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**Title:** Do dietary patterns determine levels of vitamin B\(_6\), folate, and vitamin B\(_{12}\) intakes and corresponding biomarkers in European adolescents? The HELENA study.

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Abbreviations: HELENA-CSS: Healthy Lifestyle in Europe by Nutrition in Adolescence Cross-Sectional Study; RRR: Reduced Rank Regression analyses; WHO: World Health Organization; Principal Component Analysis (PCA); HELENA-DIAT: HELENA-Dietary Assessment Tool; YANA-C: Young Adolescents’ Nutrition Assessment; 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; FFQ: Food Frequency Questionnaire.
Highlights

- Dietary patterns explain moderate variance of B-vitamins in European adolescents.
- The percentage of explained variance is higher for intakes than for biomarkers.
- Contributing food items to the patterns are quite different for males and females.

Abstract

Objective: To determine dietary patterns (DPs) explaining the highest variance of vitamin B₆, folate, and B₁₂ intake and related concentrations among European adolescents.

Methods: 2,173 adolescents participating in the HELENA (Healthy Lifestyle in Europe by Nutrition in Adolescence) study met the eligibility criteria for B-vitamins intake analysis (46 % boys) and 586 did it for biomarkers analysis (47 % boys). Two non-consecutive 24-h dietary recalls were used to assess mean intakes. Concentrations were measured by chromatography and immunoassay. Reduced rank regression was applied to elucidate the combined effect of food intakes in B-vitamins intakes and concentrations.

Results: Identified dietary patterns (one per each B-vitamin intake and biomarker and by sex) explained a variability between 34.2 % and 23.7% of the B-vitamin intakes and between 17.2 % and 7% of the biomarkers. In the reduced rank regression models, fish, eggs, cheese, and white and buttermilk intakes, loaded positively for B-vitamins intake in both sexes; in contrast, soft drinks and chocolate, loaded negatively. For biomarkers, there was higher variability in terms of loads of foods in the identified patterns, like in the case of alcoholic drinks, sugars and soft drinks. Some food items loaded differently between intakes and biomarkers, like fish products which, in girls, loaded positively for intakes, but negatively for plasma folate.

Conclusion: The identified dietary patterns explained up to 34.2% and 17.2 % of the variability of the B-vitamins intakes and plasma concentrations of European adolescents, respectively. Further studies are needed to elucidate the factors determining such patterns.
Introduction

The global burden of micronutrient deficiencies worldwide is enormous and it includes also industrialized countries and affects all population groups (1). According to WHO, micronutrient deficiencies are relevant for public health, not only in relation to specific clinical manifestations, but also as responsible for a wide range of non-specific manifestations such as infections, metabolic disorders, and delayed or impaired physical and psychomotor development (2).

In this regard, much attention has been paid to B-vitamins as key micronutrients for the maintenance of optimal health and prevention of diseases (3). Besides, there is increasing evidence of sub-deficient folate, vitamin B6, and vitamin B12 status in several population groups, including also children and adolescents (4). In fact, previous research from the HELENA study showed that 25 % of the adolescents had insufficient values of PLP (biomarker of the vitamin B6), 50 % insufficient values of folate, and 5 % of vitamin B12.

Socioeconomic family trends, (i.e., longer work hours), have changed the way adolescents obtain their meals (5) and fast food restaurants are more commonly the choice for food away from home for them (6). In fact, about a third of children and adolescents in the United States consume fast food on a typical day and they seem to visit fast food restaurants approximately twice a week (7). And these trends are similarly observed in Europe (8, 9). However, it is already known that frequent consumption of fast food may have adverse effects on their nutritional status because of excessive content of energy and fat and low nutritional value (10). All this, might be among the causes of the inadequate biomarkers values of the cited vitamins. The current public health crisis in relation to childhood obesity, demands increased funding for research. As this epidemic was not caused by inherent biological defects, the research must be focused in improving the environment with new dietary, physical activity, behavioural, environmental, and pharmacological approaches and intervention programmes. (11).

Deficiency of vitamin B6, folate and vitamin B12 can manifest in different population groups at different life stages when requirements are increased, such as during growth in
deficiency of these three vitamins at early stages of life has been related to developmental delay, feeding problems, failure to thrive, irreversible neurological damage, severe or recurrent headache or migraine, or potential implications for an altered risk for chronic disease in later life.

Nutrition research has favoured a reductionist approach emphasizing the role of single nutrients in diet-health relationships. As a matter of fact, human diet is a complex behavior. Foods are not consumed isolated and for this reason, in observational studies, it is very difficult to derive any outcome or consequence from one single food. Therefore, statistical approaches describing diet in a more holistic way have been applied to explore dietary patterns in relation to health outcomes. Among the most commonly applied techniques (namely Principal Component Analysis (PCA), Reduced Rank Regression analysis (RRR) and Cluster Analysis (CA)), RRR seems to be more flexible and powerful than other methods such as PCA it uses prior knowledge (from biologic evidence, dietary intervention studies, epidemiologic studies with biomarkers, and large prospective cohort studies) in combination with the strength of PCA in considering the correlation of dietary components and the advantage of diet-quality scores to account for current scientific evidence.

