W. Establishing a standard definition for child overweight and obesity worldwide: international survey. BMJ 2002;320:1-6.

3 Folate, vitamin B_6, and vitamin B_{12} status in European adolescents: associations with homocysteine, non-communicable factors gender, age, maturity, genetic and communicable factors body weight, supplement use, and smoking habits

3.1 Abstract

Background: The aim was to examine the association between folate, vitamin B_6, vitamin B_{12} status, and homocysteine concentrations with gender, age, maturity, genetic, body weight, supplement use, and smoking in European adolescents.

Methods: Plasma folate, red blood cell (RBC) folate, PLP, cobalamin, and holo-transcobalamin (competitive immunoassays, CVs: intraassay 1-11%, interassay 2-14%) were measured in 1097 adolescents (53%♀, 14.9±1.2 years) participating in the multi-center HELENA-CSS. To check if vitamin levels are associated with gender, age, maturity (Tanner stages), MTHFR 677C/T polymorphism, BMI, supplement use, and smoking a univariate ANOVA was done separately for each substrate.

Results: Cobalamin and homocysteine levels varied between genders (cobalamin: ♂: 329.1±125.9 versus (vs) ♀: 368.8±155.8 pmol/L; homocysteine: ♂: 8.0±4.6 vs ♀: 6.8±2.5 µmol/L; t-test, P<0.001). Higher age was associated with increased homocysteine concentrations (12.5-13.9 y: 6.5±3.2 vs 16-17.5 y: 8.6±5.3 µmol/L, P<0.001). Analogue to age homocysteine concentrations were positively associated with maturity (grade I: 5.5±1.1 vs grade V: 7.4±3.6 µmol/L, P=0.025). Maturity was inversely associated with folate (grade I: 28.6±21.9 vs grade V: 18.0±9.4 nmol/L, P=0.018), RBC folate (grade I: 1008.6±468.2 vs grade V: 790.5±352.3 nmol/L, P=0.032), and holo-transcobalamin concentrations (grade I: 75.6±42.5 vs grade V: 61.4±31.9 pmol/L, P=0.024). The MTHFR 677C/T polymorphism was associated with folate (CC: 20.2±10.1 vs TT: 15.9±10.6 nmol/L, P<0.001), RBC folate (CC: 826.2±320.5 vs TT: 805.5±413.4 nmol/L, P=0.005), cobalamin (CC: 365.5±144.0 vs TT: 336.8±140.9 pmol/L, P=0.021), holo-transcobalamin (CC: 66.7±38.8 vs TT: 57.0±22.0 pmol/L, P=0.003), and homocysteine concentrations (CC: 6.6±2.0 vs

TT: 10.2±7.4 µmol/L, P<0.001). Normal weight adolescents displayed higher folate and cobalamin concentrations than overweight adolescents (normal weight: 19.2±10.8 vs overweight: 17.1±8.6 nmol/L, P=0.030; 355.8±147.7 vs 319.9±121.3 pmol/L, P=0.001). Supplement users had higher folate (supplement users: 23.3±13.4 vs non-users: 17.9±9.5 nmol/L, P<0.001), RBC folate (supplement users: 921.1±411.9 vs non-users: 767.3±328.5 nmol/L, P<0.001), and cobalamin concentrations (supplement users: 384.2±158.0 vs non-users: 347.1±142.5 pmol/L, P=0.016) compared with non-userss. Homocysteine concentrations were lower in supplement userss than in non-userss (supplement users: 6.0±2.1 vs non-users: 7.5±3.8 µmol/L, P<0.001). Smoking was associated with folate (every day: 15.5±8.7 vs don't smoke: 19.3±10.3 nmol/L, P=0.023), RBC folate (every day: 712.7±282.3 vs don't smoke: 791.4±332.5 nmol/L, P=0.002), cobalamin (every day: 308.8±122.2 vs don't smoke: 357.0±145.4 pmol/L, P=0.029), and PLP concentrations (every day: 51.4±43.6 vs don't smoke: 61.5±49.2 nmol/L, P<0.001).

Conclusion: The present study provides possible reference data on folate, RBC folate, and PLP, cobalamin, holo-transcobalamin, and homocysteine concentrations in European adolescents. Compared with chronological age, sexual maturity seems to be a better predictor of B-vitamin status within European adolescents. Since the examined communicable factors interfere with B-vitamin and homocysteine levels, these factors should be taken into account for the development of reference values.

3.2 Introduction

Adequate intake of the water-soluble vitamins folate, vitamin B_6, and vitamin B_{12} is mandatory to ensure physiological growth and organ development. Major sources of folate, vitamin B_6, and vitamin B_{12} are vegetables, whole grain products, and animal products, respectively. However, in adolescents a decreasing consumption of nutrient dense food e.g. fruits and vegetables towards an increasing consumption of energy dense but nutrient poor food can be observed [1, 2]. Smoking, alcohol consumption and the use of oral contraceptives begin [3], and possibly other detrimental factors like sedentary lifestyle that may alter B-vitamin status are implemented [4, 5].

The sulphur-containing amino acid homocysteine is a regular intermediate in methionine metabolism [6]. Circulating concentrations of homocysteine are generally low in healthy adults (<14µmol/L) due to either a rapid remethylation to methionine or by degradation through the transsulphuration pathway, where folate, vitamin B_6, and vitamin B_{12} are required as cofactors. Elevated homocysteine concentrations are seen as a biomarker of increased oxidative stress and a higher risk for endothelial damage [7]. Homocysteine is, thus, widely accepted as an independent risk factor for cardiovascular diseases [8].

In apparently healthy adolescents folate, vitamin B_6, vitamin B_{12} status, and homocysteine concentrations have been reported only in terms of national surveys in European [9-20], Asian [21] and North American countries [22-24]. Within these studies, the examination of age and gender in relation with folate and vitamin B_{12} status is common. However, particularly in adolescents, the investigation of maturity would be more appropriate because maturity is rather correlated to metabolism than age. The SNP of the MTHFR at nucleotide position 677C/T, which results in decreased enzyme activity in the CT- and TT-genotype, is the main genetic determinant of homocysteine concentrations [25]. Body weight, supplement use, and smoking habits may reflect communicable determinants of vitamin status. The assessment of B-vitamin status and homocysteine concentrations is hampered by a lack of reference values for adolescents. Therefore, the aim was to describe the status quo, to analyse associations between B-vitamin status and homocysteine values with communicable and non-communicable factors, and to discuss possible reference data for folate, RBC folate, PLP, cobalamin, holo-transcobalamin, and homocysteine.

3.3 Methods

A description of the study design and implementation is given in the general methodology. The statistical analysis is described as follows.

3.3.1 Statistics

The dependent variables folate, RBC folate, PLP, cobalamin, holo-transcobalamin, and homocysteine concentrations were normalised by natural logarithm transformation. X^2-test was used for categorical variables and student's t-test for comparing two groups. Centre, gender, age category, grade of maturity, MTHFR 677C/T genotype, BMI category, smoking, and supplement use were defined as independent variables. For each of the dependent variables, we used univariate analysis of variance (ANOVA) to analyse how it is influenced by the independent variables. To study the associations of homocysteine, folate quintiles were added to the list of independent variables. The Bonferroni post-hoc test was used for the subgroup analysis.

3.4 Results

3.4.1 Basic characteristics

Table 3.1 (page 56) shows basic characteristics for all adolescents and by gender. The mean age of males and females was 14.9±1.2 years. The majority (43.3%) of the adolescents were situated in grade IV of maturity followed by 30.5% located in grade V. Seven adolescents were situated in grade I. Females were significantly represented with a higher number in grades III-V and with a lower number in grade I and II when compared with males (P=0.006). The average BMI was 21.4±3.6 kg/m², 22.2% of the adolescents were overweight or obese. Five point four percent of the adolescents were classified as too thin. The MTHFR 677 TT- and CT-genotype were prevalent in 14.2% and 44.5%, respectively. Vitamin and/or mineral supplements were taken by 10.8% of the adolescents during the last month before the blood drawing. Unfortunately, it was not possible to distinguish if a supplement containing folic acid, vitamin B_6 and/or vitamin B_{12} was taken or one that should not affect the B-vitamin and homocysteine concentrations. Ten point three percent smoked every day, 3.8% more than once a week but not every day, 4.7% smoked less than once a week, and 81.1% did not smoke

at all. Compared with males, females showed higher cobalamin concentrations (P<0.001) and lower homocysteine concentrations (P<0.001). Table 3.2 (page 57) describes the percentile distribution of folate, RBC folate, cobalamin, holo-transcobalamin, PLP, and homocysteine concentrations for all adolescents as well as by gender. In Table 3.3 (page 58) percentiles of normal weight and non-smokers for B-vitamins and homocysteine are given.

3.4.2 Associations of non-communicable factors age, maturity, and MTHFR 677C/T genotype with B-vitamin status as well as homocysteine levels

Tables 3.4-3.6 present associations of age, maturity, and MTHFR 677C/T genotype with B-vitamin and homocysteine concentrations. Higher age was significantly associated with increased homocysteine concentrations (P<0.001). Maturity was significantly and inversely associated with folate, RBC folate, and holo-transcobalamin concentrations (P=0.018, P=0.032, P=0.024, respectively). Analogue to the chronological age, homocysteine concentrations were positively associated with maturity (P=0.025). The MTHFR 677C/T polymorphism was significantly associated with folate, RBC folate, cobalamin, holo-transcobalamin, and homocysteine concentrations (P<0.001, P=0.005, P=0.021, P=0.003, P<0.001, respectively). Carriers of the CT-genotype displayed lowest RBC folate concentrations compared with the CC- and TT-genotype. With respect to other substrates the TT-genotype presented the lowest concentrations compared with the CC- and CT-genotype.

3.4.3 Associations of communicable factors body mass index, supplement use and smoking habits with B-vitamin status and homocysteine levels

Tables 3.7-3.9 summarise the associations of BMI, supplement use, and smoking habits with B-vitamin and homocysteine concentrations. The BMI was significantly associated with folate and cobalamin concentrations (P=0.030, P=0.001, respectively). Overweight adolescents displayed lower folate and cobalamin concentrations than normal weight adolescents. Furthermore, overweight and obese adolescents showed lower cobalamin concentrations compared with underweight adolescents. Supplement users had significantly higher folate, RBC folate, and cobalamin concentrations compared with non-users (P<0.001, P<0.001, P=0.016, respectively). At the same time homocysteine concentrations were lower

in supplement users than in non-users (P<0.001). Smoking was significantly associated with folate, RBC folate, cobalamin, and PLP concentrations (P=0.023, P=0.002, P=0.029, P<0.001, respectively).

3.5 Discussion

Strengths of the present investigation are the simultaneous analysis of gender, age, maturity, MTHFR 677C/T genotype, body weight, supplement use, and smoking habits with respect to folate, vitamin B_6, and vitamin B_{12} status as well as homocysteine concentrations. Post-hoc power analyses for our significant results showed that with a probability of >90% the effects in our sample could be found in the population.

Table 3.10 (page 62) summarises data from publications being discussed within the present chapter. Conflicting data exist with respect to gender differences in folate, vitamin B_{12} status and homocysteine concentrations. The following studies presented significantly greater homocysteine concentrations in male than in female adolescents, according to our findings: the NHANES consisting of 6461 children and adolescents aged ≤18 years (e.g. non-hispanic white: 4.96±0.07 µmol/L vs 4.69±0.07 µmol/L, P<0.0001, shown as geometric mean ± standard error) [22], the Child and Adolescent Trial for Cardiovascular Health (CATCH study) consisting of 3321 US American adolescents aged 14.1±0.5 years (5.48±1.90 µmol/L vs 5.09±1.78 µmol/L, P<0.001, shown as mean ± sd) [24], the Taipei Children Heart Study consisting of 1235 adolescents aged 12-15 years (10.50±4.13 µmol/L vs 8.95±2.61 µmol/L, P<0.05, shown as mean ± sd) [21], and a Spanish study with 165 adolescents aged 13-18.5 years (8.92 (5.51-22.94) µmol/L vs 7.91 (5.09-13.86) µmol/L, P=0.003, shown as median (2.5^{th}-97.5^{th} percentile)) [19]. Our results as presented in Table 3.1 (page 56) lie within the range of the last three publications probably due to a similar age range. On the one hand, differences in muscle mass [26] and on the other hand endogenous sex hormones [27] are discussed as an explanation for gender differences in homocysteine. The formation of creatine phosphate the muscle specific energy carrier from arginine and glycine needs SAM as methyl group donor; subsequently, SAM results in homocysteine. Andersson et al. found decreased homocysteine concentrations during pregnancy, a state characterised by elevated estrogen concentrations [28]. In women with polycystic ovary syndrome homocysteine concentrations were positively correlated with androstenedione concentrations [29]. Furthermore, greater

cobalamin concentrations in females than in males have been found in adolescents participating in the CATCH study (379.8 pmol/L vs 352.4 pmol/L, P<0.001, no measure of variation available) [24] and a Spanish study (594.82 (280.63-1,559.64) pmol/L vs 540.00 (268.00-946.47) pmol/L, P=0.003) [19] as it is the case in our study (Table 3.1, page 56). However, cobalamin concentrations in the Spanish adolescents were nearly twice the number as found in our results. We did not have significant associations between folate, RBC folate, holo-transcobalamin, PLP and gender. On the contrary, the CATCH study additionally revealed greater folate concentrations in males than in females (40.8 nmol/L vs 35.1 nmol/L, P<0.001, no measure of variation available) [24]. The British National Diet and Nutrition Survey (NDNS) of 4-18 year-old children and adolescents (n=840) found higher serum folate and RBC folate concentrations in males compared with females (folate: 21.4 (15.2-27.2) nmol/L vs 19.3 (13.6-26.1) nmol/L, P=0.025 and RBC folate: 585.0 (466.0-726.0) nmol/L vs 523.0 (413.0-667.0) nmol/L, P<0.01, shown as median (25^{th}-75^{th} percentile)) [30]. Remarkable is the relatively high range of folate concentrations found in the CATCH study which might be due to the onset of folate fortification in the US. To the best of our knowledge four publications deal with vitamin B_6 status in adolescents [30-33]. Adolescents participating in the NHANES (n=1317, 13-20 years) showed PLP concentrations of 37 (35-40) nmol/L (shown as geometric mean (95% confidence interval)) [31] which is not in line with our results (Table 3.1, page 56) possibly owing to different analytical assays. In the NHANES an enzymatic assay was utilised whereas in our and the following studies, which corroborate our findings regarding vitamin B_6 status, a HPLC method was used. Chang et al. aimed at comparing vitamin B_6 status with vitamin B_6 intake within the Nutrition and Health Survey in Taiwan (NAHSIT). In 103 adolescents aged 13-15 years [32] and 127 adolescents aged 16-18 years [33] among others plasma PLP was analysed. The younger age group showed PLP concentrations >20 nmol/L without further details. The older age group revealed PLP concentrations >35 nmol/L, except for one female adolescent (28.7 nmol/L). The average PLP concentration for males and females was 58.1±17.6 and 57.2±19.3 nmol/L shown as mean ± sd, respectively. Kerr et al. found significant gender differences for PLP within the NDNS (n=840, 4-18 years) [30]. Males had higher PLP concentrations compared with females (60.6 (43.4-78.9) nmol/L vs 54.1 (41.0-70.3) nmol/L, shown as median (25^{th}-75^{th} percentile), P<0.01). Other studies did not find significant gender differences in folate, cobalamin and homocysteine concentrations [11, 13, 14, 16, 17, 20, 34-36]. Gender differences usually appear in children and adolescents after the onset of puberty due to the release of sex

hormones estrogen, testosterone, and androgen. Depending on factors like body composition, nutritional status, and ethnicity, puberty (here: measured by the age at menarche) begins at the average age of 12.8 years in German and US American female adolescents [37, 38]. As shown in Table 3.1 (page 56), female adolescents were represented more frequently in Tanner stages III-V than male adolescents of the same age group (12.5-17.5 years) implying that female adolescents either start earlier with puberty and/or pass through quicker than male adolescents. Several authors not observing gender differences examined a sample of minimum 120 up to 264 subjects with an age span of almost twenty years (minimum age range 2-17 years and maximum 0-19 years) [11, 16, 17, 34-36]. Probably the number of pre-pubertal subjects might be disproportionately higher compared with pubertal and post-pubertal subjects. Thus, it could be difficult to demonstrate gender differences within these studies.

Nearly all currently available studies reported an age-dependent development of homocysteine [9-12, 14, 17, 20, 24, 30, 35, 39] as also found in the present work (Table 3.2, page 57). To have a better comparability, exemplary those studies are cited that presented their results by age groups. Homocysteine concentrations were significantly greater with age in 647 Belgian children and adolescents aged 5-19 years (5-9 years: 6.21 (5.14, 7.5) µmol/L, 10-14 years: 7.09 (5.69, 8.84) µmol/L, and 15-19 years: 8.84 (6.36, 12.29) µmol/L, shown as geometric mean ± sd, $P<0.001$) [9]. Huemer et al. reported a significant age-dependent homocysteine development in 264 Austrian children and adolescents aged 2-17 years (2-5 years: 5.5±1.3 µmol/L, 6-9 years: 6.2±1.9 µmol/L, 10-13 years: 7.3±1.9 µmol/L, 14-17 years: 8.6±2.3, shown as mean ± sd, $P<0.0001$) [11]. In 234 Dutch children and adolescents aged 0-19 years an age-related homocysteine increase was present (0-1 years: 5.1 (4.6, 5.6) µmol/L, 2-5 years: 4.6 (4.2, 5.1) µmol/L, 6-10 years: 6.2 (5.6, 6.9) µmol/L, 11-14 years: 7.3 (6.7, 8.0) µmol/L, 15-19 years: 8.7 (7.9, 9.6) µmol/L, shown as geometric mean (95% confidence interval), $P<0.0001$) [17]. The homocysteine ranges within the cited publications are consistent with our results. Far less authors describe an inverse correlation of folate and vitamin B_{12} status with age [9, 11, 14, 17]. De Laet et al. reported decreasing folate and cobalamin concentrations with age (5-9 years: 21.06±7.99 nmol/L folate and 517.4±172.6 pmol/L cobalamin, 10-14 years: 18.87±6.61 nmol/L and 426.1±167.3 pmol/L, 15-19 years: 15.02±6.34 nmol/L and 340.8±138.9 pmol/L, $P<0.001$) [9]. Van Beynum et al. stated the following folate and cobalamin concentrations in 0-1 year-old: 79 (60, 104) nmol/L and 439

(326, 591) pmol/L, 2-5 year-old: 24 (22, 27) nmol/L and 497 (441, 560) pmol/L, 6-10 year-old: 18 (16, 20) nmol/L and 389 (345, 438) pmol/L, 11-14 year-old: 16 (15, 18) nmol/L and 318 (284, 355) pmol/L, 15-19 year-old: 16 (14, 18) nmol/L and 242 (216, 272) pmol/L, shown as geometric mean (95% confidence interval) with P<0.0001 [17]. In our study, the decrease in folate and cobalamin concentrations with increasing age was statistically not significant (Table 3.4, page 59). Our folate concentrations are in line with ranges, but our cobalamin concentrations differ from observed ranges by De Laet et al. [9] and Van Beynum et al. [17] despite similar analytical assays. The measure of variation in both studies is relatively high.

With higher pubertal stage folate, RBC folate, and holo-transcobalamin concentrations significantly decreased and similarly to the analysis by age category homocysteine concentrations significantly increased. Only cobalamin and vitamin B_6 did not significantly decrease in our study, neither analysed by age category nor analysed by pubertal stage (Table 3.5, page 59). There could be an increased B-vitamin turnover due to the stimulation of growth hormone secretion during puberty and following growth spurt. Subsequently, elevated requirements contribute to increasing homocysteine concentrations. In addition, during these ages the change in food preferences and choice from nutrient dense to energy dense but nutrient poor food possibly could be responsible for a reduced intake of folate and might worsen the situation [40-42]. Al-Tahan et al. [19] and Hogeveen et al. [16] found no significant association between sexual maturity (assessed by Tanner and menstruation/ axillary hair growth, respectively) and folate, cobalamin and homocysteine concentrations. In the case of the Dutch study this finding might be due to the large age range (0-19 years) and the relatively low number of children and adolescents (n=186) [16]. The Spanish study comprised a similar number of adolescents (n=165), however, with a smaller age range (13-18.5 years). Ninety-three percent of the males were represented in grade IV-V and 96% of the females were situated in grade III-V. There, the association between sexual maturity and substrates was analysed with the use of a one-way ANOVA. In the present work a univariate ANOVA was performed which takes into account other potential confounders. Since age is not necessarily and precisely linked with sexual maturity the latter seems to be a better predictor of B-vitamin status and homocysteine concentrations.

To date, the MTHFR 677 C/T polymorphism in adolescents was examined in six studies [11, 13, 17-20]. Huemer et al. [11], Al-Tahan et al. [19], and Papoutsakis et al. [13] found

significantly lower folate and higher homocysteine values in carriers of the TT-genotype compared with carriers of the CC-genotype. Their findings support our results (Table 3.6, page 60). Regarding folate and homocysteine concentrations in Austrian adolescents the following results were found for CC-genotype: 22.4±7.9 nmol/L and 6.2±1.7 µmol/L, CT-genotype: 20.2±7.7 nmol/L and 6.9±2.5 µmol/L, and TT-genotype: 17.7±8.2 nmol/L and 6.7±1.7 µmol/L (shown as mean ± sd, P for folate =0.01 and P for homocysteine =0.03) [11]. In Spanish adolescents, the following folate and homocysteine concentrations were reported for CC-genotype: 13.0 (7.0-24.5) nmol/L and 7.57 (4.94-12.94) µmol/L, CT-genotype: 12.0 (4.7-19.7) nmol/L and 8.81 (5.51-21.63) µmol/L, and TT-genotype: 9.3 (6.2-17.9) nmol/L and 10.83 (7.00-22.82) µmol/L (shown as median (2.5^{th}-97.5^{th} percentile) with P for folate =0.001 and P for homocysteine <0.001) [19]. Van Beynum et al. examined only the relation between homocysteine and MTHFR 677C/T polymorphism by a multiple linear regression model [17]. After adding the interaction term *folate concentration x genotype* to the model, the result became significant (P=0.05). By contrast, Gil Prieto et al. [18] and Raslova et al. [20] did not find significant differences regarding the MTHFR 677C/T polymorphism. However, Gil Prieto et al. focused on vitamin B_{12} status which is usually not affected by the MTHFR 677 C/T polymorphism [18]. Raslova et al. firstly did not measure folate concentrations and secondly discovered this specific SNP in 4.8% of their sample which might be insufficient to find statistically significant differences for homocysteine. A power analysis was not performed [20]. In our sample, the TT-genotype occurred in 14.2% of all cases which is in line with prevalence in Spanish (15.1%) [19], Greek (16.7%) [13], Austrian (10.4%) [11], and Dutch adolescents (8.2%) [17]. In these publications, the association between vitamin B_{12} status and MTHFR 677C/T polymorphism was not significant whereas our results were significant with respect to cobalamin and holo-transcobalamin concentrations. This association can not be explained in the context of biochemical cycles. Therefore, the assumption arises that there might be a genetic linkage of MTHFR 677C/T to another SNP that affects vitamin B_{12} status. Our subgroup analysis showed a significant difference of folate and homocysteine concentrations in carriers of the TT-genotype compared with carriers of the CC- and CT-genotype (Table 3.6, page 60). Additionally, our sample revealed a significant association between RBC folate and the MTHFR 677C/T polymorphism. Unexpextedly, RBC folate concentrations were lowest in the CT-genotype and significantly different from the CC-genotype. Studies carried out with adults usually report RBC folate concentrations to be lowest in the TT-genotype. To the best

of our knowledge the present work is the only study examining the association between RBC folate concentrations and MTHFR 677C/T genotype.

