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1   **Title:** Do dietary patterns determine levels of vitamin B_6, folate, and vitamin B_{12} intakes

2   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.

2

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 ítems to the patterns are quite different for males and females.

Abstract

Objective: To determine dietary patterns (DPs) explaining the highest variance of vitamin B_6, folate, and B_{12} 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.

3

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

4

134 children and adolescence (4). Deficiency of these three vitamins at early stages of life

135 has been related to developmental delay, feeding problems, failure to thrive, irreversible

136 neurological damage (12), severe or recurrent headache or migraine (13), or potential

137 implications for an altered risk for chronic disease in later life (14).

138

139 Nutrition research has favoured a reductionist approach emphasizing the role of single

140 nutrients in diet-health relationships. As a matter of fact, human diet is a complex

141 behavior. Foods are not consumed isolated and for this reason, in observational studies,

142 it is very difficult to derive any outcome or consequence from one single food.

143 Therefore, statistical approaches describing diet in a more holistic way have been

144 applied to explore dietary patterns in relation to health outcomes. Among the most

145 commonly applied techniques (namely Principal Component Analysis (PCA), Reduced

146 Rank Regression analysis (RRR) and Cluster Analysis (CA)), RRR seems to be more

147 flexible and powerful than other methods such as PCA (15) it uses prior knowledge

148 (from biologic evidence, dietary intervention studies, epidemiologic studies with

149 biomarkers, and large prospective cohort studies) in combination with the strength of

150 PCA in considering the correlation of dietary components and the advantage of diet-

151 quality scores to account for current scientific evidence (15).

152

153 A previous analysis based on the HELENA study (16), has investigated the relationship

154 between the consumption of individual food items in this sample of European

155 adolescents in relation to the cited B-vitamins intakes and plasma concentration levels

156 (biomarkers). However, to our knowledge, apart from a German study (17)

157 investigating the associations between PCA-derived dietary patterns (DPs) and the

158 intake of some nutrients including vitamin B_6, folate, and vitamin B_{12}, there is no other

159 study addressing also B-vitamins concentrations in parallel in relation to dietary

160 patterns. Besides, up to date, there is no publication addressing the relationship between

161 the dietary patterns obtained throughout RRR analyses method and both the intakes and

162 status of vitamin B6, folate and vitamin B_{12} in European adolescents, which is precisely

163 the aim of this study.

164

165 **Material and methods**

166 *Sampling*

5

167   3,528 adolescents (47% boys) from 10 European cities in 9 countries were recruited to

168   participate in The Healthy Lifestyle in Europe by Nutrition in Adolescence Cross-

169   Sectional Study (HELENA-CSS). The cities participating in this multi-centre study

170   were Athens and Heraklion (Greece), Dortmund (Germany), Ghent (Belgium), Lille

171   (France), Pecs (Hungary), Rome (Italy), Stockholm (Sweden), Vienna (Austria), and

172   Zaragoza (Spain). The average participation rate in our study was 67%, which is

173   acceptable for this demanding epidemiological study (18). As inclusion criteria, they

174   must be 12.5-17.5 years old, not participating simultaneously in a clinical trial and not

175   having any acute infection occurring < 1 week before inclusion (19). To provide

176   rigorous quality assurance regarding ethical issues, a protocol was prepared by a

177   HELENA study centre based on its previous experience in these topics (20). It

178   conformed the good clinical practices (GCP) described at the International Conference

179   on Harmonisation (ICH), and based on concepts about ethics in biomedical research that

180   originated from the Nuremberg Code and the Declaration of Helsinki (21, 22). Informed

181   consent was obtained from all participants and their parents, and the protocol was

182   approved by the Human Research Review Committees of the corresponding centres

183   (cities) (20).

184   From the initial number of 3,528 adolescents, 2,173 (46% boys) were included based on

185   the availability of the variables of interest: complete data on two non-consecutive 24-

186   hour dietary recalls (24H-DR), maternal education as marker of socioeconomic status,

187   BMI z-score (Body Mass Index standardized values and adjusted by age and sex of the

188   adolescents), and total energy intake. Similarly, only a third (1,089, 47% boys) (19) of

189   the initial sample of 3,528 adolescents participated in blood drawings. From those, only

190   586 adolescents (45% boys) were included based on compliance with the previously

191   cited variables of interest. The first 24H-DR was completed on the same day of the

192   blood drawing while the second one was filled a fortnight after considering weekends

193   and weekdays. If the first 24H-DR was completed on Monday (weekend recall), the

194   second, was completed from Tuesday to Friday (weekday recall). Trained staff was

195   present during the assessments (23).

196   Sampling selection procedures are shown in figure 1. Further study details were

197   published elsewhere (19).

198

6

199 *Assessment of vitamin B$_6$, folate and vitamin B$_{12}$ intakes*

200 Dietary food intake was estimated by means of two self-administered computerized 24

201 HDR using the HELENA-Dietary Assessment Tool (HELENA-DIAT), adapted for

202 European adolescents (23) from a previous software (called Young Adolescents'

203 Nutrition Assessment on Computer (YANA-C)). Pitfalls were found to obtain

204 comparable measures of energy density of each food across countries by using country

205 specific food composition tables. Therefore, the obtained data was linked to the

206 German Food Code and Nutrient Data Base (BLS -Bundeslebensmittelschlüssel-,

207 version II.3.1, 2005), including 12,000 coded foods, and with up to 158 nutrient data

208 points available for each product. Afterwards, every country developed in local

209 language its own database file adapted to the respective food culture (special dishes,

210 food items, beverages, food amounts and picture links). The recorded data was linked to

211 the country-specific food codes to assign food composition data (recipes, ingredients,

212 and nutrients) from the respective national food composition databases to calculate

213 energy and nutrient intake (23, 24). Finally, the Multiple Source Method (MSM) (25)

214 was used to calculate usual nutrient intake removing the effect of day-to-day within-

215 person variability (using the adapted version of the FFQ from HBSC as a propensity

216 tool to do this) and random error in the recalls, adjusted by age, sex, and centre or

217 country (26).