A previous analysis based on the HELENA study, has investigated the relationship between the consumption of individual food items in this sample of European adolescents in relation to the cited B-vitamins intakes and plasma concentration levels (biomarkers). However, to our knowledge, apart from a German study investigating the associations between PCA-derived dietary patterns (DPs) and the intake of some nutrients including vitamin B6, folate, and vitamin B12, there is no other study addressing also B-vitamins concentrations in parallel in relation to dietary patterns. Besides, up to date, there is no publication addressing the relationship between the dietary patterns obtained throughout RRR analyses method and both the intakes and status of vitamin B6, folate and vitamin B12 in European adolescents, which is precisely the aim of this study.

Material and methods

Sampling
3,528 adolescents (47% boys) from 10 European cities in 9 countries were recruited to participate in The Healthy Lifestyle in Europe by Nutrition in Adolescence Cross-Sectional Study (HELENA-CSS). The cities participating in this multi-centre study were Athens and Heraklion (Greece), Dortmund (Germany), Ghent (Belgium), Lille (France), Pecs (Hungary), Rome (Italy), Stockholm (Sweden), Vienna (Austria), and Zaragoza (Spain). The average participation rate in our study was 67%, which is acceptable for this demanding epidemiological study (18). As inclusion criteria, they must be 12.5-17.5 years old, not participating simultaneously in a clinical trial and not having any acute infection occurring < 1 week before inclusion (19). To provide rigorous quality assurance regarding ethical issues, a protocol was prepared by a HELENA study centre based on its previous experience in these topics (20). It conformed the good clinical practices (GCP) described at the International Conference on Harmonisation (ICH), and based on concepts about ethics in biomedical research that originated from the Nuremberg Code and the Declaration of Helsinki (21, 22). 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 (cities) (20).

From the initial number of 3,528 adolescents, 2,173 (46% boys) were included based on the availability of the variables of interest: complete data on two non-consecutive 24-hour dietary recalls (24H-DR), maternal education as marker of socioeconomic status, BMI z-score (Body Mass Index standardized values and adjusted by age and sex of the adolescents), and total energy intake. Similarly, only a third (1,089, 47% boys) (19) of the initial sample of 3,528 adolescents participated in blood drawings. From those, only 586 adolescents (45% boys) were included based on compliance with the previously cited variables of interest. The first 24H-DR was completed on the same day of the blood drawing while the second one was filled a fortnight after considering weekends and weekdays. If the first 24H-DR was completed on Monday (weekend recall), the second, was completed from Tuesday to Friday (weekday recall). Trained staff was present during the assessments (23).

Sampling selection procedures are shown in figure 1. Further study details were published elsewhere (19).
Assessment of vitamin B<sub>6</sub>, folate and vitamin B<sub>12</sub> intakes

Dietary food intake was estimated by means of two self-administered computerized 24-hour dietary records (HDR) using the HELENA-Dietary Assessment Tool (HELENA-DIAT), adapted for European adolescents (23) from a previous software (called Young Adolescents’ Nutrition Assessment on Computer (YANA-C)). Pitfalls were found to obtain comparable measures of energy density of each food across countries by using country-specific food composition tables. Therefore, the obtained data was linked to the German Food Code and Nutrient Data Base (BLS-Bundeslebensmittelschlüssel-, version II.3.1, 2005), including 12,000 coded foods, and with up to 158 nutrient data points available for each product. Afterwards, every country developed in local language its own database file adapted to the respective food culture (special dishes, food items, beverages, food amounts and picture links). The recorded data was linked to the country-specific food codes to assign food composition data (recipes, ingredients, and nutrients) from the respective national food composition databases to calculate energy and nutrient intake (23, 24). Finally, the Multiple Source Method (MSM) (25) was used to calculate usual nutrient intake removing the effect of day-to-day within-person variability (using the adapted version of the FFQ from HBSC as a propensity tool to do this) and random error in the recalls, adjusted by age, sex, and centre or country (26).

Beverage and food groups

All reported 4,179 foods and beverages were categorized initially in 29 food groups based on the European Food Groups classification system (23, 27). As part of the general HELENA analysis, these foods were disaggregated into 43 food groups. For the aim of the current analysis and based on their nutritional composition some of these food groups were again aggregated. For instance, beer, wine, and spirits were aggregated into alcoholic drinks; pasta, rice, and flour into complex carbohydrates; honey and other sugar products into sugar products, oily fruits included nuts, seeds, avocado and olives; milk products grouped yogurt, white cheese, milk and yogurt beverages; and finally, other milk products merged desserts, milk-based puddings and creams. Besides, ‘products for special nutrition use’, ‘soya beverages’, and ‘miscellaneous’ were eliminated from the current analysis based on their very low consumption (0 median and mode and more than the 85% of the sample did not report...
consumption). The final number of food groups for the current analysis was 31 food
groups (table 2). Nevertheless, the resulted amount of B-vitamins intake corresponds to
the sum of the B-vitamin belongs to all the original food groups (even the one contained
in the excluded food groups in the current analyses).

