North–south gradients in plasma concentrations of B-vitamins and other components of one-carbon metabolism in Western Europe: results from the European Prospective Investigation into Cancer and Nutrition (EPIC) Study

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Abstract

Different lifestyle patterns across Europe may influence plasma concentrations of B-vitamins and one-carbon metabolites and their relation to chronic disease. Comparison of published data on one-carbon metabolites in Western European regions is difficult due to differences in sampling procedures and analytical methods between studies. The present study aimed, to compare plasma concentrations of one-carbon metabolites in Western European regions with one laboratory performing all biochemical analyses. We performed the present study in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort among 5446 presumptively healthy individuals. Quantile regression was used to compare sex-specific median concentrations between Northern (Denmark and Sweden), Central (France, Germany, The Netherlands and United Kingdom) and Southern (Greece, Spain and Italy) European regions. The lowest folate concentrations were observed in Northern Europe (men, 10·4 nmol/l; women, 10·7 nmol/l) and highest concentrations in Central Europe. Cobalamin concentrations were slightly higher in Northern Europe (men, 330 pmol/l; women, 352 pmol/l) compared with Central and Southern Europe, but did not show a clear north–south gradient. Vitamin B_2 concentrations were highest in Northern Europe (men, 22·2 nmol/l; women, 26·0 nmol/l) and decreased towards Southern Europe ($P_{trend} < 0.001$). Vitamin B_6 concentrations were highest in Central Europe in men (77·3 nmol/l) and highest in the North among women (70·4 nmol/l), with decreasing concentrations towards Southern Europe in women ($P_{trend} < 0.001$). In men, concentrations of serine, glycine and sarcosine increased from the north to south. In women, sarcosine increased from Northern to Southern Europe. These findings may provide relevant information for the study of regional differences of chronic disease incidence in association with lifestyle.

Key words: B-vitamins: Amino acids: One-carbon metabolism: Europe

One-carbon metabolism involves transfer of one-carbon units from the amino acids serine or glycine to tetrahydrofolate to form methylenetetrahydrofolate. Methylenetetrahydrofolate is either used for the synthesis of thymidine and purines, which are building blocks for DNA, or it is reduced to methyltetrahydrofolate, which re-methylates homocysteine to methionine. Methionine is activated to *S*-adenosylmethionine, the major methyl group donor for the methylation reactions of many substrates. The vitamins B_2, B_6 and B_{12} function as cofactors for enzymes, catalysing the conversion of homocysteine to methionine or cystathionine and cysteine. Choline is another source of one-carbon units. It is converted to betaine, which in the liver and kidneys serves as a substrate in another reaction re-methylating homocysteine to methionine, with dimethylglycine as a product. Sarcosine is formed from dimethylglycine or during the metabolism of excess methionine, and can be further metabolised to glycine, which is an important source of serine[1].

B-vitamins, related amino acids and variants of genes coding for enzymes involved in one-carbon metabolism may influence cellular growth, differentiation and function, which provides plausible biological background that links one-carbon metabolism to various disease outcomes. Although results from observational and experimental studies have not always been consistent, the majority of studies suggest that B-vitamins and other components related to one-carbon metabolism are associated with neural tube defects[2], the

metabolic syndrome[3,4], CVD[5], cognitive impairment[6], bone health[7] and different types of cancer[8,9].

The methylenetetrahydrofolate reductase (MTHFR) $677C \rightarrow T$ polymorphism[10] and lifestyle factors such as smoking[11] and alcohol consumption[12,13] may affect blood concentrations of B-vitamins and other components of one-carbon metabolism[14]. There is a great diversity in dietary and lifestyle patterns among European countries[15–17], which are likely to influence plasma status of nutrients and the subsequent risk of morbidity and mortality[18]. For example, a Mediterranean diet – rich in plant foods and folate – in combination with healthy lifestyle factors such as abstaining from smoking, moderate alcohol consumption and being physically active, has been associated with a lower mortality rate[19].

The European Prospective Investigation into Cancer and Nutrition (EPIC) and a number of multicentre studies have been conducted to investigate dietary intake or blood concentrations of B-vitamins as determinants of health in Europe. These studies show relatively low intake of B-vitamins in Scandinavian countries, the Netherlands, Germany and Greece[20,21]. However, no major differences in B-vitamin intake across EPIC study centres have been observed[22,23]. The SENECA (Survey in Europe on Nutrition and the Elderly, a Concerted Action) study showed large differences in plasma vitamin status between study centres, with vitamin B_6 concentrations being lowest in Greece and Denmark[24], but no clear

geographical patterns were observed[25]. A summary of thirty-one European studies on plasma/serum B-vitamins revealed relatively low folate concentrations in Norway and Sweden, while lower vitamin B_{12} concentrations were observed in the Netherlands, Germany, Czech Republic and Italy compared to other countries[21].

Data on plasma concentrations of components related to one-carbon metabolism in European countries are inconclusive. Comparison of plasma concentrations between individual European studies is difficult due to different blood sampling protocols and large inter-assay variability by laboratory methods used in different studies[26]. The present study aimed to investigate patterns in plasma B-vitamin status, amino acids and related methylamines in Western European regions, while eliminating variability in sampling procedures and assay methods, as all biochemical analyses were carried out in citrate plasma by a single laboratory. With data on 5446 individuals from the EPIC cohort, this is the largest population to date to compare patterns across European regions and in a population with no mandatory fortification with folic acid or other B-vitamins.