218

219 *Beverage and food groups*

220 All reported 4,179 foods and beverages were categorized initially in 29 food groups

221 based on the European Food Groups classification system (23, 27). As part of the

222 general HELENA analysis, these foods were disaggregated into 43 food groups. For the

223 aim of the current analysis and based on their nutritional composition some of these

224 food groups were again aggregated. For instance, beer, wine, and spirits were

225 aggregated into alcoholic drinks; pasta, rice, and flour into complex carbohydrates;

226 honey and other sugar products into sugar products, oily fruits included nuts, seeds,

227 avocado and olives; milk products grouped yogurt, white cheese, milk and yogurt

228 beverages; and finally, other milk products merged desserts, milk-based puddings and

229 creams. Besides, 'products for special nutrition use', 'soya beverages', and

230 'miscellaneous' were eliminated from the current analysis based on their very low

231 consumption (0 median and mode and more than the 85% of the sample did not report

7

232   consumption). The final number of food groups for the current analysis was 31 food

233   groups (table 2). Nevertheless, the resulted amount of B-vitamins intake corresponds to

234   the sum of the B-vitamin belongs to all the original food groups (even the one contained

235   in the excluded food groups in the current analyses).

236

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

238   Blood was obtained after an overnight fast according to a standardized blood collection

239   protocol. Further details on sample transport and quality assurance can be found

240   elsewhere (28). Briefly, to assure an adequate sample handling, storage and subsequent

241   analysis, logistics of sample transport and major parts of the analytics were centralized

242   at the Analytical Laboratory from the University of Bonn (IEL, Bonn, Germany) and

243   field-workers were previously trained. The time span between sampling and processing

244   was up to 24 h. However, a novel handling and transport system following the Good

245   Clinical Practices (29), and a novel traceability system developed at the Clinical

246   Investigation Centre in Lille based on printed documents and an electronic database,

247   together with the realization of a pilot study, confirmed the absence of any stability

248   problem (28).

249   Based on previous experiences of the research group, and after performing the pilot

250   study, it was agreed upon 30 ml of blood in order to be able to analyse all proposed

251   parameter (2 x 7.5 ml tubes for serum, 2.6 ml tube for heparin plasma, 4ml tube and 2.7

252   ml tube for EDTA for hematology) (30).

253   Concretely, pyridoxal 5′phosphate (PLP), was measured as a marker of vitamin B6

254   status, by high performance liquid chromatography (HPLC) (Varian Deutschland

255   GmbH, Darmstadt, Germany; CV = 1%) with a modified method of Kimura et al (4,

256   31). Plasma and RBC-folate and plasma vitamin B_{12}, were measured in the central

257   laboratory at the University of Bonn (IEL-Institut für Ernährungs und

258   Lebensmittelschaften-, Germany) by means of a competitive immunoassay using the

259   Immunolite 2000 analyzer (DPC Biermann GmbH, Bad Nauheim, Germany) (28).

260   Holotranscobalamin (HoloTC) was analysed in the biochemical lab at the Universidad

261   Politécnica de Madrid by microparticle enzyme immunoassay (Active B_{12} Axis-Shield

262   Ltd, Dundee, Scotland, UK) with the use of AxSym (Abbot Diagnostics, Abbott Park,

263   IL, USA) (32).

8

264

265 *Covariates*

266 As a marker of *socioeconomic status* of the families, *maternal education* was selected

267 from all the available socioeconomic related variables due to its documented association

268 with vitamin intakes and biomarkers (33). Maternal education was categorized into low,

269 medium-low, medium-high, and high, based on the information provided by the

270 adolescents through a self-administered questionnaire.

271 Weight was measured in underwear conditions with an electronic scale (Type SECA

272 861) to the nearest 0.05 kg, and height was measured barefoot in the Frankfort plane

273 with a telescopic height measuring instrument (Type SECA 225) to the nearest 0.1 cm.

274 After calculation of body mass index (BMI), z-scores of BMI were calculated via LMS

275 growth (34), and used as a covariate in the analysis, together with total *energy intake*.

276

277 *Statistical analysis*

278 The assumption of *normality* was *assessed* with *visual inspection* (histograms, boxplots

279 and *Q-Q plots*) and then with *Kolmogorov-Smirnov tes*t. All statistical tests were two-

280 sided, and p<0.05 was the threshold considered statistically significant. Statistical

281 analysis was sex stratified because most of the variables involved in this study presented

282 statistically significant differences and provided the fact that dietary patterns have been

283 shown to be different in this sample of adolescent boys and girls in Europe (35).

284 Descriptive data are presented as means and standard deviations (SD) for continuous

285 variables, and frequencies and percentages for categorical variables.