Assessment of vitamin \textit{B}_6, folate and vitamin \textit{B}_{12} biomarker concentrations

Blood was obtained after an overnight fast according to a standardized blood collection
protocol. Further details on sample transport and quality assurance can be found
elsewhere (28). Briefly, to assure an adequate sample handling, storage and subsequent
analysis, logistics of sample transport and major parts of the analytics were centralized
at the Analytical Laboratory from the University of Bonn (IEL, Bonn, Germany) and
field-workers were previously trained. The time span between sampling and processing
was up to 24 h. However, a novel handling and transport system following the Good
Clinical Practices (29), and a novel traceability system developed at the Clinical
Investigation Centre in Lille based on printed documents and an electronic database,
together with the realization of a pilot study, confirmed the absence of any stability
problem (28).

Based on previous experiences of the research group, and after performing the pilot
study, it was agreed upon 30 ml of blood in order to be able to analyse all proposed
parameter (2 x 7.5 ml tubes for serum, 2.6 ml tube for heparin plasma, 4ml tube and 2.7
ml tube for EDTA for hematology) (30).

Concretely, pyridoxal 5'-phosphate (PLP), was measured as a marker of vitamin B6
status, by high performance liquid chromatography (HPLC) (Varian Deutschland
GmbH, Darmstadt, Germany; CV = 1%) with a modified method of Kimura et al (4,
31). Plasma and RBC-folate and plasma vitamin \textit{B}_{12}, were measured in the central
laboratory at the University of Bonn (IEL-Institut für Ernährungs und
Lebensmittelschaften-, Germany) by means of a competitive immunoassay using the
Immunolite 2000 analyzer (DPC Biermann GmbH, Bad Nauheim, Germany) (28).
Holotranscobalamin (HoloTC) was analysed in the biochemical lab at the Universidad
Politécnica de Madrid by microparticle enzyme immunoassay (Active \textit{B}_{12} Axis-Shield
Ltd, Dundee, Scotland, UK) with the use of AxSym (Abbot Diagnostics, Abbott Park,
IL, USA) (32).
As a marker of socioeconomic status of the families, maternal education was selected from all the available socioeconomic related variables due to its documented association with vitamin intakes and biomarkers (33). Maternal education was categorized into low, medium-low, medium-high, and high, based on the information provided by the adolescents through a self-administered questionnaire.

Weight was measured in underwear conditions 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. After calculation of body mass index (BMI), z-scores of BMI were calculated via LMS growth (34), and used as a covariate in the analysis, together with total energy intake.

Statistical analysis

The assumption of normality was assessed with visual inspection (histograms, boxplots and Q-Q plots) and then with Kolmogorov-Smirnov test. All statistical tests were two-sided, and p<0.05 was the threshold considered statistically significant. Statistical analysis was sex stratified because most of the variables involved in this study presented statistically significant differences and provided the fact that dietary patterns have been shown to be different in this sample of adolescent boys and girls in Europe (35). Descriptive data are presented as means and standard deviations (SD) for continuous variables, and frequencies and percentages for categorical variables.

In the correlation analysis, non-parametric Spearman coefficient was used since dietary intake data and blood values were not normally distributed. Food and beverages intakes were expressed as grams or milliliters per day (g or ml/day) and B-vitamins intakes and biomarkers were expressed in their corresponding units as specified in the tables.

Reduced rank regression (RRR) was applied using the partial least square (PLS) procedure in SAS (15). RRR assumes a linear function of responses (i.e. B-vitamins intakes and statuses) with the predictors (i.e. food groups) and creates a response score that will then be projected onto the space of predictors to produce a factor score, that is, a linear function of predictors. Assessment of factors extracted by RRR are based on...
response scores rather than on factor scores. Factor loadings indicate the association
between the food groups and the derived factor and thus indicate which food groups
load highly onto the factor which explains variation in the B-vitamins intakes and
statuses. Factor loadings are equivalent to correlation coefficients (in contrast to
regression coefficients which are equivalent to quantifiable weights). Consequently,
positive factor loadings indicate that the food group is positively associated with the
factor, and negative ones show that the food group is inversely related to the factor.
Higher factor loadings indicate a greater contribution of that food group to the factor.
Data on food intake was firstly adjusted for several covariates (BMI z-scores, maternal
education and total energy intake) using mixed model analysis with a random intercept
for centre and then entered as residuals into the RRR analyses. Using RRR, the number
of extracted factors is equal to the number of selected responses, thus, one factor was
obtained for each B-vitamin (either intake or biomarker). Only food groups with factor
loadings|>0.20| were considered as relevant for the factors or patterns as has been
previously used (36). Statistical analyses were performed with SAS 9.3 for Windows
(SAS Institute Inc., Cary, NC) and SPSS 20.0 for Windows (SPSS Inc., Chicago, IL,
USA). Radar plots were performed with Excel (Microsoft).