Higher homocysteine concentrations in adolescents with a higher BMI were observed in several studies [11, 21, 24, 43]. An age and gender matched case control study including 40 obese and 20 non-obese adolescents aged 7-17 years found significantly higher homocysteine concentrations in obese than in non-obese (14.3±11.8 µmol/L vs 8.7±5.9 µmol/L, P=0.017) [43]. In the Taipei Children Heart Study 1,235 adolescents aged 12-15 years took part. Homocysteine levels were significantly correlated with BMI only in boys [21]. Huemer et al. found a significant positive correlation between homocysteine and BMI (r=0.44, P<0.001) [11]. The CATCH Study reported a significant positive correlation for homocysteine with BMI (r=0.09, P=0.001). However, after adjusting for B-vitamins the significant relationship disappeared [24] pointing out the importance of adjusting for B-vitamins when analysing homocysteine which was done in our study. With respect to homocysteine and BMI, these observations can not be confirmed by this work. Our results show a significant negative association of folate and cobalamin with BMI which is also supported by Huemer et al. (folate r=-0.27, cobalamin r=-0.28, P for both <0.001) [11]. With respect to cobalamin, this association is confirmed by results from an Israeli cohort consisting of 228 normal weight and 164 obese children and adolescents aged 6-19 years. After adjustment for age and sex, a four times higher risk to have low cobalamin concentrations (<246 pg/mL) was revealed for obese compared with non-obese (4.33 (1.54-12.2), shown as odds ratio (95% confidence interval)) [15]. Since these associations can only be found for folate and cobalamin concentrations measured in extra-cellular compartments but not for intra-cellular measured concentrations, the assumption arises that vitamin concentrations also depend on plasma volume. We did not measure plasma volume directly. Instead, we used total body water derived from BIA which comprises intra- and extra-cellular water in a bivariate correlation analysis. However, results were inconsistent as total body water was significantly correlated with folate, cobalamin, and homocysteine concentrations (folate: r=-0.132, P=0.001; cobalamin: r=-0.174, P=0.034; homocysteine: r=0.266, P<0.001) but not with RBC folate, PLP and holo-transcobalamin concentrations. Since PLP and holo-transcobalamin concentrations were also measured in plasma, these parameters should also correlate with total body water.

Three studies revealed a vitamin and/or mineral supplement use ranging from 4.6% to 19% of the respective sample [9, 24, 30, 34]. De Laet et al. [9] did not observe any difference in

B-vitamin status between supplement und non-supplement users. Kerr et al. reported significantly higher folate and RBC folate concentrations and lower homocysteine concentrations in supplement users compared with non-users (folate: 24.0 (17.0-30.2) nmol/L vs 19.5 (14.0-25.6) nmol/L, P<0.01, RBC folate: 608.0 (463.0-737.0) nmol/L vs 551.0 (435.0-673.0) nmol/L, P=0.006, and homocysteine: 5.9 (4.7-7.0) µmol/L vs 6.5 (5.1-8.2) µmol/L, P=0.005, shown as median (25^{th}-75^{th} percentile)) [30]. Osganian et al. discovered significantly higher B-vitamin concentrations and 6% lower homocysteine concentrations in supplement users than in non-supplement users (no details) [24]. These results are in agreement with our findings (Table 3.8, page 61).

An eventual influence of smoking on homocysteine concentrations was considered by several authors [12, 17, 19, 24, 30]. Except Van Beynum et al. [17] and Kerr et al. [30] all authors observed significantly higher homocysteine concentrations in smokers compared with non-smokers. One explanation might be that vitamins are oxidised by free radicals mediated through cigarette smoke. However, this hypothesis is corroborated by only two studies. Lower folate concentrations in Spanish adolescent smokers compared with non-smokers were experienced (smokers: 11.04±1.60 µmol/L homocysteine and 7.8±1.9 nmol/L folate vs non-smokers: 8.80±2.85 µmol/L homocysteine and 12.6±3.6 nmol/L folate; P=0.036 and P<0.001) [19]. Kerr et al. found lower PLP concentrations in smokers compared with non-smokers (47.2 (36.1-65.4) nmol/L vs 56.4 (42.1-77.0) nmol/L, shown as median (25^{th}-75^{th} percentile), P=0.017) [30]. We found an association between smoking and folate, RBC folate, PLP, and cobalamin, but not with homocysteine levels (Table 3.9, page 61).

The assessment of B-vitamin and homocysteine concentrations in adolescents with respect to B-vitamin deficiency or elevated risk for cardiovascular disease is difficult because sound reference values for this life stage are lacking. Based on adult threshold values, 3% of the adolescents show folate concentrations ≤7 nmol/L [44], 2% show RBC folate concentrations ≤305 nmol/L [45], 5% show PLP concentrations ≤20 nmol/L [46], 0.5% show cobalamin concentrations ≤120 pmol/L [47], 3% show holo-transcobalamin concentrations ≤29 pmol/L [48], and 3% show homocysteine concentrations ≥14 µmol/L [8]. Due to growth spurt and sexual maturation, adolescence is an energy and nutrient demanding period. Therefore, adult threshold values might not be applicable. Another approach to assess B-vitamin and homocysteine concentrations in adolescents could be to compare our percentiles (Table 3.2, page 57) with percentiles of other adolescent samples summarised in Table 3.11 (page 68).

Concluding from our results, the analysed non-communicable and communicable factors work together with B-vitamin status and homocysteine levels to some extend. In order to focus on apparently healthy adolescents without detrimental lifestyle factors, we created a second percentile compilation including normal weight adolescents and non-smokers (Table 3.3, page 58). Different grouping by gender or age makes the comparison difficult and a general statement is not possible. Further, the selection of a percentile for the assessment of sub-optimal status or deficiency e.g. the 2.5^{th}, 5^{th}, or 10^{th} percentile for B-vitamin status and equivalent for homocysteine the 97.5^{th}, 95^{th}, or 90^{th} percentile always involves a distinct arbitrariness. The linkage to a clinical outcome e.g. megaloblastic anemia for folate and/or vitamin B_{12} deficiency or cardiovascular disease for elevated homocysteine concentrations would contribute to a well-founded selection of reference percentiles. Mean corpuscular volume (MCV) together with other parameters is considered as a possible marker for macrocytosis in the context of folate or vitamin B_{12} deficiency. Thresholds vary between values >96 fL and >100 fL [49]. The maximal MCV value of our sample was 96 fL implying that folate or vitamin B_{12} deficiency is unlikely. However, a consequence of iron deficiency which is common in children and adolescents [50] is the microcytic anemia and can be followed also by MCV [51]. In the present work, the iron status was not taken into account. Thus, a definite conclusion refering to folate and/or vitamin B_{12} deficiency is not possible. More specific markers like segmentation of neutrophiles were not available within this study. Cardiovascular disease hardly appears in adolescents. Thus, a longitudinal study design would be more appropriate wherein the multifactorial causes of cardiovascular disease should be taken into account. Nevertheless, subclinical deficiency often takes place without clinical symptoms. The early identification is mandatory for the prevention.

In conclusion, the present study provides possible reference data on folate, RBC folate, and PLP, cobalamin, holo-transcobalamin, and homocysteine concentrations in European adolescents. Gender and age are still important non-communicable factors for the comparison in-between publications. In addition to homocysteine concentrations, which was associated with age in our study, folate, RBC folate, and holo-transcobalamin concentrations were associated with sexual maturity. This result indicates that sexual maturity might be a better predictor of B-vitamin and homocysteine concentrations than age. The MTHFR 677 TT-genotype was related to all parameters except PLP. Overweight adolescents had lower folate and cobalamin concentrations than normal weight. Supplement use and smoking were also

associated with B-vitamin and homocysteine concentrations. Since the communicable factors body weight, supplement use, and smoking interfere with B-vitamin and homocysteine levels, these parameters should be taken into account for the development of reference data.

Table 3.1: Basic characteristics for all and by gender

		Gender		
	All	Male	Female	P-value
Age in years (n)	14.9±1.2 (1097)	14.9±1.3 (513)	14.9±1.2 (584)	>0.05°
Grade of maturity				0.006#
I	0.7 (7)	1.5 (7)	0 (0)	
II	5.8 (61)	7.7 (37)	4.1 (24)	
III	19.7 (204)	19.3 (91)	20.0 (113)	
IV	43.3 (412)	41.6 (184)	44.8 (228)	
V	30.5 (284)	29.9 (128)	31.1 (156)	
BMI in kg/m² (n)	21.4±3.6 (1097)	21.4±3.8 (513)	21.3±3.4 (584)	>0.05°
BMI category				>0.05#
Thinness	5.4 (60)	5.1 (26)	5.7 (34)	
Normal	72.4 (791)	69.9 (357)	74.5 (434)	
Overweight	16.6 (184)	17.5 (91)	15.8 (93)	
Obese	5.6 (62)	7.5 (39)	3.9 (23)	
MTHFR 677ct genotype				>0.05#
CC	41.3 (437)	40.1 (201)	42.3 (236)	
CT	44.5 (469)	46.8 (237)	42.5 (232)	
TT	14.2 (150)	13.1 (66)	15.2 (84)	
Supplement use in % (n)	10.8% (112)	11.5 (59)	9.1 (53)	>0.05#
Smoking status in % (n)				>0.05#
Every day	10.3 (111)	9.4 (47)	11.2 (64)	
>1/week, but not every day	3.8 (41)	3.8 (19)	3.8 (22)	
<1/week	4.7 (51)	5.0 (25)	4.5 (26)	
Don't smoke	81.1 (872)	81.9 (411)	80.5 (461)	
Folate in nmol/L (n)	18.8±10.5 (1057)	18.5±10.4 (501)	19.0±10.5 (556)	>0.05*
RBC folate in nmol/L (n)	785.0±340.7 (1048)	803.2±366.5 (499)	768.4±314.8 (549)	>0.05*
Cobalamin in pmol/L (n)	349.9±143.7 (1059)	329.1±125.9 (503)	368.8±155.8 (556)	<0.001*
Holo-transcobalamin in pmol/L (n)	63.4±33.5 (1029)	62.7±31.1 (474)	64.1±35.5 (555)	>0.05*
PLP in nmol/L (n)	62.7±53.0 (877)	65.0±46.5 (407)	60.7±58.0 (470)	>0.05*
Homocysteine in µmol/L (n)	7.4±3.7 (1058)	8.0±4.6 (502)	6.8±2.5 (556)	<0.001*

Parameters are shown as mean ± sd.
* Univariate ANOVA, adjusted for center, age category, grade of maturity, BMI category, smoking status, supplement use, and MTHFR 677C/T polymorphism.
# X^2-test
° student's t-test
Abbreviations: RBC Folate – red blood cell folate, PLP – pyridoxal-5-phosphate.

Table 3.2: Percentile distribution for the whole sample and gender specific percentiles for folate, RBC folate, cobalamin, holo-transcobalamin, PLP and homocysteine

	Percentiles								
	2.5	5	10	25	50	75	90	95	97.5
Folate in nmol/L									
All (n=1057)	6.5	7.6	9.2	11.9	16.0	22.3	31.7	39.4	46.3
Males (n=502)	6.5	7.7	9.2	11.8	16.0	22.1	31.4	39.0	46.2
Females (n=555)	6.2	7.6	9.2	12.0	16.1	22.6	32.0	39.8	49.1
RBC folate in nmol/L									
All (n=1048)	320.0	380.2	430.2	553.6	721.0	942.8	1218.2	1398.1	1592.1
Males (n=499)	327.6	376.0	426.4	568.9	728.6	952.9	1246.0	1452.2	1638.2
Females (n=548)	306.7	380.6	430.3	544.2	707.4	930.5	1191.3	1349.0	1487.5
Cobalamin in pmol/L									
All (n=1059)	156.4	172.0	193.0	240.0	319.0	436.0	557.0	638.0	696.0
Males (n=503)	156.0	168.3	193.0	231.0	302.7	402.0	509.4	569.0	606.3
Females (n=556)	150.5	174.0	195.5	253.6	334.8	463.0	603.2	672.2	722.0
Holo-transcobalamin in pmol/L									
All (n=1029)	27.5	31.0	35.1	44.5	57.9	73.1	92.0	107.0	127.1
Males (n=473)	29.6	32.1	35.6	44.0	58.7	71.9	91.4	104.8	125.2
Females (n=554)	26.3	29.6	34.6	44.7	57.5	74.3	93.4	108.6	160.4
PLP in nmol/L									
All (n=877)	16.4	19.7	24.9	34.4	48.7	75.3	113.5	146.8	186.9
Males (n=404)	15.8	19.9	27.2	37.4	52.5	78.1	113.8	150.2	187.9
Females (n=468)	17.0	19.5	23.4	31.7	45.8	70.3	110.2	145.1	184.5
Homocysteine in µmol/L									
All (n=1058)	3.6	3.9	4.4	5.4	6.7	8.4	10.4	12.2	15.6
Males (n=502)	3.7	4.1	4.5	5.7	7.0	8.9	10.9	13.8	21.5
Females (n=555)	3.5	3.8	4.3	5.2	6.4	8.0	9.9	11.4	12.5

Abbreviations: RBC Folate – red blood cell folate, PLP – pyridoxal-5-phosphate.

Table 3.3: Percentile distribution for normal weight and non-smokers for folate, RBC folate, cobalamin, holo-transcobalamin, PLP and homocysteine

	Percentiles								
	2.5	5	10	25	50	75	90	95	97.5
Folate in nmol/L									
All (n=598)	7.0	8.4	9.5	12.3	17.0	24.2	32.8	39.9	46.9
Males (n=270)	7.3	8.0	9.5	12.0	16.7	24.0	31.7	38.2	44.0
Females (n=327)	6.8	8.6	9.6	12.8	17.4	24.5	33.6	41.3	51.5
RBC folate in nmol/L									
All (n=592)	330.0	379.8	430.7	557.1	732.3	959.8	1276.4	1450.3	1682.3
Males (n=269)	293.3	370.0	425.1	568.2	737.2	978.4	1346.7	1591.7	1804.4
Females (n=323)	334.9	392.5	445.0	544.9	727.6	944.7	1202.2	1367.9	1581.5
Cobalamin in pmol/L									
All (n=601)	163.1	176.6	195.7	246.0	333.0	451.0	574.3	643.6	707.0
Males (n=271)	153.1	169.8	194.0	238.3	317.0	420.1	522.3	578.0	638.3
Females (n=329)	166.2	177.0	196.0	254.1	348.6	481.0	620.1	683.7	736.6
Holo-transcobalamin in pmol/L									
All (n=585)	28.7	31.2	34.9	44.9	59.2	74.2	94.2	112.5	178.7
Males (n=262)	29.5	31.5	34.4	42.9	60.7	73.8	93.3	109.1	166.0
Females (n=323)	27.0	30.5	35.5	46.4	58.1	74.6	97.6	119.2	192.5
PLP in nmol/L									
All (n=500)	16.4	19.6	25.8	35.3	50.8	74.4	110.7	138.2	153.6
Males (n=223)	15.4	18.2	28.9	38.7	54.0	78.2	111.3	137.1	154.2
Females (n=277)	17.7	19.7	24.0	33.6	46.9	72.3	109.6	140.6	153.9
Homocysteine in µmol/L									
All (n=599)	3.6	3.8	4.4	5.2	6.6	8.3	10.3	11.8	13.4
Males (n=271)	3.7	4.1	4.7	5.7	7.0	8.8	10.9	12.6	16.0
Females (n=328)	3.4	3.7	4.2	4.9	6.2	8.0	9.7	11.3	12.5

Abbreviations: RBC Folate – red blood cell folate, PLP – pyridoxal-5-phosphate.

Table 3.4: Age specific B-vitamin and homocysteine concentrations

	Age category in years				
	12.5-13.99	14-14.99	15-15.99	16-17.49	P-value*
Folate in nmol/L (n)	20.4±10.6 (265)	19.3±9.8 (272)	18.5±10.1 (271)	16.7±11.1 (248)	>0.05
RBC folate in nmol/L (n)	786.7±343.4 (263)	808.3±336.7 (271)	763.1±302.3 (268)	781.4±380.1 (245)	>0.05
Cobalamin in pmol/L (n)	369.7±153.2 (267)	360.5±147.5 (271)	331.2±133.4 (271)	337.7±136.6 (250)	>0.05
Holo-transcobalamin in pmol/L (n)	65.2±27.8 (267)	65.9±34.8 (261)	60.2±36.6 (266)	62.2±34.2 (234)	>0.05
PLP in nmol/L (n)	65.7±69.9 (233)	59.4±36.0 (238)	60.6±45.3 (209)	65.5±54.6 (192)	>0.05
Homocysteine in µmol/L (n)	6.5±3.2 (266)[a,c]	6.8±2.5 (272)[b,d]	7.6±3.0 (271)[c,d]	8.6±5.3 (248)[a,b]	<0.001

Parameters are shown as mean ± sd.
* Univariate ANOVA, adjusted for center, gender, grade of maturity, BMI category, smoking status, supplement use, and MTHFR 677C/T polymorphism.
Abbreviations: RBC Folate – red blood cell folate, PLP – pyridoxal-5-phosphate.

P<0.05: a= 16-17.49y vs 12.5-13.99y, b= 16-17.49y vs 14-14.99y, c= 12.5-13.99y vs 15-15.99y, d= 14-14.99y vs 15-15.99y

Table 3.5: Maturity specific folate, RBC folate, cobalamin, holo-transcobalamin, PLP, and homocysteine concentrations

	Grade of maturity					
	I	II	III	IV	V	P-value*
Folate in nmol/L (n)	28.6±21.9 (7)	21.3±8.4 (55)[a,b]	18.9±9.5 (181)	17.6±10.7 (410)[a]	18.0±9.4 (284)[b]	0.018
RBC folate in nmol/L (n)	1008.6±468.2 (7)	909.2±357.0 (54)[a,c]	755.6±305.2 (179)[c]	756.6±331.5 (408)[a]	790.5±352.3 (280)	0.032
Cobalamin in pmol/L (n)	397.1±127.6 (7)	389.6±152.1 (55)	368.6±167.2 (182)	347.1±144.3 (411)	332.5±128.9 (284)	>0.05
Holo-transcobalamin in pmol/L (n)	75.6±42.5 (6)	74.4±28.2 (54)[a,b,c]	63.3±33.4 (182)[c]	61.4±30.0 (390)[a]	61.4±31.9 (278)[b]	0.024
PLP in nmol/L (n)	51.0±28.5 (4)	65.7±28.9 (47)	58.9±33.7 (154)	65.5±54.2 (333)	61.1±68.9 (232)	>0.05
Homocysteine in µmol/L (n)	5.5±1.1 (6)	6.2±5.7 (55)[a,b,c]	7.2±4.3 (182)[c,d]	7.7±3.4 (410)[a,d]	7.4±3.6 (285)[b]	0.025

Parameters are shown as mean ± sd.
* Univariate ANOVA, adjusted for center, gender, age category, BMI category, smoking status, supplement use, and MTHFR 677C/T polymorphism.
Abbreviations: RBC Folate – red blood cell folate, PLP – pyridoxal-5-phosphate.

P<0.05: a= II vs IV, b= II vs V, c= II vs III, d= III vs IV

Table 3.6: Folate, RBC folate, cobalamin, holo-transcobalamin, PLP, and homocysteine concentrations according to MTHFR 677ct genotype

	MTHFR 677 genotype			
	CC	CT	TT	P-value*
Folate in nmol/L (n)	20.2±10.1 (431)[a,c]	18.7±10.6 (459)[b,c]	15.9±10.6 (142)[a,b]	<0.001
RBC folate in nmol/L (n)	826.2±320.5 (427)[c]	751.1±332.2 (458)[c]	805.5±413.4 (142)	0.005
Cobalamin in pmol/L (n)	365.5±144.0 (431)	340.8±143.9 (462)	336.8±140.9 (143)	0.021
Holo-transcobalamin in pmol/L (n)	66.7±38.8 (409)[a]	62.3±29.0 (452)[b]	57.0±22.0 (139)[a,b]	0.003
PLP in nmol/L (n)	66.2±64.9 (361)	62.4±44.4 (373)	56.0±37.9 (122)	>0.05
Homocysteine in µmol/L (n)	6.6±2.0 (431)[a,c]	7.2±2.7 (461)[b,c]	10.2±7.4 (142)[a,b]	<0.001

Parameters are shown as mean ± sd.
* Univariate ANOVA, adjusted for center, gender, age category, grade of maturity, BMI category, smoking status, and supplement use.
Abbreviations: RBC Folate – red blood cell folate, PLP – pyridoxal-5-phosphate, MTHFR – methylenetetrahydrofolate reductase.

P<0.05: a= TT vs CC, b= TT vs CT, c= CT vs CC

Table 3.7: Folate, RBC folate, cobalamin, holo-transcobalamin, PLP, and homocysteine concentrations according to BMI category

	BMI categories in kg/m²				
	Thinness	Normal	Overweight	Obese	P-value*
Folate in nmol/L (n)	18.5±9.3 (59)	19.2±10.8 (765)[a]	17.1±8.6 (176)[a]	18.5±11.6 (57)	0.030
RBC folate in nmol/L (n)	703.5±253.0 (57)	796.1±361.9 (759)	777.4±291.8 (174)	742.0±240.1 (57)	>0.05
Cobalamin in pmol/L (n)	395.1±150.0 (58)[b,c]	355.8±147.7 (768)[a]	319.9±121.3 (177)[a,b]	318.8±126.6 (56)[c]	0.001
Holo-transcobalamin in pmol/L (n)	59.7±20.9 (57)	64.0±35.1 (743)	63.6±33.7 (175)	59.1±18.5 (53)	>0.05
PLP in nmol/L (n)	56.7±41.3 (46)	62.6±53.3 (623)	62.8±48.2 (148)	68.0±68.8 (54)	>0.05
Homocysteine in µmol/L (n)	6.8±2.9 (58)	7.3±3.5 (766)	7.6±4.8 (176)	7.5±2.9 (57)	>0.05

Parameters are shown as mean ± sd.
* Univariate ANOVA, adjusted for center, gender, age category, grade of maturity, smoking status, supplement use, and MTHFR 677C/T polymorphism.
Abbreviations: RBC Folate – red blood cell folate, PLP – pyridoxal-5-phosphate, BMI – body mass index.

P<0.05: a= overweight vs normal, b= overweight vs thinness, c= obese vs thinness

Table 3.8: Impact of supplement use on folate, RBC folate, cobalamin, holo-transcobalamin, PLP, and homocysteine status

	Supplement use		
	No	Yes	P-value*
Folate in nmol/L (n)	17.9±9.5 (893)	23.3±13.4 (110)	<0.001
RBC folate in nmol/L (n)	767.3±328.5 (885)	921.1±411.9 (107)	<0.001
Cobalamin in pmol/L (n)	347.1±142.5 (895)	384.2±158.0 (110)	0.016
Holo-transcobalamin in pmol/L (n)	62.8±32.0 (865)	69.0±40.1 (106)	>0.05
PLP in nmol/L (n)	61.4±52.6 (738)	74.4±63.5 (88)	>0.05
Homocysteine in µmol/L (n)	7.5±3.8 (893)	6.0±2.1 (110)	<0.001

Parameters are shown as mean ± sd.
* Univariate ANOVA, adjusted for center, gender, age category, grade of maturity, BMI category, smoking status, and MTHFR 677C/T polymorphism.
Abbreviations: RBC Folate – red blood cell folate, PLP – pyridoxal-5-phosphate.

Table 3.9: Impact of smoking status on folate, RBC folate, cobalamin, holo-transcobalamin, PLP, and homocysteine status

	Smoking status				
	Every day	>1/week, but not every day	<1/week	Don't smoke	P-value*
Folate in nmol/L (n)	15.5±8.7 (115)[a]	19.8±15.7 (44)	18.4±9.7 (47)	19.3±10.3 (829)[a]	0.023
RBC folate in nmol/L (n)	712.7±282.3 (114)[b]	939.6±566.7 (44)[b,c]	782.7±308.7 (47)	791.4±332.5 (820)[c]	0.002
Cobalamin in pmol/L (n)	308.8±122.2 (114)[a,b]	371.1±170.1 (44)[b]	319.0±118.4 (47)	357.0±145.4 (832)[a]	0.029
Holo-transcobalamin in pmol/L (n)	60.0±33.2 (117)	64.3±20.7 (35)	57.3±21.6 (46)	64.5±34.7 (809)	>0.05
PLP in nmol/L (n)	51.4±43.6 (90)[a,b,d]	95.3±101.6 (28)[b,c]	86.5±74.1 (38)[d]	61.5±49.2 (702)[a,c]	<0.001
Homocysteine in µmol/L (n)	8.5±5.9 (115)	7.2±2.4 (44)	7.4±2.3 (47)	7.2±3.3 (829)	>0.05

Parameters are shown as mean ± sd.
* Univariate ANOVA, adjusted for center, gender, age category, grade of maturity, BMI category, supplement use, and MTHFR 677C/T polymorphism.
Abbreviations: RBC Folate – red blood cell folate, PLP – pyridoxal-5-phosphate.

