Title: Foods contributing to vitamin B_6, folate and vitamin B_{12} intakes and biomarkers status in European adolescents: The HELENA study.

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Abstract

Purpose: To examine the association between food groups consumption and vitamin B_6, folate, and B_{12} intakes and biomarkers in adolescents.
Methods: 2,189 individuals participating in the cross-sectional HELENA (Healthy Lifestyle in Europe by Nutrition in Adolescence) study met the eligibility criteria for analysis of dietary intakes (46% males) and 632 for biomarker analysis (47% males). Food intakes were assessed by two non-consecutive 24-h recalls. Biomarkers were measured by chromatography and immunoassay. Food groups which best discriminated participants in the extreme tertiles of the distribution of vitamins were identified by discriminant analyses. Food groups with standardised canonical coefficients higher or equal to 0.3 were selected as valid *discriminators* of vitamins intake and biomarkers extreme tertiles. Linear mixed model elucidated the association between food groups and vitamins intakes and biomarkers.

Results: Vitamin B_6 intakes and biomarkers were best discriminated by meat (males and females), margarine & mixed origin lipids only in males, and breakfast cereals (females). Breakfast cereals (males), and fruits, margarine & mixed origin lipids, vegetables excluding potatoes, breakfast cereals, and soups/bouillon (females) determined the most folate intakes and biomarkers. Considering vitamin B_{12} intakes and biomarkers, meat, and white & butter milk (males and females), snacks (males), and dairy products (females) best discriminated individual in the extremes of the distribution. Fewer associations were obtained with mixed model for biomarkers than for vitamins intakes with food groups.

Conclusions: Whereas B-vitamin intakes were associated with their food sources, biomarkers did with overall food consumption. Low nutrient density foods may compromise adolescents' vitamin status.

KEYWORDS: Foods contributors, B-vitamins, adolescents.

Abbreviations: HELENA: Healthy Lifestyle in Europe by Nutrition in Adolescence; Cbl: cobalamin; MSM: Multiple Source Method; PLP: pyridoxal 5'-phosphate; EDTA: Ethylenediaminetetraacetic acid; HPLC: high performance liquid chromatography; CV: coefficient of variation; RBC-folate: red blood cell folate; HoloTC: holotranscobalamin; SD: standard deviations; BMI: Body Mass Index; FCDB: Food composition databases.

Introduction

Vitamin B_6, folate, and vitamin B_{12} deficiencies are considered as a risk factor in cardiovascular diseases, neural tube defects (NTD), and some types of cancers. They are involved in optimal cognitive function and bone health, due to their participation as co-factors in several metabolic pathways, as in the methionine cycle, among others [1, 2]. They are also key factors in growth and development because of their role in DNA-replication, and therefore important during childhood and adolescence [3]. Maintaining an optimal status of these vitamins throughout early life stages is essential in preventing long-term risks of their deficiencies like anemia [4]. Moreover, sub-clinical deficiencies of vitamin B_6, folate, and vitamin B_{12} (Cbl) status are not uncommon during adolescence [4, 5]. A recent paper based on the HELENA Study described a sub-clinical deficiency for folate and vitamin B_6 in approximately 20% of the adolescents and for red blood cell folate (RBC-folate) in 75% of the females regarding folate-related NTD [6].

Assessment of vitamin status and of its dietary correlates supports identification of population groups at risk for B-vitamin deficiencies who could then benefit from targeted public health interventions [7]. In Brazilian adolescents [8] for example, foods that made the highest contribution to the intakes of these vitamins, as assessed by a 3-day non-consecutive dietary record, included white rice (171.45g), chicken (67.46 g) and beef (61.01 g) for vitamin B_6; French bread (67.14 g), pasta (187.81 g) and beans (56.09 g) for folate; and lean beef (149.26 g), whole milk (230.44 g) and fatty beef (145.71 g) for vitamin B_{12}. On the other hand, these foods were not necessarily the main sources of B-vitamins (in terms of food composition) expressed per 100 g. Indeed, the highest positions in the rankings included breakfast cereals both for B_6 and folate and beef liver for B_{12}.

Dietary biomarkers are a good tool to more accurately assess nutritional intake/status versus self-reported methods [9]. Concentrations of blood biomarkers provide an estimation of the body vitamin status [10] interpreted as the biologic consequence of dietary intake or dietary patterns. There is a need for more evidence on the relation between biomarkers and intake, which would be useful for validation of dietary tools and in the assessment of dietary measurement-error [11].

To the author's knowledge, this is the first study addressing B-vitamins intake and status in parallel in relation to food group consumption among adolescents in Europe. The aim of this study was to examine whether consumption of different food groups discriminated between high and low tertiles of B-vitamins intakes and related blood concentrations in a large sample of European adolescents aged 12.5 to 17.5 years.

Methods

The Healthy Lifestyle in Europe by Nutrition in Adolescence Cross-Sectional Study, (HELENA-CSS), is a multi-centre study of lifestyle and nutrition among adolescents from 10 European cities from nine countries. A random cluster sampling (all adolescents from a selection of classes from all schools in the selected cities) of 3000 adolescents aged 12.5-17.5 years, stratified by geographical location, age and socioeconomic status, was carried out in Athens and Heraklion (Greece), Dortmund (Germany), Ghent (Belgium), Lille (France), Pecs (Hungary), Rome (Italy), Stockholm (Sweden), Vienna (Austria), and Zaragoza (Spain). Inclusion criteria were: not participating simultaneously in another clinical trial and being free of any acute infection occurring < 1 week before inclusion [12]. The initial number of HELENA participants was 3,528 (47% males). The average participation rate in the study was 67%, which is acceptable for this demanding epidemiological study [13]. Due to logistical reasons, participants from Heraklion and Pecs (7% of the total sample) did not provide dietary data. For the purposes of this analysis, 2,189 adolescents (46% males) were included having complete data on two non-consecutive 24-hour recalls, including Sundays, and valid data on maternal education, among others. From a random sub-sample of 941 adolescents (46% males) with available blood parameters (including adolescents from Heraklion and Pecs), a sample of 632 adolescents (47% males) was included in the current analysis having met the inclusion criteria e.g. having complete data on B-vitamins biomarkers, and data on two 24 hours dietary recalls. Further details on the sampling procedures, pilot study and reliability of the data have been published elsewhere [12]. Informed consent was obtained from all participants and their parents, and the protocol was approved by the Human Research Review Committees of the corresponding centres [14].