Results

Sensitivity analyses were performed comparing participants included vs. not included in
the study (data not shown). The proportion of boys in relation to girls included in these
analyses was 46% for the analyses with B-vitamins intake and 45% for the B-vitamins
biomarkers while for the general HELENA study sample was 47% of boys. For the
dietary analyses, included boys did not differ from those excluded in terms of age, but
had significantly lower BMI (p=0.002) and lower energy intake (p<0.05), while
included girls were significantly younger and presented higher BMI than those excluded
(p<0.05). Regarding the biomarkers analyses, included boys had significantly lower
BMI (p<0.05). Included boys and girls, significantly differed from those excluded in
terms of maternal education (p<0.05), representing mothers placed in higher categories
of education higher percentage in comparison to the ones placed in other categories than
in those excluded.
Table 1 presents the adolescents’ characteristics for both the intake and the biomarkers groups stratified by sex. In the intake group, BMI z-score, energy intake and B-vitamins intake were significantly higher (p<0.05) in boys than in girls. However, in the biomarkers group, there were significant differences for total energy intake, PLP, and plasma vitamin B\(_{12}\) between sexes.

Table 2 shows the Spearman rank correlation coefficients between the food and beverage intakes and the B-vitamins intake and biomarkers, respectively. Correlations ranged from weak (r\(_s\) = 0.06 for pasta, rice & flour for vitamin B\(_{12}\) intake in boys, and -0.06 for coffee & tea and vitamin B\(_{12}\) intake in girls) to moderate (r\(_s\) = -0.70 for margarine & lipids of mixed origins and vitamin B12 intake in boys). Food and beverage intakes were mainly correlated with B-vitamins intake. For vitamin B\(_6\) intake, both in boys and in girls, higher, significant, and positive correlations were found for vegetables excluding potatoes, starch roots and potatoes, fruits, and meat; for folate intakes, with bread and rolls, vegetables excluding potatoes, fruits, and cheese; and, for vitamin B\(_{12}\) intakes, with meat, fish products, and white and buttermilk.

In case of B-vitamins biomarkers, the foods and beverages which correlated higher, significantly and positively with them were: margarine with PLP both in boys and girls and breakfast cereals also in girls; breakfast cereals both in boys and girls with plasma folate, and in girls also chocolate, vegetable oils, margarine and mixed origin lipids, fruits, coffee and tea, and vegetable and fruit juices; for RBC-folate, potatoes were correlated to in boys, and yogurt & milk, breakfast cereals, and water in girls; plasma vitamin B\(_{12}\) correlated in boys with butter & animal fats, breakfast cereals, white & buttermilk, and savoury snacks, and in girls, with chocolate, yogurt and milk, and with white & buttermilk; and finally, with HoloTC, pulses and white & buttermilk correlated in boys, and pasta, rice and flavour, yogurt and milk, and white & buttermilk correlated in girls.

The proportions of variation in B-vitamins intake and biomarkers explained by the dietary patterns are shown in Table 3. Regarding B-vitamins intake, dietary patterns could explain between 34.2 % of the variance for folate in girls to 23.7 % of the variance for vitamin B\(_{12}\) also in girls. For B-vitamins biomarkers, the variance explained by the different dietary patterns ranged from17.2% for PLP in boys and 7.0 % for plasma vitamin B\(_{12}\) in girls. Regarding food and beverage intakes, fish, eggs, cheese,
and white and buttermilk, were the only food items showing consistent and positive loadings in the identified patterns, and soft drinks and chocolate, showed consistent and negative loadings in the identified patterns, both in boys and girls.

For biomarkers, results were less consistent in terms of foods loading direction, even loading oppositely for boys and girls in some cases. For instance, alcoholic drinks showed positive loadings for boys and negative for girls; sugars, and soft drinks both showed positive loadings for girls and negative for boys. Besides, not only differences between sexes have been found but also between intakes and biomarkers. For instance, fish products loaded positively in the identified patterns for intakes, both in boys and girls, but negatively for biomarkers, also in both sexes.

The factor loadings of each food group to the corresponding derived pattern for each B-vitamin (table 3) are representing in supplementary material as radar plots when it is possible to easily appreciate the different shapes of the patterns for boys and girls. Besides, the obtained patterns, and due to the food groups, which characterized them, might be named as follows: traditional pattern for both boys and girls in relation to vitamin B₆ intake, Mediterranean and protein-based pattern for boys and girls respectively in relation to folate intake, breakfast pattern for both boys and girls in relation to vitamin B₁₂ intake, breakfast pattern and traditional pattern for boys and girls respectively in relation to PLP, fast-food pattern and snack pattern for boys and girls respectively in relation to plasma folate, healthy-conscious pattern and desserts pattern for boys and girls respectively in relation to RBC-folate, Italian-cuisine pattern and fast-food pattern for boys and girls respectively in relation to plasma vitamin B₁₂, and finally, a breakfast dietary pattern for both boys and girls in relation to Holotranscobalamin.

**Discussion**

To our knowledge, there are no previous studies investigating the dietary patterns determining both the intake and the status of vitamin B₆, folate and vitamin B₁₂ in adolescents. Up to now, this is also the first time that RRR is used to elucidate the dietary patterns best explaining the variability in vitamin B₆, folate, and vitamin B₁₂ intake and status in a large sample of European adolescents.
The dietary patterns derived in this study were considerably different for boys and girls and the stratification of their computation was done due to previous results obtained in this respect in the literature. For instance, in Germany, Richter et al (37) it was found that there were gender differences in relation to the found dietary patterns explaining the levels of biomarkers of folate and vitamin B12. Besides, another author (35) had found statistically significant differences in the consumption of almost all the food groups in the HELENA study.