Methods

Study population

The EPIC study investigates associations between diet, lifestyle and cancer risk[27] among individuals recruited between 1992 and 2000 living in ten European countries. The present cross-sectional study comprises the control individuals who were matched to cancer cases participating in four nested case–control sub-studies on gastric- (GC, n 805), colorectal- (CRC, n 2408), lung- (LC, n 1778) and pancreatic cancer (PC, n 455). Matching criteria were sex, age group ($\pm 2\cdot5$ years), study centre and date of blood collection (all studies), and additionally for time of blood collection and fasting status in the PC study. Blood samples from EPIC-Oxford and EPIC-Norway centres were exposed to ambient temperatures for up to 48 h. As some B-vitamins and related metabolites are unstable under such conditions[26,28], all EPIC-Oxford (n 221) and EPIC-Norway (n 11) samples were excluded from the present analyses. Participants in the present study were from Greece (n 288, recruited from studies on GC (n 32), CRC (n 51), LC (n 182) and PC (n 23), Spain (n 638, recruited from studies on GC (n 107), CRC (n 238), LC (n 254) and PC (n 39)), Italy (n 799, recruited from studies on GC (n 186), CRC (n 294), LC (n 277) and PC (n 42)), France (n 133, recruited from studies on GC (n 10), CRC (n 60), LC (n 48) and PC (n 15)), Germany (n 797 recruited from studies on GC (n 117), CRC (n 313), LC (n 312) and PC (n 55)), The Netherlands (n 650, recruited from studies on GC (n 78), CRC (n 297), LC (n 237) and PC (n 38)), United Kingdom (n 919, recruited from studies on GC (n 104), CRC (n 421), LC (n 350) and PC (n 44)), Denmark (n 500, recruited from studies on GC (n 43), CRC (n 373), LC (n 0) and PC (n 84)) and Sweden (n 722, recruited from studies on GC (n 128), CRC (n 361), LC (n 118) and PC (n 115)). The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the ethical review boards from the International Agency for Research on Cancer; all local participating centres approved the study. Written informed consent was obtained from all the subjects.

Data collection

Blood sampling and laboratory analyses. In each of the recruitment centres, fasting ($42\cdot8\%$ ($6\cdot6\%$ in North, $30\cdot7\%$ in Central and $5\cdot5\%$ in Southern Europe)) or non-fasting ($47\cdot2\%$ ($6\cdot8\%$ in North, $14\cdot3\%$ in Central and $26\cdot1\%$ in Southern Europe)) blood samples of at least 30 ml were drawn at baseline between March 1991 and April 1999. In $9\cdot9\%$ ($9\cdot1\%$ in North, $0\cdot7\%$ in Central and $0\cdot1\%$ in Southern Europe) information on prandial status was missing. Samples were stored at 5–10°C, protected from light and transported to local laboratories for processing and aliquoting as previously described[27,29]. In all countries, except Denmark and Sweden, blood was separated into $0\cdot5$ ml aliquots (serum, plasma, erythrocytes and buffy coat for DNA extraction) and stored into plastic CBS straws™, which were heat sealed and stored in liquid N_2 (−196°C). Half of the aliquots were stored at the local study centre and the other half in the central EPIC biorepository at the International Agency for Research on Cancer (Lyon, France). In Denmark, aliquots of $1\cdot0$ ml were stored locally at −150°C under N_2 vapour. In Sweden, samples were stored at −80°C.

For the present study, blood samples collected into citrate plasma tubes were used for biochemical analyses in the laboratory of Bevital AS (http://www.bevital.no). The present study included B-vitamins (folate, vitamins B_2 and B_6 species, vitamin B_{12} and its marker methylmalonic acid (MMA)), methylamines (choline, betaine and dimethylglycine) and amino acids (total homocysteine (tHcy), methionine, total cysteine, cystathionine, serine, glycine and sarcosine). Vitamin B_2 measures included plasma concentrations of riboflavin and FMN; pyridoxal-5′-phosphate, pyridoxal (PL) and 4-pyridoxic acid were measures of vitamin B_6 status. Vitamin B_2 and B_6 species and methylamines were determined by LC–MS/MS[30–33], and MMA and amino acids by GC–MS/MS based on methylchloroformate derivatisation[33,34]. Vitamin B_{12} was determined with a *Lactobacillus leichmannii* microbiological method[35] and plasma folate with a *Lactobacillus casei* microbiological method, both adapted to a microtitre plate format, and analysis was carried out on a robotic workstation (Micro-lab AT plus 2; Hamilton Bonaduz AG)[36]. Within- and between-day CV for folate were 3–20%[36], 3–20% for vitamin B_2 vitamers[32], 6–22% for vitamin B_6 vitamers[32], < 5% for vitamin B_{12}, tHcy and MMA[34,35], < 5% for choline, betaine and dimethylglycine[31] and <5% for the remaining amino acids[33,34].

FMN was not measured in the LC study; serine, glycine and sarcosine were not measured in gastric- and colorectal studies; and the methylamines were not measured in LC and PC studies.

The *MTHFR* 677C → T polymorphism (rs 1801133) was determined by matrix-assisted laser desorption/ionisation

time-of-flight MS[37,38] in the studies on GC, CRC and LC. Data on the *MTHFR* 677C → T polymorphism were available for 87·8 % of the participants (664 missing).