286 In the correlation analysis, non-parametric Spearman coefficient was used since dietary

287 intake data and blood values were not normally distributed. Food and beverages intakes

288 were expressed as grams or milliliters per day (g or ml/day) and B-vitamins intakes and

289 biomarkers were expressed in their corresponding units as specified in the tables.

290 Reduced rank regression (RRR) was applied using the partial least square (PLS)

291 procedure in SAS (15). RRR assumes a linear function of responses (i.e. B-vitamins

292 intakes and statuses) with the predictors (i.e. food groups) and creates a response score

293 that will then be projected onto the space of predictors to produce a factor score, that is,

294 a linear function of predictors. Assessment of factors extracted by RRR are based on

9

295 response scores rather than on factor scores. Factor loadings indicate the association
296 between the food groups and the derived factor and thus indicate which food groups
297 load highly onto the factor which explains variation in the B-vitamins intakes and
298 statuses. Factor loadings are equivalent to correlation coefficients (in contrast to
299 regression coefficients which are equivalent to quantifiable weights). Consequently,
300 positive factor loadings indicate that the food group is positively associated with the
301 factor, and negative ones show that the food group is inversely related to the factor.
302 Higher factor loadings indicate a greater contribution of that food group to the factor.
303 Data on food intake was firstly adjusted for several covariates (BMI z-scores, maternal
304 education and total energy intake) using mixed model analysis with a random intercept
305 for centre and then entered as residuals into the RRR analyses. Using RRR, the number
306 of extracted factors is equal to the number of selected responses, thus, one factor was
307 obtained for each B-vitamin (either intake or biomarker). Only food groups with factor
308 loadings|>0.20| were considered as relevant for the factors or patterns as has been
309 previously used (36). Statistical analyses were performed with SAS 9.3 for Windows
310 (SAS Institute Inc., Cary, NC) and SPSS 20.0 for Windows (SPSS Inc., Chicago, IL,
311 USA). Radar plots were performed with Excel (Microsoft).

312

Results

314 Sensitivity analyses were performed comparing participants included vs. not included in
315 the study (data not shown). The proportion of boys in relation to girls included in these
316 analyses was 46% for the analyses with B-vitamins intake and 45% for the B-vitamins
317 biomarkers while for the general HELENA study sample was 47% of boys. For the
318 dietary analyses, included boys did not differ from those excluded in terms of age, but
319 had significantly lower BMI (p=0.002) and lower energy intake (p<0.05), while
320 included girls were significantly younger and presented higher BMI than those excluded
321 (p<0.05). Regarding the biomarkers analyses, included boys had significantly lower
322 BMI (p<0.05). Included boys and girls, significantly differed from those excluded in
323 terms of maternal education (p<0.05), representing mothers placed in higher categories
324 of education higher percentage in comparison to the ones placed in other categories than
325 in those excluded.

10

326   Table 1 presents the adolescents' characteristics for both the intake and the biomarkers

327   groups stratified by sex. In the intake group, BMI z-score, energy intake and B-vitamins

328   intake were significantly higher ($p<0.05$) in boys than in girls. However, in the

329   biomarkers group, there were significant differences for total energy intake, PLP, and

330   plasma vitamin B_{12} between sexes.

331   Table 2 shows the Spearman rank correlation coefficients between the food and

332   beverage intakes and the B-vitamins intake and biomarkers, respectively. Correlations

333   ranged from weak ($r_s= 0.06$ for pasta, rice & flour for vitamin B_{12} intake in boys, and -

334   0.06 for coffee & tea and vitamin B_{12} intake in girls) to moderate ($r_s= -0.70$ for

335   margarine & lipids of mixed origins and vitamin B12 intake in boys). Food and

336   beverage intakes were mainly correlated with B-vitamins intake. For vitamin B_6 intake,

337   both in boys and in girls, higher, significant, and positive correlations were found for

338   vegetables excluding potatoes, starch roots and potatoes, fruits, and meat; for folate

339   intakes, with bread and rolls, vegetables excluding potatoes, fruits, and cheese; and, for

340   vitamin B_{12} intakes, with meat, fish products, and white and buttermilk.

341   In case of B-vitamins biomarkers, the foods and beverages which correlated higher,

342   significantly and positively with them were: margarine with PLP both in boys and girls

343   and breakfast cereals also in girls; breakfast cereals both in boys and girls with plasma

344   folate, and in girls also chocolate, vegetable oils, margarine and mixed origin lipids,

345   fruits, coffee and tea, and vegetable and fruit juices; for RBC-folate, potatoes were

346   correlated to in boys, and yogurt & milk, breakfast cereals, and water in girls; plasma

347   vitamin B_{12} correlated in boys with butter & animal fats, breakfast cereals, white &

348   buttermilk, and savoury snacks, and in girls, with chocolate, yogurt and milk, and with

349   white & buttermilk; and finally, with HoloTC, pulses and white & buttermilk correlated

350   in boys, and pasta, rice and flavour, yogurt and milk, and white & buttermilk correlated

351   in girls.

352   The proportions of variation in B-vitamins intake and biomarkers explained by the

353   dietary patterns are shown in Table 3. Regarding B-vitamins intake, dietary patterns

354   could explain between 34.2 % of the variance for folate in girls to 23.7 % of the

355   variance for vitamin B_{12} also in girls. For B-vitamins biomarkers, the variance

356   explained by the different dietary patterns ranged from17.2% for PLP in boys and 7.0 %

357   for plasma vitamin B_{12} in girls. Regarding food and beverage intakes, fish, eggs, cheese,

11

358   and white and buttermilk, were the only food items showing consistent and positive

359   loadings in the identified patterns, and soft drinks and chocolate, showed consistent and

360   negative loadings in the identified patterns, both in boys and girls.