P<0.05: a= every day vs don't smoke, b= every day vs >1/week, but not every day, c= >1/week, but not every day vs don't smoke, d= every day vs <1/week

Table 3.10: Summary of results from several authors for associations with folate, PLP, cobalamin, and homocysteine concentrations

Author	Study design	Factors	Folate	PLP	Cobalamin	Homocysteine
Ganji et al. [22]	NHANES, n=6461, ≤18y, US, mean ± sd	Gender				m>f, 5.48±1.90 vs 5.09±1.78 µmol/L, P<0.001
Osganian et al. [24]	CATCH, n=3321, 14.1±0.5y, US, mean ± sd, after adjustment for several confounders including B-vitamins association for age and BMI n.s.	Gender	m>f, 40.8 vs 35.1 nmol/L, P<0.001		f>m, 379.8 vs 352.4 pmol/L, P<0.001	m>f, 5.48±1.90 vs 5.09±1.78 µmol/L, P<0.001
		Age				r=0.06, P<0.001
		Suppl.	User > non-user		User > non-user	User < non-user 4.82 vs 5.09 µmol/L, P=0.001
		Smoking				Smokers > non-smokers 5.19 vs 5.00 µmol/L, P=0.03
		BMI				r=0.09, P=0.001
Shen et al. [21]	Taipei Children Heart Study, n=1235, 12-15y, Taiwan, mean ± sd	Gender				m>f, 10.5±4.13 vs 8.95±2.61 µmol/L, P<0.05
		Age				
		BMI				m: sign. corr.

Author	Study design	Factors	Folate	PLP	Cobalamin	Homocysteine
Al-Tahan et al. [19]	n=165, 13-18.5y, Spain, median (2.5^{th}-97.5^{th} percentile)	Gender	n.s.		f>m, 594.82 (280.63-1,559.64) vs 540.00 (268.00-946.47) pmol/L, P=0.003	m>f, 8.92 (5.5-22.94) vs 7.91 (5.1-13.86) µmol/L, P=0.003
		Age	n.s.		n.s.	n.s.
		MTHFR 677 CC CT TT	13.0 (7.0-24.5), 12.0 (4.7-19.7), 9.3 (6.2-17.9) nmol/L, P=0.001		n.s.	7.57 (4.94-12.94), 8.81 (5.51-21.63), 10.83 (7.00-22.82) µmol/L, P<0.001
		BMI	n.s.		n.s.	n.s.
		Sexual maturity	n.s.		n.s.	n.s.
		Smoking	smokers < non-smokers, 7.8±1.9 vs 12.6±3.6 nmol/L, P<0.001		n.s.	smokers > non-smokers 11.04±1.60 vs 8.80±2.85 µmol/L, P=0.036
Kerr et al. [30]	NDNS, n=840, 4-18y, UK, median (25^{th}-75^{th} percentile)	Gender	m>f, folate: 21.4 (15.2-27.2) vs 19.3 (13.6-26.1) nmol/L, P=0.025, RBC~: 585.0 (466.0-726.0) vs 523.0 (413.0-667.0) nmol/L, P<0.01	m>f, 60.6 (43.4-78.9) vs 54.1 (41.0-70.3) nmol/L, P<0.01		
		Age	-, P<0.05	n.s.	-, P<0.05	+, P<0.05
		Suppl.	folate: 24.0 (17.0-30.2) vs 19.5 (14.0-25.6) nmol/L, P<0.01, RBC~: 608.0 (463.0-737.0) vs 551.0 (435.0-673.0) nmol/L, P=0.006			5.9 (4.7-7.0) vs 6.5 (5.1-8.2) µmol/L, P=0.005

Author	Study design	Factors	Folate	PLP	Cobalamin	Homocysteine
		Smoking		smokers<non-smokers 47.2 (36.1-65.4) vs 56.4 (42.1-77.0) nmol/L, P=0.017		n.s.
Morris et al. [31]	NHANES, n=1317, 13-20y, US, GM (CI)			37 (35-40) nmol/L		
Chang et al. [32]	NAHSIT, n=103, 13-15y, Taiwan			>20 nmol/L		
Chang et al. [33]	NAHSIT, n=127, 16-18y, Taiwan, mean ± sd			>35 nmol/L, f: 58.1±17.6 and m: 57.2±19.3 nmol/L, n.s.		
De Laet et al. [9]	n=647, 5-19y, Belgium, GM ± sd	Gender				adolescents >15y: m>f, P<0.05
		Age 5-9y: 10-14y: 15-19y:	- 21.06±7.99, 18.87±6.61, 15.02±6.34 nmol/L, P<0.001		- 517.4±172.6, 426.1±167.3, 340.8±138.9 pmol/L, P<0.001	+ 6.21 (5.14, 7.5), 7.09 (5.69, 8.84), 8.84 (6.36, 12.29) µmol/L, P<0.001
		Suppl.	n.s.		n.s.	
Huemer et al. [11]	n=264, 2-17y, Austria, mean ± sd	Gender	n.s.		n.s.	n.s.
		Age 2-5y: 6-9y: 10-13y: 14-17y:				+ 5.5±1.3, 6.2±1.9, 7.3±1.9, 8.6±2.3 µmol/L, P<0.0001
		MTHFR 677 CC CT TT	 22.4±7.9, 20.2±7.7, 17.7±8.2 nmol/L, P=0.01		n.s.	 6.2±1.7, 6.9±2.5, 6.7±1.7 µmol/L, P=0.03

Author	Study design	Factors	Folate	PLP	Cobalamin	Homocysteine
		BMI	r=-0.27, P<0.001		r=-0.28, P<0.001	r=0.09, P=0.001, after adjustment P>0.05
Van Beynum et al. [17]	n=234, 0-19y, Netherlands, GM (CI)	Gender	n.s.		n.s.	n.s.
		Age 0-1y: 2-5y: 6-10y: 11-14y: 15-19y:	- 79 (60, 104), 24 (22, 27), 18 (16, 20), 16 (15, 18), 16 (14, 18) nmol/L, P<0.0001		- 439 (326, 591), 497 (441, 560), 389 (345, 438), 318 (284, 355), 242 (216, 272) pmol/L, P<0.0001	+ 5.1 (4.6, 5.6), 4.6 (4.2, 5.1), 6.2 (5.6, 6.9), 7.3 (6.7, 8.0), 8.7 (7.9, 9.6) µmol/L, P<0.0001
		MTHFR 677 CC CT TT				n.s., but P=0.05 in the linear regression model including folate x genotype interaction as independent variable and adjusted for age
		Smoking	n.s.		n.s.	n.s.
Hogeveen et al. [16]	n=186, 0-19y, Netherlands	Gender			n.s.	n.s.
		Age			r=-0.53, P<0.05	r=0.68, P<0.05
		Sexual maturity			n.s.	n.s.
Papoutsakis et al. [13]	n=198 (92m/ 106f) 10,8-13,5y, Greece, GM	Gender	n.s.		n.s.	n.s.
		MTHFR 677 CC CT TT	 20.7 nmol/L 20.4 nmol/L 17.5 nmol/L overall P=0.028		n.s.	TT vs CT, CC 7.7 µmol/L, P=0.028 7.8 µmol/L, P=0.014 8.9 µmol/L

Author	Study design	Factors	Folate	PLP	Cobalamin	Homocysteine
Gil Prieto et al. [18]	n=313, 13-15y, Spain	Age			n.s.	
		MTHFR 677 CC CT TT			n.s.	
Raslova et al. [20]	n=386 (149m/ 237f) 11-18y, Slovakia	Gender				n.s.
		Age				n.s.
		MTHFR 677 CC CT TT				n.s.
Narin et al. [43]	Age and gender matched case control study, 40 obese, 20 non-obese, 7-17y, Turkey	Gender	n.s.		n.s.	n.s.
		BMI				obese > non-obese, 14.3±11.8 vs 8.7±5.9 µmol/L, P=0.017
Pinhas-Hamiel et al. [15]	164 obese, 228 non-obese, 6-19y, Israel, odds ratio (95% confidence interval)	BMI			risk for low cobalamin concentrations (<246 pg/mL), obese: 4.33 (1.54-12.2)	
Bates et al. [12]	NDNS, n=1193, 4-18y, UK	Age				+
		BMI	n.s.		n.s.	n.s.
		Smoking				Smokers > non-smokers

Author	Study design	Factors	Folate	PLP	Cobalamin	Homocysteine
Vilaseca et al. [35]	n=195, 0-18y	Gender				n.s.
		Age				r=0.5556, P<0.001
Bjorke Monsen et al. [34]	n=700, 0-19y	Gender	n.s.		n.s.	n.s.
Papandreou et al. [14]	n=524, 6-15y	Gender	n.s.		n.s.	n.s.
		Age	+		+	-
		BMI	n.s.		n.s.	n.s.

Abbreviations: RBC Folate – red blood cell folate, PLP – pyridoxal-5-phosphate, BMI – body mass index, MTHFR – methylenetetrahydrofolate reductase, Suppl. – Supplement use, m – males, f – females, n.s. – not significant, sign. corr. – significantly correlated, + –positively associated, - – negatively associated.

Table 3.11: Summary of percentiles from several authors for folate, RBC folate, cobalamin, PLP, homocysteine concentrations

Author	Gender	Age group	Substrate	P 2.5	P 5	P 95	P 97.5
Al-Tahan et al. [19]	Male Female	All (13-18.5y)	Folate Cobalamin Homocysteine	4.8 6.6 268.0 280.6			 22.9 13.9
De Laet et al. [9]	All	10-14y 15-19y	Homocysteine			10.2 15.2	
Hogeveen et al. [16]	All	All (0-19y)	Cobalamin Homocysteine	139.0			 13.9
Huemer et al. [11]	All	10-13y 14-17y	Folate Cobalamin Homocysteine		8.4 8.2 148.0 117.0	 11.9 12.8	
Kerr et al. [30]	All	All (4-18y)	Folate RBC Folate Cobalamin PLP Homocysteine		9.8 311.0 156.0 28.9	 11.7	
Morris et al. [31]	All	13-20y	PLP	35.0			
Osganian et al. [24]	All	All (13-14y)	Homocysteine			8.5	
Papoutsakis et al. [13]	Male Female	All (11-13.5y)	Folate Cobalamin Homocysteine	17.8 18.8 360.0 388.0			 8.6 8.1
Rauh et al. [10]	Male Female	All (6-17y)	Folate Cobalamin Homocysteine	8.6 10.0 261.0 306.0			 10.5 9.2
Van Beynum et al. [17]	All	11-14y 15-19y	Folate Cobalamin Homocysteine	15.0 14.0 284.0 216.0			 8.0 9.6

Folate, RBC folate, and PLP in nmol/L, cobalamin in pmol/L, and homocysteine in µmol/L
Abbreviations: RBC Folate – red blood cell folate, PLP – pyridoxal-5-phosphate, P – percentile, y – years.

References

1. Klepp KI, Perez-Rodrigo C, De Bourdeaudhuij I, Due PP, Elmadfa I, Haraldsdottir J, Konig J, Sjostrom M, Thorsdottir I, Vaz de Almeida MD, Yngve A, Brug J. Promoting fruit and vegetable consumption among European schoolchildren: rationale, conceptualization and design of the pro children project. Ann Nutr Metab 2005;49:212-220.

2. Libuda L, Alexy U, Buyken AE, Sichert-Hellert W, Stehle P, Kersting M. Consumption of sugar-sweetened beverages and its association with nutrient intakes and diet quality in German children and adolescents. Br J Nutr 2009;101:1549-1557.

3. Currie C, Gabhain S, Godeau E, Smith R, Curie D, Pickett W, Richter M, Morgan A. Inequalities in young people's health. HBSC international report from the 2005/2006 survey. Copenhagen: World Health Organization, Regional Office for Europe, 2008.

4. Kahn JA, Huang B, Gillman MW, Field AE, Austin SB, Colditz GA, Frazier AL. Patterns and determinants of physical activity in U.S. adolescents. J Adolesc Health 2008;42:369-377.

5. van Mechelen W, Twisk JW, Post GB, Snel J, Kemper HC. Physical activity of young people: the Amsterdam Longitudinal Growth and Health Study. Med Sci Sports Exerc 2000;32:1610-1616.

6. Selhub J. Homocysteine metabolism. Annu Rev Nutr 1999;19:217-246.

7. Papatheodorou L, Weiss N. Vascular oxidant stress and inflammation in hyperhomocysteinemia. Antioxid Redox Signal 2007;9:1941-1958.

8. Stanger O, Herrmann W, Pietrzik K, Fowler B, Geisel J, Dierkes J, Weger M. Clinical use and rational management of homocysteine, folic acid, and B vitamins in cardiovascular and thrombotic diseases. Z Kardiol 2004;93:439-453.

9. De Laet C, Wautrecht JC, Brasseur D, Dramaix M, Boeynaems JM, Decuyper J, Kahn A. Plasma homocysteine concentration in a Belgian school-age population. Am J Clin Nutr 1999;69:968-972.

10. Rauh M, Verwied S, Knerr I, Dorr HG, Sonnichsen A, Koletzko B. Homocysteine concentrations in a German cohort of 500 individuals: reference ranges and determinants of plasma levels in healthy children and their parents. Amino Acids 2001;20:409-418.

11. Huemer M, Vonblon K, Fodinger M, Krumpholz R, Hubmann M, Ulmer H, Simma B. Total homocysteine, folate, and cobalamin, and their relation to genetic polymorphisms, lifestyle and body mass index in healthy children and adolescents. Pediatr Res 2006;60:764-769.

12. Bates CJ, Mansoor MA, Gregory J, Pentiev K, Prentice A. Correlates of plasma homocysteine, cysteine and cysteinyl-glycine in respondents in the British National Diet and Nutrition Survey of young people aged 4-18 years, and a comparison with the survey of people aged 65 years and over. Br J Nutr 2002;87:71-79.

13. Papoutsakis C, Yiannakouris N, Manios Y, Papaconstantinou E, Magkos F, Schulpis KH, Zampelas A, Matalas AL. The effect of MTHFR(C677T) genotype on plasma homocysteine concentrations in healthy children is influenced by gender. Eur J Clin Nutr 2006;60:155-162.

14. Papandreou D, Mavromichalis I, Makedou A, Rousso I, Arvanitidou M. Reference range of total serum homocysteine level and dietary indexes in healthy Greek schoolchildren aged 6-15 years. Br J Nutr 2006;96:719-724.

15. Pinhas-Hamiel O, Doron-Panush N, Reichman B, Nitzan-Kaluski D, Shalitin S, Geva-Lerner L. Obese children and adolescents: a risk group for low vitamin B12 concentration. Arch Pediatr Adolesc Med 2006;160:933-936.

16. Hogeveen M, van Beynum I, van Rooij A, Kluijtmans L, den Heijer M, Blom H. Methylmalonic acid values in healthy Dutch children. Eur J Nutr 2008;47:26-31.

17. van Beynum IM, den Heijer M, Thomas CM, Afman L, Oppenraay-van Emmerzaal D, Blom HJ. Total homocysteine and its predictors in Dutch children. Am J Clin Nutr 2005;81:1110-1116.

18. Gil Prieto R, Esteban Hernandez J, Hernandez Barrera V, Cano B, de Oya M, Gil de Miguel A. [Serum vitamin B12 levels in an adolescent population in Madrid]. An Pediatr (Barc) 2008;68:474-480.

19. Al-Tahan J, Sola R, Ruiz JR, Breidenassel C, Garcia-Fuentes M, Moreno L, Castillo MJ, Pietrzik K, Gonzalez-Gross M. Methylenetetrahydrofolate reductase 677CT polymorphism and cobalamin, folate, and homocysteine status in Spanish adolescents. Ann Nutr Metab 2008;52:315-321.

20. Raslova K, Bederova A, Gasparovic J, Blazicek P, Smolkova B. Effect of diet and 677 C-->T 5, 10-methylenetetrahydrofolate reductase genotypes on plasma homocyst(e)ine concentrations in slovak adolescent population. Physiol Res 2000;49:651-658.

21. Shen M-H, Chu N-F, Wu D-M, Chang J-B. Plasma homocysteine, folate and vitamin B12 levels among school children in Taiwan: The Taipei Children Heart Study. Clinical Biochemistry 2002;35:495-498.

22. Ganji V, Kafai MR. Population references for plasma total homocysteine concentrations for U.S. children and adolescents in the post-folic acid fortification era. J Nutr 2005;135:2253-2256.

23. Ganji V, Kafai MR. Trends in serum folate, RBC folate, and circulating total homocysteine concentrations in the United States: analysis of data from National Health and Nutrition Examination Surveys, 1988-1994, 1999-2000, and 2001-2002. J Nutr 2006;136:153-158.

24. Osganian SK, Stampfer MJ, Spiegelman D, Rimm E, Cutler JA, Feldman HA, Montgomery DH, Webber LS, Lytle LA, Bausserman L, Nader PR. Distribution of and factors associated with serum homocysteine levels in children: Child and Adolescent Trial for Cardiovascular Health. JAMA 1999;281:1189-1196.

25. Kluijtmans LA, Young IS, Boreham CA, Murray L, McMaster D, McNulty H, Strain JJ, McPartlin J, Scott JM, Whitehead AS. Genetic and nutritional factors contributing to hyperhomocysteinemia in young adults. Blood 2003;101:2483-2488.

26. Elshorbagy AK, Oulhaj A, Konstantinova S, Nurk E, Ueland PM, Tell GS, Nygard O, Vollset SE, Refsum H. Plasma creatinine as a determinant of plasma total homocysteine concentrations in the Hordaland Homocysteine Study: use of statistical modeling to determine reference limits. Clin Biochem 2007;40:1209-1218.

27. Fonseca V, Guba SC, Fink LM. Hyperhomocysteinemia and the endocrine system: implications for atherosclerosis and thrombosis. Endocr Rev 1999;20:738-759.

28. Andersson A, Hultberg B, Brattstrom L, Isaksson A. Decreased serum homocysteine in pregnancy. Eur J Clin Chem Clin Biochem 1992;30:377-379.

29. Atamer A, Demir B, Bayhan G, Atamer Y, Ilhan N, Akkus Z. Serum levels of leptin and homocysteine in women with polycystic ovary syndrome and its relationship to endocrine, clinical and metabolic parameters. J Int Med Res 2008;36:96-105.

30. Kerr MA, Livingstone B, Bates CJ, Bradbury I, Scott JM, Ward M, Pentieva K, Mansoor MA, McNulty H. Folate, related B vitamins, and homocysteine in childhood and adolescence: potential implications for disease risk in later life. Pediatrics 2009;123:627-635.

31. Morris MS, Picciano MF, Jacques PF, Selhub J. Plasma pyridoxal 5'-phosphate in the US population: the National Health and Nutrition Examination Survey, 2003-2004. Am J Clin Nutr 2008;87:1446-1454.

32. Chang SJ, Hsiao LJ, Hsuen SY. Assessment of vitamin B-6 estimated average requirement and recommended dietary allowance for adolescents aged 13-15 years using vitamin B-6 intake, nutritional status and anthropometry. J Nutr 2003;133:3191-3194.

33. Chang SJ, Hsiao LJ, Lee YC, Hsuen SY. Vitamin B6 status assessment in relation to dietary intake in high school students aged 16-18 years. Br J Nutr 2007;97:764-769.

34. Bjorke Monsen AL, Refsum H, Markestad T, Ueland PM. Cobalamin Status and Its Biochemical Markers Methylmalonic Acid and Homocysteine in Different Age Groups from 4 Days to 19 Years. Clin Chem 2003;49:2067-2075.

35. Vilaseca MA, Moyano D, Ferrer I, Artuch R. Total homocysteine in pediatric patients. Clin Chem 1997;43:690-692.

36. Reddy MN. Reference ranges for total homocysteine in children. Clinica Chimica Acta 1997;262:153-155.

37. Bau AM, Ernert A, Schenk L, Wiegand S, Martus P, Grüters A, Krude H. Is there a further acceleration in the age at onset of menarche? A cross-sectional study in 1840 school children focusing on age and bodyweight at the onset of menarche. Eur J Endocrinol 2009;160:107-113.

38. Freedman DS, Khan LK, Serdula MK, Dietz W, Srinivasan SR, Berenson GS. Relation of age at menarche to race, time period, and anthropometric dimensions: the Bogalusa Heart Study. Pediatrics 2002;110:e43.

39. Brasileiro RS, Escrivao MA, Taddei JA, D'Almeida V, Ancona-Lopez F, Carvalhaes JT. Plasma total homocysteine in Brazilian overweight and non-overweight adolescents: a case-control study. Nutr Hosp 2005;20:313-319.

40. Kant AK. Reported consumption of low-nutrient-density foods by American children and adolescents: nutritional and health correlates, NHANES III, 1988 to 1994. Arch Pediatr Adolesc Med 2003;157:789-796.

41. Sichert-Hellert W, Kersting M, Manz F. Changes in time-trends of nutrient intake from fortified and non-fortified food in German children and adolescents--15 year results of the DONALD study. Dortmund Nutritional and Anthropometric Longitudinally Designed Study. Eur J Nutr 2001;40:49-55.

42. Kant AK. Consumption of energy-dense, nutrient-poor foods by adult Americans: nutritional and health implications. The third National Health and Nutrition Examination Survey, 1988-1994. Am J Clin Nutr 2000;72:929-936.

43. Narin F, Atabek ME, Karakukcu M, Narin N, Kurtoglu S, Gumus H, Coksevim B, Erez R. The association of plasma homocysteine levels with serum leptin and apolipoprotein B levels in childhood obesity. Ann Saudi Med 2005;25:209-214.

44. Jacques PF, Selhub J, Bostom AG, Wilson PW, Rosenberg IH. The effect of folic acid fortification on plasma folate and total homocysteine concentrations. N Engl J Med 1999;340:1449-1454.

45. Herbert V. Experimental nutritional folate deficiency in man. Trans Assoc Am Physicians 1962;75:307-320.

46. Lui A, Lumeng L, Aronoff GR, Li TK. Relationship between body store of vitamin B6 and plasma pyridoxal-P clearance: metabolic balance studies in humans. J Lab Clin Med 1985;106:491-497.

47. Beck WS. Neuropsychiatric consequences of cobalamin deficiency. Adv Intern Med 1991;36:33-56.

48. Herzlich B, Herbert V. Depletion of serum holotranscobalamin II. An early sign of negative vitamin B12 balance. Lab Invest 1988;58:332-337.

49. Rumsey SE, Hokin B, Magin PJ, Pond D. Macrocytosis--an Australian general practice perspective. Aust Fam Physician 2007;36:571-572.

50. Hercberg S, Preziosi P, Galan P. Iron deficiency in Europe. Public Health Nutr 2001;4:537-545.

51. England JM, Ward SM, Down MC. Microcytosis, anisocytosis and the red cell indices in iron deficiency. Br J Haematol 1976;34:589-597.

4 Homocysteine concentrations are not influenced by physical activity, cardiovascular fitness, and fatness in European adolescents

4.1 Abstract

Background: As shown in adults, homocysteine concentrations and the communicable factors PA, CVF, as well as fatness might influence each other. Inconsistent results have been found regarding this association in studies with adolescents. Thus, the aim was to examine the association between homocysteine levels and PA, CVF, and fatness in European adolescents.

Methods: In European adolescents (n=1097, 14.9±1.2 years, 53%♀, MTHFR 677 genotype: CC 41%, CT 45%, TT 14%) participating in the multicenter HELENA-CSS PA (accelerometer), CVF (20-m shuttle run test), and fatness (skinfold thicknesses and body fat) were assessed; plasma folate, cobalamin, and homocysteine concentrations were measured (competitive immunoassays, CVs: intraassay 5-7%, interassay 8-13%). Multiple regressions examined the association between tHcy and PA, CVF, and fatness (confounders: age, maturity, folate, cobalamin, creatinine, smoking, supplement use, and MTHFR 677C/T genotype).