The RRR-derived dietary patterns could account for higher variability (up to 34.2 %) of the B-vitamins intake than for the B-vitamins concentrations (up to 17.2 %). In a recent manuscript (16) we also observed fewer and weaker associations for B-vitamins biomarkers than for their intakes, in relation with food groups, based also in HELENA study. The different proportions of the variance explained by dietary patterns, when considering either B-vitamins intake or their biomarkers might be due to the fact that biomarkers concentrations could be related with long-term food consumption patterns, whereas the available information related to B-vitamins intake was recorded using two 24H-DR. Pointing in this direction, in a previous analysis by Vandevijvere et al., considering different biomarkers of micronutrients intake (vitamin C, β-carotene, docosohexaenoic acid, eicosapentaenoic acid, vitamin B_{12} and folate), correlations were higher when considering the food and beverage consumption frequencies (from the food frequency questionnaire -FFQ-) as compared to mean food and beverages intakes (from the 24H-DR) and the same concentration biomarkers (38). But sometimes, the differences can be due to the time lap between intakes and the blood drawings, mainly for food groups which are not consumed often such us fish products, for instance.

In another study from Germany by Ritcher et al. (17), in a similar population group, dietary patterns were obtained using Principal Components Analyses (PCA) and, in boys, vitamin B_{6} diet density increased with increasing scores of the ‘healthy’ pattern. Folate diet density was also related to this “healthy” pattern in both sexes (17). This “healthy pattern” consisted of high consumption frequencies of fruits, vegetables, legumes, mushrooms, chicken, rice, vegetable oil, soup, and grains in boys; and of rice, vegetable oil, soup, chicken, legumes, vegetables, fruits, vegetarian dishes, eggs, fish,
water, warm sauces and mushrooms in girls. In our study, the intake of this B-vitamin was mainly explained for a traditional dietary pattern both in boys and girls, containing some of the food groups which determine the healthy pattern in the German study. In our study, boys’ patterns presented some similarities, at least regarding vitamin B6 and folate, as the observed patterns related with these vitamins consisted of vegetables excluding potatoes, starch roots and potatoes, fruits, meat, fish products, and white and buttermilk in detriment of chocolate, soft drinks for vitamin B6; and of vegetable oils, pulses, vegetables excluding potatoes, fruits, and fruit and vegetable juices in detriment of meat for folate. These patterns also featured foods considered to be healthy.

Besides, the German study reported a traditional pattern that related to vitamin B12 intake density in boys (17), consisted of processed meat, potatoes, white bread, margarine, meat (except chicken), eggs, cheese, and fish, while in our study, vitamin B12 intake was related to a breakfast pattern consisted mainly of white and buttermilk, cheese and eggs. In girls, vitamin B12 intake density was associated with the so-called traditional and western’ pattern in the cited German study by Ritcher et al. (17), which involved potatoes, warm sauces, meat (except chicken), white bread, processed meat, as well as pizza, French fries, sausages, soft drinks, confectionary, cake/cookies and negatively correlated with water. In our study, vitamin B12 intake in girls was determined also by breakfast pattern like in boys, characterized by the consumption of bread and rolls, yogurt and milk, eggs, white and buttermilk, and cheese. It is worth to highlight the fact that in our study, meat, which is consider one of the main sources, scores negatively in the patterns that precisely explained the highest variance in the vitamin B12 intake both in boys and girls. These differences might be obtained due to the difference in the statistical approach to derive the dietary patterns (in PCA used in this German study, the linear combination of the food groups is explained, but not the variation in a response variable -B-vitamins-).

All in all, while for boys, B-vitamins intakes were determined by similar patterns in both studies, we found larger differences in girls. However, providing that the statistical techniques to extract the dietary patterns in these two studies have different purposes, we must be cautious in comparing the results: PCA searches for the highest variability
among the food intake, while RRR looks at the highest variability in explaining the
differences for each outcome variable (39).

Up to now, there is no similar study performed to which compare our results for B-
vitamins biomarkers. In Indian children in a study by Kehoe et al. (40), a lacto-
vegetarian dietary pattern was related to folate status and negatively related to vitamin
B\textsubscript{12} status. In USA Health-Professionals, folate status was negatively correlated with a
‘Western’ dietary pattern (41). However, food accessibility and food preferences in the
first case, and the population group in the second one, made these results non-
comparable to ours.

Surprisingly, we have identified different patterns determining B-vitamins biomarkers
for boys and for girls, apart the 'breakfast pattern' which explained a variance of 17.2 %
and 10.7 % for boys and girls, respectively for PLP.

For instance, a dietary pattern that could be considered as fast-food and snack patterns
might explain between 12.8 % and 9.0 % of the variance in plasma folate concentrations
in boys and girls, respectively. In boys, a healthy-conscious dietary pattern determined
RBC-folate, while in girls it was determined by a dessert dietary pattern including
several snacking food items. Similarly, a dietary pattern characterized by food items
typical of the 'Italian cuisine' and another one characterized by food served in fast-food
restaurants explained the variance of the plasma vitamin B\textsubscript{12} in boys and girls,
respectively. A dietary pattern characterized by food items served at breakfast explained
the variance for HoloTC in both sexes.