Assessment of vitamin B_6, folate, vitamin B_{12} intakes and total energy intake

Dietary intakes were examined using the computerized 24-hour recall, self-administered HELENA-Dietary Assessment Tool (HELENA-DIAT), adapted for European adolescents from the Young Adolescents' Nutrition Assessment on Computer (YANA-C) software [15]. The adolescents completed the 24-hour

recalls two times in a two-week period. Trained staff was present during this assessment [15]. More information about this tool can be found elsewhere [15, 16]. Difficulties in obtaining comparable measures of energy density of each food across countries precluded the use of country specific food composition tables. For this reason, data were linked to the German Food Code and Nutrient Data Base (BLS -Bundeslebensmittelschlüssel-, version II.3.1, 2005), which includes 12,000 coded foods, and with up to 158 nutrient data points available for each product. The Multiple Source Method (MSM) [17] was used to calculate usual nutrient intake removing the effect of day-to-day within-person variability and random error in the recalls. For that purpose, the default model was used and consequently, all the surveyed adolescents were considered as habitual consumers assuming indeed that most nutrients are consumed on a daily basis in contrast as happens with foods that might be consumed from time to time only. The lack of food frequency information detemined that step 2 was not included in the model.

Moreover, the method to identify under-reporters is described elsewhere [18]. For the purposes of this analysis, it was decided to not exclude under-reporters based on the assumption that some under-reporters were potentially adolescents restricting their intakes.

All reported 4,179 foods and beverages, as part of recipes or as individual food, were aggregated in initial 29 food groups based on the European Food Groups classification system [15, 19]. As part of the general HELENA analysis, these foods were disaggregated into 43 food groups. For the purposes of the current analysis and based on their nutritional composition some of these food groups were further aggregated. Among those aggregated were alcoholic drinks (beer, wine, others…), complex carbohydrates (pasta, rice, flour…), sugar products (honey, and other sugar products…), oily fruits (nuts & seeds, avocado & olives…), milk products (yogurt & white cheese and milk & yogurt beverages), and other milk products (desserts & puddings milk based, creams…). In Annex 1, there is an explanation of all the milk-based related categories. Four food groups were eliminated from the current analysis namely 'products for special nutrition use', 'soya beverages', 'miscellaneous', and 'meat substitutes'). This was done on the basis of very low consumption (0 median and mode and more than the 85% of the sample did not report consumption). So, in the end, the final number of food groups for this analysis was 31 food groups which are fully presented in table 4. In any case, for the estimation of vitamin intakes, all the food groups were included so as not to underestimate them even when the vitamins provided by the eliminated food groups were almost insignificant.

Inadequate intakes of each B-vitamin was evaluated, using the EAR reference from the DRIs [3] shown in table 1, as having 1.3 and 1.2 mg/d for vitamin B_6 in males and females, respectively; 400 µg/d for folate in both sexes; and 2.4 µg/d for vitamin B_{12} also for both sexes. To evaluate the adequacy of intakes of B-vitamins, the full amount of vitamins coming from all the food groups (even those from excluded food groups) were taking into account.

Assessment of vitamin B_6, folate and vitamin B_{12} biomarkers concentrations

At school, early in the morning, and following an overnight fast, 30 ml of blood was drawn according to a standardized blood collection protocol by a certified phlebotomist. More details on sample transport and quality assurance can be found elsewhere [20]. For the measurement of pyridoxal 5´phosphate (PLP), biomarker of vitamin B_6, ethylene diamine tetra acetic acid (EDTA) whole blood was centrifuged at 3,500×g for 15 min. The supernatant fluid was transported at a stable temperature of 4-7°C to the central laboratory at the University of Bonn (IEL-Institut fuer Ernährungs und Lebensmittelschaften-, Germany) and stored at -80°C until analysed. PLP was measured by high performance liquid chromatography (HPLC) (Varian Deutschland GmbH, Darmstadt, Germany; CV = 1%) with a modified method of Kimura et al [6, 21].

For the measurement of plasma folate and Cbl, heparinised tubes were collected, placed immediately on ice, and centrifuged within 30 min (3,500 g for 15 min). The supernatant fluid was transported at a stable temperature of 4-7°C to the central laboratory at the University of Bonn (IEL-Institut fuer Ernährungs und Lebensmittelschaften-, Germany) and stored at -80°C until assayed. After measuring the hematocrit in situ, EDTA whole blood was used for the red blood cell folate (RBC-folate) analysis. EDTA whole blood was diluted 1:5 with freshly prepared 0.1% ascorbic acid for cell lysis and incubated for 60 min in the dark before storage at -80°C. Plasma and RBC-folate and plasma cobalamin were measured by means of a competitive immunoassay using the Immunolite 2000 analyzer (DPC Biermann GmbH, Bad Nauheim, Germany) (CV for plasma folate = 5.4%, RBC folate = 10.7%, Cobalamin = 5.0%) [20]. Serum for measuring holotranscobalamin (HoloTC) were obtained by centrifuging blood collected in evacuated tubes without anticoagulant at 3,500 g for 15 min within 1 hour. Once send to IEL, the sera were aliquoted and stored at -80°C until transport in dry ice to the biochemical lab at the Universidad Politécnica de Madrid for

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analysis (Laboratory number 242 of the Laboratory Network of the Region of Madrid). HoloTC was measured by microparticle enzyme immunoassay (Active B_{12} Axis-Shield Ltd, Dundee, Scotland, UK) with the use of AxSym (Abbot Diagnostics, Abbott Park, IL, USA) (CV = 5.1%) [22].

Confounders

Maternal education was used as socioeconomic confounding variable, obtained via a self-administered questionnaire, completed by the adolescent. This variable was one of the most related socioeconomic factors associated with the studied B-vitamins [23]. This variable was assessed using four levels: elementary, lower secondary, higher secondary or tertiary education. Anthropometry battery measurements were assessed following standardized procedures. Weight was measured in underwear and without shoes with an electronic scale (Type SECA 861) to the nearest 0.05 kg, and height was measured barefoot in the Frankfort plane with a telescopic height measuring instrument (Type SECA 225) to the nearest 0.1 cm. Body mass index (BMI) was calculated using the Quetelet formula (kg/m^2) and used as a covariate.