Results: Compared with males (t-test, P<0.001), females displayed significantly lower PA (134.6±36.2 vs 158.1±44.6 min/d), CVF (34.7±5.8 vs 50.5±8.9 mL O_2 /kg/min) and greater skinfold thickness (102.8±35.7 vs 76.0±39.2 mm) and body fat (27.5±7.6 vs 21.1±10.6%). Homocysteine (♂: 8.0±4.6 vs ♀: 6.8±2.5 µmol/L) and cobalamin (♂: 329.1±125.9 vs ♀: 368.8±155.8 pmol/L) concentrations varied between genders (t-test, P<0.001), but folate was similar (♂:18.5±10.4 vs ♀: 19.0±10.5 nmol/L). Homocysteine concentrations were not significantly associated with PA, CVF, or fatness (P>0.05) after controlling for potential confounders, neither in males nor females.

Conclusion: Homocysteine concentrations in European adolescents included in the HELENA Study could not be related to PA, CVF, or fatness.

4.2 Introduction

The sulphur-containing amino acid homocysteine is a regular intermediate in the methionine cycle [1]. Circulating concentrations of homocysteine are generally low in the healthy population (<14µmol/L) due to either a rapid remethylation to methionine or by degradation through the transsulphuration pathway. Elevated homocysteine concentrations are seen as a biomarker of increased oxidative stress which is associated with an increased risk for endothelial damage [2] and cardiovascular diseases [3]. Moreover, unphysiologically high homocysteine concentrations may contribute to the development of dementia and Alzheimer's disease [4, 5], osteoporosis [6-8], and oral clefts [9, 10].

Individual homocysteine concentrations depend on non-communicable factors like age, gender, and genetics [11-16], but can be effectively modified by lifestyle, especially eating behaviour. In adults, inadequate folate and vitamin B_{12} intake [13, 15-18], excessive alcohol intake, smoking, and hyper-energetic nutrition resulting in obesity can increase homocysteine above acceptable concentrations (≥14µmol/L) [18-20]. In children and adolescents, similar results have been observed [21]. The effect of PA and fitness in adults has been reported in few studies. On the one hand, lack of PA might worsen the situation [22] and on the other hand, subjects with a highly physically active lifestyle (>758 minutes per week) might have increased homocysteine concentrations due to elevated vitamin requirements [23].

Whether PA and CVF are associated with homocysteine concentrations already in childhood and adolescence is not clear. In a small sample of Spanish adolescents from the AVENA study (Alimentación y Valoración del Estado Nutricional de los Adolescentes) an inverse association between homocysteine and CVF was observed in females [15]. In contrast, results from the Swedish part of the European Youth Heart Study (EYHS) do not support these previous findings [16]. The purpose of this study was, thus, to examine the association of homocysteine concentrations with objectively measured PA, CVF and fatness after controlling for potential confounders in a large sample of European adolescents.

4.3 Methods

A description of the study design and implementation is given in the general methodology. Below, the assessment of PA, CVF and anthropometry is described.

4.3.1 Assessment of physical activity

A uni-axial accelerometer (Actigraph MTI, model GT1M, Manufacturing Technology Inc., Fort Walton Beach, FL, USA) was used to assess PA. Adolescents were instructed to place the monitor underneath the clothing, at their lower back, using an elastic waist band and to wear it for seven consecutive days. They were also instructed to wear the accelerometer at all times except during water-based activities and periods of sleep. At least three days of recording with a minimum of eight hours registration per day was set as an inclusion criterion; the time-sampling interval was 15 seconds. A measure of total volume of activity was expressed as the sum of recorded counts per epoch divided by the total daily registered time in minutes. The time engaged in moderate PA and vigorous PA was calculated and presented as the average time per day during the entire recording. The time engaged at moderate PA [3-6 metabolic equivalents (METs)] was calculated based upon a blanket cut-off of 2,000 counts per minute − approximately equivalent to the intensity of a brisk walk (4.5 km/h). Periods of vigorous PA (>6 METs) were based upon a blanket cut-off of 4,000 counts per minute. Also, the time spent in at least moderate intensity level (>3 METs) was calculated as the total time spent in moderate and vigorous physical activity (MVPA, min/day). Each minute spent above the specific cut-off was summarised in the corresponding intensity level group. Time spent in light and low PA was defined as the sum of time per day in which counts per minute were <2000 and <100, respectively. Within this study the sum of minutes spent in low, moderate, and vigorous PA was used and is hereafter called total PA.

4.3.2 Assessment of cardiovascular fitness

Cardiovascular fitness was assessed by a 20-m shuttle run test. Adolescents were instructed to run in a straight line and to pace themselves according to the audio signals emitted from a pre-recorded cassette tape. The initial speed was 8.5 km/h, which was increased by 0.5 km/h per minute (one minute equal to one stage). The tape used was calibrated over one minute. The test was finished when the subject either failed to reach the end lines concurrent with the audio signals on two consecutive occasions or stopped because of fatigue. Cardiovascular fitness was qualified as the number of stages completed (precision of 0.5 steps). In addition, to facilitate comparison with previous studies maximal oxygen consumption (VO_2max, mL O_2 /kg/min) was estimated using Ruiz's equation [24].

4.3.3 Anthropometry

The anthropometric methodological approach within the HELENA-CSS was described in detail by Nagy et al. [25]. Briefly, body weight was measured in kg using a standard beam balance (Seca, precision 100 g, range 0-150 kg). Height was measured in cm using a precision stadiometer (Seca, precision 0.2 cm, range 70-200 cm). Body mass index was calculated with the equation weight in kg divided by height in m squared (kg/m^2). Skinfold thicknesses (triceps, biceps, subscapular, suprailiac, thigh, and calf) were repeatedly measured at the left side of the body using a Holtain caliper (Crymych, UK, range 0–40 mm) and the mean calculated. Only adolescents having data of all six skinfolds (hereafter referred to as 'skinfold thickness') were included for the analyses. Percent body fat was calculated with the use of the following equation based upon Slaughter et al. [26]: fat (%) = 0.61*(triceps skinfold in mm + calf skinfold in mm) + 5.1 for females and fat (%) = 0.735*(triceps skinfold in mm + calf skinfold in mm) + 1 for males.

4.3.4 Statistics

Blood homocysteine, folate, vitamin B_{12} concentrations, levels of CVF (stages and VO_2max), and BMI were normalised by natural logarithm transformation.

To test gender specific differences student's t-test was used for metric variables and X^2-test for categorical variables. A bivariate correlation analysis was performed to check the associations between homocysteine and PA, CVF (stages and VO_2max), and fatness (BMI, skinfold thickness, and body fat) by gender. Separate multiple regressions were executed split by gender to study the relation between homocysteine and PA, fitness and fatness after controlling for potential confounders: age, maturity, folate, vitamin B_{12}, creatinine, smoking, supplement use, and MTHFR 677C/T.

4.4 Results

Table 4.1 (page 81) reveals the gender specific characteristics of the study population (n=1097). Females (n=584, 53%) were in a more advanced pubertal stage and displayed significantly greater skinfold thickness and body fat compared with males. Physical activity as well as CVF expressed in stages and VO_2max was significantly higher in males than females. Eleven percent of the adolescents smoked every day, 4% at least once a week, 5%

less than once a week, 80% didn't smoke. A vitamin and/or mineral supplement was taken by 11% of the adolescents. Smoking behaviour and supplement use did not vary between genders. Table 4.2 (page 82) presents bivariate correlations between homocysteine concentrations and PA, CVF (stages and VO_2max), BMI, skinfold thickness, and body fat. In males, PA was negatively associated with homocysteine concentrations. In females, CVF variables were negatively and BMI was positively associated with homocysteine. Table 4.3 (page 83) shows the gender specific relationship between homocysteine and PA, fitness, and fatness after checking for age, maturity, folate, vitamin B_{12}, and creatinine concentrations, smoking, supplement use, and MTHFR 677C/T polymorphism, respectively. Variation in homocysteine concentrations could not be explained by PA, CVF, or parameters of fatness.

4.5 Discussion

Strengths of the present research are the inclusion of a relatively large number of adolescents and several potential confounders including the MTHFR 677C/T genotype. Cardiovascular fitness was objectively measured by the 20-m shuttle run test and herewith VO_2max was estimated for a better comparability with other studies. The indirect measurement of VO_2max is feasible within epidemiologic studies: it is not only practical, time-efficient, low in cost and equipment requirements, but can also performed on large numbers of adolescents simultaneously [27]. In addition to BMI, body composition was measured by skinfold thickness which is suggested to be a better predictor of body fatness in later life than BMI [28].

Table 4.4 (page 84) summarises results from several studies discussed in this investigation. To encourage an active lifestyle, the international and national public health organisations recommend at least 30 minutes of PA with at least moderate intensity on most days per week [29, 30]. The WHO proposes that school-aged children should complete at least 60 minutes of moderate to vigorous intensity PA each day to ensure a healthy development [31]. Adolescents participating in the HELENA Study achieved 145.0±41.8 min/d. However, within this study, low, moderate, and vigorous PA were pooled together. Physical activity on a moderate to vigorous intensity will probably form the smaller part. Few studies targeted the relationship of PA with homocysteine; most of them were carried out on adults. The first hint that regular PA may significantly reduce homocysteine concentrations was provided by a study performed on 21 overweight women with polycystic ovary syndrome aged 29.7±6.8 years [32]. Brisk walking three times a week over a period of six months led to decreased

homocysteine concentrations (baseline: 10.06±3.22 vs after exercise: 7.36±1.96 µmol/L, generalised linear hierarchical analysis: P<0.001) and increased VO_2max values compared with baseline values (baseline: 2.50±0.31 vs after exercise: 2.60±0.35 L/min, P=0.019). Studies with apparently healthy adults did not clearly support this hypothesis. Independent of B-vitamin status, homocysteine concentrations were similar in physically less active (≤420 min/week, n=40) compared with active (>420 min/week, n=36) adults aged 26±5 years (low PA: 7.5±1.6 vs high PA: 7.7±1.6 µmol/L, P=0.36). Moreover, the extremely high active group (>750 min/week, n=11) showed significantly higher homocysteine concentrations compared with extremely low physically active participants (<130 min/week, n=9) (extremely high PA shown as mean (range): 8.6(6.1-12.3) vs extremely low PA: 6.9(2.9-9.1) µmol/L, t-test: P<0.001) [23]. Results from the Women's Health Study including 27,158 women aged 54.7±7.1 years showed a significant association between homocysteine concentrations and quintiles of PA without adjustment for B-vitamin status (highest quintile (>1574 kcal/week) shown as median: 10.4 vs lowest quintile (<145 kcal/week): 10.8µmol/L, Cusick's nonparametric test for trend: P<0.001). Odds ratios for the association of quintiles of PA with homocysteine concentrations did not significantly differ (P for linear trend =0.49) [33]. A one year follow up based on a sample of 915 Danish adults aged 30-60 years indicated that homocysteine may not be reduced by general lifestyle interventions such as quitting smoking habits, reducing the consumption of alcoholic beverages, coffee and/or tea, increasing PA, or modifying dietary habits [34]. With regards to children and adolescents the EYHS (n=680, 9-10 and 15-16 years) [16] was the first to examine the association between homocysteine and PA. However, no significant results were found (multiple regressions: P=0.30 in adolescents) after adjustment for gender, pubertal development, socioeconomic status, folate and vitamin B_{12} intake, and MTHFR 677C/T genotype being in accordance with our findings.

Cardiovascular fitness is defined as the ability of active skeletal muscle to utilise oxygen during exercise. Recent data suggest that fitness is an important marker for several health outcomes in young people like obesity, cardiovascular risk, skeletal and psychological health [35]. Changes in tissues and systemic vasculature may deteriorate the physiological capacity and subsequently may also negatively affect the CVF. To some extent, elevated homocysteine concentrations may attribute to these pathological changes by the generation of reactive oxygen species and impairing the nitric oxide production and bioavailability [2]. High homocysteine concentrations have been associated with poor CVF in women participating in the NHANES (n=1,444, aged 20-49 years) with available measures of CVF and homocysteine [36]. Multiple logistic regressions were done for homocysteine as continuous variable

(P=0.003), as quartiles (P<0.001) as well as odds ratios (P<0.001) after adjustment for potential confounders including folate and vitamin B_{12} concentrations. In former male athletes aged 35-62 years, homocysteine concentrations were associated with current levels of PA [37]. Exercising ex-athletes showed lower homocysteine concentrations than sedentary ex-athletes (PA (n=52): 9.43±2.12 vs sedentary (n=25): 12.32±4.49 µmol/L, ANOVA: P<0.001). Regarding adolescents, CVF was significantly and inversely associated with homocysteine concentrations in Spanish female adolescents from the AVENA Study (n=156, aged 14.8±1.4 years) [15]. Regression models included folate and vitamin B_{12} concentrations as well as MTHFR 677C/T genotype (P=0.007). In contrast, results from the EYHS did not indicate a significant association between homocysteine and fitness in children and adolescents [16] which is in line with our results. The difference between results from the AVENA and the EYHS as well as our study might be due to the smaller sample size, a power analysis was not performed. Eventually, it must be considered that usually healthy children and adolescents do not show cardiovascular pathologies. Higher homocysteine concentrations in adults due to advanced age and longer exposure of homocysteine on the tissue might be an explanation for differences between adults and adolescents.

Associations between BMI and homocysteine concentrations were frequently investigated in adolescents. In an Austrian sample of 264 children and adolescents aged 2-17 years, significant, positive correlations were found between homocysteine levels and BMI (r=0.44, P<0.001) [38]. The cross-sectional Taipei Children Heart Study including 1,235 adolescents aged 12-15 years revealed a positive association between homocysteine quintiles and BMI only in males after adjusting for age (P for trend <0.001) [39]. Narin et al. examined 40 obese and 20 non-obese children and adolescents aged 7-17 years. Obese had significantly higher homocysteine levels than non-obese from the age- and sex-matched control group (obese: 14.3±11.8 µmol/L vs non-obese: 8.7±5.9 µmol/L, P=0.017) [40]. Osganian et al. found a positive association between homocysteine concentrations and BMI in 3,524 adolescents aged 13-14 years, but after adjustment for potential confounders, particularly blood vitamin concentrations, which had the strongest effect the association was no longer significant [41]. These findings stress the importance of adjusting for B-vitamin status when examining associations between homocysteine and other parameters. Associations between body fat and homocysteine concentrations were hardly investigated in adolescents. Gallistl et al. examined homocysteine concentrations before and after a three-week weight loss program in 37 obese girls aged 12.0±1.8 years and 19 obese boys aged 11.9±1.7 years (BMI, ♀: 26.9±5.25, ♂: 26.2±5.2 kg/m²). Instead of body fat, lean body mass (LBM) was chosen that is calculated by

subtracting body fat from body weight. Multiple linear regression analysis indicated that only baseline LBM was positively associated with homocysteine concentrations (P=0.002) [42]. We adjusted for B-vitamin status and MTHFR 677C/T genotype and did not find an association between homocysteine concentrations and fatness (expressed as BMI, skinfold thickness, and body fat). This is in accordance with findings reported by several European cross-sectional studies including the EYHS [16], the British National Diet and Nutrition Survey (n=922, 4-18 years) [43], a Belgian (n=647, 5-19 years) [44], a Spanish (n=165, 13-18.5 years) [45], and a Greek study (n=524, 6-15 years) [46], as well as a Brazilian case control study (n=239, 5-19 years) [47].

In conclusion, the results of the present study suggest that objectively measured PA, CVF, and fatness were not associated with homocysteine levels in European adolescents, even after controlling for several potential confounders including the MTHFR 677C/T genotype.

Table 4.1: Gender specific characteristics of the study sample

	All	Male	Female	*P*-value
Age in years (n)	14.9±1.2 (1097)	14.9±1.3 (513)	14.9±1.2 (584)	0.810°
Maturity in stages % (n)				0.019#
I	0.7 (7)	1.5 (7)	0 (0)	
II	5.8 (61)	7.7 (37)	4.1 (24)	
III	19.7 (204)	19.3 (91)	20.0 (113)	
IV	43.3 (412)	41.6 (184)	44.8 (228)	
V	30.5 (284)	29.9 (128)	31.1 (156)	
MTHFR 677C/T in % (n)				
CC	41.3 (437)	40.1 (201)	42.3 (236)	0.222#
CT	44.5 (469)	46.8 (237)	42.5 (232)	
TT	14.2 (150)	13.1 (66)	15.2 (84)	
Physical activity in min/day (n)	145.0±41.8 (730)	158.1±44.6 (324)	134.6±36.2 (406)	<0.001°
Cardiovascular fitness in stages (n)	4.79±2.75 (841)	6.32±2.78 (405)	3.36±1.78 (436)	<0.001°
Cardiovascular fitness in mL O_2 /kg/min (n)	42.3±10.9 (841)	50.5±8.9 (405)	34.70±5.77 (436)	<0.001°
Body mass index in kg/m² (n)	21.4±3.6 (1097)	21.4±3.8 (513)	21.3±3.4 (584)	0.689°
Skinfold thickness in mm (n)	90.2±39.6 (1023)	76.0±39.2 (480)	102.8±35.7 (543)	<0.001°
Body fat in % (n)	24.5±9.7 (1054)	21.1±10.6 (494)	27.5±7.6 (560)	<0.001°

Parameters are shown as mean ± sd.
° student's t-test
X^2-test
Abbreviations: MTHFR – methylenetetrahydrofolate reductase.

Table 4.2: Bivariate correlations between homocysteine and independent variables by gender

	Males		Females	
	Pearson	*P*-value	Pearson	*P*-value
Physical activity in min/day	-0.223	0.000	-0.027	>0.05
Cardiovascular fitness in stages	0.019	>0.05	-0.112	0.024
Cardiovascular fitness in mL O_2 /kg/min	-0.029	>0.05	-0.099	0.046
Body mass index in kg/m²	0.084	>0.05	0.114	0.007
Skinfold thickness in mm	-0.079	>0.05	0.045	>0.05
Bodyfat in %	-0.086	>0.05	0.022	>0.05

Table 4.3: Standardised multiple regression coefficients (**ß**), standard error (SE), and semipartial correlation (sr) examining the association of physical activity, cardiovascular fitness, and fatness with homocysteine after controlling for age, maturity, folate, vitamin B_{12} and creatinine concentrations, smoking, supplement use, and MTHFR 677C/T polymorphism

	Males				Females			
	ß	SE	sr	P-value	ß	SE	sr	P-value
Physical activity in min/day	-0.069	0.000	-0.083	>0.05	-0.020	0.000	-0.025	>0.05
Cardiovascular fitness in stages	0.062	0.035	0.079	>0.05	-0.034	0.030	-0.040	>0.05
Cardiovascular fitness in mL O_2 /kg/min	0.062	0.100	0.078	>0.05	-0.014	0.106	-0.016	>0.05
Body mass index in kg/m²	-0.029	0.097	-0.037	>0.05	0.004	0.091	0.004	>0.05
Skinfolds thickness in mm	-0.033	0.000	-0.042	>0.05	-0.005	0.000	-0.005	>0.05
Bodyfat in %	-0.010	0.036	-0.013	>0.05	-0.011	0.052	-0.013	>0.05

Table 4.4: Summary of results from several authors for associations between physical activity, cardiovascular fitness, and fatness and homocysteine concentrations

Author	Study design	Factors	Homocysteine
Randeva et al. [32]	Intervention, 21 overweight women with polycystic ovary syndrome, 29.7±6.8y, brisk walking 3x per week over six months	PA	baseline: 10.06±3.22 vs after exercise: 7.36±1.96 µmol/L, P<0.001
Joubert et al. [23]	less active (<420 min/week, n=40) compared with active (>420 min/week, n=36) adults, 26±5y, mean (range)	PA	low PA: 7.5±1.6 vs high PA: 7.7±1.6 µmol/L, P=0.36 extremely high PA (>750 min/week, n=11): 8.6 (6.1-12.3) vs extremely low PA (<130 min/week, n=9): 6.9 (2.9-9.1) µmol/L, P<0.001
Mora et al. [33]	Women's Health Study, n=27,158, 54.7±7.1y, quintiles of PA, median	PA	highest quintile (>1574 kcal/week): 10.4 vs lowest quintile (<145 kcal/week): 10.8µmol/L, P<0.001 Odds ratios for the association of quintiles of PA with homocysteine concentrations n.s.
Husemoen et al. [34]	general lifestyle intervention, one-year follow up, n=915, 30-60y	PA	n.s.
Ruiz et al. [16]	EYHS, n=680, 9-10 and 15-16y, adjustments for gender, maturity, socioeconomic status, folate and vitamin B_{12} intake, and MTHFR 677C/T genotype	PA	n.s.
		CVF	n.s.
		BMI	n.s.
Kuo et al. [36]	NHANES, n=1444, 20-49y, multiple logistic regressions, adjustments include folate and vitamin B_{12} concentrations	CVF	as continuous variable (P=0.003), as quartiles (P<0.001) as odds ratios (P<0.001)
Unt et al. [37]	Currently active ex-athletes (n=52) compared with sedentary ex-athletes (n=25), 35-62y	CVF	active: 9.43±2.12 vs sedentary: 12.32±4.49 µmol/L, P<0.001
Ruiz et al. [15]	AVENA study, n=156, 14.8±1.4y, regression models included folate and vitamin B_{12} levels as well as MTHFR 677C/T genotype	CVF	- in females, P=0.007
Huemer et al. [48]	n=264, 2-17y, Austria, mean ± sd	BMI	r=0.09, P=0.001, after adjustment P>0.05
Shen et al. [39]	Taipei Children Heart Study, n=1235, 12-15y, Taiwan, mean ± sd	BMI	m: sign. corr.
Osganian et al. [41]	CATCH, n=3321, 14.1±0.5y, US, mean ± sd	BMI	+, after adjustment for blood vitamin concentrations P>0.05
Gallistl et al. [42]	three-week weight loss intervention, 37 obese females and 19 obese males, 11.9±1.7y	LBM	+ baseline LBM, P=0.002
Bates et al. [43]	NDNS, n=1193, 4-18y, UK	BMI	n.s.

Author	Study design	Factors	Homocysteine
De Laet et al. [44]	n=647, 5-19y, Belgium, GM±sd	BMI	n.s.
Al-Tahan et al. [45]	n=165, 13-18.5y, Spain, median (2.5^{th}-97.5^{th} percentile)	BMI	n.s.
Papandreou et al. [46]	n=524, 6-15y, Greece	BMI	n.s.
Brasileiro et al. [47]	case control study, n=239, 5-19y, Brazil	BMI	n.s.

Abbreviations: PA – physical activity, CVF – cardiovascular fitness, BMI – body mass index, MTHFR – methylenetetrahydrofolate reductase, n.s. – not significant, + –positively associated, - – negatively associated.

References

1. Selhub J. Homocysteine metabolism. Annu Rev Nutr 1999;19:217-246.

2. Papatheodorou L, Weiss N. Vascular oxidant stress and inflammation in hyperhomocysteinemia. Antioxid Redox Signal 2007;9:1941-1958.

3. Stanger O, Herrmann W, Pietrzik K, Fowler B, Geisel J, Dierkes J, Weger M. Clinical use and rational management of homocysteine, folic acid, and B vitamins in cardiovascular and thrombotic diseases. Z Kardiol 2004;93:439-453.

4. Haan MN, Miller JW, Aiello AE, Whitmer RA, Jagust WJ, Mungas DM, Allen LH, Green R. Homocysteine, B vitamins, and the incidence of dementia and cognitive impairment: results from the Sacramento Area Latino Study on Aging. Am J Clin Nutr 2007;85:511-517.

5. Seshadri S, Beiser A, Selhub J, Jacques PF, Rosenberg IH, D'Agostino RB, Wilson PW, Wolf PA. Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. N Engl J Med 2002;346:476-483.

6. McLean RR, Jacques PF, Selhub J, Tucker KL, Samelson EJ, Broe KE, Hannan MT, Cupples LA, Kiel DP. Homocysteine as a predictive factor for hip fracture in older persons. N Engl J Med 2004;350:2042-2049.

7. Gjesdal CG, Vollset SE, Ueland PM, Refsum H, Meyer HE, Tell GS. Plasma homocysteine, folate, and vitamin B 12 and the risk of hip fracture: the hordaland homocysteine study. J Bone Miner Res 2007;22:747-756.

