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Iris Iglesia Altaba

Ingesta y estatus de vitamina b6, folato y vitamina b12 en adolescentes europeos: determinantes y consecuencias

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Director/es
Moreno Aznar, Luis

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Autor

Iris Iglesia Altaba

Director/es

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DIRECTORES DE LA TESIS DOCTORAL

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Facultad de Ciencias de la Salud
Universidad de Zaragoza

Dra. Marcela González Gross, PhD
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Departamento de Salud y Rendimiento Humano
Facultad de Ciencias de la Actividad Física y del Deporte (INEF)

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Dra. Theodora Mouratidou
Universidad de Zaragoza
Departamento de Fisiatría y Enfermería
Facultad de Ciencias de la Salud

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Fdo. Theodora Mouratidou
En Zaragoza, a 7 de marzo de 2017

A mis abuelos.

Nunca podré dedicarles tanto como ellos me han dedicado a mí.

“Cada minuto del Camino, sueñas con llegar a Santiago, y cuando por fín llegas, te das cuenta de que lo único que te queda en el corazón, es el Camino.”

(Adaptación de una frase de un peregrino del *Camino de Santiago* en la etapa entre *Arzúa* y *Santiago*).

Indice

1. Lista de publicaciones (list of publications)

La presente Tesis Doctoral está basada en un compendio de publicaciones de contribuciones científicas originales previamente publicadas o sometidas a revisión. Las referencias completas de los artículos contenidos pasan a detallarse a continuación:

I. Iglesia, I., Mouratidou, T., González-Gross, M., Novakovic, R., Breidenassel, C., Jiménez-Pavón, D., Huybrechts, I., De Henauw, S., Geelen, A., Gottrand, F., Kafatos, A., Mistura, L., de Heredia, F.P., Widhalm, K., Manios, Y., Molnar, D., Stehle, P., Gurinovic, M., Cavelaars, A.E.J.M., Veer, P.V.t, Moreno, L.A. Socioeconomic factors are associated with folate and vitamin B12 intakes and related biomarkers concentrations in European adolescents: The HELENA Study. Nutr Res2014 Mar;34(3):199-209.

II. Iglesia, I., Mouratidou, T., Gonzalez-Gross, M., Huybrechts, I., Breidenassel, C., Santabárbara, J., Díaz, L.E., Hallstrom, L., De Henauw, S., Gottrand, F., Kafatos, A., Widhalm, K., Manios, Y., Molnár, D., Stehle, P., Moreno, L.A. Foods contributing to vitamin B6, folate, and vitamin B12 intakes and biomarkers status in European adolescents: The HELENA study. Eur J Nutr2016 May 25.

III. Iglesia, I., Huybrechts,I., González-Gross, M., Mouratidou, T., Santabárbara, J., Chajès,V., González-Gil, E.M., Park, J. Y., Bel-Serrat, S., Cuenca-García, M., Castillo, M., Kersting, M., Widhalm, K., De Henauw, S., Sjöström, M., Gottrand, F., Molnár, D., Manios, Y., Kafatos, A., Ferrari, M., Stehle, P., Marcos, A., Sánchez-Muniz, F.J., and Moreno, L.A. Folate and vitamin B12 concentrations are associated with plasma

docosahexaenoic and eicosapentaenoic fatty acids in European adolescents: the HELENA study. Br J Nutr2017 Jan;117(1):124-33.

IV. Iglesia, I., González-Gross, M., Huybrechts, I., De Miguel-Etayo, P., Molnár, D., Manios, Y., Widhalm, K., Gottrand, F., Kafatos, A., Marcos, A., de la O Puerta, A., Leclercq, C., De Henauw, S., Stehle, P., Kersting, M., Mouratidou, T., Moreno L.A. on behalf of the HELENA study group. Associations between insulin resistance and three B-vitamins in European adolescents: The HELENA study. Accepted for publication in *Nutrición Hospitalaria*.