Descriptive variables. Data on age, sex and information on lifestyle factors known to affect B-vitamin status, such as smoking status[11] (never, former, current, missing) and alcohol consumption, were collected at enrolment in the study. Total alcohol consumption (pure ethanol in g/d) at baseline, which represented consumption over the 12 months before enrolment in the EPIC cohort, was determined from lifestyle questionnaires. The present study distinguishes between abstainers, very light or occasional consumers (0·1– < 4·9 g/d), moderate consumers (4·9– < 30 g/d) and heavy consumers (≥ 30 g/d). Data on total alcohol intake were available for 99·6 % of the participants (twenty-two missing).

Statistical methods

Statistical analyses were conducted for the total study population, as well as within each European region (North: Sweden and Denmark; Central: France, United Kingdom, the Netherlands and Germany; South: Italy, Spain and Greece) and country. Using the PROC CORR SAS procedure, we calculated Spearman rank correlation coefficients between metabolites, adjusting (using a PARTIAL statement) for age, sex, sub-study and prandial status. Because riboflavin may be formed from FMN[39], we considered the sum of riboflavin and FMN as a measure for vitamin B_2 status. As PL may be formed from pyridoxal-5'-phosphate in blood and 4-pyridoxic acid from PL in the liver[40], we considered the sum of pyridoxal-5'-phosphate, PL and 4-pyridoxic acid to be a measure of vitamin B_6 status. Some metabolites were not normally distributed. Therefore, differences in crude concentrations of the B-vitamins between demographic and lifestyle groups were assessed by non-parametric tests, Mann–Whitney U tests or χ^2 tests where appropriate.

Because blood concentrations of several metabolites varied according to sub-studies as well as age groups, medians of one-carbon-related nutrients across European regions were described and estimated by using a direct standardisation method[41]. First, the total population was considered as 'a standard' and was distributed into all possible combinations of three age groups (≤ 60, 60– ≤ 70 and > 70 years), four sub-studies (GC, CRC, PC and LC) and three groups of prandial status (fasting, non-fasting and missing information on prandial status), which comprises thirty-six subgroups altogether. For each combination, we estimated the relative frequency or weight (w) from the total population. Second, the median (m) of each metabolite was estimated for each combination of sub-study and age in each region by using quantile regression models. Finally, the standardised median of each metabolite within each region was defined as the weighted average of the respective median m, weighted by w. This procedure was also used for calculating the 5th and the 95th percentiles of the metabolites. Tests for trends in plasma concentrations of the one-carbon metabolites across the European regions were assessed by median regression (i.e. quantile regression), in which we regressed

crude blood concentrations of the metabolites against the European region, adjusted for age groups, sub-studies and prandial status. P for difference between regions was assessed by the Wald test.

We further investigated proportional differences in metabolite concentrations across the three European regions at the 0·025, 0·05, 0·25, 0·50, 0·75, 0·95 and 0·975 quantiles of the outcome variables[42]. The results were plotted graphically, displaying the percentage differences between the European regions (with Southern Europe as the reference) *v.* the metabolite concentrations at each quantile cut-off. These models are adjusted for age, sex, sub-study and prandial status. In addition, we investigated whether relations of plasma folate and tHcy with the MTHFR 677C → T polymorphism differed across regions by plotting proportional differences across MTHFR genotypes against metabolite concentrations at each quantile cut-off of the outcome for each region separately.

Statistical analyses were performed using SAS version 9.2 for Windows (SAS Institute, Inc.) and the open-source statistical program environment R with the package 'quantreg' to obtain quantile regression results[43].

Results

Characteristics of the study population and data integrity

The average age of the total study population (n 5446) was 59·0 years, and 46·1 % were women. The UK study population had the highest mean age (65·4 years) and the Spanish study population had the lowest (54·2 years). The French study population only included women and the German study population represented the largest proportion of men (76·7 %) (data not shown). The proportion of current smokers was lowest in the Central European countries (16·4 %), and the proportion of individuals consuming ≥ 30 g alcohol/d was highest in the Southern European countries (22·2 %) (Table 1). The prevalence of the *MTHFR* 677TT genotype was 17·9 % in Southern Europe compared with 12·2 % in Central Europe and 8·2 % in Northern Europe (Table 1).

Spearman correlation coefficients (ρ_s), adjusted for age, sex, sub-study and prandial status between the vitamin B_6 species, ranged from 0·61 to 0·71, riboflavin correlated with FMN ($\rho_s = 0·26$), vitamin B_{12} with MMA ($\rho_s = -0·26$) and tHcy ($\rho_s = -0·24$) and folate with tHcy ($\rho_s = -0·30$, all P values < 0·01). These correlations were essentially similar across the European regions and individual countries (data not shown).

Table 2 shows concentrations of B-vitamins and tHcy according to relevant demographic and lifestyle factors. Women had higher concentrations of vitamins B_2 and B_{12} compared with men, whereas vitamin B_6 and tHcy were higher in men. Furthermore, concentrations of vitamin B_2, folate and tHcy were higher, whereas vitamin B_{12} was lower, in individuals older than 60 years compared with younger participants. Concentrations of all vitamins were lower among smokers compared with ex- and never smokers, whereas tHcy was lowest in never smokers. Those consuming ≥ 30 g alcohol/d had lower vitamin B_2, B_{12} and folate concentrations, but higher vitamin B_6 and tHcy concentrations, compared with