8. van Meurs JB, Dhonukshe-Rutten RA, Pluijm SM, van der Klift M, de Jonge R, Lindemans J, de Groot LC, Hofman A, Witteman JC, van Leeuwen JP, Breteler MM, Lips P, Pols HA, Uitterlinden AG. Homocysteine levels and the risk of osteoporotic fracture. N Engl J Med 2004;350:2033-2041.

9. Little J, Gilmour M, Mossey PA, Fitzpatrick D, Cardy A, Clayton-Smith J, Hill A, Duthie SJ, Fryer AE, Molloy AM, Scott JM. Folate and clefts of the lip and palate--a U.K.-based case-control study: Part II: Biochemical and genetic analysis. Cleft Palate Craniofac J 2008;45:428-438.

10. Knott L, Hartridge T, Brown NL, Mansell JP, Sandy JR. Homocysteine oxidation and apoptosis: a potential cause of cleft palate. In Vitro Cell Dev Biol Anim 2003;39:98-105.

11. Gonzalez-Gross M, Marcos A, Pietrzik K. Nutrition and cognitive impairment in the elderly. Br J Nutr 2001;86:313-321.

12. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJ, den Heijer M, Kluijtmans LA, van den Heuvel LP, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10:111-113.

13. Papoutsakis C, Yiannakouris N, Manios Y, Papaconstantinou E, Magkos F, Schulpis KH, Zampelas A, Matalas AL. The effect of MTHFR(C677T) genotype on plasma homocysteine concentrations in healthy children is influenced by gender. Eur J Clin Nutr 2006;60:155-162.

14. Balasa VV, Gruppo RA, Glueck CJ, Stroop D, Becker A, Pillow A, Wang P. The relationship of mutations in the MTHFR, prothrombin, and PAI-1 genes to plasma levels of homocysteine, prothrombin, and PAI-1 in children and adults. Thromb Haemost 1999;81:739-744.

15. Ruiz JR, Sola R, Gonzalez-Gross M, Ortega FB, Vicente-Rodriguez G, Garcia-Fuentes M, Gutierrez A, Sjostrom M, Pietrzik K, Castillo MJ. Cardiovascular fitness is negatively associated with homocysteine levels in female adolescents. Arch Pediatr Adolesc Med 2007;161:166-171.

16. Ruiz JR, Hurtig-Wennlof A, Ortega FB, Patterson E, Nilsson TK, Castillo MJ, Sjostrom M. Homocysteine levels in children and adolescents are associated with the methylenetetrahydrofolate reductase 677C>T genotype, but not with physical activity, fitness or fatness: the European Youth Heart Study. Br J Nutr 2007;97:255-262.

17. Selhub J, Jacques PF, Wilson PW, Rush D, Rosenberg IH. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. JAMA 1993;270:2693-2698.

18. Jacques PF, Bostom AG, Wilson PW, Rich S, Rosenberg IH, Selhub J. Determinants of plasma total homocysteine concentration in the Framingham Offspring cohort. Am J Clin Nutr 2001;73:613-621.

19. Nygard O, Refsum H, Ueland PM, Vollset SE. Major lifestyle determinants of plasma total homocysteine distribution: the Hordaland Homocysteine Study. Am J Clin Nutr 1998;67:263-270.

20. Silaste ML, Rantala M, Alfthan G, Aro A, Kesaniemi YA. Plasma homocysteine concentration is decreased by dietary intervention. Br J Nutr 2003;89:295-301.

21. Kerr MA, Livingstone B, Bates CJ, Bradbury I, Scott JM, Ward M, Pentieva K, Mansoor MA, McNulty H. Folate, related B vitamins, and homocysteine in childhood and adolescence: potential implications for disease risk in later life. Pediatrics 2009;123:627-635.

22. Chrysohoou C, Panagiotakos DB, Pitsavos C, Zeimbekis A, Zampelas A, Papademetriou L, Masoura C, Stefanadis C. The associations between smoking, physical activity, dietary habits and plasma homocysteine levels in cardiovascular disease-free people: the 'ATTICA' study. Vasc Med 2004;9:117-123.

23. Joubert LM, Manore MM. The Role of Physical Activity Level and B-Vitamin Status on Blood Homocysteine Levels. Med Sci Sports Exerc 2008.

24. Ruiz JR, Ramirez-Lechuga J, Ortega FB, Castro-Pinero J, Benitez JM, Arauzo-Azofra A, Sanchez C, Sjostrom M, Castillo MJ, Gutierrez A, Zabala M. Artificial neural network-based equation for estimating VO(2max) from the 20m shuttle run test in adolescents. Artif Intell Med 2008.

25. Nagy E, Vicente-Rodriguez G, Manios Y, Beghin L, Iliescu C, Censi L, Phillipp K, Ortega Porcel F, De Vriendt T, Moreno L, Molnar D, group. ObotHs. Harmonization process and reliability assessment of anthropometric measurements in a multicenter study in adolescents. Int J Obes 2008;32:S58-S65.

26. Slaughter MH, Lohman TG, Boileau RA, Horswill CA, Stillman RJ, Van Loan MD, Bemben DA. Skinfold equations for estimation of body fatness in children and youth. Hum Biol 1988;60:709-723.

27. Ruiz JR, Ortega FB, Gutierrez A, Sjostrom M, Castillo MJ. Health-related physical fitness assessment in childhood and adolescence; A European approach based on the AVENA, EYHS and HELENA studies. J Public Health 2006;14:269-277.

28. Nooyens AC, Koppes LL, Visscher TL, Twisk JW, Kemper HC, Schuit AJ, van Mechelen W, Seidell JC. Adolescent skinfold thickness is a better predictor of high body fatness in adults than is body mass index: the Amsterdam Growth and Health Longitudinal Study. Am J Clin Nutr 2007;85:1533-1539.

29. Haskell WL, Lee IM, Pate RR, Powell KE, Blair SN, Franklin BA, Macera CA, Heath GW, Thompson PD, Bauman A. Physical activity and public health: updated recommendation for adults from the American College of Sports Medicine and the American Heart Association. Med Sci Sports Exerc 2007;39:1423-1434.

30. Strong WB, Malina RM, Blimkie CJ, Daniels SR, Dishman RK, Gutin B, Hergenroeder AC, Must A, Nixon PA, Pivarnik JM, Rowland T, Trost S, Trudeau F. Evidence based physical activity for school-age youth. J Pediatr 2005;146:732-737.

31. Global Strategy on Diet, Physical Activity and Health. Physical Activity and Young People.: Word Health Organization, 2009.

32. Randeva HS, Lewandowski KC, Drzewoski J, Brooke-Wavell K, O'Callaghan C, Czupryniak L, Hillhouse EW, Prelevic GM. Exercise decreases plasma total homocysteine in overweight young women with polycystic ovary syndrome. J Clin Endocrinol Metab 2002;87:4496-4501.

33. Mora S, Lee IM, Buring JE, Ridker PM. Association of physical activity and body mass index with novel and traditional cardiovascular biomarkers in women. JAMA 2006;295:1412-1419.

34. Husemoen LL, Thomsen TF, Fenger M, Jorgensen T. Changes in lifestyle and total homocysteine in relation to MTHFR(C677T) genotype: the Inter99 study. Eur J Clin Nutr 2006;60:614-622.

35. Ortega FB, Ruiz JR, Castillo MJ, Sjostrom M. Physical fitness in childhood and adolescence: a powerful marker of health. Int J Obes (Lond) 2008;32:1-11.

36. Kuo HK, Yen CJ, Bean JF. Levels of homocysteine are inversely associated with cardiovascular fitness in women, but not in men: data from the National Health and Nutrition Examination Survey 1999-2002. J Intern Med 2005;258:328-335.

37. Unt E, Zilmer K, Magi A, Kullisaar T, Kairane C, Zilmer M. Homocysteine status in former top-level male athletes: possible effect of physical activity and physical fitness. Scand J Med Sci Sports 2008;18:360-366.

38. Huemer M, Vonblon K, Fodinger M, Krumpholz R, Hubmann M, Ulmer H, Simma B. Total Homocystine, Folate, and Cobalamin, and Their Relation to Genetic Polymorphisms, Lifestyle and Body Mass Index in Healthy Children and Adolescents. Pediatr Res 2006;60:764-769.

39. Shen M-H, Chu N-F, Wu D-M, Chang J-B. Plasma homocysteine, folate and vitamin B12 levels among school children in Taiwan: The Taipei Children Heart Study. Clinical Biochemistry 2002;35:495-498.

40. Narin F, Atabek ME, Karakukcu M, Narin N, Kurtoglu S, Gumus H, Coksevim B, Erez R. The association of plasma homocysteine levels with serum leptin and apolipoprotein B levels in childhood obesity. Ann Saudi Med 2005;25:209-214.

41. Osganian SK, Stampfer MJ, Spiegelman D, Rimm E, Cutler JA, Feldman HA, Montgomery DH, Webber LS, Lytle LA, Bausserman L, Nader PR. Distribution of and factors associated with serum homocysteine levels in children: Child and Adolescent Trial for Cardiovascular Health. JAMA 1999;281:1189-1196.

42. Gallistl S, Sudi KM, Erwa W, Aigner R, Borkenstein M. Determinants of homocysteine during weight reduction in obese children and adolescents. Metabolism 2001;50:1220-1223.

43. Bates CJ, Mansoor MA, Gregory J, Pentiev K, Prentice A. Correlates of plasma homocysteine, cysteine and cysteinyl-glycine in respondents in the British National Diet and Nutrition Survey of young people aged 4-18 years, and a comparison with the survey of people aged 65 years and over. Br J Nutr 2002;87:71-79.

44. De Laet C, Wautrecht JC, Brasseur D, Dramaix M, Boeynaems JM, Decuyper J, Kahn A. Plasma homocysteine concentration in a Belgian school-age population. Am J Clin Nutr 1999;69:968-972.

45. Al-Tahan J, Sola R, Ruiz JR, Breidenassel C, Garcia-Fuentes M, Moreno L, Castillo MJ, Pietrzik K, Gonzalez-Gross M. Methylenetetrahydrofolate reductase 677CT polymorphism and cobalamin, folate, and homocysteine status in Spanish adolescents. Ann Nutr Metab 2008;52:315-321.

46. Papandreou D, Rousso I, Makedou A, Arvanitidou M, Mavromichalis I. Association of blood pressure, obesity and serum homocysteine levels in healthy children. Acta Paediatr 2007;96:1819-1823.

47. Brasileiro RS, Escrivao MA, Taddei JA, D'Almeida V, Ancona-Lopez F, Carvalhaes JT. Plasma total homocysteine in Brazilian overweight and non-overweight adolescents: a case-control study. Nutr Hosp 2005;20:313-319.

48. Huemer M, Vonblon K, Fodinger M, Krumpholz R, Hubmann M, Ulmer H, Simma B. Total homocysteine, folate, and cobalamin, and their relation to genetic polymorphisms, lifestyle and body mass index in healthy children and adolescents. Pediatr Res 2006;60:764-769.

5 Bone mineral density in association with folate, vitamin B_6, vitamin B_{12} status and homocysteine concentrations in Spanish adolescents

5.1 Abstract

Background: As shown in elderly, homocysteine is associated with a higher risk for osteoporosis and fractures. Independent of age, in vitro studies support the hypothesis that folate, vitamin B_{12} status, and homocysteine concentrations might be associated with BMD. In studies with adolescents, inconsistent results have been found. Thus, the aim was to examine the association between B-vitamin and homocysteine concentrations with bone area (BA), bone mineral content (BMC), and BMD in Spanish adolescents.

Methods: In Spanish adolescents (n=114, 14.8±1.0 years, 54%♀, MTHFR 677 genotype: CC 39%, CT 42%, TT 19%) participating in the multicenter HELENA-CSS BA, BMC, and BMD were assessed (DXA); plasma folate, RBC folate, PLP, cobalamin, holo-transcobalamin, and homocysteine concentrations were measured (competitive immunoassays, CVs: intraassay 1-11%, interassay 2-14%). Multiple regressions examined the association of folate, vitamin B_6, vitamin B_{12} status, and homocysteine concentrations with whole body and lumbar spine BA, BMC, and BMD (confounders: sex, age, height, weight, maturity, creatinine, vitamin D, MTHFR 677C/T polymorphism, calcium intake, and PA).

Results: Males showed greater BA (♂: 1967.1±294.3 vs ♀: 1803.8±164.6 cm², t-test: P=0.001), BMC (♂: 2138.3±540.4 vs ♀: 1870.0±298.5 g, P=0.003), and were physically more active (♂: 142.6±35.0 vs ♀: 125.5±32.0 min/d, P=0.013) compared with females. Variations in whole body or lumbar spine BMC and BMD could not be explained by folate, vitamin B_6, vitamin B_{12} status, and homocysteine concentrations (P>0.05) after controlling for potential confounders.

Conclusion: Folate, vitamin B_6, vitamin B_{12} status, and homocysteine concentrations in Spanish adolescents were not associated with BMC or BMD.

5.2 Introduction

Bone mineral density is predominantly influenced by adequate calcium intake, endogenous vitamin D availability, intake and synthesis, and PA throughout the whole life [1]. Based on recent studies in elderly it is hypothesised that also folate and vitamin B_{12} status as well as homocysteine concentrations are associated with BMD [2]. In addition, polymorphisms like the MTHFR 677C/T may play a further role [3]. These hypotheses were motivated by observations in patients with homocystinuria, a severe inborn error of metabolism which is characterised by unphysiologically high concentrations of homocysteine (>100µmol/L). Among other clinical manifestations, patients suffer from premature osteoporosis and fractures [4]. The main nutritional and genetic determinants of homocysteine, that is folate, vitamin B_6, and vitamin B_{12} and the MTHFR 677C/T polymorphism, may also have an independent effect on bone tissue. Independent of age, in vitro studies support the hypothesis that homocysteine disturbs the collagen cross-linking in bone [5]. Low concentrations of cobalamin have been associated with suppressed osteoblast activity [6] and low concentrations of folate, PLP, and cobalamin have been associated with stimulated osteoclast activity [7].

Low BMD and BMC have been associated with low vitamin B_{12} status in former macrobiotic adolescents [8]. The MTHFR 677 TT-genotype was significantly associated with lower spinal BMD within the Avon Longitudinal Study of Parents and Children (ALSPAC) in 3,196 9-year-old children [9]. It remains unclear whether BMD is associated with folate, vitamin B_6, vitamin B_{12} status, and homocysteine concentrations in apparently healthy adolescents. Thus, the purpose of this study was to examine the interaction of B-vitamin status and homocysteine concentrations with BA, BMC, and BMD after controlling for potential confounders in Spanish adolescents.

5.3 Methods

A detailed description of the study design and implementation is given in the general methodology. The measurement of bone-related variables, vitamin D, PA, and calcium intake is described as follows.

5.3.1 Study design and subjects

Data collection took place within a European multicenter CSS that was performed within the 6th European Union (EU) framework programme to assess a "Healthy Lifestyle in Europe by Nutrition in Adolescence" (HELENA) [10]. This work is confined to a subset of 114 adolescents aged 12.5-17.49 years from Saragossa with available data for BMD as well as folate, vitamin B_6, vitamin B_{12} status, and homocysteine concentrations. All protocols and informed consents for this study were reviewed and approved by the Research Ethics Committee of the Government of Aragón in Spain, according to the Declaration of Helsinki 1961 (revision of Edinburgh 2000) and International Conferences on Harmonization for Good Clinical Practice [11].

5.3.2 Dual energy X-ray absorptiometry measurement

The total BA (cm^2) and BMC (g) was measured by DXA using a paediatric version of the software QDR-Explorer (Hologic Corp., Software version 12.4, Waltham, MA); the BMD (g/cm^2) was also calculated. The DXA was calibrated using a lumbar spine phantom as recommended by the manufacturer. Subjects were scanned in supine position and the scans were performed at high resolution [12].

5.3.3 Biochemistry

Vitamin D was analysed with the use of IDS OCTEIA 25-hydroxy vitamin D kit. The kit is an enzyme immunoassay for the quantification of 25-hydroxy vitamin D and other hydroxylated metabolites. Calibrators, controls and samples (25 µL) were diluted with 1 mL biotin-labelled 25-hydroxy vitamin D and thoroughly vortexed for 10 seconds. The diluted samples (200 µL) were incubated for two hours at room temperature (18-25°C) in microtitre wells coated with a highly specific sheep 25-hydroxy vitamin D antibody. All wells were washed three times with 300 µL wash solution; a possible excess of wash solution was removed before proceeding to the next step. Enzyme-labelled avidin (200 µL), in this case horseradish peroxidase binding selectively to complexed biotin, was added using a multichannel pipette to guarantee a simultaneous onset of reaction. The microtitre plate was incubated for 30 minutes at room temperature. Past another wash step (3x, 300 µL), colour is developed by adding 200 µL chromogenic substrate (tetramethylbenzidine, TMB) and after an incubation time of 30 minutes at room temperature. After adding 100 µL stop solution (hydrochloric acid, HCl)

within 30 minutes the absorbance of the stopped reaction mixtures were read in a microtitre plate reader at 450 nm (reference 650 nm). The colour intensity is inversely proportional to the concentration of 25-hydroxy vitamin D. Intra- and interassay CVs were 6.7% and 8.7%, respectively. The sensitivity of vitamin D was ≤5 nmol/L. With respect to specificity the manufacturer observed a cross reactivity of <0.01% between cholecalciferol and 25-hydroxyvitamin D3, and <0.3% between ergocalciferol and 25-hydroxyvitamin D3.

5.3.4 Assessment of physical activity and calcium intake

A uni-axial accelerometer (Actigraph MTI, model GT1M, Manufacturing Technology Inc., Fort Walton Beach, FL, USA) was used to assess PA. Adolescents were instructed to place the monitor underneath the clothing, at their lower back, using an elastic waist band and to wear it for seven consecutive days. They were also instructed to wear the accelerometer at all times except during water-based activities and periods of sleep. At least three days of recording with a minimum of eight hours registration per day was set as an inclusion criterion; the time-sampling interval was 15 seconds. A measure of total volume of activity was expressed as the sum of recorded counts per epoch divided by the total daily registered time in minutes. The time engaged in moderate PA and vigorous PA was calculated and presented as the average time per day during the entire recording. The time engaged at moderate PA [3-6 metabolic equivalents (METs)] was calculated based upon a blanket cut-off of 2,000 counts per minute − approximately equivalent to the intensity of a brisk walk (4.5 km/h). Periods of vigorous PA (>6 METs) were based upon a blanket cut-off of 4,000 counts per minute. Also, the time spent in at least moderate intensity level (>3 METs) was calculated as the total time spent in moderate and vigorous physical activity (MVPA, min/day). Each minute spent above the specific cut-off was summarised in the corresponding intensity level group. Time spent in light and low PA was defined as the sum of time per day in which counts per minute were <2000 and <100, respectively. Within this study the sum of minutes spent in low, moderate, and vigorous PA was used and is hereafter called total PA.

Food consumption was assessed by a computer-assisted, self-administered 24h-recall. Data were linked to national food composition databases to calculate calcium intake [13].

5.3.5 Statistics

Folate, RBC folate, PLP, cobalamin, holo-transcobalamin, homocysteine, creatinine, and vitamin D concentrations were normalised by logarithmic transformations.

Gender-specific differences were tested using student's t-test for metric variables and X^2-test for categorical variables. A bivariate correlation analysis was performed to study the correlations between BA, BMC, BMD and B-vitamin and homocysteine concentrations. Separate multiple regression models were built to evaluate the association between folate, vitamin B_6, vitamin B_{12} status, and homocysteine concentrations and whole body BMC and BMD; potential confounders were: sex, age, height, weight, maturity, creatinine, MTHFR 677C/T, vitamin D, calcium intake, and total PA. The analysis was repeated with lumbar spine BMC and BMD as dependent variables to examine if other regions of the body are more sensitive to folate, vitamin B_6, vitamin B_{12} status, and/or homocysteine concentrations. In order to have a higher statistical impact, both genders were analysed together while gender was chosen as a covariate. Evaluating the effect of homocysteine variables of folate, PLP, and cobalamin concentrations were added as covariates.

5.4 Results

Table 5.1 (page 97) shows the gender specific characteristics of the study population (n=114). All subjects were situated in Tanner stages III (n=11), IV (n=19), and V (n=84). Females (n=62, 54%) were in a more advanced pubertal stage compared with males (P=0.016). The TT-genotype of the MTHFR 677C/T polymorphism was present in 19.3% of the adolescents. Males had higher creatinine concentrations, were significantly taller, had a higher calcium intake, showed a greater BA and BMC, and were physically more active compared with females (P<0.05). Seven percent of the males smoked every day, 10% less than once a week, 83% didn't smoke. Their smoking behaviour differed significantly (P=0.042) from females of which 10% smoked every day, 3% at least once a week, and 87% did not smoke. A vitamin and/or mineral supplement was taken by 16% of the males and 2% of the females (P=0.011). Table 5.2 (page 98) presents bivariate correlations between BA, BMC, BMD and independent variables. Age, maturity, height, weight, BMI, and creatinine concentration were positively correlated with whole body BA, BMC and BMD (all P<0.05). Table 5.3 (page 99) shows multiple regressions studying the association of folate, vitamin B_6, vitamin B_{12} status, and homocysteine concentrations with whole body and lumbar spine BMC and BMD after controlling for potential confounders. Variations in whole body or lumbar spine BMC and

BMD could not be explained by folate, vitamin B_6, vitamin B_{12} status, and homocysteine concentrations.

5.5 Discussion

Strengths of our study are the use of a combination of two biomarkers for the measurement of folate and vitamin B_{12} status (plasma folate and RBC folate, cobalamin and holo-transcobalamin). To prevent misinterpretations folate and cobalamin concentrations were added as covariates to the multiple regression analysis when examining associations between homocysteine and bone parameters.

Our results do not support the hypothesis that in early ages BMD is associated with folate, vitamin B_6, and vitamin B_{12} status or homocysteine concentrations in apparently healthy adolescents. Beside BMD (g/cm²) we included BMC (g) in the analyses because no assumptions are made about the relationship between BMC and BA; potential size-related artefacts are avoided [14]. Kalkwarf et al. published BMD percentile curves based on three annual measurements for 1, 554 healthy children aged 6-16 years [15]. Percentiles were modelled by sex, race, and age. In 12-16 year-old males the median ranges from 0.886-1.098 g/cm² and in females from 0.900-1.079 g/cm². Data is comparable with our results (Table 5.1, page 97).To achieve a higher sample size, we omitted gender specific analyses of our data. Therefore, gender was added as a confounder to the multiple regression analysis. A possibly varying sensitivity to vitamin concentrations of different parts of the body we additionally considered BMD and BMC of the lumbar spine as dependent variables for multiple regression analysis, but associations could not be shown either (Table 5.3, page 99).

Up to date, there are two publications examining the relationship between B-vitamin status or B-vitamin related genetic and BMD in children [9] and adolescents [8]. The ALSPAC published data about the MTHFR 677C/T genotype and spinal BMD from 3,196 children aged 9 years [9]. Genetic effects were assessed using a recessive model for the minor T allel (TT versus CT+CC) and a dose model (reflecting the amount of T alleles) for the child genotype. Analyses were adjusted for age, height, weight, and sex. With a power of 80% the TT-genotype was significantly associated with lower BMD (recessive: $P=0.009$, dose: $P<0.001$). Usually a borderline folate and vitamin B_{12} status leads to increased homocysteine concentrations especially in the TT-genotype. Therefore, this result argues indirectly for an association between folate, cobalamin and/ or homocysteine concentrations and BMD in

children but not necessarily for an association with genetics. However, in this study, folate, riboflavin, vitamin B_6, and vitamin B_{12} intakes were estimated using standard portion sizes of typical foods eaten in the UK. Since blood vitamin concentrations reflect vitamin status more precisely than dietary intake, the direct relationship was not corroborated. To ensure that only vitamin status is observed we adjusted for the MTHFR 677C/T polymorphism in our analysis.