Statistical analysis

The Statistical Package for Social Sciences version 17.0 (SPSS Inc., Chicago, IL, USA) was used for analyses. All statistical tests and corresponding p values were two-sided, and $p<0.05$ was considered statistically significant. All analyses were sex-specific. Descriptive data are presented as medians, means and standard deviations (SD), and also tertiles in case of B-vitamins intake and biomarkers concentrations. Tertile distribution of B-vitamins intake and biomarkers was used to examine major food sources based on discriminant function analyses. This method tests whether cases (B-vitamins intakes and biomarkers of the adolescents) are well classified from a set of discriminating variables (all 31 food groups) into predefined groups of a criterion variable (the B-vitamins tertiles).

Ideally, data to run discriminant analyses should be normally distributed. However, it is recognized as a robust method and can be used also with non-parametric data as in this analysis [24]. Moreover, it has been already used for similar purposes in the literature [25].

The relationships between the discriminant food groups resulted from the discriminant analysis regarding vitamin B_6, folate and vitamin B_{12} intakes and biomarkers concentrations (dependent variables) were examined using linear mixed model analysis including random effects for centre. Age, maternal education, BMI, and total energy intake were included in the model as covariates. All non-parametric variables liable to be normalized were log-transformed, such as BMI and B-vitamins intake and related biomarkers variables. Food group consumption and energy intake variables were not able to be normalized using the conventional techniques.

Results

Figure 1 shows the sampling procedure. The ratio males/females was significantly lower in the sample included in the analyses (for both dietary and biomarker) than those who were not (p=0.004). Males included in the dietary analysis had significantly lower energy intake (p=0.001) than those excluded. Both included males and females, significantly differed from those who were excluded in terms of maternal education (p<0.001), being the percentage of mothers in higher categories of education higher in those included (these data is not presented). Similarly, adolescents included in the analyses for biomarkers, had significantly lower energy intake, and more mothers positioned in the higher categories of education than those who did not (p<0.05), with the exception of the ratio between males and females for which no statistical difference was found. Tables 1 and 2, present the adolescents' characteristics stratified by sex correspondingly to those in the dietary and the biomarker groups' analysis. There were no statistical significant difference in the mean intakes of all three B-vitamins between the sample included for dietary and biomarkers analyses. As expected, most of the adolescents met the recommendations for vitamin B_6 and B_{12}, oppositely as occurred with folate.

To identify the food items which best distinguished between lower and upper tertiles of distribution both for B-vitamins intakes and biomarkers, discriminant analysis was performed. When the standardised canonical function coefficients were higher or equal to 0.3, they were selected as discriminating food groups [26]. The interpretation of the discriminant coefficients (or weights) is the same to that of standardized regression coefficients (beta's) in multiple regression which implies magnitude of the association. The sign indicates the direction of the relationship [27]. A further way of interpreting discriminant analysis results is to describe each group in terms of its profile, using the group means of the predictor variables.

These results are presented in table 3. For vitamin B_6 intakes, food groups which best discriminated individuals in the lowest and highest tertile of the distribution included meat and starch roots & potatoes for both males and females; and breakfast cereals for females and margarine & mixed origin lipids for males considering PLP concentrations. For folate intake, those food groups which better determined it, included fruit & vegetable juices (males), fruits (females), and vegetables (excluding potatoes) for both sexes; whereas for folate biomarkers, were breakfast cereals and fruits for males and females, and, margarine & mixed origin lipids (females). High and low tertiles of vitamin B_{12} intake, were best discriminated by meat and white & butter milk for both sexes, and for vitamin B_{12} biomarkers, they were white & butter milk for males and females, and savoury snacks (males), and dairy products (females).

Table 4 presents changes in B-vitamins values (both intakes and statuses) per 10 grams increases in the corresponding food group. In summary, fewer significant changes were observed for biomarkers than for vitamin intakes. The distribution and strength of changes in B-vitamins derivate from the increase in 10 grams of the food groups' consumption were very similar in males and females.

Significant lower vitamin B_6 intake was observed with the increase in intake of sugared products such as cakes, soft drinks, confectionary products, and chocolate in both males and females. In contrast, significant upper vitamin B_6 intake were observed with the increase in intake of fish products, fruits, meat, starch roots and potatoes, vegetables, white & butter milk, and yogurt & white cheese in both males and females.

Lower folate intake were observed in regards to intake of cakes, chocolate, soft drinks, confectionary products, meat, snacks, and sugar products in males and females. Equally, significantly higher folate intake was observed for bread & rolls, cheese excluding quark, eggs, fish products, fruits, vegetable juices, oily fruits, pulses, soups & bouillon (in males), starch roots & potatoes, vegetable oils, coffee/tea (in females), vegetable oils (in females), vegetables excluding potatoes, vegetarian products, water, white and butter milk, and yogurts in both males and females. Likewise, plasma folate and RBC-folate increased significantly in the same manner with breakfast cereals, cereal products and fruits in both sexes.

Dairy products, eggs, fish products, meat, starch roots & potatoes, water, white & butter milk and yogurt significantly increased the vitamin B_{12} intakes in both males and females. Moreover, in males, the consumption of breakfast cereals, soups & bouillon and vegetables excluding potatoes significantly increased the intake of vitamin B_{12}, contrarily as it does with bread & rolls, and oily fruits. In addition, for females, cheese excluding quark significantly increased vitamin B_{12} intake and was decreased with soft drinks and vegetarian products. Vitamin B_{12} biomarkers as well as intake, increased with meat, soups & bouillon and white and butter milk (males), and with dairy products and white & butter milk (females). In males, soft drinks, chocolate and oily fruits decreased vitamin B_{12} biomarkers, similarly as with intakes, as occurred with chocolate and sauces in females.