Table 1. Characteristics of the study population

	Total population	Region*			
		North	Central	South	P for difference
n	5446	1222	2499	1725	
Sex					
Female (%)	46·1	45·5	50·4	40·9	<0·001†
Age (years)					
Mean	59·0	59·7	60·5	57·1	<0·001‡
Range	28·5–78·6	30·0–73·3	34·5–73·3	28·5–78·6	
Smoking (%)					
Unknown	1·3	0·6	1·1	2·0	
Never	42·4	38·8	43·1	44·2	
Ex	34·3	31·8	39·4	28·6	
Current	22·0	28·9	16·4	25·2	<0·001†
Alcohol consumption (g/d)§					
Abstainers: 0 g/d (%)	14·3	9·7	12·7	19·8	
≥ 0·1 g/d < 4·9 (%)	28·5	29·7	32·3	22·1	
≥ 4·9 g/d < 30 (%)	41·8	46·9	43·5	35·9	
≥ 30 g/d (%)	15·4	13·7	11·5	22·2	<0·001†
MTHFR 677 C → T§					
CC (%)	41·8	47·8	44·3	34·3	
CT (%)	45·0	44·0	43·5	47·8	
TT (%)	13·2	8·2	12·2	17·9	<0·001†

MTHFR, methylenetetrahydrofolate reductase.

* North: Sweden and Denmark; Central: France, United Kingdom, the Netherlands and Germany; South: Italy, Spain and Greece.

† P for difference by χ^2 test.

‡ P for difference by ANOVA.

§ Data on alcohol intake and *MTHFR* 677C→T genotype data were available for 99·6 % (twenty-two missing) and 87·8 % (664 missing), respectively.

abstainers and very light or occasional consumers of alcohol. Furthermore, plasma folate concentrations were lower among those with the MTHFR 677TT genotype compared with the CC and CT genotypes, whereas tHcy concentrations were higher in the MTHFR 677TT genotype. Concentrations of B-vitamins according to these demographic and lifestyle variables were similar within each region (data not shown). Finally, concentrations of vitamin B_{12} were lower, whereas concentrations of vitamins B_2, B_6, and tHcy were higher among those with fasting blood samples.

Patterns of B-vitamins, amino acids and related nutrients in Europe

We investigated patterns across three European regions (North: Sweden and Denmark; Central: France, United Kingdom, the Netherlands and Germany; South: Italy, Spain and Greece). Sex-specific standardised median (5th–95th percentile) concentrations (Table 3) showed that folate concentrations were lowest in Northern Europe in both men (10·4 (4·82–24·2) nmol/l) and women (10·7 (4·73–31·5) nmol/l) and highest concentrations were seen in Central Europe in both men (14·6 (6·40–41·8) nmol/l) and women (13·9 (6·05–44·8) nmol/l). However, the geographic pattern in tHcy concentrations – an inverse marker of folate and vitamin B_{12} status – did not mirror patterns of folate concentrations, and tHcy concentrations were lowest in Northern Europe. Vitamin B_{12} status was generally better in Northern Europe among men and women, as reflected by higher vitamin B_{12}

and lower MMA concentrations, but did not show a clear north–south gradient. Highest vitamin B_2 concentrations were observed in men (22·2 (11·1–85·0) nmol/l) and women (26·0 (12·5–69·5) nmol/l) in the North, with decreasing concentrations from the north to south ($P_{trend} < 0.001$ in both sexes). Likewise, we observed that vitamin B_6 concentrations were highest in Northern European women (74·4 (33·8–528) nmol/l) and decreased from the north to south ($P_{trend} < 0.001$), whereas vitamin B_6 concentrations in men were highest in Central Europe (77·3 (41·8–207) nmol/l) (Table 3). In men, standardised median concentrations of the amino acids sarcosine, serine and glycine were lowest in Northern Europe, and showed increasing concentrations towards Southern Europe ($P_{trend} < 0.01$ for all). No such clear patterns in amino acid concentrations were observed in women. Furthermore, Southern European men had the highest concentrations of the nutrients choline and betaine, with a significant north–south gradient for both nutrients, whereas these patterns were not observed for women (Table 3). After further adjustment for season of blood collection, smoking and alcohol intake, the standardised medians remained essentially the same (data not shown).

We also compared relative differences in B-vitamin concentrations between regions at the 0·025, 0·05, 0·1, 0·25, 0·5, 0·75, 0·9, 0·95 and 0·975 percentiles of the B-vitamin distributions (Fig. 1). Relative to Southern Europe, folate concentrations in Central and the Northern Europe showed a shift to higher levels of the upper part of the distribution (for Central Europe) and a shift to lower levels of the lower part of the distribution (for Northern Europe). Differences in tHcy

Table 2. Plasma concentrations of vitamins B$_2$, B$_6$, B$_{12}$ and folate by demography, lifestyle and methylenetetrahydrofolate reductase (MTHFR)* genotype

(Medians and 5th–95th percentiles)