Around 16 % of the girls, of the 584 adolescents which had blood drawings, (42) used
contraceptives, and B-vitamins status might be affected from them as was suggested for
the literature, at least for vitamin B\textsubscript{6} (PLP). However, it is precisely for this vitamin for
which we have obtained more similar patterns between boys and girls.

Different dietary patterns were obtained for B-vitamins intakes and for their
 corresponding biomarkers as already expected owing to previous results obtained for
the same sample when analysing only food items individually with these B-vitamins
(16). Reasons for these differences might be attributed to differences in metabolism,
interference with other nutrients, and physiologic status, nutrients variation in same
food items depending on where or how the food was grown or how it was processed, and lack of data regarding supplements use or fortified food items, as previously suggested (16). In addition, two types of biomarkers were used in our study: those which reflect the recent intakes (such as plasma folate and plasma vitamin B_{12}), but also PLP, RBC-folate, and HoloTC which are more focused on detecting the corresponding vitamins storages (12). In general, dietary patterns are considered to reflect long-term food intake rather than short-term food intake, and consequently might better explain biomarkers’ storage rather than punctual intake markers, as this was the case for folate in our study, but not for vitamin B_{12} biomarkers.

The fact that the dietary patterns obtained in this study had been adjusted for centre, could prevent to obtain higher proportions of the variance explained as compared with studies performed in single countries. However, identifying common dietary patterns independently of the different countries and socioeconomic status, could facilitate the approach for future interventions trying to ameliorate the nutritional status or avoiding deficiencies of young population groups.

The characterization of adolescents based on their dietary patterns while accounting for their socioeconomic status, helps to address adolescents with higher risk of inadequate intakes and vitamin status and to highlight the priorities for health promotion and, also, provides a better understanding of the role of diet in relation to disease.

Strengths and limitations

In this instance, what empowered considerably this study is the use of RRR, instead of the traditional \textit{a posteriori} methods like PCA or cluster analyses, to determine the dietary patterns which best explain the variability in the B-vitamins intakes and statuses of European adolescents for the first time in the literature. The dietary patterns derived from \textit{a posteriori} methods explain the variation in food groups intake, which may be appropriate to characterize existing basic dietary patterns in a population but may not optimally represent patterns relevant for the aetiology of specific diseases (43, 44); however, \textit{a priori} methods do not consider the correlated aspects of some food groups (45). New hybrid methods use a combination of \textit{a priori} knowledge on nutrient intake or risk factors for disease and the underlying dietary data to derive dietary patterns (46), and RRR is among them (15).
Apart from that, the use of a large and culturally diverse sample of European adolescents from 9 European countries is also another important strength (19). Moreover, the questionnaires used to assess maternal education, were previously validated (47). Another important strength of the study is the correction procedures used to avoid the limitations of the 24H-DR. These were, for instance, the use of the MSM method to correct the crude intake data values for within-person variation (48), and the use of the correspondent vitamins biomarkers. However, correlations between biomarkers and usual food intakes obtained from the recalls were low in this sample (38). This could be due to the fact that dietary intakes correlate better with biomarkers when the number of days covered by the reference method increases (49). On the other hand, due to standardization reasons, the use of the German food composition table provides differences in comparison with national food composition tables, small, and for most nutrients negligible, which implies to be a reliable approach (26). Blood biomarkers were analysed in the same centre, strengthening the reliability of the lab-results (28).

As a limitation, food fortification was not included in the German food composition database, and the analyses were not controlled for dietary B-vitamins supplement use. However, only 5% of our sample was shown to use them, so no important differences in the interpretation of our results would be expected.

The nature of this cross-sectional design of HELENA study, does not allow establishing causality in the associations found.

**Conclusion**

Dietary patterns obtained by reduced rank regression analyses explained between 23.7% and 34.2% of the variability of B-vitamins intake, and between 7.0% and 17.2% of the variability of B-vitamins concentrations in European adolescents. Apart from the patterns obtained to determine plasma folate and plasma vitamin B_{12}, which were mainly unfavorable, the dietary patterns determining the levels (both intake and status) of vitamins B_{6}, folate and vitamin B_{12}, included mainly healthy food groups. In consequence, there is an urgent need for classifying vulnerable population groups in terms of dietary patterns, such as adolescents, to identify those at increased risk of low
B-vitamin levels and to determine what are the main determinants of these dietary patterns.
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References


31. Kimura M, Kanhira K, Yokoi K. Highly sensitive and simple liquid chromatographic


Figure 1. Sampling selection process
Table 1. Characteristics of adolescents with complete information on B-vitamins intake and biomarkers stratified by sex.