Discussion

The results showed that intakes and biomarkers levels of B-vitamins are associated with overall dietary patterns and not only with their main dietary sources. Such observations provide useful information for the development of public health interventions aiming to increase B-vitamins intakes and levels in adolescents, mainly in the case of folate for which more than 90% of both males and females did not meet the current recommendations. However, in a previous report [6] resulted from the HELENA study, only 35% of the adolescents had PF values (recent intakes) under the recommendations, and 27% did it for RBC-folate (stores). Comparing with the same report, differences between vitamin B_6 and B_{12} intakes in relation with their respective biomarkers were not such pronounced. The proportion of adolescents who did not meet the recommended values for PLP were 20% (compared with our 0% regarding intakes of vitamin B_6), and between 2-5% of our sample of adolescents did not meet the recommended values of vitamin B_{12} biomarkers accordingly as what we have obtained in our study

for this vitamin. This is not surprising as in this sample, correlation between micronutrient intakes and concentrations in blood were not very high [28]. These differences found between intakes and status among the B-vitamins we are concerning about in this study, may be given by the differences in the bioavailability [29-31] of all of them. For instance, for folate bioavailability ranges from around 30% - 50 [29]; for vitamin B_{12} between 42-66%, considering that its bioavailability significantly decreases with increasing intake of vitamin B_{12} per meal [31]; and for vitamin B_6, 75% on average [30]. Besides, folate fortification of foods might be more common than for the other B-vitamins, or even underestimated [32].

To the authors' knowledge, this is the first study to address the relationship between food groups identified from discriminant analysis and vitamin B_6, folate and vitamin B_{12} intakes and associated biomarkers levels. Even though, a number of studies have looked into the main food contributors of individual B-vitamins intakes in young populations [8, 33, 34] and related biomarkers [35], also in adults or elderly people [7, 36]. The findings of a Brazilian study [8], which looked into the known contributors, the highest contributors to vitamin B_6 intake were white rice, chicken and beef. In In the current study, meat, breakfast cereals (in females), starch roots & potatoes in both sexes were the foods which discriminated best. Regarding folate, French bread, pasta and beans were the highest contributors in Brazil (as Brazilians have mandatory fortification of flours with folic acid), while in the European sample, fruits & vegetables (also juices), cereals and breakfast cereals, fish products, and margarine & lipids of mixed origin were those foods whose consumption was positively related with folate intake and biomarkers. Beef and whole milk contributed the most to vitamin B_{12} intake in terms of natural sources in Brazilian adolescents; and similarly, in the current sample, those, which discriminate best, were meat, fish, white & butter milk, dairy products, and vegetable oils.

The majority of food groups found to discriminate between low and high tertiles of B-vitamins intake and biomarkers, even if they were not the main sources, were strongly associated with them. However, there were some exceptions with some non-main sources mainly with dairy & milky products, juices, and cereal products. A plausible explanation for that includes the way in which foods were aggregated in our study, that is based on the proportion of individual foods into recipes and that such foods may be often supplemented and fortified [37] with B-vitamins. Apart from this fact, the non-main sources found to be related in this study with B-vitamins, dairy & milky products, juices, and cereal products are frequently consumed by adolescents [38]. The acceptance of such products is determined by factors like familiarity, personal perception, health claim or functional ingredient used [39]. It should also be considered that these food groups specifically are really prone to be supplemented or fortified and this is usually a claim for the consumers due to the excessive advertising blitz [40].

231 Foods items which corresponded with the highest tertiles of B-vitamins intake were disaggregated in order to have a better understanding of the particular food
232 item contributing to the intakes or biomarkers of the vitamins. In that way, dairy products were able to discriminate in tertiles of intakes of vitamin B_6 probably
233 due to the presence of fruit milk-shakes (as some fruits are relevant sources of vitamin B_6), and milk in cereals (cereals are also important sources), as occurred
234 with the food group "white & butter milk". An important observation of this study, was the positive significant changes observed between soft drinks and PLP
235 in females most likely due to the addition of B-vitamins in energy drinks [41]. It is also worth noting that PLP was positively associated with soft drinks in
236 females but negatively with vitamin B_6 intake and could be due to the possibly hidden amounts of, e.g. B-vitamins on them, their addition to energy drinks,
237 included in our soft drinks category, could result in positive associations with PLP.

238 A higher number of significant changes were observed for intakes than for blood concentrations. This is no surprising as blood concentrations could be influenced
239 by other mechanisms such as cooking method, metabolism, interference with other nutrients, and physiologic status [42-44], among others. B-vitamins
240 concentrations are not only related with foods containing these vitamins but more with dietary patterns [11], as also reported Vandevijvere et al [28]. They
241 reported that correlations found were better between food frequency consumption and concentration biomarkers than between food intakes (and concentration
242 biomarker, also for folate and vitamin B_{12} biomarkers. This is most likely because food frequency consumption represents usual intake while 24 h recalls
243 represent current intake, in particular for foods that are generally not consumed daily. Another plausible explanation for these differences between intakes and
244 biomarkers can be the fact that nutrients and food components can vary considerably for the same food depending on where or how the food was grown or how
245 it was processed [11]. Besides, the biomarkers used in our study do not always reflect the recent anecdotic intakes, but rather the usual intake and therefore do
246 not necessarily match with the results from the 24 hours recalls that were used for this study. For instance, PLP, RBC-folate, and HoloTC are not the most
247 suitable biomarkers to detect changes in day-to-day variation intakes [3]. We should also consider that B-vitamins supplement use was not assessed in the
248 HELENA study and fortification of the products has not been taking into account in the Food Composition DataBases (FCDBs) and these can also explain these
249 differences [28]. In this sense, it is important to mention that the consumption of breakfast cereals (often fortified product) [37] was more strongly associated
250 with B-vitamin biomarkers than with their intakes. This finding suggests that the quantification of vitamins used in the food composition table could
251 underestimate the real amount of these vitamins because of the fortifications as occurs with folate and white & butter milk, or for vitamin B_{12} and fruit and

252 vegetable juices, etc, or that the BLS FCDB (the Bundeslebensmittelschlüssel, the german food composition database used to assess all the dietary intake sample
253 with the 24 hours recalls) gives lower nutrient values than other national databases as was already shown by our colleagues Julián-Almárcegui et al [45].