	Folate (nmol/l)		Vitamin B$_{12}$ (pmol/l)		Vitamin B$_2$ (nmol/l)†		Vitamin B$_6$ (nmol/l)‡		tHcy (μmol/l)	
	Median	5th–95th percentiles	Median	5th–95th percentiles	Median	5th–95th percentiles	Median	5th–95th percentiles	Median	5th–95th percentiles
Sex										
Male (n 2889)	12·6	5·44–34·5	297	161–523	18·9	9·03–70·2	70·7	34·7–175	11·1	7·04–19·1
Female (n 2557)	12·7	5·66–38·6	324	174–589	21·6	10·1–76·3	64·3	31·4–265	9·56	6·16–16·0
P for difference§		0·046		<0·001		<0·001		<0·001		<0·001
Age (years)										
< 60 (n 3005)	12·2	5·58–33·3	316	175–563	19·8	9·39–72·3	66·8	33·2–199	9·80	6·28–16·7
> 60 (n 2441)	13·0	5·61–39·1	298	157–550	20·7	9·54–76·1	68·6	33·0–213	11·1	7·0–18·8
P for difference§		<0·001		<0·001		0·014		0·126		<0·001
Smoking										
Never (n 2313)	13·2	5·98–38·4	314	169–589	21·2	10·1–76·8	68·4	35·0–222	10·1	6·32–16·5
Ex (n 1867)	13·0	6·01–37·4	305	162–533	20·8	9·41–85·4	72·8	36·2–205	10·7	6·75–17·5
Current (n 1196)	10·7	4·66–30·7	304	160–539	17·8	8·49–57·2	59·4	27·6–199	10·7	6·56–20·5
P for trend‖		<0·001		0·002		<0·001		<0·001		<0·001
Alcohol¶										
Abstainer (n 774)	13·3	5·65–39·0	315	160–614	20·3	9·71–72·3	62·0	30·0–201	10·5	6·71–18·8
0·1–4·9 g/d (n 1544)	12·1	5·31–37·1	318	168–563	21·0	9·82–65·4	61·9	31·4–203	10·2	6·35–17·2
4·9–30 g/d (n 2271)	12·7	5·66–34·7	304	165–549	20·5	9·41–76·1	70·6	34·6–219	10·3	6·54–17·2
≥ 30 g/d (n 835)	12·4	5·89–35·5	300	164–518	19·0	8·42–92·5	76·5	38·0–196	10·8	6·65–19·5
P for trend‖		0·005		<0·001		0·021		<0·001		<0·001
MTHFR 677*¶										
CC (n 1999)	13·0	5·93–36·3	304	167–530	19·8	9·51–68·9	67·6	33·3–208	10·4	6·42–17·0
CT (n 2152)	12·5	5·59–34·2	306	162–573	19·8	9·19–65·9	67·0	32·5–207	10·4	6·59–17·3
TT (n 631)	11·0	5·02–37·2	298	163–536	19·2	8·49–89·8	70·4	32·7–200	11·5	6·98–23·9
P for trend‖		<0·001		0·475		0·764		0·278		<0·001
Prandial status										
Fasting (n 2333)	12·8	5·63–37·6	295	160–528	20·8	9·78–75·7	71·5	33·5–240	10·7	6·67–19·5
Non-fasting (n 2572)	13·1	5·74–36·4	316	167–587	19·1	8·94–66·5	64·2	32·4–178	10·3	6·32–18·5
Unknown (n 541)	11·0	5·05–40·4	314	177–549	24·2	12·3–101	74·8	37·8–267	10·8	6·91–18·0
P for difference§		0·059		<0·001		<0·001		<0·001		<0·001

tHcy, total homocysteine.

* Not measured in controls belonging to the pancreatic cancer study.

† Vitamin B$_2$: riboflavin + FMN.

‡ Vitamin B$_6$: pyridoxal-5′-phosphate + pyridoxal + 4-pyridoxic acid.

§ P for difference by Mann–Whitney U, unknown prandial status excluded from statistical test.

‖ P for trend by Wald test.

¶ Data on alcohol intake and MTHFR 677C→T genotype data were available for 99·6 % (twenty-two missing) and 87·8 % (664 missing), respectively.

Table 3. Standardised plasma concentrations and trends across European regions* of B-vitamins and related metabolites

(Medians and 5th−95th percentiles)