<table>
<thead>
<tr>
<th>Variables in this study</th>
<th>B-vitamins intake</th>
<th>B-vitamins biomarkers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males (N=999)</td>
<td>Females (N=1184)</td>
</tr>
<tr>
<td>Age (years old)</td>
<td>Mean</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>Standard Deviation</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>p-value</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI z-scores</td>
<td>14.8</td>
<td>14.7</td>
</tr>
<tr>
<td></td>
<td>1.3</td>
<td>1.2</td>
</tr>
<tr>
<td>&lt;0.001†</td>
<td></td>
<td></td>
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<tr>
<td>Energy intake (kcal/d)</td>
<td>2,482.4</td>
<td>1,892.6</td>
</tr>
<tr>
<td></td>
<td>808.7</td>
<td>580.1</td>
</tr>
<tr>
<td>&lt;0.001*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal education</td>
<td>Low</td>
<td>Medium-low</td>
</tr>
<tr>
<td>(levels, n (%))</td>
<td>63</td>
<td>274</td>
</tr>
<tr>
<td></td>
<td>(6.3)</td>
<td>(27.4)</td>
</tr>
<tr>
<td>&lt;0.001*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;6&lt;/sub&gt; intake (µg/d)</td>
<td>1,782.3</td>
<td>1,430.3</td>
</tr>
<tr>
<td></td>
<td>613.8</td>
<td>474.1</td>
</tr>
<tr>
<td>&lt;0.001*</td>
<td></td>
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<tr>
<td>Folate intake (µg/d)</td>
<td>211.3</td>
<td>177.4</td>
</tr>
<tr>
<td></td>
<td>74.8</td>
<td>59.2</td>
</tr>
<tr>
<td>&lt;0.001*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B&lt;sub&gt;12&lt;/sub&gt; intake (µg/d)</td>
<td>6.0</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>2.3</td>
<td>4.6</td>
</tr>
<tr>
<td>&lt;0.001*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;6&lt;/sub&gt; (piridoxalphosphate)(nmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol/L)</td>
<td>69.1</td>
<td>62.4</td>
</tr>
<tr>
<td>&lt;0.001*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma folate (nmol/L)</td>
<td>18.3</td>
<td>18.4</td>
</tr>
<tr>
<td>RBC-folate (nmol/L)</td>
<td>806.7</td>
<td>762.1</td>
</tr>
<tr>
<td>Plasma vitamin B&lt;sub&gt;12&lt;/sub&gt; (pmol/L)</td>
<td>335.5</td>
<td>380.3</td>
</tr>
<tr>
<td>HoloTC(pmol/L)</td>
<td>60.8</td>
<td>62.6</td>
</tr>
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</table>

†Based on T-test statistics
*Based on Mann-Whitney test statistic
Based on chi-square test statistic
Table 2. Spearman correlations coefficients between food intake (g/d) and B-vitamins intake and biomarkers.

<table>
<thead>
<tr>
<th>Food groups</th>
<th>Vitamin B&lt;sub&gt;6&lt;/sub&gt; intake (µg/d)</th>
<th>Folate intake (µg/d)</th>
<th>B&lt;sub&gt;12&lt;/sub&gt; intake (µg/d)</th>
<th>Vitamin B&lt;sub&gt;6&lt;/sub&gt; (piridoxalphosphate) (nmol/L)</th>
<th>Plasma folate (nmol/L)</th>
<th>RBC-folate (nmol/L)</th>
<th>Plasma vitamin B&lt;sub&gt;12&lt;/sub&gt; (pmol/L)</th>
<th>HoloTC (pmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bread &amp; rolls</td>
<td>0.29†</td>
<td>0.20†</td>
<td>0.42†</td>
<td>0.35†</td>
<td>-0.04</td>
<td>-0.03</td>
<td>-0.16†</td>
<td>-0.84</td>
</tr>
<tr>
<td>Chocolate</td>
<td>0.08†</td>
<td>0.11†</td>
<td>0.15†</td>
<td>0.11†</td>
<td>-0.03</td>
<td>-0.05</td>
<td>0.01†</td>
<td>0.15†</td>
</tr>
<tr>
<td>Pasta, rice, flour</td>
<td>0.06</td>
<td>0.02</td>
<td>0.20†</td>
<td>0.12†</td>
<td>0.06*</td>
<td>0.07†</td>
<td>-0.17†</td>
<td>-0.01</td>
</tr>
<tr>
<td>Nuts, seeds, avocados</td>
<td>0.03</td>
<td>0.1†</td>
<td>0.21†</td>
<td>0.21†</td>
<td>0.01</td>
<td>0.03</td>
<td>-0.06</td>
<td>-0.03</td>
</tr>
<tr>
<td>Alcoholic drinks</td>
<td>0.08†</td>
<td>-0.01</td>
<td>0.06</td>
<td>0.02</td>
<td>0.06</td>
<td>0.07†</td>
<td>-0.10</td>
<td>0.04</td>
</tr>
<tr>
<td>Sugars</td>
<td>0.13†</td>
<td>0.05</td>
<td>0.07*</td>
<td>0.07*</td>
<td>0.07</td>
<td>0.01</td>
<td>-0.10</td>
<td>-0.07</td>
</tr>
<tr>
<td>Vegetable oils</td>
<td>0.14†</td>
<td>0.09†</td>
<td>0.24†</td>
<td>0.16†</td>
<td>0.08</td>
<td>0.05</td>
<td>-0.04</td>
<td>-0.15†</td>
</tr>
<tr>
<td>Yogurt, milk, margarine &amp; lipids of mixed origins</td>
<td>0.14†</td>
<td>0.14†</td>
<td>0.14†</td>
<td>0.14†</td>
<td>0.18</td>
<td>0.18†</td>
<td>-0.02</td>
<td>0.1</td>
</tr>
<tr>
<td>Dairy Dessert &amp; cream</td>
<td>-0.05</td>
<td>-0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>-0.70*</td>
<td>-0.07*</td>
<td>0.14*</td>
<td>0.11*</td>
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<tr>
<td>Butter and animal fats</td>
<td>0.10†</td>
<td>0.06*</td>
<td>0.02</td>
<td>0.05</td>
<td>0.08</td>
<td>0.08†</td>
<td>-0.06</td>
<td>-0.02</td>
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<tr>
<td>Salty sauces</td>
<td>0.01</td>
<td>0.08†</td>
<td>0.09</td>
<td>0.09</td>
<td>0.07</td>
<td>0.04</td>
<td>0.01</td>
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<tr>
<td>Pulses</td>
<td>0.09†</td>
<td>0.16†</td>
<td>0.09†</td>
<td>0.10†</td>
<td>0.15</td>
<td>0.15†</td>
<td>0.02</td>
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<tr>
<td>Vegetables excluding potatoes</td>
<td>0.08*</td>
<td>0.05</td>
<td>0.08*</td>
<td>0.03</td>
<td>0.08</td>
<td>0.08†</td>
<td>-0.03</td>
<td>-0.02</td>
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<tr>
<td>Starch roots,</td>
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<td>0.32†</td>
<td>0.47†</td>
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<td>0.21</td>
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<td>0.13</td>
<td>0.19†</td>
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<td>-0.17†</td>
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* indicates p < 0.05; † indicates p < 0.01.
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<th>0.09</th>
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<th>0.12</th>
<th>0.04</th>
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* p<0.05; † p<0.01