254 Apart from the positive changes found for B-vitamins (both intakes and biomarkers) and produced by their main food sources, other clearly stated changes (in
255 this case negative), were the ones with SSB, confectionary products, chocolates, other sugared products, cakes, and savoury snacks. These results indicate that
256 consumption of such foods could compromise vitamin intakes and statuses of this sample of European adolescents by substitution of other foods with a higher
257 B-vitamins density [46].

258 Another important finding of this study is that white & butter milk appears to be a good discriminator for low and high B-vitamin intakes and of some biomarkers
259 both in males and females. Moreover, it was also associated with vitamin B_6 intake, folate intake (only in males), and with vitamin B_{12} intakes and biomarkers,
260 both in males and females. This could indicate a potential link of these products with good nutritional status of the studied B-vitamins. This association was also
261 found in a Dutch study conducted in 1995 [47].

262

263 *Strengths and limitations*

264 The main strength of this study is the use of harmonised and standardised procedures in a large sample of adolescents from various European cities [12].
265 Questionnaires used in the study were previously validated [48]. Another important strength of the study is the correction procedures used to avoid the limitations
266 of the 24 hours recalls; the use of the MSM method to correct the crude intake data values for within-person variation [49], and the use of the correspondent B-
267 vitamins biomarkers. Blood biomarkers were analysed in a centralized laboratory, strengthening the reliability of the results [20]. However, correlations between
268 biomarkers and usual food intakes obtained from the recalls were low in this sample [28]. This could be due to the fact that dietary intakes correlate better with
269 biomarkers when the number of days covered by the reference method increases [50]. On the other hand, due to standardization reasons, the use of the German
270 food composition table, provided differences in B-vitamins composition when compared with national food composition tables. These differences were small,

and for most nutrients negligible, which implies a reliable estimation [45]. Moreover, local adaptations to foods and recipes were done based on a protocol which was developed to make locally culture-specific food pictures. Each centre contributed to the upgrade of the tool to a European level by making inventories of country-specific food lists and by providing pictures of typical recipes [15]. An additional limitation is that food fortification is not included the the German food composition database.

A weakness in the dietary data in this study is that the analyses were not controlled for dietary B-vitamins supplement use as they were not asked to adolescents. However, findings of the DONALD study [51] and other studies [33, 52, 53] indicated that breakfast cereal intakes (normally fortified with B-vitamins, among others), determined more folate intakes than supplements. The intake of pharmacological micronutrient supplements was assessed in our sample for those who did the blood drawing, and they represented 11.5% of males and 9.1% of females [6]. This slightly difference between males (7.1%) and females (8.0%) was already observed in the DONALD study [54].

Conclusions

This study makes an important contribution in providing evidence of the association of food group and vitamin B_6, folate and vitamin B_{12} intakes and status in European adolescents. The results of this study indicate that B-vitamins intakes were associated with intakes of their main food sources and that biomarker concentration with the overall food consumption pattern. Moreover, the obtained disagreement between the results found with B-vitamins intakes and biomarkers needs further research with large healthy population samples to determine if long-term proposed dietary recommendations converge in adequate nutritional biomarkers concentrations in blood.

In this study, findings suggest that special attention considering B-vitamins status should be put in adolescents who are used to consume food groups with low micronutrient density like savoury snacks, and not consuming a healthy varied food items more prone to have required micronutrients, like fruit and vegetables.

289 This study shows the importance of consuming a variety of foods from all food groups to assure an adequate micronutrient status. The study of dietary patterns,
290 which examines the effects of overall diet and settles down a wider frame of food and nutrient consumption, may be more predictive of disease risk than
291 individual foods or nutrients and thus, it could be a future approach for investigating diet-status-health relationships.

292

293 **Conflict of interest**

294 On behalf of all authors, the corresponding author states that there is no conflict of interest.

295

296 **Ethical Standard Disclosure**

297 This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by
298 the Human Research Review Committees of the corresponding centres. Informed written consent was obtained from all the adolescents and their parents.

299

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513

514 Table 1. Adolescents´ characteristics belonging to the HELENA study with complete information on dietary intake.

Characteristics	Males (1004)						Females (1185)					
	% PII	Mean ± SD	Median	T1	T2	T3	% PII	Mean ± SD	Median	T1	T2	T3
Age	-	14.8 ± 1.3	14.8	-	-	-	-	14.7 ± 1.2	14.7	-	-	-
BMI (kg/m^2)	-	21.3 ± 3.9	20.5	-	-	-	-	21.2 ± 3.5	20.	-	-	-
Energy intake (kcal/d)	-	2493.5 ± 828.1	2371.0	-	-	-	-	1894.4 ± 583.2	1830.8	-	-	-
Vitamin B_6 intake (µg/d)	0.0	1797.5 ± 649.0	1700.3	412.8-1450.6	1450.6-1994.2	1994.2-5035.5	0.0	1432.9± 482.3	1379.1	439.2-1195.5	1195.5-1562.1	1562.1-4516.5
Folate intake (µg/d)	98.0	212.0 ± 75.5	199.6	44.0-171.8	171.8-236.1	236.1-487.0	100.0	177.5 ± 59.4	170.1	50.2-147.0	147.0-195.1	195.1-491.7
B_{12} intake (µg/d)	2.9	6.0 ± 2.4	5.7	0.6-4.8	4.8-6.7	6.7-15.6	6.0	4.6 ± 1.8	4.2	0.9-3.7	3.69-5.0	5.0-14.9

515 PII, prevalence of inadequate intake based on the values provided by the Food and Nutrition Board of the Institute of Medicine (IOM); SD, standard deviation,
516 T, tertile; BMI, Body Mass Index

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527 Table 2. Adolescents´ characteristics belonging to the HELENA study with complete information on vitamin B-biomarkers.

