Folate and vitamin B₁₂ concentrations are associated with plasma DHA and EPA fatty acids in European adolescents: the Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) study

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Abbreviations: DQI, Diet Quality Index; FA, fatty acids; HELENA, Healthy Lifestyle in Europe by Nutrition in Adolescence; PC, phosphatidylcholine; PF, plasma folate; PL, phospholipids; PLP, pyridoxal 5'-phosphate; SAH, *S*-adenosyl-ʟ-homocysteine; tHcy, homocysteine.

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Abstract

This study aimed to examine the association between vitamin B_6, folate and vitamin B_{12} biomarkers and plasma fatty acids in European adolescents. A subsample from the Healthy Lifestyle in Europe by Nutrition in Adolescence study with valid data on B-vitamins and fatty acid blood parameters, and all the other covariates used in the analyses such as BMI, Diet Quality Index, education of the mother and physical activity assessed by a questionnaire, was selected resulting in 674 cases (43 % males). B-vitamin biomarkers were measured by chromatography and immunoassay and fatty acids by enzymatic analyses. Linear mixed models elucidated the association between B-vitamins and fatty acid blood parameters (changes in fatty acid profiles according to change in 10 units of vitamin B biomarkers). DHA, EPA) and n-3 fatty acids showed positive associations with B-vitamin biomarkers, mainly with those corresponding to folate and vitamin B_{12}. Contrarily, negative associations were found with n-6:n-3 ratio, *trans*-fatty acids and oleic:stearic ratio. With total homocysteine (tHcy), all the associations found with these parameters were opposite (for instance, an increase of 10 nmol/l in red blood cell folate or holotranscobalamin in females produces an increase of 15·85 µmol/l of EPA (*P* value <0·01), whereas an increase of 10 nmol/l of tHcy in males produces a decrease of 2·06 µmol/l of DHA (*P* value <0·05). Positive associations between B-vitamins and specific fatty acids might suggest underlying mechanisms between B-vitamins and CVD and it is worth the attention of public health policies.

Key words: Fatty acids: B-vitamins: Adolescents: Europe

Atherosclerotic CVD is the main cause of death worldwide[1]. Over the last few decades, an increase in CVD risk factors such as excess body weight, insulin resistance or glucose intolerance among others has been observed in both children and adolescents[2]. Such evidences support claims to independently assess the effect of different CVD risk factors in these age groups and promote strategies to prevent future events of atherosclerosis[3].

Cardiovascular risk factors including high serum concentrations of total cholesterol, TAG or LDL-cholesterol or low levels of HDL-cholesterol are associated with the development of arterial fatty streaks[4] and increased total homocysteine (tHcy) concentrations. The latter is considered an independent predictor of cardiovascular mortality or of all-cause mortality[5] because of its role in oxidative stress, endothelium damage and thrombogenicity enhancement[6]. However, a debate exists about whether tHcy should be interpreted as a risk factor or a biomarker of cardiovascular events.

Results from large prospective studies suggest that elevated circulating concentrations of tHcy are associated with increased risk of CVD, regardless of other well-known risk factors such as smoking, high blood pressure, high serum lipids and obesity[7]. At the same time, results of a recent meta-analysis showed that tHcy-lowering interventions did not reduce the occurrence of cardiovascular events[8]. It could be explained by the fact that homozygous carriers of the *677C-T* polymorphism on the methylenetetrahydrofolate reductase gene have higher CVD risk, which implies higher tHcy circulating levels[7,9].

A recent systematic review examining the impact of homocysteine-lowering interventions via increased vitamins B_6, folate or B_{12} supplement uptake on cardiovascular events failed to identify any relationship[10]. This fact set out the possibility that the positive associations between serum homocysteine and CVD risk may be confounded by other cardiovascular risk factors[11] such as low plasma (n-3) long-chain PUFA levels. The main (n-3) long-chain PUFA such as EPA and DHA are suggested to act as cardioprotective elements by lowering blood pressure and heart rate, reducing serum TAG, thrombotic tendency, inflammation and arrhythmias and by improving endothelial function, insulin sensitivity, paraoxonase concentrations and plaque stability[12]. Previous animal[13–15] and human[16–18] studies have reported that some changes in plasma fatty acid (FA) concentrations might be explained by

B-vitamin biomarkers because of their role as cofactors in the methylation of phosphatidylethanolamine (PE) to phosphatidylcholine (PC), crucial in the transport of PUFA in the blood. The latter could explain the controversial results observed regarding the association between tHcy and CVD risk. However, results obtained in such studies were also inconsistent among them[13–18].

Assumptions that B-vitamin biomarkers involved in tHcy regulation are linked to serum phospholipid (PL) FA profiles have not been studied in young populations yet. The aim of this study was to examine the association between vitamin B_6, folate and vitamin B_{12} biomarkers (including tHcy) and fasting lipid profile and serum PL FA profile (focusing on n-3 FA) in healthy European adolescents.