HoloTC: Holotranscobalamin
Table 3. Percentage of variation in B-vitamins intake and biomarkers explained by dietary patterns defined by food groups with factor loadings with value ≥0.2, stratified by sex.

<table>
<thead>
<tr>
<th>Food Group</th>
<th>Vitamin B6 intake (µg/d)</th>
<th>Folate intake (µg/d)</th>
<th>B12 intake (µg/d)</th>
<th>Vitamin B6 (piridoxal phosphate) (nmol/L)</th>
<th>Plasma folate (nmol/L)</th>
<th>RBC-folate (nmol/L)</th>
<th>Plasma vitamin B12 (pmol/L)</th>
<th>HoloTC (pmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>♂</td>
<td>29.5</td>
<td>27.9</td>
<td>30.7</td>
<td>17.2</td>
<td>12.8</td>
<td>9.0</td>
<td>16.4</td>
<td>10.7</td>
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<td>25.7</td>
<td>34.2</td>
<td>30.7</td>
<td>23.7</td>
<td>17.2</td>
<td>9.0</td>
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</tbody>
</table>

Percent variation accounted for by RRR factor loadings of food groups:

- Bread & rolls: -0.29
- Chocolate: 0.28
- Pasta, rice, flour: -0.21
- Nuts, seeds, olives, avocado: -0.23
- Alcoholic drinks: 0.29
- Sugars: 0.28
- Vegetable oils: 0.29
- Yogurt, milk: 0.34
- Margarine & lipids of mixed origins: 0.35
- Dairy Dessert & cream: 0.53
- Butter and animal fats: 0.29
- Salty sauces: -0.21
<table>
<thead>
<tr>
<th>Category</th>
<th>RRR</th>
<th>HoloTC</th>
<th>Reduced-Rank regression analyses</th>
<th>Holotranscobalami</th>
</tr>
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<tbody>
<tr>
<td>Pulses</td>
<td>0.27</td>
<td>-0.22</td>
<td>0.22</td>
<td>-0.21</td>
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<td>Vegetables excluding potatoes</td>
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<td>0.53</td>
<td>0.38</td>
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<td>Starch roots, potatoes</td>
<td>0.26</td>
<td>0.26</td>
<td>-0.38</td>
<td>-0.43</td>
</tr>
<tr>
<td>Breakfast cereals</td>
<td>0.29</td>
<td>0.38</td>
<td>0.44</td>
<td>-0.26</td>
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<tr>
<td>Fruits</td>
<td>0.29</td>
<td>0.38</td>
<td>0.44</td>
<td>-0.26</td>
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<tr>
<td>Soups, bouillon</td>
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<td>0.38</td>
<td>0.44</td>
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<td>Water</td>
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<td>Coffee, tea</td>
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<td>0.38</td>
<td>0.44</td>
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<td>0.44</td>
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</tbody>
</table>
3,528 adolescents (47 % males) in the whole HELENA study sample

2,173 adolescents (46 % males) with 2X24 HDR\(^+\) + available data in covariates

1,089 adolescents (47 % males) with data on blood parameters

586 adolescents (45 % males) with 2X24 HDR\(^+\) and available data in covariates

\(^+\)HDR: Hours Dietary Recalls