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Characteristics	Males (295)						Females (337)					
	% PII	Mean ± SD	Median	T1	T2	T3	% PII	Mean ± SD	Median	T1	T2	T3
Age		14.84± 1.3	14.8	-	-	-		14.8 ± 1.18	14.8	-	-	-
BMI (kg/m^2)		21.1 ± 3.8	20.4	-	-	-		21.16 ± 3.40	20.6	-	-	-
Energy intake (kcal/d)		2551.1 ± 876.9	2418.6	-	-	-		1896.0 ± 555.5	1852.5	-	-	-
Vitamin B_6 intake (µg/d)	0.0	1839.0 ± 652.9	1795.1	495.5-1469.9	1469.9-2075.8	2075.8-5035.2	0.0	1453.2 ± 508.2	1402.1	439.2-1225.1	1225.1-1584.0	1584.0-4516.5
Folate intake (µg/d)	98.3	215.1 ± 73.74	205.7	54.7-174.1	174.1-244.6	244.6-472.6	100.0	180.7 ± 58.0	175.3	50.2-154.1	154.1-201.2	201.2-491.7
B_{12} intake (µg/d)	1.4	6.2 ± 2.5	5.9	0.7-4.7	4.7-6.7	6.74-15.6	4.2	4.6 ± 1.7	4.3	0.9-3.8	3.8-5.0	5.0-13.0
Serum vitamin B_6 (nmol/L)(264, 317)		68.7 ± 45.3	55.9	13.2-44.8	44.8-69.7	69.3-315.7		62.5 ± 64.5	47.1	5.8-39.1	39.1-61.7	61.7-899.1
Plasma folate (nmol/L) (295, 337)		18.2 ± 10.3	15.3	4.9-12.8	12.8-19.0	19.0-82.9		18.3 ± 9.7	15.9	4.8-13.3	13.3-20.1	20.1-70.5
RBC-folate (nmol/L) (293, 332)		819.0 ± 378.5	742.4	228.32-630.4	630.4-868.0	868.0-3673.3		766.7 ± 305.8	727.9	241.3-622.5	622.5-855.4	855.4-2361.9

Serum vitamin B_{12} (pmol/L) (295, 336)		333.6 ± 130.5	306.0	114.0-247.0	247.0-375.3	375.3-725.0		378.0 ± 158.3	348.0	110.0-282.3	282.3-432.0	432.0-1036.0
HoloTC (pmol/L) (272, 325)		65.0 ± 31.8	61.1	16.23-50-9	50.9-67.3	67.3-265.0		64.5 ± 33.9	59.1	18.4-50.0	50.0-67.5	67.5-325.5

529 SD, standard deviation, T, tertile; BMI, Body Mass Index. Numbers between brackets in the column of characteristics represent the males and females,
530 respectively, with information on any type of biomarker correspondingly.

531

532 Table 3. Summary of interpretative measures for stepwise two-group discriminant analysis of study participants in the high and low tertiles of vitamin
533 B_6,folate and vitamin B_{12} intakes and biomarkers.

	Males		Females	
	Univariate F ratio*	Standardized canonical coefficients†	Univariate F ratio*	Standardized canonical coefficients†
Vitamin B_6 intakes ($+3^{\infty}$, $+3^{\infty}$)				
Fish products (g/day)	28.25	0.34	41.70	0.34
Fruits (g/day)	100.72	0.45	92.53	0.38
Fruit & vegetable juices (g/day)	31.25	0.31	40.37	0.31
Meat (g/day)	320.76	0.80	280.80	0.65
Starch roots & potatoes (g/day)	117.52	0.45	152.84	0.45
Vegetables (excluding potatoes) (g/day)			105.92	0.36
White & butter milk (g/day)	70.99	0.31		

Serum B_6 (-3^{∞}, -3^{∞})				
Breakfast cereals (g/day)			10.11	-0.58
Cereals (g/day)	6.97	0.79		
Margarina and mixed lipids (g/day)	4.36	-0.63		
Oily fruits (g/day)			4.52	0.44
Starch roots & potatoes (g/day)			7.89	0.58
Vegetable oils (g/day)			3.44	0.43
Folate intakes ($+3^{\infty}$, $+3^{\infty}$)				
Bread & rolls (g/day)			131.61	0.40
Cheese excluding quark (g/day)	105.97	0.35		
Fruits (g/day)	32.20	0.39	143.76	0.46
Fruit & vegetable juices (g/day)	65.63	0.43	56.74	0.36
Pulses (g/day)			29.14	0.34
Vegetables (excluding potatoes) (g/day)	204.04	0.41	273.93	0.54
White&butter milk (g/day)	30.56	0.31		
Plasma folate (-3^{∞}, $+3^{\infty}$)				
Bread & rolls (g/day)			2.37	-0.43
Breakfast cereals (g/day)	10.87	-0.55	12.60	0.51
Cereals (g/day)	13.86	0.76		

Carbonated/ Soft drinks (g/day)	3.63	0.49		
Chocolate (g/day)			2.27	0.37
Fish products (g/day)			2.68	0.36
Fruits (g/day)			9.80	0.49
Margarina and mixed lipids (g/day)			6.22	0.47
Savoury snacks (g/day)			2.94	-0.39
RBC-Folate (-3^{∞}, -3^{∞})				
Breakfast cereals (g/day)	8.15	-0.52		
Carbonated/ Soft drinks (g/day)	4.67	0.49		
Cereals (g/day)	7.92	0.58		
Dairy products (g/day)	1.62	0.46		
Fruits (g/day)	6.70	-0.40	8.60	-0.56
Margarina and mixed lipids (g/day)			7.24	-0.58
Savoury snacks (g/day)			2.25	0.39
Soups/bouillon (g/day)			6.42	0.53
Sugar products (g/day)			3.33	0.44
Vitamin B_{12} intake ($+3^{\infty}$, $+3^{\infty}$)				
Cheese excluding quark (g/day)			48.88	0.46

Dairy products (g/day)	48.22	0.40	53.81	0.37
Fish products (g/day)	49.87	0.37	53.55	0.48
Meat (g/day)	280.48	0.77	234.63	0.70
White & butter milk (g/day)	126.65	0.50	147.65	0.53
Plasma vitamin B_{12} ($+3^{\infty}$, $+3^{\infty}$)				
Cereals (g/day)			3.74	0.41
Dairy products (g/day)			7.08	0.59
Savoury snacks (g/day)	5.66	0.68		
White & butter milk (g/day)	8.57	0.81	12.43	0.66
Other milk products			5.76	0.41
Holotranscobalamin ($+3^{\infty}$, $+3^{\infty}$)				
Cakes (g/day)	3.15	-0.44		
Carbonated/ Soft drinks (g/day)	8.47	-0.53		
Dairy products (g/day)			9.91	0.74
Savoury snacks (g/day)	0.01	0.41		
White & butter milk (g/day)	26.87	0.80	9.37	0.73

534 *Univariate F ratio: ratio of between-groups variability to the within-groups variability. Large F values indicate greater discriminating power.
535 †Standardized canonical coefficients: partial contribution (discriminating power) of each variable to the discriminate function controlling for all other
536 variables in the equation
537 The number 3 between parenthesis next to the vitamin intake or biomarker represent the sign of the canonical coefficient in relation to the position of centroids
538 in the discriminant function for the third tertile, for males and females, respectively. The +3 in Holotranscobalamin in males, together with a negative

standardized canonical coefficient for cakes, means that those subjects with higher intake of cakes are more probably settled down in the first tertile of the biomarker.