361   For biomarkers, results were less consistent in terms of foods loading direction, even

362   loading oppositely for boys and girls in some cases. For instance, alcoholic drinks

363   showed positive loadings for boys and negative for girls; sugars, and soft drinks both

364   showed positive loadings for girls and negative for boys. Besides, not only differences

365   between sexes have been found but also between intakes and biomarkers. For instance,

366   fish products loaded positively in the identified patterns for intakes, both in boys and

367   girls, but negatively for biomarkers, also in both sexes.

368

369   The factor loadings of each food group to the corresponding derived pattern for each B-

370   vitamin (table 3) are representing in supplementary material as radar plots when it is

371   possible to easily appreciate the different shapes of the patterns for boys and girls.

372   Besides, the obtained patterns, and due to the food groups, which characterized them,

373   might be named as follows: *traditional pattern* for both boys and girls in relation to

374   vitamin B_6 intake, *Mediterranean and protein-based pattern* for boys and girls

375   respectively in relation to folate intake, *breakfast pattern* for both boys and girls in

376   relation to vitamin B_{12} intake, *breakfast pattern and traditional pattern* for boys and

377   girls respectively in relation to PLP, *fast-food pattern and snack pattern* for boys and

378   girls respectively in relation to plasma folate, *healthy-conscious pattern and desserts*

379   *pattern* for boys and girls respectively in relation to RBC-folate, *Italian-cuisine pattern*

380   and *fast-food pattern* for boys and girls respectively in relation to plasma vitamin B_{12},

381   and finally, a *breakfast dietary pattern* for both boys and girls in relation to

382   Holotranscobalamin.

383

384

385   **Discussion**

386

387   To our knowledge, there are no previous studies investigating the dietary patterns

388   determining both the intake and the status of vitamin B_6, folate and vitamin B_{12} in

389   adolescents. Up to now, this is also the first time that RRR is used to elucidate the

390   dietary patterns best explaining the variability in vitamin B_6, folate, and vitamin B_{12}

391   intake and status in a large sample of European adolescents.

12

392

393 The dietary patterns derived in this study were considerably different for boys and girls

394 and the stratification of their computation was done due to previous results obtained in

395 this respect in the literature. For instance, in Germany, Richter et al (37) it was found

396 that there were gender differences in relation to the found dietary patterns explaining the

397 levels of biomarkers of folate and vitamin B12. Besides, another author (35) had found

398 statistically significant differences in the consumption of almost all the food groups in

399 the HELENA study.

400

401 The RRR-derived dietary patterns could account for higher variability (up to 34.2 %) of

402 the B-vitamins intake than for the B-vitamins concentrations (up to 17.2 %). In a recent

403 manuscript (16) we also observed fewer and weaker associations for B-vitamins

404 biomarkers than for their intakes, in relation with food groups, based also in HELENA

405 study.

406 The different proportions of the variance explained by dietary patterns, when

407 considering either B-vitamins intake or their biomarkers might be due to the fact that

408 biomarkers concentrations could be related with long-term food consumption patterns,

409 whereas the available information related to B-vitamins intake was recorded using two

410 24H-DR. Pointing in this direction, in a previous analysis by Vandevijvere et al.,

411 considering different biomarkers of micronutrients intake (vitamin C, β-carotene,

412 docosohexaenoic acid, eicosapentaenoic acid, vitamin B_{12} and folate), correlations were

413 higher when considering the food and beverage consumption frequencies (from the food

414 frequency questionnaire -FFQ-) as compared to mean food and beverages intakes (from

415 the 24H-DR) and the same concentration biomarkers (38). But sometimes, the

416 differences can be due to the time lap between intakes and the blood drawings, mainly

417 for food groups which are not consumed often such us fish products, for instance.

418

419 In another study from Germany by Ritcher et al. (17), in a similar population group,

420 dietary patterns were obtained using Principal Components Analyses (PCA) and, in

421 boys, vitamin B_6 diet density increased with increasing scores of the 'healthy' pattern.

422 Folate diet density was also related to this "healthy" pattern in both sexes (17). This

423 "healthy pattern" consisted of high consumption frequencies of fruits, vegetables,

424 legumes, mushrooms, chicken, rice, vegetable oil, soup, and grains in boys; and of rice,

425 vegetable oil, soup, chicken, legumes, vegetables, fruits, vegetarian dishes, eggs, fish,

13

426 water, warm sauces and mushrooms in girls. In our study, the intake of this B-vitamin
427 was mainly explained for a *traditional dietary pattern* both in boys and girls, containing
428 some of the food groups which determine the *healthy pattern* in the German study. In
429 our study, boys' patterns presented some similarities, at least regarding vitamin B_6 and
430 folate, as the observed patterns related with these vitamins consisted of vegetables
431 excluding potatoes, starch roots and potatoes, fruits, meat, fish products, and white and
432 buttermilk in detriment of chocolate, soft drinks for vitamin B_6; and of vegetable oils,
433 pulses, vegetables excluding potatoes, fruits, and fruit and vegetable juices in detriment
434 of meat for folate. These patterns also featured foods considered to be healthy.