Methods

Study design

The Healthy Lifestyle in Europe by Nutrition in Adolescence Cross-Sectional Study (HELENA-CSS) obtained standardised, reliable and comparable data on relevant nutrition and health-related parameters from a large sample of European adolescents[19]. Fieldwork took place from November 2006 to December 2007 in ten cities (Vienna, Ghent, Lille, Dortmund, Athens, Heraklion, Pecs, Rome, Zaragoza and Stockholm) of nine European countries (two in Greece).

Subjects

Adolescents aged 12·5–17·5 years were randomly recruited from selected schools. The HELENA-CSS study population consisted of 3528 adolescents (response rate 61·3 %)[20]. Of those, a subsample of 1089 adolescents was randomly selected for blood sampling. For the purposes of this study, only adolescents with available information on B-vitamin biomarkers, lipid profile, serum PL FA, BMI and Diet Quality Index (DQI) were included, resulting in 674 cases (43 % males). Sampling selection procedures can be observed in Fig. 1.

The present study was performed following the ethical guidelines of the Declaration of Helsinki, the Good Clinical Practice rules and the legislation about clinical research in
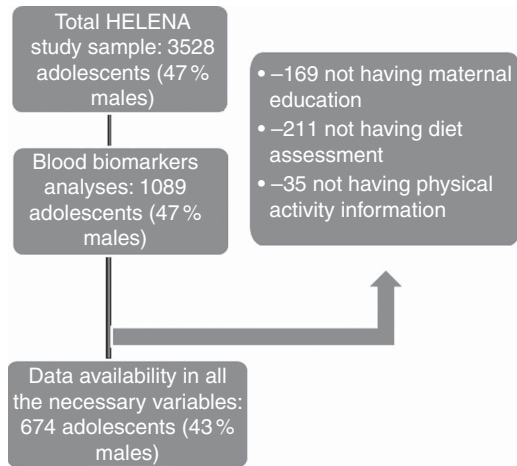
Fig. 1. Flow chart of the selection process from the Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) study initial sample.

human subjects of the participating countries. The Ethics Committees of the involved centres approved the study protocol and procedures, and all subjects and their parents or legal guardians signed an informed written consent form[21].

Assessment of vitamin B₆, folate and vitamin B₁₂ biomarker concentrations as well as serum phospholipid fatty acids

Assessment and analysis of biological samples in the study have already been described elsewhere[22,23]. In brief, fasting blood samples were collected by venepuncture at school. Plasma folate (PF), serum vitamin B_{12}, tHcy and red blood cell folate (RBC-folate) were measured by competitive immunoassay (Immulite 2000; DPC Biermann GmbH) (CV for PF = 5·4 %, RBC-folate = 10·7 %, serum vitamin B_{12} = 5·0 %). Pyridoxal 5'-phosphate (PLP) was measured by HPLC (Varian Deutschland GmbH; CV = 1 %). Serum holotranscobalamin (HoloTC) was assayed by microparticle enzyme immunoassay (Active B12; Axis-Shield Ltd, CV = 5·1 %) using AxSYM (Abbott Diagnostics)[22].

TAG as well as HDL- and LDL-cholesterol concentrations were enzymatically assayed by the Dimension RxL clinical chemistry system (Dade Behring).

Serum FA composition from PL was determined by capillary GC (model 3900; Varian GmbH) after extraction performed by TLC using the standard 1,2 Dipentadecanoyl-sn-glycero-3-phosphocholine. The total amount of FA was obtained as a result of the percentage area by integrating the area under the peak and dividing it by the total area for all FA. The CV was 4·4 % for all serum PL FA analyses[24]. The headings 'saturated fatty acids', 'MUFA', 'PUFA', 'trans', 'ω3' and 'ω6' include all types of these FA identified during blood analyses.

Diet Quality Index for adolescents

DQI was computed from data obtained from two non-consecutive, 24-h recalls performed via a computer-based,

self-reported tool called the HELENA Dietary Assessment Tool[25]. This tool was based on a previous version developed for Flemish adolescents, the Young Adolescents' Nutrition Assessment on Computer valid for twenty-nine food groups aggregated according to the European Food Groups classification system[26] and for several nutrients apart from energy, including macronutrients, fibre, Ca, Fe and vitamin C[25]. Total daily food intake was distributed into six meal occasions. For each occasion, the user was invited to select all the consumed food items and beverages from a standardised food list. If any food or beverage was not included in the list, they could be added manually at any time. Household measurements or pictures of portion sizes were used to obtain information on quantities. The DQI used in this study was originally developed for preschool-aged children[27], and was later adapted and validated for use in adolescents[28] and consists of three components: dietary quality, dietary diversity and dietary equilibrium. This index is used as an indicator of compliance with the Flemish Food Based Dietary Guidelines, which are very similar to dietary guidelines in other countries including the WHO CINDI (Countrywide Integrated Non-communicable Disease Intervention programme) pyramid, making it applicable for a European population[28].

Physical examination

Weight and height were measured with an electronic scale (SECA 861; Seca Ltd) and with a telescopic stadiometer (SECA 225; Seca Ltd) to the nearest 0·1 kg and to the nearest 0·1 cm, respectively[29]. BMI was calculated as body weight[30] divided by the square of height (m). In addition, BMI was adjusted for age and sex providing a BMI standard deviation score using the British 1990 growth reference data from the Child Growth Foundation[31].