Ninety-three former macrobiotic adolescents compared with 102 omnivorous adolescents aged 9-15 years were examined to elucidate the relationship between vitamin B_{12} status and BMD [8]. In the whole sample, after adjusting for height, weight, BA, percent LBM, age, puberty, and calcium intake, MMA was significantly higher in subjects with a low BMD (P=0.0003) than in subjects with normal BMD. Cobalamin was significantly lower in the group with low BMD (P=0.0035) or BMC (P=0.0038) than in the group with normal BMD or BMC. The group of ex-macrobiotic adolescents displayed a significant inverse association between MMA and BMD but not between vitamin B_{12} and BMD ($P<0.05$). This result indicated that MMA may be a more specific and sensitive marker than serum cobalamin for vitamin B_{12} deficiency. The present study chose holo-transcobalamin as a functional parameter also known to be a better indicator for vitamin B_{12} deficiency than serum cobalamin [16, 17]. Though, we did not observe any significant association between cobalamin concentrations and BMD or between holo-transcobalamin concentrations and BMD. Better sensitivity and specificity of MMA compared with holo-transcobalamin might be possible but is still discussed in relevant publications [18]. In general, the macrobiotic diet is characterised by reduced intake of animal products. As a long-term effect of this diet a suboptimal or even deficient vitamin B_{12} status is likely. Furthermore, low calcium intake might have contributed to low BMD in the macrobiotic group. Dhonukshe-Rutten et al. [8] reported that 41% of the macrobiotic group had cobalamin concentrations below the 5^{th} percentile of the control group (<229 pmol/L). In our sample of apparently healthy adolescents, the 5^{th} percentile of cobalamin concentrations (202 pmol/L) was lower than that of a Dutch control group. Besides deviating dietary habits, the different age range within these two studies (9-15 years vs 12.5-17.49 years) might be responsible since age seems to be inversely associated with B-vitamin status [19, 20].

Other studies primarily focused on elderly in the context of osteoporosis and fractures. Concerning folate, vitamin B_{12} status and homocysteine concentrations these studies have found inconsistent results. Folate status was associated with vertebral BMD in 117 postmenopausal women aged 54.4±0.5 years [21]. Within the Hordaland Homocysteine Study (HHS) including 3,070 subjects aged 47-50 and 71-75 years, the relationship between folate

and hip BMD was observed only in females [22]. The Framingham study comprising 1,002 subjects aged 75.3±4.9 years confirmed the association between folate and femoral neck BMD in males and females [2]. A significant association between vitamin B_{12} and BMD has been shown by several studies [2, 23, 24]. A Dutch study with 194 participants older than 69 years found this association only in women but not in men. No association between other B-vitamins or homocysteine and BMD could be shown either [23]. In men and women this association could be corroborated by the Framingham Offspring Osteoporosis Study [2, 24]. With respect to homocysteine, the HHS showed only in females an inverse association between BMD and homocysteine [22]. An inverse association was also found in the Framingham Study but could not be maintained after adjustment for potential confounders [2]. This study pointed out the importance of adjusting by all influencing variables including folate and cobalamin whenever homocysteine were the target variable.

This study has some limitations. The HELENA-CSS was not primarily designed to examine the influence of B-vitamin status on BMD and subsequently the number of participants might be too small to detect an influence.

In conclusion, this study provides data on the relationship between BMD and folate, vitamin B_6, and vitamin B_{12} status and homocysteine concentrations. None of these vitamins or metabolites could be related to whole body or lumbar spine BMD and BMC suggesting that these bone parameters are not affected by vitamin status in early ages.

Table 5.1: Characteristics of the study sample by gender

	All	Male	Female	*P*-value
Age in years (n)	14.8±1.0 (114)	14.9±1.1 (52)	14.7±0.9 (62)	>0.05°
Pubertal stage in % (n)				0.016#
III	9.1 (11)	12.5 (7)	6.3 (4)	
IV	16.3 (19)	26.2 (14)	8.0 (5)	
V	74.5 (84)	61.3 (31)	85.7 (53)	
MTHFR 677 C/T in % (n)				>0.05#
CC	38.8 (43)	41.0 (21)	36.9 (22)	
CT	41.9 (46)	42.9 (22)	41.0 (24)	
TT	19.3 (21)	16.1 (8)	22.1 (13)	
Height in cm (n)	165.1±9.3 (114)	169.4±9.9 (52)	161.5±7.2 (62)	0.000°
Body mass index in kg/m² (n)	20.8±2.8 (114)	21.0±3.1 (52)	20.7±2.6 (62)	>0.05°
Creatinine in mg/dL (n)	0.72±0.14 (111)	0.77±0.14 (52)	0.68±0.12 (59)	0.001°
Vitamin D in nmol/L (n)	63.1±19.1 (107)	63.4±22.1 (49)	62.8±16.3 (58)	>0.05°
Physical activity in min (n)	133.4±34.4 (100)	142.6±35.0 (46)	125.5±32.0 (54)	0.013°
Calcium intake in mg/day (n)	747.8±335.4 (51)	874.5±388.2 (23)	648.8±252.9 (28)	0.017°
Whole body bone area in cm² (n)	1879.9±246.9 (110)	1967.1±294.3 (51)	1803.8±164.6 (59)	0.001°
Whole body bone mineral content in g (n)	1995.1±447.1 (110)	2138.3±540.4 (51)	1870.0±298.5 (59)	0.003°
Whole body bone mineral density in g/cm² (n)	1.051±0.108 (110)	1.072±0.120 (51)	1.031±0.092 (59)	0.050°

Parameters are shown as mean ± sd.
° student's t-test
X^2-test
Abbreviations: MTHFR – methylenetetrahydrofolate reductase.

Table 5.2: Bivariate correlations between whole body bone area, bone mineral content, and -density and independent variables

	Whole body bone area		Whole body bone mineral content		Whole body bone mineral density	
	Pearson	P-value	Pearson	P-value	Pearson	P-value
Age in years	0.280**	0.003	0.273**	0.004	0.261**	0.006
Maturity in stages	0.387**	0.000	0.413**	0.000	0.441**	0.000
Height in cm	0.886**	0.000	0.820**	0.000	0.614**	0.000
Weight in kg	0.852**	0.000	0.793**	0.000	0.598**	0.000
BMI in kg/m²	0.421**	0.000	0.393**	0.000	0.309**	0.001
Physical activity	0.059	>0.05	0.075	>0.05	0.066	>0.05
Calcium intake in mg/day	0.254	>0.05	0.259	>0.05	0.195	>0.05
log Vitamin D	0.063	>0.05	0.050	>0.05	0.020	>0.05
log folate	0.060	>0.05	0.049	>0.05	0.014	>0.05
log RBC folate	0.128	>0.05	0.130	>0.05	0.101	>0.05
log cobalamin	-0.177	>0.05	-0.129	>0.05	-0.047	>0.05
log holo-transcobalamin	-0.029	>0.05	0.018	>0.05	0.088	>0.05
log PLP	0.044	>0.05	0.008	>0.05	-0.038	>0.05
log homocysteine	0.118	>0.05	0.119	>0.05	0.125	>0.05
log creatinin	0.400**	0.000	0.457**	0.000	0.502**	0.000
MTHFR 677C/T polymorphism	0.160	>0.05	0.166	>0.05	0.161	>0.05
Smoking	-0.027	>0.05	-0.013	>0.05	-0.016	>0.05
Supplements	0.132	>0.05	0.122	>0.05	0.052	>0.05

* P<0.05 (2-tailed). ** P<0.01 (2-tailed).
Abbreviations: RBC folate – red blood cell folate, PLP – pyridoxal-5-phosphate, MTHFR – methylenetetrahydrofolate reductase.

Table 5.3: Standardised multiple regression coefficients (ß), standard error (SE), and partial correlation (partial) examining the association of folate, vitamin B_6, and vitamin B_{12} status as well as homocysteine concentrations with whole body and lumbar spine bone mineral density and –content after controlling for sex, age, height, weight, maturity, creatinine, vitamin D, MTHFR 677C/T polymorphism, calcium intake, and physical activity

	ß	SE	Partial	P-value
Whole body bone mineral density				
log folate	0.206	0.026	0.258	>0.05
log RBC folate	0.121	0.034	0.154	>0.05
log cobalamin	0.008	0.039	0.009	>0.05
log holo-transcobalamin	0.148	0.036	0.199	>0.05
log PLP	-0.164	0.018	-0.240	>0.05
log homocysteine	0.040	0.049	0.047	>0.05
Whole body bone mineral content				
log folate	0.122	63.817	0.243	>0.05
log RBC folate	0.096	83.042	0.194	>0.05
log cobalamin	0.026	94.083	0.051	>0.05
log holo-transcobalamin	0.008	92.435	0.016	>0.05
log PLP	-0.079	43.027	-0.191	>0.05
log homocysteine	0.082	117.412	0.156	>0.05
Lumbar spine bone mineral density				
log folate	0.150	0.034	0.177	>0.05
log RBC folate	0.134	0.044	0.161	>0.05
log cobalamin	-0.128	0.051	-0.146	>0.05
log holo-transcobalamin	-0.021	0.049	-0.026	>0.05
log PLP	-0.226	0.023	-0.308	>0.05
log homocysteine	0.152	0.062	0.170	>0.05
Lumbar spine bone mineral content				
log folate	0.108	2.707	0.155	>0.05
log RBC folate	0.100	3.488	0.146	>0.05
log cobalamin	0.099	4.071	0.136	>0.05
log holo-transcobalamin	-0.077	4.003	-0.112	>0.05
log PLP	-0.063	1.908	-0.105	>0.05
log homocysteine	0.076	4.895	0.105	>0.05

Abbreviations: RBC folate – red blood cell folate, PLP – pyridoxal-5-phosphate.

References

1. Institute of Medicine. Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride. Washington, DC: Food and Nutrition Board, National Academy Press, 1997.

2. McLean RR, Jacques PF, Selhub J, Fredman L, Tucker KL, Samelson EJ, Kiel DP, Cupples LA, Hannan MT. Plasma B vitamins, homocysteine, and their relation with bone loss and hip fracture in elderly men and women. J Clin Endocrinol Metab 2008;93:2206-2212.

3. Shiraki M, Urano T, Kuroda T, Saito M, Tanaka S, Miyao-Koshizuka M, Inoue S. The synergistic effect of bone mineral density and methylenetetrahydrofolate reductase (MTHFR) polymorphism (C677T) on fractures. J Bone Miner Metab 2008;26:595-602.

4. Brenton DP. Skeletal abnormalities in homocystinuria. Postgrad Med J 1977;53:488-496.

5. Kang AH, Trelstad RL. A collagen defect in homocystinuria. J Clin Invest 1973;52:2571-2578.

6. Carmel R, Lau KH, Baylink DJ, Saxena S, Singer FR. Cobalamin and osteoblast-specific proteins. N Engl J Med 1988;319:70-75.

7. Herrmann M, Schmidt J, Umanskaya N, Colaianni G, Al Marrawi F, Widmann T, Zallone A, Wildemann B, Herrmann W. Stimulation of osteoclast activity by low B-vitamin concentrations. Bone 2007;41:584-591.

8. Dhonukshe-Rutten RA, van Dusseldorp M, Schneede J, de Groot LC, van Staveren WA. Low bone mineral density and bone mineral content are associated with low cobalamin status in adolescents. Eur J Nutr 2005;44:341-347.

9. Steer C, Emmett P, Lewis S, Smith GD, Tobias J. The Methylenetetrahydrofolate Reductase (MTHFR) C677T Polymorphism is Associated with Spinal BMD in Nine-Year-Old Children. J Bone Miner Res 2008.

10. Moreno L, de Henauw S, Gonzalez-Gross M, Kersting M, Molnar D, Gottrand F, Barrios L, Sjostrom M, Manios Y, Gilbert C, Leclercq C, Widhalm K, Marcos A, Group obotHS. Design and implementation of the Healthy Lifestyle by Nutrition in Adolescence Cross-Sectional Study. Int J Obes 2008;32:S4-S11.

11. Beghin L, Castera M, Manios Y, Gilbert C, Kersting M, de Henauw S, Kafatos A, Gottrand F, Molnar D, Sjostrom M, Leclercq C, Widhalm K, Mesana M, Moreno L, Libersa C, group. ObotHs. Quality assurance of ethical issues and regulatory aspects relating to Good Clinical Practices in the HELENA cross sectional study. Int J Obes 2008;in press.

12. Calbet JA, Dorado C, Diaz-Herrera P, Rodriguez-Rodriguez LP. High femoral bone mineral content and density in male football (soccer) players. Med Sci Sports Exerc 2001;33:1682-1687.

13. Kersting M, Sichert-Hellert W, Vereecken CA, Diehl J, Beghin L, De Henauw S, Grammatikaki E, Manios Y, Mesana MI, Papadaki A, Phillipp K, Plada M, Poortvliet E, Sette S. Food and nutrient intake, nutritional knowledge and diet-related attitudes in European adolescents. Int J Obes (Lond) 2008;32 Suppl 5:S35-41.

14. Prentice A, Parsons TJ, Cole TJ. Uncritical use of bone mineral density in absorptiometry may lead to size-related artifacts in the identification of bone mineral determinants. Am J Clin Nutr 1994;60:837-842.

15. Kalkwarf HJ, Zemel BS, Gilsanz V, Lappe JM, Horlick M, Oberfield S, Mahboubi S, Fan B, Frederick MM, Winer K, Shepherd JA. The bone mineral density in childhood study: bone mineral content and density according to age, sex, and race. J Clin Endocrinol Metab 2007;92:2087-2099.

16. Clarke R, Sherliker P, Hin H, Nexo E, Hvas AM, Schneede J, Birks J, Ueland PM, Emmens K, Scott JM, Molloy AM, Evans JG. Detection of vitamin B12 deficiency in older people by measuring vitamin B12 or the active fraction of vitamin B12, holotranscobalamin. Clin Chem 2007;53:963-970.

17. Obeid R, Herrmann W. Holotranscobalamin in laboratory diagnosis of cobalamin deficiency compared to total cobalamin and methylmalonic acid. Clin Chem Lab Med 2007;45:1746-1750.

18. Hvas AM, Nexo E. Diagnosis and treatment of vitamin B12 deficiency--an update. Haematologica 2006;91:1506-1512.

19. van Beynum IM, den Heijer M, Thomas CM, Afman L, Oppenraay-van Emmerzaal D, Blom HJ. Total homocysteine and its predictors in Dutch children. Am J Clin Nutr 2005;81:1110-1116.

20. Bjorke Monsen AL, Refsum H, Markestad T, Ueland PM. Cobalamin Status and Its Biochemical Markers Methylmalonic Acid and Homocysteine in Different Age Groups from 4 Days to 19 Years. Clin Chem 2003;49:2067-2075.

21. Cagnacci A, Bagni B, Zini A, Cannoletta M, Generali M, Volpe A. Relation of folates, vitamin B12 and homocysteine to vertebral bone mineral density change in postmenopausal women. A five-year longitudinal evaluation. Bone 2008;42:314-320.

22. Gjesdal CG, Vollset SE, Ueland PM, Refsum H, Drevon CA, Gjessing HK, Tell GS. Plasma total homocysteine level and bone mineral density: the Hordaland Homocysteine Study. Arch Intern Med 2006;166:88-94.

23. Dhonukshe-Rutten RA, Lips M, de Jong N, Chin APMJ, Hiddink GJ, van Dusseldorp M, De Groot LC, van Staveren WA. Vitamin B-12 status is associated with bone mineral content and bone mineral density in frail elderly women but not in men. J Nutr 2003;133:801-807.

24. Tucker KL, Hannan MT, Qiao N, Jacques PF, Selhub J, Cupples LA, Kiel DP. Low plasma vitamin B12 is associated with lower BMD: the Framingham Osteoporosis Study. J Bone Miner Res 2005;20:152-158.

6 General discussion and conclusion

The HELENA study as a whole is the first study that provides data on various markers for the assessment of health and nutrition measured in one sample of European adolescents. To guarantee a high level of quality, a pilot study was performed to check if standard operating procedures were adopted by all research partners. Work package 2 monitored the compliance with guidelines for Good Clinical Practise and was responsible for the quality control throughout the whole project [1]. The present work is confined to a subgroup within the HELENA-CSS. In this subgroup, blood samples were taken for the measurement of various health and nutrition related parameters, therefore, stability tests were carried out [2]. Within this work, the B-vitamin status and their associations to homocysteine and selected non-communicable and communicable factors were investigated. The simultaneous analysis of gender, age, maturity, MTHFR 677C/T genotype, body weight, supplement use, and smoking habits with respect to folate, vitamin B_6, vitamin B_{12}, status and homocysteine concentrations (univariate ANOVA) presents a clear strength. To avoid misinterpretations, we took into account folate and cobalamin concentrations as confounders when homocysteine was concerned. We did not include dietary intake of B-vitamins because these data were not yet available. However, blood vitamin concentrations are widely presumed to reflect dietary intake more accurately because usually food databases disregard the intake of fortified food items. The additional measurement of two biomarkers for folate and vitamin B_{12} status complete the measurements and may confirm results from just one parameter. Another strength is the inclusion of a large number of adolescents based on a multinational European population. The post-hoc power analyses performed for our significant results showed that with a probability of >90% the effects in our sample could be found in the population.

In the following, the associations between non-communicable factors gender, age, sexual maturity, and MTHFR 677C/T genotype and B-vitamin status as well as homocysteine concentrations are recapitulated. Especially gender, age, and maturity seem to be closely related in adolescence. Usually, gender differences with respect to B-vitamin status and homocysteine concentrations become more apparent after the onset of sexual maturation. To the best of our knowledge one publication evaluated B-vitamin status and homocysteine concentrations by sexual maturity measured by Tanner stages [3]. The sample of 165 Spanish

adolescents was predominantly situated in grades III-V. Their age range (13-18.5 years) is comparable to ours (12.5-17.49 years). Here, gender differences with respect to cobalamin and homocysteine concentrations were found (both P=0.003). Differences by grades of sexual maturity could not be shown, possibly due to the proceeding sexual maturity in this sample, a relatively low number of participants, and the statistical analysis (one-way ANOVA) not taking into account other potential confounders. An other study surveyed puberty features (menstruation/ axillary hair growth, respectively) in a Dutch sample of 186 children and adolescents aged 0-19 years without finding gender differences or variations by puberty features [4]. With an age range of almost 20 years it might be difficult to find gender differences which can be supported by studies performed on infants and children [5, 6]. A higher grade of sexual maturity is not necessarily but somehow linked with higher age. Puberty measured by the age at menarche began at the average age of 12.8 years in German and US American female adolescents. The age may vary depending on factors like body composition, nutritional status, and ethnicity [7, 8]. The older an adolescent the more probable is the complete sexual maturation. But sexual maturation does not take a linear course in terms of age. Hence, the evaluation by sexual maturity becomes vital for adolescents due to its link to physiology. However, most of the current publications omitted this important non-communicable factor [9-18]. The evaluation of B-vitamin and homocysteine concentrations by gender and age is common, but published results were ambiguous. According to our findings, significantly higher cobalamin concentrations in females compared with males were observed in two studies from the US and Spain [3, 9] and significantly higher homocysteine concentrations in males compared with females were reported in four publications [3, 9, 10, 19]. Increasing homocysteine concentrations with increasing age were present in nearly all presently available publications [9, 11, 12, 14, 16, 17, 20-24]. Significantly higher folate [9, 11] and PLP concentrations [11] in males compared with females were reported not being in line with our findings. The association between lower folate and higher homocysteine values in carriers of the MTHFR 677 TT-genotype and compared with carriers of the CC-genotype was supported by four out of six studies [3, 14, 16, 18]. However, Gil Prieto et al. [25] did not measure folate or homocysteine concentrations and Raslova et al. [17] did not measure folate concentrations and discovered a prevalence of 4.8% for the TT-genotype which might be too small to detect an association. In studies performed on adults, usually RBC folate concentrations were lowest in the TT-genotype [26, 27]. Though, in our study RBC folate levels were lowest in the CT-genotype. Other studies performed on adolescents did not investigate RBC folate levels together with MTHFR 677C/T genotype.

Several associations between the communicable factors BMI, body fat, supplement use, smoking habits, PA, and CVF and B-vitamin and homocysteine concentrations have been identified within this work. In line with our results, a negative association between BMI and folate concentrations was reported [14]; a negative association between BMI and cobalamin concentrations was described in the same Austrian sample and furthermore in an Israeli sample [14, 28]. Not in accordance with our findings, higher homocysteine concentrations in adolescents with a higher BMI were observed in several studies from the US, Austria and Turkey [9, 10, 14, 29]. Associations between body fat and homocysteine concentrations were hardly investigated in adolescents. Gallistl et al. carried out a three-week weight loss program in 37 obese girls aged 12.0±1.8 years and 19 obese boys aged 11.9±1.7 years with homocysteine and LBM measurements before and after the intervention [30]. Therefore, results are difficult to compare. Two authors reported a significantly higher folate status and lower homocysteine concentrations in supplement users compared with non-users [9, 11] which is in agreement with our findings. We found an association of smoking habits with folate, RBC folate, PLP, and cobalamin, but not with homocysteine concentrations. These findings are supported to some extent by results from a Spanish study and the British NDNS [3, 11]. Significantly lower folate [3] and PLP [11] concentrations in adolescent smokers compared with non-smokers were experienced. Significantly higher homocysteine concentrations in smokers compared with non-smokers were observed in three publications [3, 9, 22]. Few studies targeted the relationship of PA with homocysteine in adolescents. With respect to children and adolescents the EYHS (n=680, 9-10 and 15-16 years) [31] was the first study wherein the association between homocysteine and PA was examined. However, no significant results were found after adjustment for gender, pubertal development, socioeconomic status, folate and vitamin B_{12} intake, and MTHFR 677C/T genotype. Regarding cardiorespiratory fitness significant and inverse association with homocysteine concentrations was found in Spanish female adolescents from the AVENA Study (n=156, aged 14.8±1.4 years) [32]. Regression models included folate and vitamin B_{12} levels as well as MTHFR 677C/T genotype. In contrast, results from the EYHS did not indicate a significant association between homocysteine and fitness in children and adolescents [31]. In the present work, a comparable age group was evaluated and similar statistics were applied. Thus, the main determinants of homocysteine remain folate and cobalamin concentrations as well as the MTHFR 677C/T genotype as demonstrated by Kluijtmans et al. [33].

The association of B-vitamin status and homocysteine concentrations with bone parameters was examined in a Spanish adolescent sub-sample from the HELENA study. However, no associations were found concerning whole body BMD and lumbar spine BMD. Up to date, there are two publications examining the relationship between B-vitamin status or B-vitamin related genetic and BMD in children [34] and adolescents [35]. A British study reported data from 3196 children aged nine years about the MTHFR 677C/T genotype and spinal BMD [34]. With a power of 80% the TT-genotype was significantly associated with lower BMD. Since no adjustments were made for folate and vitamin B_{12} status the association might be ambiguous because the altered physiology of carriers of the TT-genotype also depends on the folate and vitamin B_{12} status. A Dutch study compared former macrobiotic adolescents with omnivorous adolescents aged 9-15 years to elucidate the relationship between vitamin B_{12} status and BMD [35]. After adjusting for several confounders, in the whole sample MMA was significantly higher in subjects with a low BMD than in subjects with normal BMD. Cobalamin was significantly lower in the group with low BMD or BMC than in the group with normal BMD or BMC. In the group of ex-macrobiotic adolescents a significant inverse association between MMA and BMD but not between vitamin B_{12} and BMD was observed. A macrobiotic diet during childhood might leave its mark by depleted cobalamin stores and decreased BMD through low calcium intake. Alternative diets were not checked within the present work. Participants from the HELENA study were apparently healthy. Therefore, the amplitude of vitamin concentrations in our population might be too narrow and the range concentrations too high to detect an association.