435

436 Besides, the German study reported a *traditional pattern* that related to vitamin B_{12}
437 intake density in boys (17), consisted of processed meat, potatoes, white bread,
438 margarine, meat (except chicken), eggs, cheese, and fish, while in our study, vitamin
439 B_{12} intake was related to a *breakfast pattern* consisted mainly of white and buttermilk,
440 cheese and eggs. In girls, vitamin B_{12} intake density was associated with the so-called
441 *traditional and western' pattern* in the cited German study by Ritcher et al. (17), which
442 involved potatoes, warm sauces, meat (except chicken), white bread, processed meat, as
443 well as pizza, French fries, sausages, soft drinks, confectionary, cake/cookies and
444 negatively correlated with water. In our study, vitamin B_{12} intake in girls was
445 determined also by *breakfast pattern* like in boys, characterized by the consumption of
446 bread and rolls, yogurt and milk, eggs, white and buttermilk, and cheese. It is worth to
447 highlight the fact that in our study, meat, which is consider one of the main sources,
448 scores negatively in the patterns that precisely explained the highest variance in the
449 vitamin B_{12} intake both in boys and girls. These differences might be obtained due to
450 the difference in the statistical approach to derive the dietary patterns (in PCA used in
451 this German study, the linear combination of the food groups is explained, but not the
452 variation in a response variable -B-vitamins-).

453

454

455 All in all, while for boys, B-vitamins intakes were determined by similar patterns in
456 both studies, we found larger differences in girls. However, providing that the statistical
457 techniques to extract the dietary patterns in these two studies have different purposes,
458 we must be cautious in comparing the results: PCA searches for the highest variability

14

459   among the food intake, while RRR looks at the highest variability in explaining the

460   differences for each outcome variable (39).

461

462   Up to now, there is no similar study performed to which compare our results for B-

463   vitamins biomarkers. In Indian children in a study by Kehoe et al. (40), a lacto-

464   vegetarian dietary pattern was related to folate status and negatively related to vitamin

465   B_{12} status. In USA Health-Professionals, folate status was negatively correlated with a

466   'Western' dietary pattern (41). However, food accessibility and food preferences in the

467   first case, and the population group in the second one, made these results non-

468   comparable to ours.

469

470   Surprisingly, we have identified different patterns determining B-vitamins biomarkers

471   for boys and for girls, apart the 'breakfast pattern' which explained a variance of 17.2 %

472   and 10.7 % for boys and girls, respectively for PLP.

473   For instance, a dietary pattern that could be considered as *fast-food and snack patterns*

474   might explain between 12.8 % and 9.0 % of the variance in plasma folate concentrations

475   in boys and girls, respectively. In boys, a *healthy-conscious dietary pattern* determined

476   RBC-folate, while in girls it was determined by a *dessert dietary pattern* including

477   several snacking food items. Similarly, a dietary pattern characterized by food items

478   typical of the *'Italian cuisine'* and another one characterized by food served in *fast-food*

479   *restaurants* explained the variance of the plasma vitamin B_{12} in boys and girls,

480   respectively. A dietary pattern characterized by food items served at *breakfast* explained

481   the variance for HoloTC in both sexes.

482

483   Around 16 % of the girls, of the 584 adolescents which had blood drawings, (42) used

484   contraceptives, and B-vitamins status might be affected from them as was suggested for

485   the literature, at least for vitamin B_6 (PLP). However, it is precisely for this vitamin for

486   which we have obtained more similar patterns between boys and girls.

487

488   Different dietary patterns were obtained for B-vitamins intakes and for their

489   corresponding biomarkers as already expected owing to previous results obtained for

490   the same sample when analysing only food items individually with these B-vitamins

491   (16). Reasons for these differences might be attributed to differences in metabolism,

492   interference with other nutrients, and physiologic status, nutrients variation in same

15

493 food items depending on where or how the food was grown or how it was processed,
494 and lack of data regarding supplements use or fortified food items, as previously
495 suggested (16). In addition, two types of biomarkers were used in our study: those
496 which reflect the recent intakes (such us plasma folate and plasma vitamin B_{12}), but also
497 PLP, RBC-folate, and HoloTC which are more focused on detecting the corresponding
498 vitamins storages (12). In general, dietary patterns are considered to reflect long-term
499 food intake rather than short-term food intake, and consequently might better explain
500 biomarkers' storage rather than punctual intake markers, as this was the case for folate
501 in our study, but not for vitamin B_{12} biomarkers.

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

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

512

513 *Strengths and limitations*

514 In this instance, what empowered considerably this study is the use of RRR, instead of
515 the traditional *a posteriori* methods like PCA or cluster analyses, to determine the
516 dietary patterns which best explain the variability in the B-vitamins intakes and statuses
517 of European adolescents for the first time in the literature. The dietary patterns derived
518 from *a posteriori* methods explain the variation in food groups intake, which may be
519 appropriate to characterize existing basic dietary patterns in a population but may not
520 optimally represent patterns relevant for the aetiology of specific diseases (43, 44);
521 however, *a priori* methods do not consider the correlated aspects of some food groups
522 (45). New hybrid methods use a combination of *a priori* knowledge on nutrient intake
523 or risk factors for disease and the underlying dietary data to derive dietary patterns (46),
524 and RRR is among them (15).