Socio-economic status and self-reported physical activity

Maternal education was used as proxy for socio-economic status obtained via a self-administered questionnaire. This variable was assessed using four categories (elementary, lower secondary, higher secondary or tertiary education)[32].

The level of physical activity was obtained using the validated International Physical Activity Questionnaire for Adolescents (IPAQ-A), adapted and validated for the HELENA study[33]. The IPAQ-A covers four physical activity domains during the whole week: school-related physical activity (including activity during physical education classes and breaks), transportation, housework and activities during leisure time. Activities were later classified into low, moderate and vigorous activities on the basis of guidelines for data processing and analyses of the IPAQ-A[34]. Total time spent on moderate-to-vigorous activity was summed and truncated to avoid overestimation[35].

Statistical analyses

Population characteristics are presented as means and standard deviations unless otherwise stated. All analyses were stratified by sex. Baseline characteristics were compared between sexes

using Student's t test or the Mann–Whitney U test for continuous variables and by Pearson's χ^2 test for categorical variables. Tests for normality were performed using the Kolmogorov–Smirnov test. B-vitamin biomarkers, fasting lipids and serum PL FA-related variables were logarithmically transformed to obtain a normal distribution.

Associations between either fasting lipid profile or serum PL FA and B-vitamin biomarkers (vitamin B_6, folate, vitamin B_{12} and tHcy as independent variables) were tested with linear mixed-effects models to control for study design (clustering of cases within cities), including z-scores of BMI, DQI, maternal education and physical activity. Associations are presented in terms of change in 10 units of the independent variables (B-vitamin biomarkers) to ease interpretation and to present biologically relevant results. A coefficient lower than 10 implies a negative association, whereas a coefficient higher than 10 implies a positive one. In addition, single univariate tests controlling for the false discovery rate, using the widely agreed 0·25 cut-off point[36] with the Benjamini–Hochberg method[37], were performed to control for the type I error inflation associated with multiple testing. Significance was established according to the new P values obtained after applying the Benjamini–Hochberg method. PASW 20.0 for Windows (IBM SPSS Inc.) was used to run the analyses.

Results

Several significant differences in terms of descriptive characteristics were observed between included and excluded participants (data not shown). In the whole HELENA study sample, males represented 48 %, whereas for the sample included in this analysis males represent 43 %. For instance, included males had significantly lower BMI and higher DQI scores, and there were significant differences in terms of maternal education ($P < 0.001$) as compared with those excluded. No differences were observed in females. Included males had lower levels of DHA ($P < 0.05$), whereas included females had higher levels of palmitoleic acid and palmitoleic:palmitic ratio ($P < 0.05$). Both male and female participants had higher levels of α-linolenic fatty acid compared with adolescents excluded from the analysis. No differences were found between included and excluded adolescents regarding B-vitamin biomarkers.

Table 1 presents adolescents' characteristics stratified by sex. Females had significantly higher DQI scores and lipid parameters as opposed to males. Males had significantly higher weekly moderate-to-vigorous physical activity, lower levels of serum vitamin B_{12}, and higher PLP and tHcy levels ($P < 0.01$). The mean palmitoleic:palmitic acid ratio was identical for both sexes.

Associations presented in Table 2 reflect changes in 10 units of the independent variables (B-vitamin biomarkers). A coefficient lesser than 10 implies a negative association. Overall, more significant associations were found between folate and vitamin B_{12} biomarkers and fasting lipid profile and serum PL FA status as compared with PLP or tHcy concentrations. All significant associations found with tHcy were negative, except

for the oleic:stearic ratio where a positive significant association was observed in males with an increase of 10 µmol in tHcy concentrations, resulting in a decrease in the PL FA in serum. The associations found between either fasting lipid profile or serum PL FA and B-vitamin biomarkers were always in the opposite direction as those found with tHcy. Moreover, the association found between TAG and serum vitamin B_{12} was positive for males and negative for females. Most of the significant associations were observed between B-vitamin biomarkers and DHA, n-3 FA and n-3:n-6 ratio as the dependent variables.

Discussion

To our knowledge, this is the first study investigating the associations between B-vitamin biomarkers and serum PL FA in a large sample of adolescents and one of the few available in humans. Our results primarily showed that folate and vitamin B_{12} biomarkers and homocysteine concentrations were associated with serum PL FA, mainly with the n-3 pathway. Evidence of associations in the literature is unclear and often debatable. Some studies have reported associations between B-vitamin biomarkers and PUFA both in animals[15,38] and in humans[15,18,39], whereas others have failed to observe any associations in elderly and pregnant women[16,40]. However, this is the first time that such associations are explored in a population of healthy adolescents. It is of great importance to further investigate such associations in future studies because of their role in CVD. It would also be relevant to evaluate the link between both B-vitamins and FA and DNA methylation and future conditions such as cancer or other metabolic diseases[41].

Another HELENA-based subanalysis by Vyncke et al.[42] showed that food intake could explain a limited variance of serum PL FA, being maximal for the n-3 FA (14·2 %). We have also observed that the highest variance of B-vitamin concentrations explained by the habitual combination of foods consumed by a subject or a population group, referring to the dietary patterns[43], consists of only 17 % (unpublished results).