	Standardised median plasma concentrations																	
	Men									Women								
	All men (n 2889)		North (n 666)		Central (n 1204)		South (n 1019)			All (n 2557)		North (n 556)		Central (n 1295)		South (n 706)		
	Median	5th−95th percentiles	Median	5th−95th percentiles	Median	5th−95th percentiles	Median	5th−95th percentiles	P_{trend}†	Median	5th−95th percentiles	Median	5th−95th percentiles	Median	5th−95th percentiles	Median	5th−95th percentiles	P_{trend}†
Vitamins																		
Folate (nmol/l)	12·7	5·63−34·8	10·4	4·82−24·2	14·6	6·40−41·8	12·3	5·45−30·8	<0·001	13·0	5·72−39·0	10·7	4·73−31·5	13·9	6·05−44·8	13·7	6·29−34·1	<0·001
B_{12} (pmol/l)	298	164−509	330	198−564	286	157−462	290	156−544	<0·001	326	177−571	352	186−595	311	173−559	330	182−583	0·037
B_2 (nmol/l)‡	19·1	9·59−71·2	22·2	11·1−85·0	20·5	10·5−76·8	14·6	7·62−47·4	<0·001	21·8	10·9−76·5	26·0	12·5−69·5	23·0	12·2−77·9	17·2	8·11−84·2	<0·001
B_6 (nmol/l)§	71·2	36·7−180	75·1	36·5−209	77·3	41·8−207	61·6	32·2−134	<0·001	65·6	33·7−292	74·4	33·8−528	67·8	36·8−320	55·1	28·8−111	<0·001
Amino acids (μmol/l)																		
tHcy	11·1	7·73−19·4	10·3	7·32−16·8	11·4	7·91−20·2	11·4	7·82−20·1	<0·001	9·66	6·51−17·3	8·85	6·06−14·7	10·4	7·02−18·6	9·09	6·27−15·0	0·853
Cys	273	228−328	278	232−337	272	227−324	270	227−321	0·001	262	219−322	269	225−327	260	218−320	258	214−316	<0·001
Met	26·6	18·2−39·6	25·9	17·7−38·4	25·1	17·5−38·6	28·7	20·8−41·9	<0·001	23·9	17·0−36·3	23·0	16·7−35·0	23·4	16·1−36·7	25·6	19·1−36·6	<0·001
Ser‖	133	91·4−188	123	85·5−176	125	88·5−183	142	104−194	<0·001	143	95·8−206	129	88·5−183	147	97·9−211	143	98·3−209	0·003
Gly‖	256	182−371	234	177−367	251	174−377	263	198−370	<0·001	293	190−507	275	174−496	301	195−521	285	194−486	0·965
Sar‖	1·60	0·98−2·89	1·44	0·96−2·57	1·53	0·98−2·90	1·71	1·00−2·94	<0·001	1·34	0·81−2·47	1·31	0·81−2·11	1·29	0·78−2·33	1·46	0·94−2·89	0·022
Nutrients or intermediates (μmol/l)																		
MMA	0·18	0·12−0·36	0·17	0·11−0·34	0·18	0·12−0·39	0·17	0·11−0·33	0·999	0·17	0·11−0·33	0·17	0·11−0·28	0·18	0·12−0·36	0·17	0·11−0·31	0·999
Choline¶	9·76	6·57−15·1	9·04	6·27−13·5	9·82	6·46−15·6	10·5	7·28−16·3	<0·001	9·17	6·19−14·5	8·65	5·45−12·5	9·37	6·29−15·1	9·26	6·54−15·1	0·038
Betaine¶	35·9	23·1−58·2	34·9	23·1−57·7	35·5	22·9−56·9	38·3	23·1−61·6	<0·001	29·2	16·8−48·1	29·0	16·7−46·4	28·1	16·8−46·7	31·9	16·7−51·1	0·007
DMG¶	3·72	2·45−6·05	3·67	2·33−5·64	3·68	2·42−5·96	3·86	2·61−6·49	0·012	3·24	2·10−5·42	3·21	2·17−5·39	3·26	2·09−5·21	3·28	1·96−5·88	0·478
Cystathionine	0·18	0·09−0·46	0·20	0·10−0·63	0·18	0·10−0·43	0·17	0·09−0·39	<0·001	0·16	0·08−0·42	0·17	0·08−0·51	0·16	0·08−0·42	0·16	0·09−0·36	0·048

tHcy, total homocysteine; MMA, methylmalonic acid; DMG, dimethylglycine.
* North: Sweden and Denmark; Central: France, United Kingdom, the Netherlands and Germany; South: Italy, Spain and Greece.
† By Quantile regression, Wald test.
‡ Vitamin B_2: riboflavin + FMN not measured in controls matched to lung cancer.
§ Vitamin B_6: pyridoxal-5′-phosphate + pyridoxal + 4-pyridoxic acid.
‖ Not measured in controls matched to gastric and colorectal cancer.
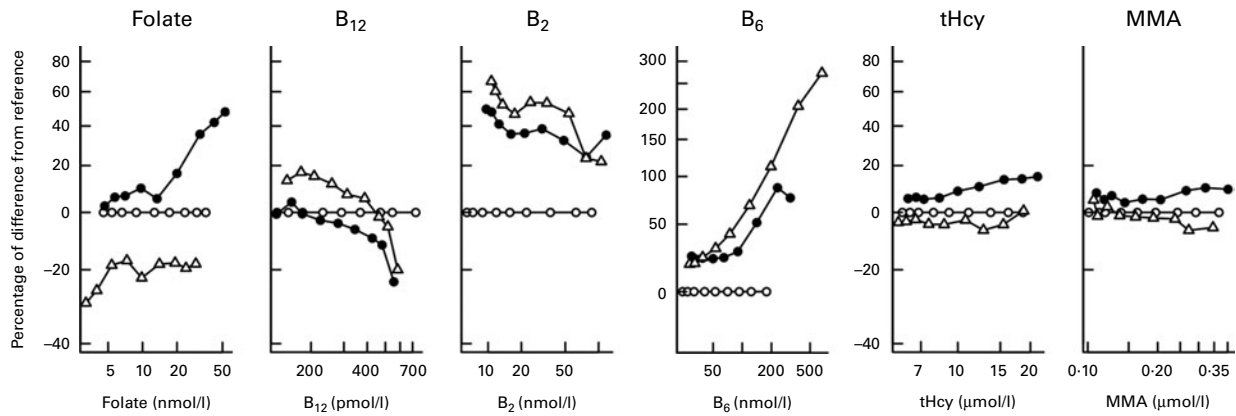¶ Not measured in controls matched to lung and pancreas cancer.

Fig. 1. Plasma concentrations of B-vitamins across Western European regions by quantile regression. Concentrations of the vitamins and their markers are shown on the x-axis, and the differences between the European regions are shown on the y-axis. The 0·025, 0·05, 0·1, 0·25, 0·5, 0·75, 0·9, 0·95 and 0·975 percentiles are reflected by the dots. The model is adjusted for age, sex and sub-study and prandial status. Southern Europe is the reference category. tHcy, total homocysteine; MMA, methylmalonic acid. –○–, South (reference); –●–, Central; –△–, North.

concentrations were small. The profiles for markers of vitamin B_{12} status were complementary to each other, with the lowest vitamin B_{12} and highest MMA concentrations in Central Europe. The relative regional differences in vitamin B_2 concentrations over the whole distribution range were symmetrical with higher levels for Central and the Northern Europe. There were regional differences in vitamin B_6 concentrations, with highest levels in Central and the Northern Europe. The distribution curve was asymmetrical and showed most pronounced differences at higher concentrations.