16

525 Apart from that, the use of a large and culturally diverse sample of European
526 adolescents from 9 European countries is also another important strength (19).
527 Moreover, the questionnaires used to assess maternal education, were previously
528 validated (47). Another important strength of the study is the correction procedures used
529 to avoid the limitations of the 24H-DR. These were, for instance, the use of the MSM
530 method to correct the crude intake data values for within-person variation (48), and the
531 use of the correspondent vitamins biomarkers. However, correlations between
532 biomarkers and usual food intakes obtained from the recalls were low in this sample
533 (38). This could be due to the fact that dietary intakes correlate better with biomarkers
534 when the number of days covered by the reference method increases (49). On the other
535 hand, due to standardization reasons, the use of the German food composition table
536 provides differences in comparison with national food composition tables, small, and
537 for most nutrients negligible, which implies to be a reliable approach (26). Blood
538 biomarkers were analysed in the same centre, strengthening the reliability of the lab-
539 results (28).

540 As a limitation, food fortification was not included in the German food composition
541 database, and the analyses were not controlled for dietary B-vitamins supplement use.
542 However, only 5% of our sample was shown to use them, so no important differences in
543 the interpretation of our results would be expected.
544 The nature of this cross-sectional design of HELENA study, does not allow establishing
545 causality in the associations found.
546
547

548 **Conclusion**

549 Dietary patterns obtained by reduced rank regression analyses explained between 23.7
550 % and 34.2 % of the variability of B-vitamins intake, and between 7.0 % and 17.2 % of
551 the variability of B-vitamins concentrations in European adolescents. Apart from the
552 patterns obtained to determine plasma folate and plasma vitamin B_{12}, which were
553 mainly unfavorable, the dietary patterns determining the levels (both intake and status)
554 of vitamins B_6, folate and vitamin B_{12}, included mainly healthy food groups. In
555 consequence, there is an urgent need for classifying vulnerable population groups in
556 terms of dietary patterns, such as adolescents, to identify those at increased risk of low

17

557 B-vitamin levels and to determine what are the main determinants of these dietary
558 patterns.

559

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829   Figure 1. Sampling selection process

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832   Table 1. Characteristics of adolescents with complete information on B-vitamins intake and biomarkers stratified by sex.

Variables in this study		B-vitamins intake				
		Males (N=999)		Females (N=1184)		
		Mean	Standard Deviation	Mean	Standard Deviation	p-value
Age (years old)		14.8	1.3	14.7	1.2	0.27
BMI z-scores		0.6	1.2	0.3	1.1	<0.001†
Energy intake (kcal/d)		2,482.4	808.7	1,892.6	580.1	<0.001*
Maternal education (levels, n (%))	Low	63	(6.3)	80	(6.8)	0.60$
	Medium-low	274	(27.4)	306	(25.8)	
	Medium-high	294	(29.4)	375	(31.7)	
	High	368	(36.8)	423	(35.7)	
Vitamin B_6 intake (µg/d)		1,782.3	613.8	1,430.3	474.1	<0.001*
Folate intake (µg/d)		211.3	74.8	177.4	59.2	<0.001*
B_{12} intake (µg/d)		6.0	2.3	4.6	1.8	<0.001*
		B-vitamins biomarkers				
		Males (N=265)		Females (N=321)		
Age (years old)		14.4	1.2	14.4	1.2	0.93
BMI z-scores		0.5	1.2	0.3	1.0	0.10†
Energy intake (kcal/d)		2,549.6	876.1	1,897.3	557.8	<0.001†
Maternal education (levels, n (%))	Low	19	(7.2)	29	(9.0)	0.51$
	Medium-low	60	(22.6)	76	(23.7)	
	Medium-high	81	(30.6)	107	(33.3)	
	High	105	(39.6)	109	(34.0)	
Vitamin B_6 (piridoxalphosphate)(nmol/L) (nmol/L)		69.1	45.9	62.4	64.2	0.001*
Plasma folate (nmol/L)		18.3	9.9	18.4	9.9	0.70*
RBC-folate (nmol/L)		806.7	350.0	762.1	302.5	0.22*
Plasma vitamin B_{12} (pmol/L)		335.5	132.2	380.3	165.0	0.001*
HoloTC(pmol/L)		60.8	36.8	62.6	36.7	0.94*

833   †Based on T-test statistics

834   *Based on Mann-Whitney test statistic

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835 $^{\$}$Based on chi-square test statistic

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839   Table 2. Spearman correlations coefficients between food intake (g/d) and B-vitamins intake and biomarkers.