In previous studies, the DQI showed positive associations with HoloTC and with n-3 FA[28]. In our study, associations between B-vitamin biomarkers, FA and lipids fraction were adjusted for DQI scores to mitigate the effect of diet in observed associations. However, we cannot preclude that some of the observed associations may be due to the combination of both B-vitamins and FA contained in the same food items. For instance, previous analyses showed that fish products are important contributors of both DHA[44] and folate[45], and such products were found to be positively associated with the DQI, whereas less healthy foods showed negative associations. In this sense, it is important to consider the DQI dietary equilibrium component as it might influence negatively the total score if a food group is consumed in excess[28].

Two physiological pathways that may explain such associations have been described[15,18]. However, despite the plausibility of both mechanisms, the specific metabolic pathway linking both B-vitamins and FA has not been clearly established. First, B-vitamins act as cofactors in the methylation process as

Table 1. Characteristics of the study participants
(Mean values and standard deviations)

Characteristics (*n* of males, *n* of females)	Male		Female		
	Mean	SD	Mean	SD	*P*
Age (years)*	14·78	1·26	14·78	1·18	0·95
BMI *z*-score (319, 403)†*	0·47	1·14	0·31	1·06	0·47
DQI (319, 403)‡*	52·46	15·46	55·07	14·97	0·02
Moderate–vigorous physical activity (min/d) (319, 403)*	123·10	92·42	90·61	77·22	<0·001
Maternal education (319, 403) (*n*)§*					0·71
High education	119		120		
Medium–high education	95		134		
Medium–low education	82		103		
Low education	23		46		
B-vitamins-related biomarkers					
PLP (nmol/l) (289, 379)*	65·53	43·23	60·72	61·74	0·004
PF (nmol/l) (319, 403)*	18·17	9·99	18·69	9·65	0·21
RBC-folate (nmol/l) (317, 398)*	806·32	388·90	758·23	297·97	0·34
Serum vitamin B_{12} (pmol/l) (319, 401)*	328·81	128·33	367·34	158·41	0·003
HoloTC (pmol/l) (300, 392)*	63·43	31·15	62·67	32·42	0·59
tHcy (μmol/l) (318, 401)*	7·98	4·83	6·87	2·54	0·001
Fasting lipid profile					
TAG (mmol/l) (319, 403)*	1·69	0·81	1·94	1·01	<0·001
Total cholesterol (mmol/l) (319, 403)*	3·99	0·70	4·32	0·71	<0·001
HDL-cholesterol (mmol/l) (319, 403)*	1·37	0·25	1·48	0·28	<0·001
LDL-cholesterol (mmol/l) (319, 403)*	2·36	0·67	2·54	0·66	<0·001
Serum PL fatty acids					
15 : 0 pentadecanoic (μmol/l) (319, 403)*	1·86	0·79	1·91	0·79	0·40
16 : 0 palmitic (μmol/l) (319, 403)*	1137·37	190·48	1228·95	218·85	<0·001
16 : 1 (*n*-7) palmitoleic (μmol/l) (319, 403)*	22·17	9·91	25·39	11·76	<0·001
18 : 0 stearic (μmol/l) (319, 403)*	490·52	94·18	526·43	80·56	<0·001
18 : 1 (*n*-9) oleic (μmol/l) (319, 403)*	252·64	73·43	262·43	80·56	0·07
18 : 2 (*n*-6) linoleic (μmol/l) (319, 403)	810·68	164·84	887·07	182·02	<0·001
18 : 3 (*n*-6) γ-linolenic (μmol/l) (319, 403)*	3·62	2·15	3·63	1·67	0·05
18 : 3 (*n*-3) ALA (μmol/l) (319, 403)*	5·55	3·25	6·06	3·93	0·09
20 : 0 eicosanoic (μmol/l) (319, 403)*	19·10	4·23	21·71	4·50	<0·001
20 : 5 (*n*-3) EPA (μmol/l) (319, 403)*	18·39	12·99	19·07	12·96	0·65
22 : 6 (*n*-3) DHA (μmol/l) (319, 403)*	91·31	37·09	110·04	39·25	<0·001
Trans (μmol/l) (303, 390)*	1·93	0·72	2·17	0·79	<0·001
SFA (μmol/l) (319, 403)*	2185·53	305·05	2327·06	323·14	<0·001
MUFA (μmol/l) (319, 403)*	357·08	90·55	379·40	101·53	0·001
PUFA (μmol/l) (319, 403)*	1402·58	259·13	1530·46	276·35	<0·001
n-3 PUFA (μmol/l) (319, 403)*	115·26	46·73	135·17	49·63	<0·001
n-6 PUFA (μmol/l) (319, 403)*	1287·32	240·24	1395·28	260·07	<0·001
Ratio *n*-6:*n*-3 PUFA (319, 403)*	9·21	2·69	8·82	2·97	0·02
Ratio oleic:stearic fatty acids (oleoyl-CoA activity) (319, 403)*	0·52	0·13	0·50	0·11	0·008
Ratio palmitoleic:palmitic fatty acids (stearoyl-CoA activity) (319, 403)*	0·02	0·01	0·02	0·01	0·003