Analyses of the proportion of individuals with deficient B-vitamin status, as based on the 2·5th percentile distribution of the total study population, revealed that deficiencies of vitamin B_2 (6·2 % with concentrations <7·9 nmol/l) and vitamin B_6 (4·5 % with concentrations <28·9 nmol/l) were most prevalent in Southern European countries, whereas vitamin B_{12} and folate deficiency were more frequently observed in Central (2·9 % with concentrations <140 pmol/l) and Northern Europe (4·6 % with concentrations <4·6 nmol/l), respectively (data not shown).

Distribution of folate according to methylenetetrahydrofolate reductase 677C → T genotype

There was an increasing north–south gradient for the number of T-alleles of the MTHFR 677C → T genotype (Table 1). We investigated the distribution of folate and tHcy according to the MTHFR 677C → T genotype across regions by quantile regression (Table 4 and Fig. S1, available online). In the total study population and in each region, there was a trend towards lower folate and higher tHcy concentrations according to the number of T-alleles (Table 4). Further, the distribution was asymmetric, with the largest differences in the lower quantiles of folate and the highest quantiles in tHcy. The asymmetric profile and differences were most pronounced in Southern Europe (Fig. S1, available online). Thus, the MTHFR 677TT genotype was associated with an increased prevalence of subjects with low folate and high tHcy, suggesting impaired folate status, and exerted the largest differences on distribution of these indices in populations with the highest MTHFR 677 T allele frequency.

Table 4. Plasma folate and total homocysteine (tHcy) concentrations according to the methylenetetrahydrofolate reductase (*MTHFR*) 677C → T genotype across European regions*

(Medians and 5th–95th percentiles)

MTHFR 677	North (n 1221)		Central (n 1221)		South (n 935)		P for difference†
	Median	5th–95th percentiles	Median	5th–95th percentiles	Median	5th–95th percentiles	
Plasma folate concentrations							
CC	9·93	4·78–31·9	13·1	6·23–39·4	13·5	6·10–31·2	
CT	10·0	4·24–26·5	12·6	5·95–36·5	12·6	5·66–28·4	
TT	9·02	4·09–23·5	11·4	4·96–45·7	10·4	4·64–26·3	<0·0001
Plasma tHcy concentrations							
CC	9·65	6·23–16·8	9·75	6·57–15·6	9·16	5·92–14·8	
CT	9·76	6·51–18·0	10·0	6·75–15·6	8·85	6·05–15·4	
TT	10·6	6·62–20·4	10·7	6·73–21·0	9·82	6·21–23·7	<0·0001

* North: Sweden and Denmark; Central: France, United Kingdom, the Netherlands and Germany; South: Italy, Spain and Greece.
† P for difference by Kruskal–Wallis test.

Discussion

Principal findings

The present study is the largest to date to investigate geographical patterns in blood concentrations of B-vitamins, amino acids and other metabolites related to one-carbon metabolism. Folate concentrations were lowest in Northern European countries, with increasing concentrations from north to south in women. Vitamins B_2 and B_6 concentrations were highest in Northern European countries and showed a clear decreasing north–south gradient in both men and women. Concentrations of tHcy, sarcosine, serine and glycine in men were lowest in Northern Europe, and increased towards Southern Europe. The highest concentrations of the nutrients choline and betaine were observed in Southern Europe, but there was no significant north–south gradient for choline in men.

B-vitamin status

Food patterns, traditions of vitamin supplementation and lifestyle factors differ greatly across European regions. Compared with the overall EPIC study population, the dietary pattern of Italy and Greece is characterised by a higher consumption of plant foods – the main source of folate – and a lower consumption of animal – the main source of vitamin B_{12} – and processed foods. Diets in France and Spain contain relatively high amounts of plant foods and animal products, whereas the UK general population has a relatively high consumption of tea, sauces, cakes, soft drinks, margarine and butter. Finally, the diet in the Nordic countries, The Netherlands, Germany and the UK general population is relatively high in potatoes and animal, processed and sweetened/refined foods[44,45].

A lower intake of fruits and vegetables in Northern Europe may explain the tendency towards lower folate concentrations in the Northern region, and is in line with the previously reported lower folate intake[20,23] and plasma levels[21] in Northern countries. The higher plasma vitamin B_{12} concentrations in Northern Europe agree with higher vitamin B_{12} intake in Northern countries[46] and probably reflect a higher meat intake in this region[44]. Lower vitamin B_2 concentrations observed in Southern countries could be partially explained by a lower consumption of dairy products[47]. These findings are in line with results from the SENECA study showing a higher consumption of dairy products in Northern countries compared with Southern countries[48], although the EPIC population had higher dairy intakes in Southern countries[47], as well as higher intakes of vegetables, fruits and cereals, which are also sources of vitamin B_2.[49] Vitamin B_6 concentrations were lower in Southern countries compared with Northern countries, and differences between regions became larger at the higher end of the vitamin B_6 distribution, where the concentrations are similar to concentrations obtained by vitamin B_6 supplements[50,51].