Food groups	Vitamin B$_6$ intake (µg/d) ♂	♀	Folate intake (µg/d) ♂	♀	B$_{12}$ intake (µg/d) ♂	♀	Vitamin B$_6$ (piridoxalphosphate) (nmol/L) ♂	♀	Plasma folate (nmol/L) ♂	♀	RBC-folate (nmol/L) ♂	♀	Plasma vitamin B$_{12}$ (pmol/L) ♂	♀	HoloTC (pmol/L) ♂	♀
Bread & rolls	0.29†	0.20†	0.42†	0.35†	0.17†	0.15†	-0.04	-0.03	-0.16†	-0.84	-0.15*	-0.05	-0.07	-0.10	0.02	-0.06
Chocolate	0.08†	0.11†	0.15†	0.11†	0.03	0.1†	-0.03	-0.05	0.01	0.15†	0.01	0.06	-0.02	0.12*	-0.07	0.00
Pasta, rice, flour	0.06	0.02	0.20†	0.12†	0.06*	0.07*	-0.17†	-0.01	-0.26†	-0.11†	-0.17†	-0.04	0.06	0.07	0.02	0.13*
Nuts, seeds, olives, avocado	0.03	0.1†	0.21†	0.21†	0.01	0.03	0.03	-0.06	-0.03	0.03	0.02	0.10	-0.03	0.05	0.00	-0.00
Alcoholic drinks	0.08†	-0.01	0.06	0.02	0.02	-0.02	0.06	0.07	-0.10	0.04	-0.00	0.05	-0.05	-0.02	-0.23†	0.01
Sugars	0.13†	0.05	0.07*	0.07*	0.07*	0.02	0.07	0.01	-0.10	-0.07	0.01	-0.12*	0.08	-0.04		
Vegetable oils	0.14†	0.09†	0.24†	0.16†	0.08*	0.05	-0.04	-0.15†	0.04	0.19†	0.00	0.10	-0.04	0.02	0.03	-0.09
Yogurt, milk	0.14†	0.14†	0.14†	0.14†	0.18†	0.18†	-0.02	0.1	0.08	0.05	0.12	0.26*	0.07	0.17†	0.03	0.23†
Margarine & lipids of mixed origins	-0.05	-0.05	0.05	0.05	-0.70*	-0.07*	0.14*	0.11*	0.05	0.22†	-0.00	-0.09	-0.05	-0.06	-0.01	0.05
Dairy Dessert & cream	0.10†	0.06*	0.02	0.05	0.08†	0.08†	-0.06	-0.02	-0.02	-0.03	0.06	-0.05	0.09	0.11	0.01	0.07
Butter and animal fats	0.01	0.08†	0.09	0.09	0.07*	0.05	0.04	0.07	0.01	-0.08	0.03	0.08	0.14*	0.10	0.13*	0.05
Salty sauces	0.09†	0.16†	0.09†	0.10†	0.15†	0.15†	0.02	0.09	0.02	0.02	-0.04	-0.12*	-0.02	-0.07	-0.01	0.01
Pulses	0.08*	0.05	0.08*	0.03	0.08†	0.08†	-0.03	-0.02	0.11	-0.11	0.07	0.08	0.12	0.10	0.21†	0.05
Vegetables excluding potatoes	0.30†	0.32†	0.47†	0.49†	0.21†	0.16†	-0.05	-0.02	0.05	0.10	-0.06	-0.05	0.07	0.01	0.03	0.06
Starch roots,	0.30†	0.36†	0.14†	0.16†	0.13†	0.19†	-0.01	-0.17†	-0.04	-0.03	0.14*	0.11	0.05	0.07	0.02	0.08

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potatoes															
Breakfast cereals	0.14†	0.10†	0.09†	0.10†	0.15†	0.12†	0.04	0.21†	0.18†	0.10	0.11	0.19†	0.14*	0.06	0.12	-0.03
Fruits	0.32†	0.28†	0.33†	0.33†	0.06	0.05	0.08	0.09	0.13*	0.16†	-0.04	-0.21†	0.06	0.07	0.08	0.02
Soups, bouillon	0.13†	0.06	0.03	-0.02	0.16†	0.09†	-0.07	-0.00	-0.07	-0.11	0.09	0.04	0.08	0.06	0.08	-0.01
Water	0.08*	0.01	0.08*	0.08†	0.05	0.01	0.02	-0.07	-0.05	-0.07	0.04	0.14*	0.01	-0.08	-0.01	-0.04
Coffee, tea	0.04	-0.01	0.05	0.07*	-0.04	-0.06*	0.10	0.04	-0.14†	-0.16†	-0.04	-0.01	-0.06	-0.14*	-0.22†	-0.12*
Fruit & veg juices	0.19	0.19†	0.27†	0.23†	-0.04	-0.01	-0.01	-0.02	0.17†	0.10	0.04	0.02	-0.05	0.10	0.06	-0.01
Soft drinks	0.02	0.03	0.01	0.04	-0.04	-0.03	0.09	0.07	-0.12	-0.13*	-0.09	-0.13*	-0.01	-0.14*	-0.24†	-0.16†
Meat	0.57†	0.49†	0.17†	0.12†	0.52†	0.44†	0.06	-0.01	-0.14*	-0.09	-0.13*	-0.01	0.12	0.30	0.06	0.04
Fish products	0.14†	0.15†	0.06*	0.08†	0.26†	0.27†	-0.04	-0.07	0.03	0.03	-0.04	-0.02	0.04	0.11	0.05	0.09
Eggs	0.13†	0.08†	0.19†	0.21†	0.19†	0.15†	-0.06	-0.01	0.03	0.03	-0.01	-0.03	0.03	0.08	0.03	0.00
White milk & buttermilk	0.26†	0.21†	0.19†	0.16†	0.42†	0.34†	-0.03	0.00	-0.03	-0.08	0.11	0.01	0.24†	0.18†	0.34†	0.16†
Cheese	0.11†	0.08†	0.37†	0.29†	0.17†	0.20†	-0.04	-0.03	-0.03	-0.01	-0.03	0.06	-0.02	-0.10	-0.06	0.09
Meat sustitutes	0.02	0.02	0.06	0.09†	0.04	-0.03	0.06	0.03	0.00	-0.24†	-0.03	-0.19†	0.12	0.10	0.03	-0.03
Cakes, pies, biscuits	0.14†	0.15†	0.18†	0.15†	0.14†	0.12†	0.00	-0.09	0.06	0.10	0.09	0.07	0.06	-0.01	-0.09	0.03
Savoury snacks	0.12†	0.08†	0.14†	0.11†	0.07*	0.02	-0.04	-0.02	-0.10	-0.01	0.01	0.02	0.14*	0.08	-0.08	-0.01
Confectionary products	0.07*	0.07*	0.10†	0.06	-0.07	0.03	0.08	0.09	0.04	0.05	0.00	0.08	0.05	0.08	-0.03	-0.04

840 * p<0.05; † p<0.01

841 HoloTC: Holotranscobalamin

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844 Table 3. Percentage of variation in B-vitamins intake and biomarkers explained by dietary patterns defined by food groups with factor loadings with value
845 ≥0.2, stratified by sex.