DQI, Diet Quality Index; PLP, pyridoxal 5'-phosphate; PF, plasma folate; HoloTC, holotranscobalamin; tHcy, total homocysteine; PL, phospholipids; ALA, α-linolenic fatty acid.
* Values between brackets following the B-vitamin biomarkers are the number of males and females, respectively, with available information for these biomarkers and the rest of variables used in the analyses.
† *P* value based on *t* test. All other *P* values refer to the Mann–Whitney *U* test.
‡ DQI was calculated following the formula previously described[28].
§ *P* values based on the χ^2 test.

described in Fig. 2. The methylation of PE to PC by phosphatidylethanolamine methyltransferase (PEMT) requires the methyl donor *S*-adenosylmethionine (SAM). This reaction is inhibited by *S*-adenosyl-L-homocysteine (SAH). SAH is hydrolysed to homocysteine throughout a reversible reaction: homocysteine will result in increased SAH, thereby inhibiting PEMT[15]. In the liver, PC is crucial for the transport of PUFA in the blood, particularly DHA.

Nevertheless, in a randomised controlled trial (RCT) of 253 elderly participants[16], results did not support this mechanism, as the proportion of EPA, docosapentaenoic acid and DHA acids in plasma PC did not differ between the B-vitamin supplementation and the placebo groups after 2 years of intervention. This lack of agreement in the results could be due to differences in the study design (RCT against cross-sectional) and population group (elderly against adolescents), as several studies have suggested that *n*-3 PUFA metabolism changes with age, provided that Δ6 desaturase (a PLP-dependent enzyme) decreases with age[47]. This fact would affect the second proposed mechanism.

The second plausible mechanism is based on the impairment of hepatic Δ6 desaturase activity. The Δ6 desaturase is a PLP-dependent enzyme, required for the interconversion of high-unsaturated fatty acids (HUFA)[18] into PUFA such as

Table 2. Changes in fatty acid profiles according to changes in 10 units of B-vitamin biomarkers† in HELENA adolescent males and females (controlled for the influence of the centre as random effect)
(β-Coefficients with their standard errors)