Up to date sound reference values for adolescents are lacking for intake recommendations as well as for vitamin concentrations measured e.g. in blood. Subsequently, the assessment of B-vitamin and homocysteine concentrations in adolescents with respect to B-vitamin deficiency or elevated risk for cardiovascular diseases remains difficult. Adolescents have their own specific needs and the comparison with adult reference values is probably inappropriate [36]. Without linking population reference data like ours to markers for B-vitamin deficiency it would be unadvised to derive recommendations for the maintenance of health. In the present work MCV was used as a marker for macrocytosis, a clinical outcome of folate and/or vitamin B_{12} deficiency. The maximal MCV value of our sample was 96 fL (cut-off varies between >96 and >100 fL [37]) implying that folate or vitamin B_{12} deficiency is unlikely. However, a definite conclusion is not possible since the iron status was not checked. Iron deficiency, which is frequently prevalent in adolescents [38], lowers MCV

values and might veil folate and/or vitamin B_{12} deficiency [39, 40]. Studies with artificially induced vitamin deficiencies are unethical. So the current state of the art persists namely the development of reference values e.g. by establishing population reference data and out of it defining percentiles. Though, the determination of percentiles e.g. the 2.5^{th}, 5^{th}, or 10^{th} percentiles to define suboptimal supply or vitamin deficiency, or the respective 90^{th}, 95^{th}, or 97.5^{th} percentiles to define an elevated risk for cardiovascular disease imply a distinct arbitrariness. This was stated by Kromeyer-Hauschild et al. in conjunction with the development of reference values for BMI [41]. As a prerequisite for the development of sound reference values, possible associations between B-vitamins, homocysteine and non-communicable as well as communicable factors were analysed within the present work. Hence, the question arises how to integrate non-communicable or communicable factors in the development of reference values. Regarding the development of reference values for BMI Cole et al. subdivided their sample into gender and age categories [42, 43]. In our study some but not all of the examined substrates were associated with gender, age, sexual maturity, MTHFR 677C/T polymorphism, BMI, supplement usage, and smoking behaviour. To some extent B-vitamin concentrations were significantly lower in overweight/ obese adolescents and smokers. Therefore, based on reference percentiles by Cole et al. [42, 43] we generated another percentile compilation (Table 3.3, page 58) only including data from normal weight and non-smoking adolescents. These percentiles were slightly higher compared with percentiles including the whole sample. Furthermore, our data suggest an integration of sexual maturity because most of the examined substrates are associated with this non-communicable factor. Atkinson et al. dealt with the derivation of nutrient intake values and arrived at the conclusion that the standardization of age groups should be based on a biological basis like growth and pubertal stages with consideration of relevant developmental milestones throughout childhood [36]. Concurrently, one should keep in mind that population reference data can underlie temporal changes. In the case of BMI upward trends have been observed by Gulliford et al. and Lahti-Koski et al. over the past decades [44, 45].

This study has some limitations. In general, the cross-sectional design does not allow the drawing of causal conclusions. Some prospective longitudinal studies on the development of cardiovascular risk factors from adolescence to adulthood were implemented; however, up to date most of these studies omitted the relatively new risk factor homocysteine. One main difficulty for assessing our results in the context of other findings is the lacking comparability in-between studies. Many research groups use different age groups and age ranges or merge

children and adolescents. To evaluate by age categories is widespread and simple. Though, the evaluation by sexual maturity would be more plausible in a physiological context. Another step forward could be to involve the sex hormone status. Especially in adolescents it would have been interesting to examine the association between endogenous sex hormones and folate, vitamin B_6, vitamin B_{12} status, and homocysteine concentrations which were not analysed in our study. Then, the target group of the HELENA study were apparently healthy adolescents. One needs to consider that usually healthy adolescents do not show manifest cardiovascular pathologies. A suboptimal intake especially with folate is widely presumed throughout all ages and social classes but vitamin deficiencies usually do not occur in industrial countries. Subsequently, the range of vitamin concentrations in our population might be too narrow and the concentrations too high to detect an influence.

In conclusion, the underlying current folate, vitamin B_6, vitamin B_{12} status, and homocysteine concentrations in European adolescents participating in the HELENA study were described. Due to the cross-sectional design, these data solely reflect the current B-vitamin status and homocysteine concentrations in European adolescents. In order to explore the influence of B-vitamin status and homocysteine concentrations in adolescence for the later life, a longitudinal study design would be necessary. Within this work selected non-communicable and communicable factors in relation with folate, vitamin B_6, and vitamin B_{12} status and homocysteine concentrations were analysed and several associations were identified. The commonly considered non-communicable and communicable factors gender, age, and BMI should be maintained for future research though not all parameters were associated with gender, age, and BMI in our study. Our results pointed out that sexual maturity is an important determinant in adolescents with respect to folate and vitamin B_{12} status as well as homocysteine levels. The importance of MTHFR 677C/T polymorphism was corroborated also for adolescents. Likewise supplement usage and smoking habits were relevant determinants in adolescents. Physical activity and CVF was not associated with homocysteine concentrations. Bone parameters could not be related to folate, vitamin B_6, vitamin B_{12} status and/or homocysteine concentrations. Finally, this work provides potential reference data that might be a starting point for the further development of sound reference values.

References

1. Beghin L, Castera M, Manios Y, Gilbert C, Kersting M, de Henauw S, Kafatos A, Gottrand F, Molnar D, Sjostrom M, Leclercq C, Widhalm K, Mesana M, Moreno L, Libersa C, group. ObotHs. Quality assurance of ethical issues and regulatory aspects relating to Good Clinical Practices in the HELENA cross sectional study. Int J Obes 2008;32:S12-S18.

2. Gonzalez-Gross M, Breidenassel C, Gomez S, Ferrari M, Beghin L, Spinneker A, Diaz L, Maiani G, Demailly A, Al-Tahan J, Albers U, Warnberg J, Stoffel-Wagner B, Jiménez-Pavón D, Libersa C, Pietrzik K, Marcos A, Stehle P, Group obotHS. Sampling and processing of fresh blood samples within a European multicenter nutritional study: evaluation of biomarker stability during transport and storage. Int J Obes 2008;32:S66-S75.

3. Al-Tahan J, Sola R, Ruiz JR, Breidenassel C, Garcia-Fuentes M, Moreno L, Castillo MJ, Pietrzik K, Gonzalez-Gross M. Methylenetetrahydrofolate reductase 677CT polymorphism and cobalamin, folate, and homocysteine status in Spanish adolescents. Ann Nutr Metab 2008;52:315-321.

4. Hogeveen M, van Beynum I, van Rooij A, Kluijtmans L, den Heijer M, Blom H. Methylmalonic acid values in healthy Dutch children. Eur J Nutr 2008;47:26-31.

5. Karikas GA, Kriebardis A, Samara I, Schulpis K, Papachristodoulou M, Fytou-Pallikari A. Serum homocysteine levels and paraoxonase 1 activity in preschool aged children in Greece. Clin Chem Lab Med 2006;44:623-627.

6. Hay G, Johnston C, Whitelaw A, Trygg K, Refsum H. Folate and cobalamin status in relation to breastfeeding and weaning in healthy infants. Am J Clin Nutr 2008;88:105-114.

7. Bau AM, Ernert A, Schenk L, Wiegand S, Martus P, Grüters A, Krude H. Is there a further acceleration in the age at onset of menarche? A cross-sectional study in 1840 school children focusing on age and bodyweight at the onset of menarche. Eur J Endocrinol 2009;160:107-113.

8. Freedman DS, Khan LK, Serdula MK, Dietz W, Srinivasan SR, Berenson GS. Relation of age at menarche to race, time period, and anthropometric dimensions: the Bogalusa Heart Study. Pediatrics 2002;110:e43.

9. Osganian SK, Stampfer MJ, Spiegelman D, Rimm E, Cutler JA, Feldman HA, Montgomery DH, Webber LS, Lytle LA, Bausserman L, Nader PR. Distribution of and factors associated with serum homocysteine levels in children: Child and Adolescent Trial for Cardiovascular Health. JAMA 1999;281:1189-1196.

10. Shen M-H, Chu N-F, Wu D-M, Chang J-B. Plasma homocysteine, folate and vitamin B12 levels among school children in Taiwan: The Taipei Children Heart Study. Clinical Biochemistry 2002;35:495-498.

11. Kerr MA, Livingstone B, Bates CJ, Bradbury I, Scott JM, Ward M, Pentieva K, Mansoor MA, McNulty H. Folate, related B vitamins, and homocysteine in childhood and adolescence: potential implications for disease risk in later life. Pediatrics 2009;123:627-635.

12. De Laet C, Wautrecht JC, Brasseur D, Dramaix M, Boeynaems JM, Decuyper J, Kahn A. Plasma homocysteine concentration in a Belgian school-age population. Am J Clin Nutr 1999;69:968-972.

13. Chang SJ, Hsiao LJ, Lee YC, Hsuen SY. Vitamin B6 status assessment in relation to dietary intake in high school students aged 16-18 years. Br J Nutr 2007;97:764-769.

14. Huemer M, Vonblon K, Fodinger M, Krumpholz R, Hubmann M, Ulmer H, Simma B. Total homocysteine, folate, and cobalamin, and their relation to genetic polymorphisms, lifestyle and body mass index in healthy children and adolescents. Pediatr Res 2006;60:764-769.

15. Morris MS, Picciano MF, Jacques PF, Selhub J. Plasma pyridoxal 5'-phosphate in the US population: the National Health and Nutrition Examination Survey, 2003-2004. Am J Clin Nutr 2008;87:1446-1454.

16. van Beynum IM, den Heijer M, Thomas CM, Afman L, Oppenraay-van Emmerzaal D, Blom HJ. Total homocysteine and its predictors in Dutch children. Am J Clin Nutr 2005;81:1110-1116.

17. Raslova K, Bederova A, Gasparovic J, Blazicek P, Smolkova B. Effect of diet and 677 C-->T 5, 10-methylenetetrahydrofolate reductase genotypes on plasma homocyst(e)ine concentrations in slovak adolescent population. Physiol Res 2000;49:651-658.

18. Papoutsakis C, Yiannakouris N, Manios Y, Papaconstantinou E, Magkos F, Schulpis KH, Zampelas A, Matalas AL. The effect of MTHFR(C677T) genotype on plasma homocysteine concentrations in healthy children is influenced by gender. Eur J Clin Nutr 2006;60:155-162.

19. Ganji V, Kafai MR. Population references for plasma total homocysteine concentrations for U.S. children and adolescents in the post-folic acid fortification era. J Nutr 2005;135:2253-2256.

20. Vilaseca MA, Moyano D, Ferrer I, Artuch R. Total homocysteine in pediatric patients. Clin Chem 1997;43:690-692.

21. Papandreou D, Mavromichalis I, Makedou A, Rousso I, Arvanitidou M. Total serum homocysteine, folate and vitamin B12 in a Greek school age population. Clin Nutr 2006;25:797-802.

22. Bates CJ, Mansoor MA, Gregory J, Pentiev K, Prentice A. Correlates of plasma homocysteine, cysteine and cysteinyl-glycine in respondents in the British National Diet and Nutrition Survey of young people aged 4-18 years, and a comparison with the survey of people aged 65 years and over. Br J Nutr 2002;87:71-79.

23. Rauh M, Verwied S, Knerr I, Dorr HG, Sonnichsen A, Koletzko B. Homocysteine concentrations in a German cohort of 500 individuals: reference ranges and determinants of plasma levels in healthy children and their parents. Amino Acids 2001;20:409-418.

24. Brasileiro RS, Escrivao MA, Taddei JA, D'Almeida V, Ancona-Lopez F, Carvalhaes JT. Plasma total homocysteine in Brazilian overweight and non-overweight adolescents: a case-control study. Nutr Hosp 2005;20:313-319.

25. Gil Prieto R, Esteban Hernandez J, Hernandez Barrera V, Cano B, de Oya M, Gil de Miguel A. [Serum vitamin B12 levels in an adolescent population in Madrid]. An Pediatr (Barc) 2008;68:474-480.

26. Smulders YM, Smith DE, Kok RM, Teerlink T, Gellekink H, Vaes WH, Stehouwer CD, Jakobs C. Red blood cell folate vitamer distribution in healthy subjects is determined by the methylenetetrahydrofolate reductase C677T polymorphism and by the total folate status. J Nutr Biochem 2007;18:693-699.

27. Golbahar J, Fathi Z, Tamadon M. Distribution of 5,10-methylenetetrahydrofolate reductase (C667T) polymorphism and its association with red blood cell 5-methyltetrahydrofolate in the healthy Iranians. Clin Nutr 2005;24:83-87.

28. Pinhas-Hamiel O, Doron-Panush N, Reichman B, Nitzan-Kaluski D, Shalitin S, Geva-Lerner L. Obese children and adolescents: a risk group for low vitamin B12 concentration. Arch Pediatr Adolesc Med 2006;160:933-936.

29. Narin F, Atabek ME, Karakukcu M, Narin N, Kurtoglu S, Gumus H, Coksevim B, Erez R. The association of plasma homocysteine levels with serum leptin and apolipoprotein B levels in childhood obesity. Ann Saudi Med 2005;25:209-214.

30. Gallistl S, Sudi KM, Erwa W, Aigner R, Borkenstein M. Determinants of homocysteine during weight reduction in obese children and adolescents. Metabolism 2001;50:1220-1223.

31. Ruiz JR, Hurtig-Wennlof A, Ortega FB, Patterson E, Nilsson TK, Castillo MJ, Sjostrom M. Homocysteine levels in children and adolescents are associated with the methylenetetrahydrofolate reductase 677C>T genotype, but not with physical activity, fitness or fatness: the European Youth Heart Study. Br J Nutr 2007;97:255-262.

32. Ruiz JR, Sola R, Gonzalez-Gross M, Ortega FB, Vicente-Rodriguez G, Garcia-Fuentes M, Gutierrez A, Sjostrom M, Pietrzik K, Castillo MJ. Cardiovascular fitness is negatively associated with homocysteine levels in female adolescents. Arch Pediatr Adolesc Med 2007;161:166-171.

33. Kluijtmans LA, Young IS, Boreham CA, Murray L, McMaster D, McNulty H, Strain JJ, McPartlin J, Scott JM, Whitehead AS. Genetic and nutritional factors contributing to hyperhomocysteinemia in young adults. Blood 2003;101:2483-2488.

34. Steer C, Emmett P, Lewis S, Smith GD, Tobias J. The Methylenetetrahydrofolate Reductase (MTHFR) C677T Polymorphism is Associated with Spinal BMD in Nine-Year-Old Children. J Bone Miner Res 2008.

35. Dhonukshe-Rutten RA, van Dusseldorp M, Schneede J, de Groot LC, van Staveren WA. Low bone mineral density and bone mineral content are associated with low cobalamin status in adolescents. Eur J Nutr 2005;44:341-347.

36. Atkinson SA, Koletzko B. Determining life-stage groups and extrapolating nutrient intake values (NIVs). Food Nutr Bull 2007;28:S61-76.

37. Rumsey SE, Hokin B, Magin PJ, Pond D. Macrocytosis--an Australian general practice perspective. Aust Fam Physician 2007;36:571-572.

38. Hercberg S, Preziosi P, Galan P. Iron deficiency in Europe. Public Health Nutr 2001;4:537-545.

39. England JM, Ward SM, Down MC. Microcytosis, anisocytosis and the red cell indices in iron deficiency. Br J Haematol 1976;34:589-597.

40. d'Onofrio G, Chirillo R, Zini G, Caenaro G, Tommasi M, Micciulli G. Simultaneous measurement of reticulocyte and red blood cell indices in healthy subjects and patients with microcytic and macrocytic anemia. Blood 1995;85:818-823.

41. Kromeyer-Hauschild K, Wabitsch M, Kunze D, Geller F, Geiß HC, Hesse V, von Hippel A, Jaeger U, Johnsen D, Korte W, Menner K, Müller G, Müller JM, Niemann-Pilatus A, Remer T, Schaefer F, Wittchen H-U, Zabransky S, Zellner K, Ziegler A, Hebebrand J. Perzentile für den Body-mass-index für das Kindes- und Jugendalter unter Heranziehung verschiedener deutscher Stichproben. Monatsschr Kinderheilkd 2001;149:807-818.

42. Cole T, Bellizzi M, Flegal M, Dietz W. Establishing a standard definition for child overweight and obesity worldwide: international survey. BMJ 2002;320:1-6.

43. Cole TJ, Flegal KM, Nicholls D, Jackson AA. Body mass index cut offs to define thinness in children and adolescents: international survey. BMJ 2007;335:194.

44. Gulliford MC, Rona RJ, Chinn S. Trends in body mass index in young adults in England and Scotland from 1973 to 1988. J Epidemiol Community Health 1992;46:187-190.

45. Lahti-Koski M, Vartiainen E, Mannisto S, Pietinen P. Age, education and occupation as determinants of trends in body mass index in Finland from 1982 to 1997. Int J Obes Relat Metab Disord 2000;24:1669-1676.

Appendix

Healthy Lifestyle in Europe by Nutrition in Adolescence in 13-16 years

Adolescents across Europe.

Cross-sectional study (HELENA-CSS) and Pilot Study.

CASE REPORT FORM

Subject number : H2	

Subjects initials : ....
first name/second name /family name

GENERAL INSTRUCTIONS FOR THE INVESTIGATOR

General Instructions for the Investigator (see SOP of the CRF)

When completing this CRF, please
- Use a ballpoint, fine-tip pen (permanent ink), black or blue.
- Make sure that all entries are clear and legible, preferably in block letters.
- Use English and specific medical terminology.
- Make corrections by drawing a single line through the incorrect item, which must remain legible, and write the correct data next to it.
- Do not use correction fluids or any other method of erasure!
- Date and initial for all corrections and changes.
- Tick closed boxes and circles ☒

Do not use
- abbreviations and acronyms

Subject Initials : first name-middle-family/last name (ex)
Jean Marcel DUPONT J M D
- If, there is no second name, put a "-"
- Subject initials must remain consistent throughout the CRF.
-

Enrolment Code
- Subjects will be assigned an enrolment code number at inclusion : study code Helena CSS – H1 - city number (see Appendix) -number of study inclusion

Subject number: H2 ....
Number of centre | Number of school | Number of the class | Number of subject

Exemple **H2** 0 4 0 1 0 5 1 0

Missing Data
- If some information has been impossible to obtain, please strike out the field/box and explain briefly next to the field/box why the information is missing.

Historical dates
- If a historical date, or part of a historical date, has been impossible to obtain, please fill in as much as is known and strike out the remaining field(s)

Sic
- Indicate data which are unusual or unexpected but still correct by noting 'Sic' next to that data. Sic can be understood as 'so is correct.'

SELECTION OF SUBJECTS

Inclusion Criteria

	Yes	No
Male and female subjects aged 13 – 16 ± 2 years	☐	☐
Schooling in one of the participating cities	☐	☐
Informed consent form signed by the parents and/or the legal representative.	☐	☐

Exclusion Criteria

Subject participating simultaneously in another research trial	☐	☐

Subject number : ☐

Date of consent : ☐☐ day ☐☐ month 2 0 0 year

Visit Date : ☐☐ day ☐☐ month 2 0 0 year

Name and signature of the investigator :

☐

PHYSICAL EXAMINATION

1) Date of Birth : ⌊__⌋ ⌊__⌋ **1 9** ⌊__⌋⌊__⌋
day month year

2) Sex : Male ☐(1) Female ☐(2)

3) Medical personal history (including traumatic accidents):

None ☐(0)

Or Medical diagnosis (see coding annex): (1)

	Year of diagnosis /or intervention	Past medical or surgical history (coding)
1	⌊__⌋⌊__⌋⌊__⌋⌊__⌋	/
2	⌊__⌋⌊__⌋⌊__⌋⌊__⌋	/
3	⌊__⌋⌊__⌋⌊__⌋⌊__⌋	/
4	⌊__⌋⌊__⌋⌊__⌋⌊__⌋	/
5	⌊__⌋⌊__⌋⌊__⌋⌊__⌋	/
6	⌊__⌋⌊__⌋⌊__⌋⌊__⌋	/
7	⌊__⌋⌊__⌋⌊__⌋⌊__⌋	/
8	⌊__⌋⌊__⌋⌊__⌋⌊__⌋	/

4) Current treatment (medication or supplements)

Has the subject taken any treatment for more than 7 days during the last 30 days ?

Yes ☐(1) No ☐ (0)

If yes, please specify when and what was taken):

	During of medication			What Medication (INNs)	CODE (see appendix IV)
	Start date (dd/mm/year)	End date (dd/mm/year)	On going		
1	/ /	/ /	☐		/
2	/ /	/ /	☐		/
3	/ /	/ /	☐		/
4	/ /	/ /	☐		/
5	/ /	/ /	☐		/
6	/ /	/ /	☐		/
7	/ /	/ /	☐		/
8	/ /	/ /	☐		/

Has the subject taken any treatment in the last 24 hours? (except when on going is ticked)

Yes ☐(1) No ☐ (0)

Fever (>38°C) during the last 24 hours Yes ☐ (1) No ☐ (0)

5) Allergy

Has patient any allergy? Yes ☐ (1) No ☐ (0)

If yes, please indicate which type of allergen?

- ❍ Pollen
- ❍ Dust
- ❍ Food
- ❍ Animal
- ❍ Other:

If Other, please specify

.....

(one letter per case)

Which is/are the clinical signs ?

- ❍ Atopic dermatitis
- ❍ Urticaria
- ❍ Allergic rhinitis
- ❍ Asthma
- ❍ Other

If Other, please specify

.....

(one capital letter per case)

6) Clinical examination of following items and tick the appropriate box :

	Normal	*Abnormal*	*If abnormal findings, specify*
a) General condition	☐ (1)	☐ (0)	……………………………
b) Abdominal palpation	☐(1)	☐(0)	……………………………
c) Pulmonary auscultation	☐(1)	☐(0)	…………………………...
d) Cardiac auscultation	☐(1)	☐(0)	…………………………...

e) Other abnormalities if detected

|_|_|_|_|_|_|_|_|_|_|_|_|

(one capital letter per case)

7) Pubertal status (see appendix I)

Girls

a) breast					**b) pilosity**				
1	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

Boys

a) gonade					**b) pilosity**				
1	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

Only for girls, if adaptable

Have you already your menses?

Yes ☐ (1) No ☐ (0)

Age of menarchy: |_|_| Months |_|_|_|_| Year

Date of last menstruation : |_|_| dd |_|_| mm |_|_|_|_| Year

Daily oral contraception ? Yes ☐(1) No ☐ (0)
Or
Patch contraception? Yes ☐(1) No ☐ (0)

CLINICAL PARAMETERS

8) Anthropometry

a) Height : ⌞__⌟__⌟__⌟ **cm**

b) Weight : ⌞__⌟__⌟__⌟ **kg**

9) <u>Bio-impedancemetry</u> :

measurement

Resistance **Reactance**

⌞__⌟__⌟__⌟ ⌞__⌟__⌟__⌟

- **<u>% of Body Density by Bop Pod (optional):</u>**

measurement

⌞__⌟__⌟__⌟, ⌞__⌟

10) SKIN FOLD THICKNESS

	1st measurement	2nd measurement	3rd measurement
a) Bicipital (mm)	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋
b) Tricipital (mm)	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋
c) Sub-scapular (mm)	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋
d) Suprailiac (mm)	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋
e) Thigh (mm)	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋
f) Calf (mm)	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋

11) CIRCUMFERENCES

	1st measurement	2nd measurement	3rd measurement
a) Arm (mm)	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋
b) Biceps (mm)	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋
c) Waist (mm)	⌊__.__.__⌋, ⌊__⌋	⌊__.__.__⌋, ⌊__⌋	⌊__.__.__⌋, ⌊__⌋
d) Hip (mm)	⌊__.__.__⌋, ⌊__⌋	⌊__.__.__⌋, ⌊__⌋	⌊__.__.__⌋, ⌊__⌋
e) Proximal thigh (mm)	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋

12) VITAL SIGNS

a) Blood pressure (mmHg) :

Systolic 1	Diastolic 1	Systolic 2	Diastolic 2
⌊_ _ _⌋	⌊_ _ _⌋	⌊_ _ _⌋	⌊_ _ _⌋

b) Heart Rate (beats/min) : ⌊_ _ _⌋

13) Do the subject participate to accelerometry assessment ? Yes ☐(1) No☐

BLOOD SAMPLES

Laboratory Assessments, blood

	Yes	No
Subject allocated to a blood samples class ?	☐	☐

	Yes	No
Blood samples have been collected (including specific questionnary ?)	☐	☐

Sample Date ⌊_ _⌋ / ⌊_ _⌋ / ⌊_ _ _ _⌋
dd mm Year

Did the adolescent have fever/cold the day of blood samples? ☐ ☐

Please fill the appropriate Laboratory request form HELENA-CSS

STUDY TERMINATION

Has the subject completed all exams and questionnaires of the study?