	Vitamin B$_6$ intake (µg/d)		Folate intake (µg/d)		B$_{12}$ intake (µg/d)		Vitamin B$_6$ (piridoxalphosphate)(nmol/L)		Plasma folate (nmol/L)		RBC-folate (nmol/L)		Plasma vitamin B$_{12}$ (pmol/L)		HoloTC(pmol/L)	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Percent variation accounted for by RRR factor	29.5	27.9	25.7	34.2	30.7	23.7	17.2	10.7	12.8	9.0	16.4	9.4	10.7	7.0	8.6	9.9
Factor loadings of food groups																
Bread & rolls						0.28	0.25	-0.21	0.24							
Chocolate	-0.29	-0.25					-0.23		0.22			-0.27				
Pasta, rice, flour													0.31			
Nuts, seeds, olives, avocado									0.34	-0.24		0.30				
Alcoholic drinks													0.21	-0.23		
Sugars											-0.33					0.44
Vegetable oils			0.29										0.24			
Yogurt, milk						0.22							-0.27		0.28	0.43
Margarine & lipids of mixed origins							0.29		0.35				-0.29			
Dairy Dessert & cream								0.53				-0.30				-0.23
Butter and animal fats														-0.21	0.34	
Salty sauces												-0.25				

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Pulses			0.27	-0.22				0.22			-0.21				
Vegetables excluding potatoes	0.26	0.53	0.38	-0.41			-0.47	-0.22			0.22			0.46	
Starch roots, potatoes	0.26	0.26			-0.38	-0.43		-0.29	0.22			0.47		-0.23	
Breakfast cereals							0.23				0.27		-0.25		0.27
Fruits	0.29	0.38	0.44	-0.26		-0.20						-0.34			-0.21
Soups, bouillon							0.21			-0.32	0.26		0.20		
Water							-0.25				0.38				-0.28
Coffee, tea							0.26							0.21	0.23
Fruit & veg juices			0.38	-0.26				-0.25							
Soft drinks	-0.27	-0.20				-0.20					-0.33			0.38	
Meat	0.53	0.22	-0.40	0.56	-0.39	-0.33				0.27	0.22			0.23	
Fish products	0.20	0.27		0.24						-0.34				-0.32	-0.29
Eggs					0.35	0.31				-0.37		0.24			0.21
White milk & buttermilk	0.29			0.20	0.29	0.25		-0.28			0.27				
Cheese					0.39	0.38		0.31		0.28	0.27	-0.26	-0.20		
Meat sustitutes							0.26		-0.58	-0.32	0.34				
Cakes, pies, biscuits		-0.23								0.38	0.26				
Savoury snacks						-0.28				-0.27					
Confectionary products							-0.29					-0.22		-0.33	

846   RRR:Reduced-Rank regression analyses; HoloTC: Holotranscobalami

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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
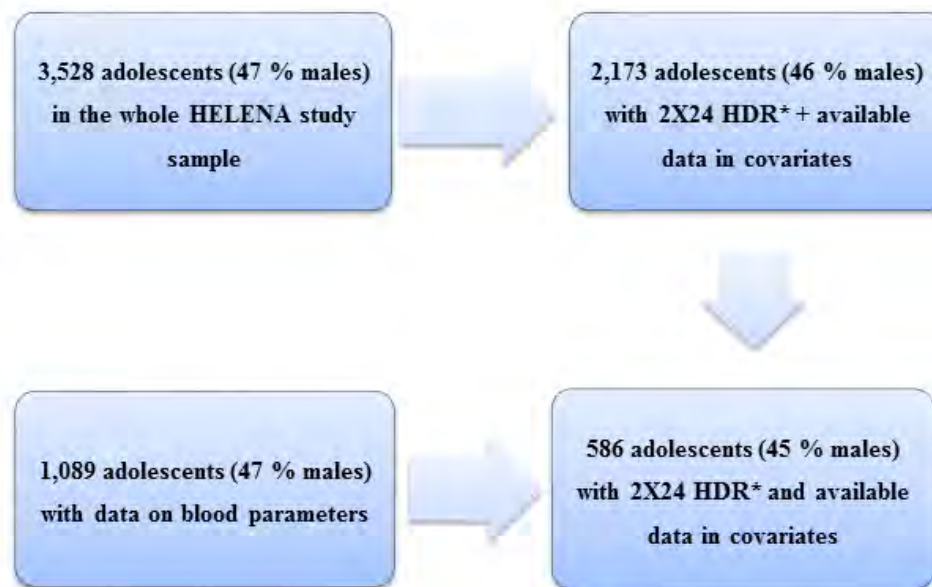
*HDR: Hours Dietary Recalls

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851   Figure_IrisIglesia_Nutrition_bestsetConverted.png

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