	PLP (nmol/l)		PF (nmol/l)		RBC-folate (nmol/l)		tHcy (μmol/l)		Serum vitamin B₁₂ (pmol/l)		HoloTC (pmol/l)	
	β	SE	β	SE	β	SE	β	SE	β	SE	β	SE
Fasting lipid profiles: males												
TAG (mmol/l)	9.93	11.07	10.02	11.27	11.75	11.40	11.22	11.46	13.18*	11.56	12.02	11.53
Total cholesterol (mmol/l)	10.23	10.42	10.72	10.52	10.47	10.57	9.12	10.57	11.22*	10.62	11.75*	10.59
HDL-cholesterol (mmol/l)	11.22*	10.45	10.23	10.52	10.23	10.59	9.12	10.59	10.23*	10.64	10.72	10.62
LDL-cholesterol (mmol/l)	9.55	10.69	10.96	10.81	10.47	10.91	9.55	10.96	11.75*	11.04	11.48	10.96
Serum PL fatty acids												
Pentadecanoic (μmol/l)	10.47	11.02	9.95	11.25	11.75	11.40	8.32	11.40	17.78*	11.46	18.62*	11.43
Palmitic (μmol/l)	10.23	10.40	9.55	10.50	10.00	10.54	10.00	10.54	10.96*	10.59	10.47	10.57
Palmitoleic (μmol/l)	10.47	10.84	8.71	11.07	10.00	11.17	10.47	11.19	11.22	11.27	10.00	11.22
Stearic (μmol/l)	10.47	10.42	10.00	10.54	10.23	10.62	8.71*	10.64	11.48*	10.67	11.22*	10.64
Oleic (μmol/l)	9.55	10.64	7.94*	10.79	9.33	10.89	11.22	10.89	9.55	10.96	8.71*	10.94
Linoleic (μmol/l)	10.47	10.47	9.55	10.59	9.77	10.64	9.33	10.67	10.96*	10.69	10.47	10.67
γ-Linolenic (μmol/l)	9.95	11.07	7.76*	11.35	10.00	11.51	10.72	11.56	10.72	11.67	14.45	11.59
ALA (μmol/l)	10.47	11.14	8.51	11.40	11.75	11.56	8.51	11.56	12.30*	11.67	9.77	11.61
Eicosanoic (μmol/l)	10.05	10.76	8.91	10.94	9.33	11.04	9.77	11.04	8.71*	11.12	9.55	11.12
EPA (μmol/l)	12.30	11.27	10.47	11.59	14.79*	11.75	8.91	11.83	14.45*	11.89	14.79*	11.83
DHA (μmol/l)	12.30*	10.79	12.88*	10.96	14.13*	11.07	7.94*	11.09	13.18*	11.17	13.18*	11.14
Trans (μmol/l)	9.33	10.84	8.13*	11.02	9.12	11.14	11.22	11.14	8.32*	11.22	7.59*	11.17
SFA (μmol/l)	10.17	10.30	9.86	10.40	10.16	10.45	9.66	10.45	10.96*	10.47	10.57	10.47
MUFA (μmol/l)	9.77	10.54	8.32*	10.72	9.55	10.79	10.47	10.76	10.23	10.86	9.55	10.84
PUFA (μmol/l)	10.47	10.42	9.93	10.52	10.12	10.59	9.33	10.59	11.22*	10.64	10.96*	10.62
n-3 PUFA (μmol/l)	12.30*	10.81	12.30*	10.99	15.85*	11.09	7.94*	11.12	13.18*	11.19	13.49*	11.14
n-6 PUFA (μmol/l)	10.47	10.47	9.77	10.52	9.77	10.59	9.33	10.59	11.22*	10.64	10.72	10.62
Ratio n-6:n-3 PUFA	8.51*	10.62	7.94*	10.74	7.08*	10.81	11.48	10.84	8.32*	10.89	8.13*	10.86
Ratio oleic:stearic (oleoyl-CoA activity)	9.12	10.57	7.76*	10.69	8.91	10.76	12.88*	10.76	8.32*	10.81	7.76*	10.81
Ratio palmitoleic:palmitic (stearoyl-CoA activity)	9.12	10.72	7.76	10.89	8.91	10.99	12.88	10.99	8.32	11.07	7.76	11.02
Fasting lipid profile: females												
TAG (mmol/l)	10.09	10.84	10.23	11.14	12.02	11.40	8.32	11.61	7.41*	11.27	9.33	11.30
Total cholesterol (mmol/l)	10.05	10.33	10.47	10.42	11.48*	10.52	10.05	10.59	10.72	10.47	12.02*	10.47
HDL-cholesterol (mmol/l)	10.72	10.38	9.77	10.50	10.14	10.59	10.72	10.69	11.22	10.54	11.48*	10.54
LDL-cholesterol (mmol/l)	9.55	10.52	11.22	10.69	12.59*	10.84	9.77	10.99	10.72	10.79	12.30*	10.76
Serum PL fatty acids												
Pentadecanoic (μmol/l)	10.96	10.84	10.96	11.14	13.49*	11.38	6.76*	11.59	12.59	11.25	15.49*	11.25
Palmitic (μmol/l)	10.00	10.33	9.77	10.45	11.22*	10.52	10.00	10.62	9.33	10.50	10.47	10.50
Palmitoleic (μmol/l)	10.23	10.76	11.22	11.04	11.48	11.25	9.55	11.43	9.55	11.14	10.96	11.14
Stearic (μmol/l)	10.23	10.35	9.77	10.47	10.47	10.57	9.12	10.67	9.77	10.52	10.96*	10.52
Oleic (μmol/l)	10.23	10.52	9.12	10.72	10.96	10.86	9.77	10.99	8.71	10.76	10.23	10.76
Linoleic (μmol/l)	10.47	10.38	9.33	10.52	10.96	10.62	10.00	10.72	9.12	10.57	10.23	10.57
γ-Linolenic (μmol/l)	9.77	10.76	8.91	11.04	10.47	11.27	7.59*	11.46	9.33	11.17	11.75	11.17
ALA (μmol/l)	11.48	10.91	9.91	11.25	12.59*	11.51	7.08*	11.72	10.96	11.35	10.47	11.32
Eicosanoic (μmol/l)	8.71	10.59	8.91	10.81	10.72	10.99	7.94*	11.14	9.77	10.89	10.96	10.86
EPA (μmol/l)	11.48	11.04	10.72	11.46	15.85*	11.75	7.94	12.05	12.88	11.59	15.85*	11.56
DHA (μmol/l)	10.23	10.64	12.88*	10.84	15.14*	11.02	8.32	11.17	10.23	10.94	13.49*	10.91
Trans (μmol/l)	9.33	10.69	8.13*	10.91	10.96	11.12	11.48	11.30	9.12	11.04	9.12	11.02
SFA (μmol/l)	10.06	10.26	9.89	10.35	10.76*	10.42	9.84	10.47	9.51	10.40	10.62	10.40
MUFA (μmol/l)	10.09	10.45	9.55	10.62	10.96	10.74	9.77	10.84	9.12	10.67	10.72	10.67
PUFA (μmol/l)	10.23	10.35	9.77	10.47	11.22*	10.54	9.42	10.64	11.22	10.52	10.72	10.52
n-3 PUFA (μmol/l)	10.47	10.67	12.5*	10.89	15.85*	10.96	7.94	11.25	10.47	10.99	14.13*	10.96
n-6 PUFA (μmol/l)	10.23	10.35	9.55	10.47	10.96*	10.57	9.55	10.64	9.33	10.52	10.47	10.52
Ratio n-6:n-3 PUFA	9.55	10.47	7.76*	10.72	7.24*	10.86	11.48	10.99	8.91	10.79	7.94*	10.79
Ratio oleic:stearic (oleoyl-CoA activity)	9.93	10.42	9.33	10.57	10.23	10.69	10.72	10.79	9.12	10.62	9.33	10.62
Ratio palmitoleic:palmitic (stearoyl-CoA activity)	9.93	10.62	9.33	10.86	10.23	11.04	10.72	11.19	9.12	10.94	9.33	10.94