Yes ☐ No ☐

If no, please specify:

Essential for each subject:

	Yes	**No**
1. Inclusion criteria	☐	☐
2. Weight and height	☐	☐
3. Informed consent signed	☐	☐

Optional (each subject must complete at least 75% of the following, i.e. 12 measurements)

1. Clinical examination (validity assessed by the Medical Doctor)

   Yes ☐ No ☐

2. Anthropometric measurements (all the measurements)

   Yes ☐ No ☐

3. BIA

Yes ☐ No ☐

4. General Questionnary for adolescents Self European Socio-economic Status (not fully blank)

Yes ☐ No ☐

5. Nutrition Knowledge (NKT-C)(75% of the questions responded)

Yes ☐ No ☐

6. Eating Behaviour (EWI-C) (75% of the questions responded)

Yes ☐ No ☐

7. YANA-C (2 days)

Yes ☐ No ☐

8. Food choice and preference (75% of the questions responded)

Yes ☐ No ☐

9. Determinants of Healthy Eating (HE) + Determinants of physical activity (PA)

Yes ☐ No ☐

10. Physical Activity (PAQ)

Yes	No
☐	☐

11. Questionnary for parents (QP) (75% of the questions responded)

Yes	No
☐	☐

12. Blood Sampling (including questionnaire)

Yes	No
☐	☐

13. Accelerometry assessment (minimum criteria defined into the protocol)

Yes	No
☐	☐

14. Physical fitness tests

Yes	No
☐	☐

Main reason (only one) for premature discontinuation

Withdrawal of the subject ☐

Adverse event ☐
Please make sure that AE form is completely filled

Subject lost of follow-up ☐

Other ☐
Please specify.....

Name and signature of the person checking the CRF

Date and signature

└┴┴┘/ └┴┴┘/ └┴┴┴┴┴┴┘

Name of the investigator: ……
Date and signature

└┴┴┘/ └┴┴┘ / └┴┴┴┴┴┴┘

Healthy Lifestyle in Europe by Nutrition in Adolescence

Healthy Lifestyle in Europe by Nutrition in Adolescence in 13-16 years adolescents across Europe.

Cross-sectional study (HELENA-CSS) and Pilot Study.

BLOOD SAMPLING

QUESTIONNAIRE

From UL2/Bonn November 2005

Subject number: H 1 ....
Number of centre | Number of school | Number of class | Number of subject

Subjects initials: .....
first name/second name /family name

Evaluation date: ....
Day | Month | Year

Please ask the adolescent **before** taking the blood samples and tick the appropriate box to answer each question.

1. Has the adolescent fasted for at least 10 hours?

Yes ☐ (1) No ☐ (0)

Please specify what time he had his last meal, snack or drink (other than water):

└─┴─┘ └─┴─┘
hh mm

2. Did the adolescent come to school by motorised transport (car, bus, …)?

Yes ☐ (1) No ☐ (0)

Or walking?

Yes ☐ (1) No ☐ (0)

3. Did the adolescent have a common cold or other infectious disease during the last week?

Yes ☐ (1) No ☐ (0)

If yes, please specify type of disease

└……┴……┴……┴……┴……┴……┴……┴……┴……┴……┴……┘

(one letter per case)

Answer will be coded (Coding instructions will be provided) by investigator before electronic data capture.

└……┴……┘

If yes, please indicate for how long: └……┴……┘ days

4. Did the adolescent have any symptoms of allergy during the last week?

Examples of symptoms: Red itchy, watery eyes or sneezing/congestion/runny nose or urticaria or abdominal pain or asthma or other.

Yes ☐ (1) No ☐ (0)

If yes, please indicate for how long: ..... days

If yes, please indicate which kind of allergy?

- ❍ Pollen
- ❍ Dust
- ❍ Food allergens
- ❍ Animals
- ❍ Others:

for others, please specify :

.....

one letter per case

Answer will be coded (Coding instructions will be provided) by investigator before electronic data capture

.....

5. Did the adolescent take any medication during the last month?

Yes ☐(1) No ☐ (0)

If yes, please specify when, what was taken and doses (table):

	Duration (days)	Name of the medication	Quantity per day
1			
2			
3			
4			
5			

6. Did the adolescent take any supplement (vitamin/minerals) during the last month?

Yes ☐(1) No ☐ (0)

If yes, please specify when, what was taken and doses (table):

	Duration (days)	Name of the supplement	Dose per day
1			
2			
3			
4			
5			

7. Has the adolescent been vaccinated during the last two weeks?

Yes ☐(1) No ☐ (0)

If yes, please specify when and what vaccine:

Date of vaccination: ....
dd mm year

Type:

.....

one letter per case

Answer will be coded (Coding instructions will be provided) by investigator before electronic data capture

.....

8. (Only for girls) Is the girl menstruating at the moment?

Yes ☐ (1) No ☐ (0)

Please specify first day of last menstruation:

Date: ..... dd ..... mm ..... year

9. Has there been any complications during the blood extraction?

Yes ☐ (1) No ☐ (0)

If yes, specify the problem:

.....

one letter per case

Answer will be coded (Coding instructions will be provided) by investigator before electronic data capture

.....

And fill an adverse event form in the Case Report Form !!!!

END OF THE BSQ

Healthy Lifestyle in Europe by Nutrition in Adolescence in 13-16 years adolescents across Europe.

Cross-sectional study (HELENA-CSS)

MANUAL FOR BLOOD SAMPLING

WP9

Bonn, September 2006

CHANGES SINCE THE PILOT STUDY

Since the end of the Pilot study we had to change some aspects. We are sorry for that, but now we think we simplified some parts of the manual. We already included the changes in the general manual below, so that you just have to follow the instructions. But for a better understanding, here we pointed out all changes:

A.) First of all, we changed the amount of the haematology tube:

EDTA 1.2 ml *(old)* ⇒ EDTA 2.7 ml *(new)*

B.) Next, we **removed** 2 tubes:

CITRATE 3 ml and **HEPARIN 4.5 ml**

C.) We added another SERUM FOR GEL tube, and changed the amount of the tubes from 9 mL to 7.5 mL

Serum 9 ml (old) ⇒ 2 x Serum 7.5 ml (new)

D.) We had to add another Eppendorf (GREEN cap) for 1 ml whole EDTA blood. You have to **cool** it together with the other Eppendorfs (Red, Blue, Yellow).

Green Eppi: 1 mL whole blood

E.) The Eppendorf with the **BLUE** cap contains **only 400 µl** (instead of 500 µl) **metaphosphoric solution** and **only 400 µl plasma** (instead of 500 µl).

F.) The Eppendorf with the **Red** cap contains **only 400 µl** (instead of 500 µl) EDTA whole blood and we already pipetted **400 µl Cytochex** in it.

G.) There are bio-bottles with two sizes. The volume is 2.5 L or 3 L. But there is no difference for the transport or handling, you can use them equally.

H.) Empty tubes (Heparin tube, Serum tubes): We don't need them anymore. You **don't** have to send them to Bonn, they are waste like the needles.

I.) Transport:

The non-cooled transport contains only **two** (instead of three) bio-bottles.
(reason is the missing empty tubes.)

The cooled transport contains a bio-bottle with **four** (instead of three) plastic bags [see point D].

MANUAL FOR BLOOD SAMPLING

A. AT LEAST THREE DAYS IN ADVANCE

- Please confirm with us the address of the school and time of the pick up for the courier

B. THE DAY BEFORE

a. The day before blood sampling, one researcher must go to school in order to **remind the adolescents** that the next morning at 8 a.m. they will have to donor a blood sample and that they have to be fasted.

b. If requested, EMLA patches will be provided. The subject has to fix them on both arms in the morning before leaving the house.

c. At the lab:

Preparing of the metaphosphoric solution: Pour the liquid content of bottle A into bottle B (with the solid content). Shake it gently until all solid substance is solved. Then pipette 400 µl of the metaphosporic solution in the corresponding eppendorfs (**BLUE cap**). It must be stored in the refrigerator (4-8°C) overnight.

d. You already have **400 µl cytochex** in corresponding eppendorfs (**RED cap).** Storage in a refrigerator is not necessary but possible.

e. Please **prepare all the materials** you need the next morning for blood sampling. Make sure that the labelled tubes you take with you correspond to the class where blood is sampled.

f. Please **freeze all cooling elements** for the transport to Bonn at least one day in advance.

g. Make sure that you have **crushed ice** for the next day!

h. Please **remove all stickers** from the cardboard boxes before you use them for the transport.

C. MATERIALS FOR BLOOD SAMPLING

1. medical gloves
2. tourniquet and cushion
3. disinfectant spray
4. gauze swabs
5. butterfly´s
6. labelled tubes (5 tubes each scholar) + some reserves
7. labelled empty eppendorfs (yellow, green, white) + some reserves
8. labelled prepared eppendorfs (blue, red) + some reserves
9. labelled plastic tube (for SERUM) + some reserves
10. 4 plastic bags for separating the different eppendorfs
11. cryobox for the serotec/fatty acids
12. pipettes and tipps
13. crushed ice
14. emla-patches
15. waterproof pens
16. adhesive strips
17. small waste container

18. yellow waste bin for contaminated waste (used butterfly´s...)
19. transportable centrifuge
20. transport boxes, shipping address, all label elements and tape for the transport
21. telephone number of the Bonn group
22. cooling elements
23. blood sampling questionnaires and fax of traceability for Bonn

D. PERSONS FOR BLOOD MANAGEMENT

For the blood drawing day, you need at least:

a. **1 medical doctor/nurse** for the blood drawing itself

b. **1 person** for the tube shaking, tube/rack handling and centrifugations (this work is connected to the doctor, with 2 doctors you need 2 persons)

c. **1 person** for blood separation and pipetting

d. **1 person** as interviewer for the BSQ/breakfast

E. PREVIOUS CONDITIONS

All the subjects must fulfil the following conditions:

a. The subjects participating in the blood sampling must have signed the informed written consent (and their parents).

b. 10/12 h fasted. The last food should be eaten at 8 p.m. the day before blood sampling. During the fasting period, only water intake is permitted.

F. ANALYTICAL CONDITIONS

1. Blood samples must be taken **between 8 and 10 a.m**.
2. Before taking the blood sample, the subject must answer the "Blood sampling questionnaire" and take it with him/her to the doctor. Please make sure that you get a very specific answer to these topics included in the BSQ:
    a. Having or not practiced vigorous exercise 24 h before blood sampling.
    b. Following or not their normal diet (Remember special holidays).
    c. Suffering or not from any acute infectious diseases during the last week.
    d. Suffering or not from any allergic symptoms (red itchy, watery eyes or sneezing/ congestion/ runny nose) during the last week.
    e. Consuming or not any medication during the fasting period (exception made for those strictly necessary, for example, insulin).
3. Before blood sampling, the subject should rest (sitting) about 5 minutes and take off the EMLA patches.
4. Blood samples must be taken while the subject is sitting.
5. All researchers, including the person who is going to take the blood sample, must wear gloves.
6. Please make sure before starting that the labelled tubes correspond to the subject whose blood is going to be taken.

7. Blood should be taken using the Sarstedt® system provided by the Bonn group.
8. The total amount of blood will be around 25 ml.
9. The tubes should be filled-in in the **following order**:
    a. EDTA 2.7 ml
    b. SERUM 7.5 ml
    c. SERUM 7.5 ml
    d. HEPARIN 2.6 ml
    e. EDTA 4 ml

10. **PLEASE NOTE THAT ALL THE TUBES MUST BE FULL!!**
11. The tubes should be managed in the following way:
    a. EDTA 2.7 ml (turn it up and down carefully three times, then place it in the rack)
    b. SERUM tube 7.5 ml (turn it up and down carefully three times, then place it in the rack)
    c. SERUM tube 7.5 ml (turn it up and down carefully three times, then place it in the rack)
    d. HEPARIN 2.6 ml (BLUE labelled) (turn it up and down carefully 3 times, place it **immediately on ice!)**
    e. EDTA 4 ml (turn it up and down carefully three times, then place it directly into a bio bottle)

12. Once the subject has finished, put at first a gauze swab and tell him/her to press on the arm for 2 minutes if required replace it with a plaster.
13. The subject should leave the room and be told where the breakfast is going to be served.
14. For the following, please make sure that the labelled eppis correspond to the subject.

Further blood handling:

1. From the EDTA 2.7 ml tube:
    - take 0.4 ml with the micropipette and put it in the eppendorf already filled-in with 0.4 ml of cytochex (**RED cap**). Close properly and mix carefully.
    - take another 1 ml with the micropipette and put it in the eppendorf (**GREEN cap**). Close properly.
    - Close the EDTA 2.7 ml tube and send the remaining blood to the local lab for the hemogram.
2. Once there are enough serum tubes, centrifuge them at room temperature for 10 minutes at 3500 rpm (aprox. 2500 x g). (we will send each centre the centrifugation speed to there corresponding centrifuge)
    - After centrifugation, put **1 mL** of **serum** in the corresponding **eppendorf (WHITE)** for the serotec and put it in the cryobox.

- Then fill **another eppi (WHITE)** with **1 ml** of serum for the fatty acid determination and put it in the same cryobox. These eppendorfs have to be frozen locally **as soon as possible**.
- Decant the **rest of both serum tubes** into the corresponding **plastic tube**. Close properly. Throw away the empty SERUM tubes with the remaining RBC!

3. Once there are enough **HEPARIN 2.6 ml** tubes, centrifuge them at room temperature for 10 minutes at 3500 rpm (aprox. 2500 x g).
   - After centrifugation take 400 µl of the supernatant and put it in the eppendorf already filled with 400 µl of metaphosphoric solution (**BLUE cap**). Close the eppi properly and mix carefully.
   - Decant the rest of the plasma into the corresponding eppendorf (or pipette it) (**YELLOW cap**). **Throw away** the empty HEPARIN tube with the RBC!
4. Please put as soon as possible all eppendorfs (**BLUE, GREEN, RED, YELLOW**) in the corresponding plastic bags.
5. **For the cooling:** Collect all plastic bags with the coloured eppis and place them in the bio-bottle. Then put the bio-bottle in the Styrofoam box and place the cooling elements around (Details see below at point 32 “COOLING”).
6. The **EDTA 4 ml** tube will be packed in one bio-bottle (may be directly after blood drawing) and sent to Bonn without further manipulation.
7. Once finished, you must have (for each subject):
   a. EDTA 4 ml tube
   b. EDTA 2.7 ml tube containing 1.3 ml of whole blood
   c. 1 eppendorf (RED cap) containing 0.4 ml of EDTA blood mixed with 0.4 ml cytochex
   d. 1 eppendorf (BLUE cap) containing 0.4 ml heparin plasma mixed with 0.4 ml metaphosphoric solution
   e. 1 eppendorf (YELLOW cap) containing the rest of heparin plasma (aprox. 1 ml)
   f. 1 eppendorf (GREEN cap) containing 1 ml of EDTA blood.
   g. 1 eppendorf (WHITE cap) containing 1 ml serum for the local serotec
   h. 1 eppendorf (WHITE cap) containing 1 ml serum for the fatty acid determination
   i. 1 plastic tube containing the rest of serum (aprox. 6 ml)
8. All tubes must be sent to Bonn, with the exception of number **b, g and h.**
9. Take all the EDTA 2.7 ml tubes containing 1.3 ml to the local lab for the **hemogram**/haematology. Once you have the results, make a copy and send the copy to Bonn. (See shipping address Bonn or per fax +49-228-733217). Keep the original with all the other documents.

- Try to get back the EDTA 2.7 ml tube from the local lab after analysing the blood! Then freeze it together with the cryobox (serotec/fatty acids).

10. Take the cryobox with all the eppendorfs containing 1 ml of serum to your local lab and freeze them at –20°/-80° C. These will be sent to Bonn once all together after the study.
11. For the rest of the tubes, please proceed in the following way:

COOLING:

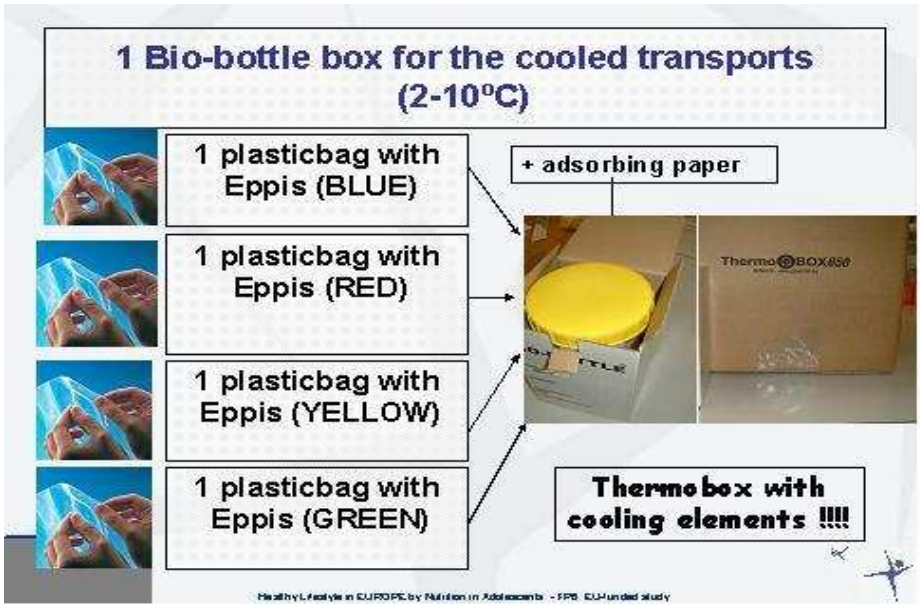
j. Take one Bio-bottle and place the adsorbing paper (from the inside of the bottle) on the ground. Put the four plastic bags inside.

k. Close the Bio-bottle and put it back into the original box.

l. Then place this box into the styrofoam transport box. Take the cooling elements out of the freezer and place them around and on top of the Bio-bottle box.

m. Close the Styrofoam transport box and put it into its cardboard box for transportation.

Please be sure that everything is properly closed.



Tubes:

a. Take another Bio-bottle (with adsorbing paper inside) and fill in all EDTA 4 ml tubes.

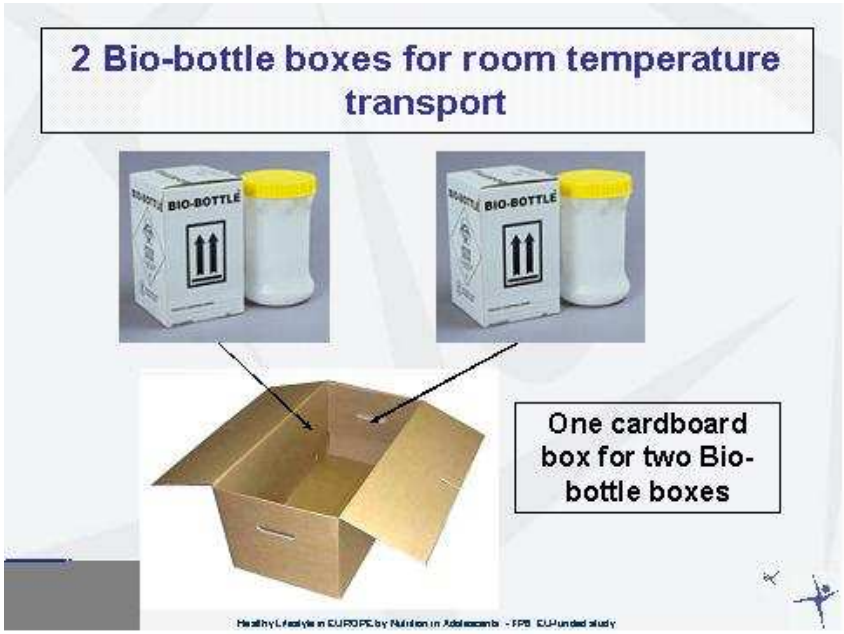
b. Take another Bio-bottle (with adsorbing paper inside) and fill in all plastic tubes with SERUM.

c. Put the closed Bio-bottles into the original Bio-bottle cartons and then into the second larger cardboard box.

NOTE: It is not important that exactly all tubes for the **non-cooled** transport are in the corresponding Bio-bottle as long as every tube will be sent to Bonn.

PLEASE BE SURE THAT EVERYTHING IS PROPERLY CLOSED.


2 Bio-bottle boxes for room temperature (20-25°C) transport
BIO-BOTTLE
BIO-BOTTLE
1 box for all EDTA-tubes (4 ml)
1 box for all Serum plastic tubes


2 Bio-bottle boxes for room temperature transport
BIO-BOTTLE
BIO-BOTTLE
One cardboard box for two Bio-bottle boxes

Transport:

1. Just put the **shipping address** and the **UN 3373 sticker** on each cardboard box.

Please be sure that you **removed all old stickers** from the cardboard boxes before you use them for the transport.

2. Fill out the way bill and the exemption letter.

3. The courier (**GO!**) will come at the agreed time to take the two cardboard boxes (one box for the cooled transport, one for the non-cooled transport) directly from the schools.

4. If you have problems with the courier please call us we have to organise it from Bonn, also if it seems a little bit complicated.

Shipping address Bonn:

Christina Breidenassel

IEL-Ernährungsphysiologie

Rheinische Friedrichs-Wilhelms Universität

Endenicher Allee 11-13

D-53115 Bonn

GERMANY

Office Bonn: +49 228 733767

Fax Bonn: +49 228 733217

Danksagung

An erster Stelle möchte ich Herrn Prof. Dr. Peter Stehle für die Ermöglichung dieser Arbeit am Institut für Ernährungs- und Lebensmittelwissenschaften (IEL) – Humanernährung danken. Insbesondere die zahlreichen, wissenschaftlichen Gespräche empfand ich als überaus bereichernd. Vielen Dank für die Unterstützung!

Frau Prof. Dr. Marcela González-Gross verdanke ich, dass das HELENA-Projekt in Kooperation mit dem IEL zustande kam. Außerdem möchte ich mich bei Ihr herzlich für die langjährige Betreuung und die Übernahme des Korreferats bedanken.

Frau PD Dr. Reinhild Prinz-Langenohl danke ich für die spontane Übernahme des Korreferats und die wissenschaftlichen Gespräche!

Der Europäischen Kommission danke ich für die finanzielle Förderung des HELENA-Projektes.

Prof. Dr. Klaus Pietrzik und seiner Arbeitsgruppe möchte ich für die gute Zusammenarbeit danken und für die Messung der Proben, die die Basis dieser Arbeit bilden!

Bei der gesamten HELENA-Arbeitsgruppe will ich mich für ihren tatkräftigen Einsatz bedanken! Mein besonderer Dank gilt hier dem Projekt Koordinator Prof. Luis A. Moreno, und meinen Tutoren Dr. Jonatan R. Ruiz, Dr. Christina Breidenassel, Dr. German Vicente Rodriguez und Dr. Jean Dallongeville.

Auch persönlich möchte ich mich bei Frau Dr. Christina Breidenassel bedanken. Sie hat in der Planung, Organisation und Durchführung der HELENA-Studie für die Arbeitsgruppe 9 Enormes geleistet. Stets trug sie mit ihren wertvollen Ideen zur Verbesserung der Studie und meiner Arbeit bei.

Christel Bierschbach, Erika Blatzheim, Adelheid Schuch, Anke Berchtold, Petra Pickert, Ulrike Albers, Miriam Segoviano und Jara Valtueña arbeiteten sehr gewissenhaft unsere HELENA-Blutproben im Labor auf. Christel und Anke haben mich stets geduldig in die HPLC-Analytik eingewiesen und Petra hat mich mit dem Immulite 2000 vertraut gemacht. Wenn der -80°C-Gefrierschrank Alarm schlug, kam Margret Schüller manchmal sogar am Wochenende ins Institut, um unsere HELENA-Blutproben vor dem Auftauen zu retten. Vielen Dank für die Einsatzbereitschaft!

Oliver Melsheimer, Dr. Julia Anna Knoll und Prof. Dr. Joachim Kunert danke ich herzlich für die statistische Beratung und für die geduldige Beantwortung meiner Fragen!

Meinen Arbeitskollegen, insbesondere Andre Spinneker danke ich für die schöne Zeit am Institut und die erfrischenden Diskussionen!

Mein Dank fürs Korrekturlesen geht an Zsófia Potak und Gudrun Frings!

Meiner Familie, meinen Freunden und Vertrauten, meinem Erfolgsteam, und insbesondere meinem Ernst, danke ich für das Verständnis, die Hilfsbereitschaft und Unterstützung.