Table 4. Changes in vitamin B_6. folate. and vitamin B_{12} intake and biomarker levels with increases of 10 grams/day of specified food groups stratified by gender.

Food groups	Vitamin B_6				Folate						Vitamin B_{12}					
(1 unit = 10g/day)	Intakes (µg/d)		Serum vitamin B_6 (nmol/L)		Intakes (µg/d)		Plasma folate (nmol/L)		RBC-Folate (nmol/L)		Intakes (µg/d)		Plasma vitamin B_{12} (pmol/L)		HoloTC (pmol/L)	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Alcoholic drinks	0.010	-0.008	-0.011	-0.085	0.007	0.002	0.003	0.023	-0.029	-0.036	0.015	-0.012	0.000	0.003	-0.008	0.002
Bread & rolls	-0.005	-0.015	**-0.149**[a]	**-0.171**[a]	**0.074**[b]	**0.094**[b]	**-0.110**[a]	-0.055	-1.019	-0.052	**0.000**[b]	0.029	-0.050	-0.073	-0.027	0.008
Breakfast cereals	0.041	0.048	0.091	**0.740**[b]	0.049	**0.112**[a]	**0.409**[b]	**0.786**[b]	4.196	0.444	**0.094**[a]	0.032	0.056	0.250	0.116	0.237
Butter & animal fats	**-0.135**[a]	**-0.192**[a]	0.145	-0.084	-0.008	-0.045	0.033	-0.097	0.282	-0.195	-0.092	-0.033	0.030	0.295	0.004	0.477
Cakes	**-0.080**[b]	**-0.103**[b]	0.089	-0.017	**-0.055**[b]	**-0.129**[b]	-0.004	-0.018	0.067	0.003	-0.052	**-0.110**[b]	-0.028	-0.045	-0.063	-0.019
Carbonated/ soft drinks	-0.013	**-0.010**[a]	0.011	0.029	**-0.022**[b]	**-0.024**[b]	**-0.019**[a]	-0.026	-0.023	-0.027	**-0.015**[b]	**-0.015**[b]	-0.005	-0.015	**-0.025**[b]	-0.019
Cereals products	**-0.071**[b]	**-0.076**[b]	-0.081	-0.019	-0.003	-0.018	**-0.084**[a]	-0.069	-0.043	-0.025	**-0.061**[b]	**-0.043**[a]	0.017	0.021	-0.012	0.031
Cheese excluding quark	**-0.142**[b]	**-0.123**[a]	-0.120	-0.125	0.195[b]	**0.134**[b]	0.069	0.001	0.046	0.063	0.061	**0.170**[b]	-0.038	-0.158	0.053	0.050

Chocolate	**-0.168[b]**	**-0.202[b]**	**-0.223[a]**	-1.645	**-0.113[b]**	**-0.179[b]**	-0.016	0.034	-0.052	0.018	**-0.159[b]**	**-0.118[b]**	**-0.158[a]**	0.040	-0.076	**-0.226[a]**
Coffee/ tea	-0.001	-0.002	0.049	0.007	**0.017[a]**	**0.024[b]**	-0.020	**0.078[b]**	0.020	0.055	-0.008	-0.005	0.010	0.026	-0.006	**0.055[a]**
Confectionary products	-0.186	**-0.157[b]**	0.024	0.415	**-0.117[a]**	**-0.238[b]**	0.199	-0.254	0.187	0.046	**-0.362[b]**	-1.170	0.069	0.341	-0.007	-0.131
Dairy products	0.004	0.000	0.003	**0.069[a]**	0.013	0.011	-0.006	0.034	-0.021	0.027	**0.034[b]**	**0.050[b]**	0.008	**0.047[a]**	-0.028	**0.085[b]**
Eggs	0.026	-0.052	-0.295	-0.120	**0.321[b]**	**0.327[b]**	0.223	-0.092	0.155	-0.158	**0.311[b]**	**0.222[b]**	0.012	0.207	-0.128	0.074
Fish products	**0.145[b]**	**0.223[b]**	0.273	0.033	**0.079[a]**	**0.099[b]**	0.153	**0.222[a]**	0.085	0.169	**0.295[b]**	**0.305[b]**	0.012	0.047	-0.036	0.006
Fruits	**0.068[b]**	**0.078[b]**	0.027	0.074	**0.090[b]**	**0.101[b]**	**0.052[a]**	**0.064[a]**	0.050	0.046	-0.018	-0.012	0.002	0.014	0.009	0.016
Fruit & vegetable juices	**0.018[b]**	**0.018[b]**	-0.008	0.018	**0.042[b]**	**0.036[b]**	**0.055[b]**	0.019	0.022	-0.001	**-0.028[b]**	**-0.026[b]**	-0.005	-0.015	0.025	-0.034
Margarine & mixed origin lipids	-0.075	**-0.252[a]**	0.596	-0.012	-0.038	-0.032	**0.464[a]**	0.493	0.231	0.546	0.058	0.080	0.321	0.353	0.305	0.476
Meat	**0.163[b]**	**0.143[b]**	0.018	-0.023	**-0.041[b]**	**-0.059[b]**	-0.062	-0.050	-0.068	-0.008	**0.170[b]**	**0.144[b]**	**0.060[a]**	0.005	0.045	0.034
Oily fruits	-0.122	0.160 b	-0.047	-0.407	**0.028[b]**	**0.336[b]**	-0.186	0.087	-0.172	-0.041	**-0.269[a]**	-0.079	-0.253	0.236	**-0.823[a]**	-0.135
Other milk products	-0.026	**-0.070[a]**	-0.098	0.041	-0.038	**-0.073[a]**	0.118	0.046	0.111	-0.025	0.067	-0.023	0.108	**0.212[a]**	0.004	0.095
Pulses	**0.071[b]**	0.037	0.081	-0.040	**0.170[b]**	**0.199[b]**	-0.035	-0.131	-0.058	-0.003	-0.040	-0.029	-0.005	-0.113	-0.082	0.068
Sauces	**-0.097[b]**	-0.031	-0.231	0.171	**-0.084[b]**	-0.069	-0.069	-0.133	-0.029	0.024	0.058	0.046	-0.031	-0.205	-0.086	-1.660
Savoury snacks	0.004	0.057	-0.199	-0.216	**-0.199[b]**	**-0.135[a]**	-14.922	**-0.521[b]**	-0.087	-0.430	**-0.143[a]**	**-2.826[b]**	0.172	-0.200	-0.008	-0.325
Soups/ bouillon	**0.046[b]**	0.016	-0.048	-0.026	**0.026[a]**	-0.018	0.024	-0.047	0.035	-0.060	**0.055[b]**	0.024	0.029	0.014	**0.075[a]**	-0.013