PLP, pyridoxal 5'-phosphate; PF, plasma folate; HoloTC, holotranscobalamin; tHcy, total homocysteine; ALA, α-linolenic fatty acid.
* Coefficients are statistically significant based on the raw P values and using the Benjamini–Hochberg procedure with a false discovery rate of 0·25.
† Coefficients are the result of multiplying by 10 the real coefficients provided by the linear mixed model analysis including random effects for centre and represent the change resulting from an increase of 10 nmol/l or pmol/l of indicated B-vitamin biomarker. Analyses are adjusted by z-scores of BMI, Diet Quality Index, physical activity and maternal education level and false discovery rate control by the Benjamini–Hochberg method (refs).

arachidonic acid (20 : 4 n-6) and DHA (22 : 6 n-3). This was also observed in one *in vitro* study with cultured cells[48] in which a reduction in vitamin B₆ levels led to changes in the n-3 and n-6 long-chain PUFA profile in parallel with reductions in the rate of the desaturation processes. They also found that concentrations of total FA and PL species were higher in vitamin B₆-deficient cells, as it was also observed in some studies in rats[49,50]. This might be because of the reduction in FA oxidation rates and
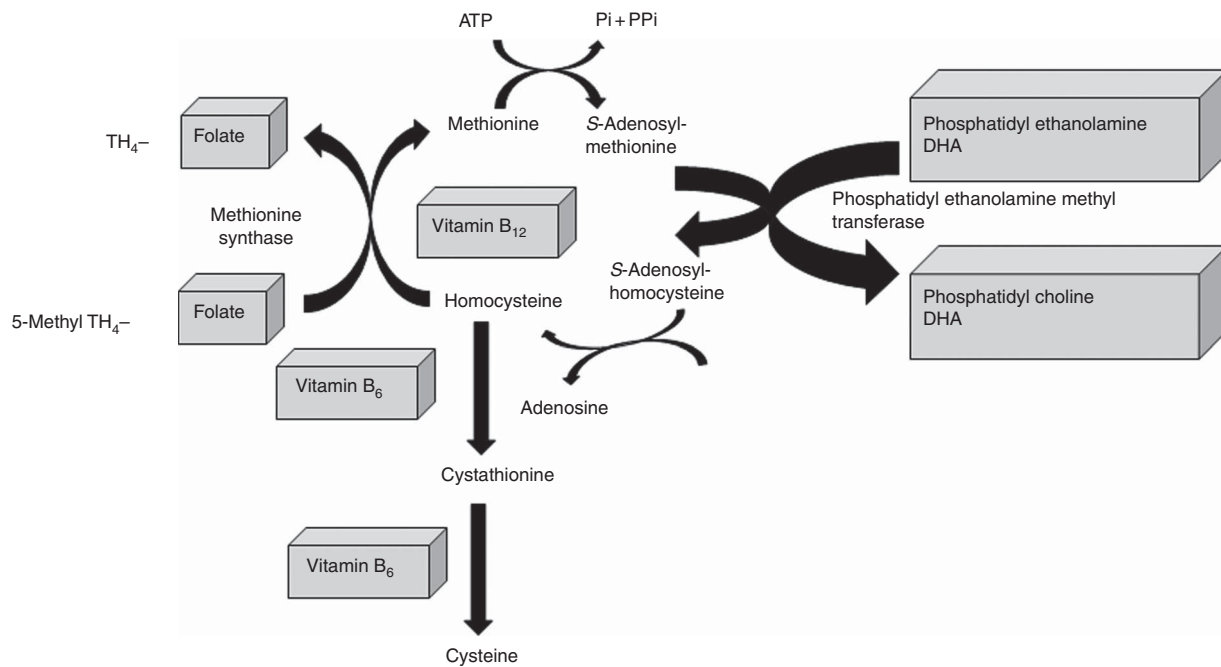
Fig. 2. One-carbon cycle: interactions between B-vitamins and DHA (adapted from Kulkarni *et al.*[46]).

their accumulation into cells because of the impairment of carnitine whose synthesis might be affected in case of vitamin B_6 deficiency[51].

In view of the observed associations, this second pathway is less likely to explain our results, as more consistent associations were found for folate and vitamin B_{12} biomarkers than for PLP (vitamin B_6 biomarker). However, it is suggested elsewhere[48] that this pathway is more probable than the first one as SAM concentrations were higher, but SAH was lower in the cultured cells with vitamin B_6 restriction. However, the study did not consider the interaction between folate and vitamin B_{12}.

In our study, the pro-inflammatory index *n*-6:*n*-3 PUFA ratio was significantly lower in adolescents with higher concentrations of B-vitamin biomarkers; however, the *n*-6:*n*-3 PUFA ratio was significantly higher in those with higher tHcy concentrations. PLP concentrations were also reported to be inversely associated with C-reactive protein among other pro-inflammatory markers, independent of plasma homocysteine concentrations[52]. This association has been speculated to reflect the mobilisation of this coenzyme into inflammatory sites. Nevertheless, the underlying mechanisms remain unclear.