A subsample of the EPIC population revealed a north–south gradient in vitamin supplement use, with higher consumption in Northern European countries than in Southern European countries, and higher consumption for women than for men[52], which could also partially explain the higher concentrations observed for vitamins B_2, B_6 and B_{12}, but not for folate in the Northern region.

Mandatory folic acid fortification has not been implemented in Western European countries. However, national policies on voluntary folic acid fortification vary considerably[53], which may also affect folate status in individuals who do not use supplements[54]. In addition to higher folate status, mandatory[55] or voluntary food folic acid fortification[54] also influences the concentrations of other B-vitamins, tHcy and nutrients involved in one-carbon metabolism[3]. However, it is unclear to what extent any voluntary fortification might have affected the between-region differences in the present study.

One study observed a lower folate intake in Southern European countries in the summer compared with Northern countries[23]. This may be explained by the fact that green leafy vegetables, one of the major dietary sources of folate in those countries, are broadly known as an early-spring or late autumn crop[23]. Adjustment for season of blood collection did not affect the trends observed in the present study. Smoking behaviour[11] and alcohol consumption[13] are known lifestyle factors that affect B-vitamin status. Even though there were differences in smoking behaviour and alcohol consumption across regions, additional adjustment for these lifestyle factors did not materially alter the estimated patterns across regions.

Amino acids and other nutrients

Folate-dependent one-carbon metabolism is closely linked to pathways involving amino acids like homocysteine, methionine, serine and glycine, and donors of one-carbon units such as choline, betaine and related methylamines[3]. Therefore, it is important to consider not only B-vitamins, but also amino acids, and other nutrients and metabolites in epidemiological studies on one-carbon metabolism. Patterns of plasma concentrations of the amino acids tHcy, methionine, sarcosine, serine and glycine were consistent, with the lowest concentrations in Northern Europe and increasing concentrations towards Southern Europe in men. Likewise, men in Southern Europe had the highest concentrations of the nutrients choline and betaine, whereas in women, betaine and choline concentrations were highest in Southern and the Central Europe, respectively. The high choline concentrations are likely to be attributed to high egg consumption[56], which has been reported to be highest in Spain[57].

Methylenetetrahydrofolate reductase 677C → T genotype and folate status

Prevalence of the *MTHFR* 677TT genotype in white populations in Europe, North America and Australia varies from 8 to 20 %, and a trend of increasing frequency of the TT genotype has been observed from north to south Europe[58], which agrees with the present study. The *TT* variant, which was most prevalent in Southern Europe, was associated with lower folate and higher tHcy concentrations, as has previously been shown[59]. Folate and tHcy are the metabolites that are most strongly

influenced by MTHFR genotype[10]. Although one would thereby expect lower folate status in the South as well, we observed that folate concentrations were higher in the South, suggesting that dietary factors are stronger determinants for folate status than this specific genetic factor.

Limitations and strengths

The EPIC study investigates the relation of diet and lifestyle with cancer incidence, and was as such not designed to be representative of the general European population. The study cohort comprised individuals recruited from the general population, blood donors (Spain and Italy), teachers and school workers (France), a health-conscious population (UK) and women participating in a breast cancer screening programme (The Netherlands and Italy)[23]. The present study population may therefore reflect a more health-conscious part of the European population. The patterns observed may consequently have been under- or overestimated as a result of potential selection bias. An important strength of the present study is the large sample size of individuals recruited from several Western European countries. Nevertheless, the present study cannot provide any estimates of potential differences in disease risk profiles across the European regions as we lack data on disease status or use of medication that may affect metabolite concentrations. Furthermore, the impact of alcohol consumption on the observed patterns could not be investigated in further detail as we did not have data specifically on the consumption of beer, wine or spirits. Finally, it is known that menopausal status affects choline concentrations[60]. However, the present study lacked statistical power to investigate whether postmenopausal status affected choline concentrations across the European regions.

A standardised protocol for the collection and storage of blood was used in the majority of participating study centres; all blood samples were collected in citrate plasma and all biochemical analyses were performed in one laboratory. This reduced pre-analytical variability due to differences in sample handling and eliminated inter-assay variations. Recently, the same laboratory investigated the stability of metabolites related to one-carbon metabolism at room temperature and during long-term storage[26]. The results of that study underscore the importance of adequate sample handling and storage in epidemiological and clinical studies[26]. Folate, species of vitamins B_2 and B_6 and choline appear to be unstable during storage[26]. However, the sum of riboflavin and FMN and the sum of pyridoxal-5′-phosphate, PL and 4-pyridoxic acid are stable, and we therefore presented the sum of these species as measures of vitamins B_2 and B_6 status in the present investigation. In addition, concentrations of choline, but not betaine, increase with increasing storage time[26]. Therefore, higher concentrations of both choline and betaine observed in Central European countries are more likely explained by a higher dietary intake of choline than by pre-analytical errors. The observed correlations between metabolites were essentially the same when calculated within countries, regions or the total study population, observations that support data integrity.

Conclusions

The present study covers a large geographical area in Europe, and presents a comprehensive spectrum of B-vitamins, amino acids and other nutrients involved in one-carbon metabolism. The study reveals clear decreasing north–south gradients of vitamins B_2 and B_6, and increasing gradients of folate and the majority of amino acids included in the present study. These differences may reflect distinct dietary and lifestyle patterns between regions, and may be relevant for the study of regional differences in chronic disease incidence in Western Europe.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0007114512004990

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