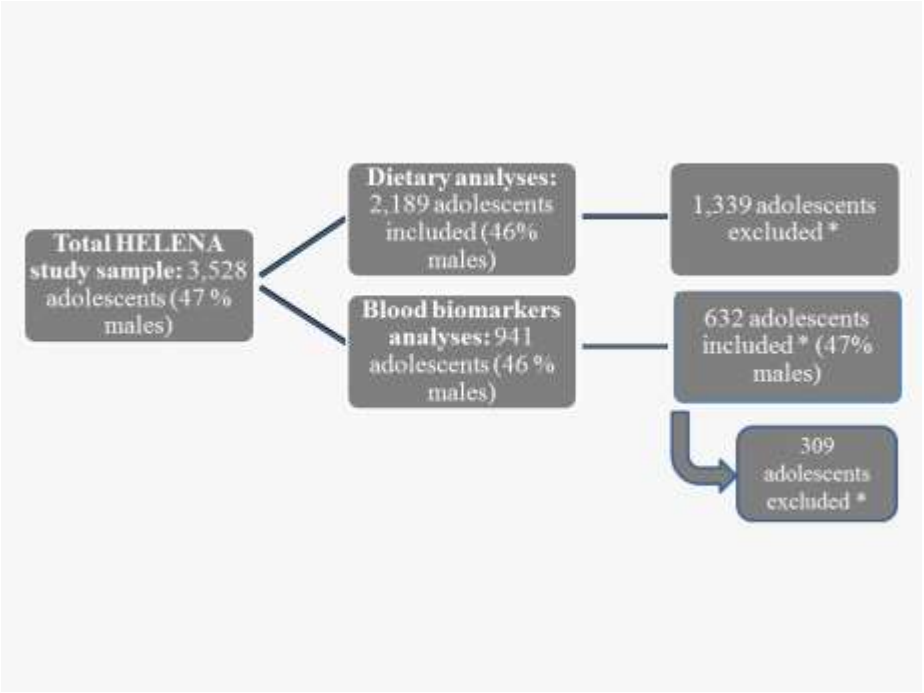
Starch roots & potatoes	**0.204[b]**	**0.229[b]**	0.022	**-0.196[a]**	**0.066[b]**	**0.046[a]**	0.000	-0.109	-0.051	-0.082	0.049	0.045	-0.002	0.019	-0.051	0.008
Sugar products	-0.041	-0.049	0.047	-0.269	-0.051	-0.004	-0.016	-0.269	0.088	-0.338	-0.065	-0.103	0.135	-0.299	**0.331[b]**	-0.237
Vegetable oils	-0.078	**0.253[a]**	0.038	-0.700	**0.666[b]**	**0.624[b]**	-0.328	-0.514	0.098	-0.078	-0.210	-0.178	-0.039	0.280	-0.265	0.223
Vegetables (excluding potatoes)	**0.067[b]**	**0.133[b]**	-0.027	0.063	**0.283[b]**	**0.308[b]**	0.071	0.130	0.071	0.075	0.031	0.023	0.009	0.021	0.012	0.014
Vegetarian products	0.065	-0.019	0.267	-0.270	**0.208[a]**	**0.209[b]**	0.057	-0.106	-0.160	-0.174	0.077	**-0.255[b]**	-0.001	-0.021	-0.098	-0.056
Water	0.003	-0.004	0.003	-0.008	0.003	**0.005[b]**	0.005	-0.005	0.003	0.002	**0.004[a]**	**0.004[a]**	0.002	-0.004	0.005	-0.001
White & butter milk	**0.019[b]**	**0.016[a]**	0.003	0.044	**0.039[b]**	**0.044[b]**	0.024	0.053	0.039	0.015	**0.053[b]**	**0.055[b]**	0.015	0.055 b	**0.032[b]**	**0.052[b]**
Yogurt & white cheese	**0.031[a]**	**0.024[b]**	-0.011	0.066	**0.050[b]**	**0.071[b]**	0.051	0.066	0.038	0.011	**0.065[b]**	**0.062[b]**	-0.009	0.069	-0.005	**0.116[b]**

These coefficients are the result of multiplying by 10 the real coefficients providing by linear mixed model analysis including random effects for centre and represent the change resulting from an increase of 10 grams of indicated food group consumption. Analyses are adjusted by age. BMI, total energy intake, and maternal education level.

[a] Statistically significant at the 0.05 level

[b] Statistically significant at the 0.01 level

Figure 1. Sampling selection.



*Based on the exclusion criteria and the data availability in the covariates of the analysis and the two 24 hours recalls and biomarkers, respectively.

Food group	Items included, description
Dairy products	Milk and yogurt beverages
Other milk products	Desserts& puddings milk based and creams with extra amount of sugars and fats
White & butter milk	Milk (any kind of fat content), and also the liquid left behind after churning butter out of cream, which is typical for northern countries in Europe
Yogurt & white cheese	All kind of yogurts and low fat cheese

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