It is worth noticing that we observed an inverse association between PF concentrations and *trans*-FA. *Trans*-FA can inhibit the activity of Δ6 desaturase in the liver and subsequently decrease the efficacy of HUFA interconversion[18] into PUFA. *Trans*-FA are a special type of unsaturated fatty acids with one double bond in the trans position and they can be naturally or industrially present in food products. Their presence in food products is highly variable (from 1 to 50% industrially, to 2 to 9% naturally)[53]. Because of the established association between *trans*-FA and CVD[54], the food industry has drastically decreased the content of these *trans*-FA in their products in the

last decade, but there are still some European countries, especially in Eastern Europe, where levels are still elevated[53]. Our study was performed in 2006; at the time, the vast majority of *trans*-FA intakes came from industrial products and their level in blood was already low. The levels of these FA would be even lower, and it is very likely that such an association would not have been found if our study was reproduced today.

The ratios product:precursor of $18:1n-9cis:18:0$ (oleic:stearic) (oleoyl-CoA desaturase activity) and $16:1n-7cis:16:0$ (palmitoleic:palmitic) (stearoyl-CoA desaturase activity) measured in plasma PL are integrated markers of SFA intake[55]. These ratios are relevant as they are associated with increasing risks of obesity, diabetes or cancer[56,57]. We found positive associations of oleic:stearic acid with homocysteine and negative associations with PF and the biomarkers of vitamin B_{12} in males. This might be because of the fact that B-vitamins play a role as cofactors in the metabolism of sulphur-containing amino acids such as homocysteine and these are related with lipid metabolism as reported in several studies[58]; however, this explanation needs to be confirmed. Recently, several studies have suggested that sulphur-containing amino acids modulate the expression of stearoyl-CoA desaturase-1, a key enzyme in the hepatic synthesis of MUFA[59].

Considerations of the study

This study has several strengths including the use of harmonised, standardised and validated tools and procedures in a large sample of European adolescents[12]. Another important strength is the availability of B-vitamin biomarkers, accepted in the literature (PLP, PF, RBC-folate, HoloTC, tHcy) or widely used (serum B_{12})[22], which accurately reflect B-vitamins' status and complement dietary assessment methods. Moreover, all these biomarkers were centrally analysed, increasing the

accuracy of the laboratory results[21]. Finally, the HELENA study sample size was calculated on the basis of the variance of BMI, which is a variable with the greatest variability in studies assessing the nutritional status in adolescents, which reinforces the representativeness of the data. Besides, different geographical areas across Europe and different socio-demographic groups were recruited. Sexual maturation of this sample of European adolescents has been previously described[20], and the distribution of Tanner[60] categories, both in males and females, was between the third and the fifth stage. Therefore, our results could be extrapolated to similar groups of healthy adolescents living in European cities.

On the other hand, the present study has several limitations. The sources of *trans*-FA isomers (natural or industrial) were not analysed in this study, and therefore we can only consider *trans*-FA in total, and the authors assume that the majority came from industrial sources. Similarly, choline, which might also influence our results, was not measured.

In addition, the reduction of the sample from the original 1089 adolescents with blood samples, due to the unavailability of the variables of interest for this analysis, poses a study limitation. Besides, the cross-sectional design of the study does not allow us to establish cause–consequence relationships from the variables of interest.

Only as a consideration, previous reports based on the HELENA study have shown that the association between FA intake and plasma FA cannot be assumed, except for several long-chain *n*-3 PUFA[42]. In fact, it seems that plasma FA concentrations in adolescents could be seldom explained by diet, being maximal at 14·2 % for the *n*-3 FA. This lack of association between FA intake and biomarkers may be explained by the different routes followed by FA once metabolised in the human body (oxidation, elongation and desaturation, to be incorporated in cell membranes, or stored in the adipose tissue[42]). This is why we have used the DQI as a covariate to control for diet instead of FA intake. In addition, a better overall dietary quality, assessed with the DQI, was associated with a higher total fat intake (expressed as % energy) in males and females in a previous study[61].

Conclusion

B-vitamin-related biomarkers, especially those corresponding to folate and vitamin B_{12}, were positively associated with serum PL FA, mainly with the *n*-3 pathway, whereas these associations were negative with homocysteine levels. Among the possible mechanisms that might explain our results, the influence of these vitamins in the conversion of PE to PC, which is the main membrane PL in transporting actively the PUFA, seems to be the most likely route. However, additional experiments are required in which additional parameters should be measured – for example, plasma and tissue levels of SAM or SAH.

To conclude, our findings suggest that higher B-vitamin biomarker concentrations might be related to higher PUFA concentrations (mainly *n*-3 series). Besides, the pro-inflammation marker *n*-6:*n*-3 PUFA ratio decreased with increasing values of B-vitamin biomarkers, as with *trans*-FA (probably because of different dietary sources in this case) and

the oleic:stearic acid ratio. Consequently, assuring a good status of B-vitamin biomarkers is very important as it might influence cardiovascular health by lowering homocysteine levels, but also by increasing circulating *n*-3 PUFA and decreasing the pro-inflammatory marker *n*-6:*n*-3 PUFA ratio.

Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114516004414

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