V. Iglesia I., Huybrechts, I., Mouratidou, T., Santabárbara, J., Fernández-Alvira, J.M., Santaliestra-Pasías, A.M. Manios, Y., De la O Puerta, A., Kafatos, A., Gottrand, F., Marcos, A., Sette, S., Plada, M., Stehle, P., Molnár, D., Widhalm, K., Kersting, M., De Henauw, S., Moreno, L.A., González-Gross, M., on behalf of the HELENA study group. Do dietary patterns determine levels of vitamin B6, folate, and vitamin B12 intakes and corresponding biomarkers in European adolescents? The HELENA study. Second revision in *Nutrition.*

2. Proyectos de investigación (Research Projects)

1.- Estudio HELENA (Healthy Lifestyle in Europe by Nutrition in Adolescence)

Financiado por la Comisión Europea: European Sixth RTD Framework Programme (CONTRACT FOOD-CT-2005-007034)

Página web: www.helenastudy.com

Coordinador General: Luis Alberto Moreno Aznar (Universidad de Zaragoza)

Las investigaciones subsecuentes se han realizado gracias al Ministerio Español de Ciencia e Innovación (JCI-2010-07055) con la contribución del Fondo Europeo Regional de Desarrollo (FEDER).

3. Lista de abreviaturas (List of abbreviations)

ADN: Ácido desoxirribonucleico

AGCL: Ácidos Grasos de Cadena Larga

AI: Adequate Intakes

ANCOVA: Análisis de la Covarianza

ATP: Adenosine Triphosphate

CEICA: Comité Ético de Investigación Clínica de Aragón

CV: Coefficient of Variation

DACH: Deutchland (D), Austria (A) y Suiza (CH)

DGCE: Dirección General de la Comisión Europea

DISH: Determinants -Intake-Status-Health

DQI: Diet Quality Index

DRI: Dietary Reference Intakes

EAR: Estimated Average Requirement

EDTA: Ethilene-Diamine-Tetraacetic acid

EURRECA: EURopean RECcomendations Aligned

FAO: Food and Agriculture Organization

FAS: Family Affluenze Scale

FFQ: Food Frequency Questionnaire

FI: Factor Intrínseco

FMI: Fat Mass Index

FNB-IOM: Food and Nutrition Board of the American Institute of Medicine

GENUD: Growth, Exercise, NUtrition and Development

HDL: High Density Lipoprotein

HELENA: Healthy Lifestyle in Europe by Nutrition in Adolescence

HELENA-DIAT: HELENA-Dietary Assessment Tool

HoloTC: Holotranscobalamin

HOMA: Homeostatic Model Assessment

IDR: Ingestas Dietéticas de Referencia

IMC: Índice de Masa Corporal

IMG: Índice de Masa Grasa

IPAQ: International Physical Atctivity Questionnaire

ISAK: International Society for the Advancement of Kinanthropometry

ISCO: International Standard Classification of Occupations

JCR: Journal Citations Report

LDL: Low Density Lipoproteins

MSM: Multiple Source Method

NoE: Network of Excellence

OMS: Organización Mundial de la Salud

PASW: Predictive Analytics SoftWare

PC: Phosphatidyl-choline

PCA: Principal Component Analysis

PE: Phosphatidyl-Ethalolamine

Pi: pyrophosphate

PLP: Pyridoxal-5-phosphate

Ppi: inorganic pyrophosphate

PUFAs: Polyunsaturated Fatty Acids

RBC-folate: Red blood cell folate

RDA: Recommended Dietary Allowances

RNI: Recommended Nutritional Intakes

RRR: Reduced Rank Regression

SAH: S-Adenosyl-Homocysteine

SAM: S-Adenosil-Metionine

SAS: Statistical Analysis Software

TG: Triglycerides

Thcy: Total Homocysteine

THF: Tetrahidrofolato

UL: Tolerable upper intake level

YANA-C: Young Adolescents' Nutrition Assessment on Computer

4. Resumen

La adolescencia es un período caracterizado por un aumento de las necesidades de nutrientes debido a un ritmo rápido de crecimiento y desarrollo. A ello se le suma el hecho de que sea un período en el que los adolescentes cambian sus hábitos alimentarios, encaminados a consumir alimentos de mayor densidad energética y menor densidad nutricional, a saltarse comidas, etc. Todo ello, hace que sea un periodo de vulnerabilidad a nivel nutricional, tal y como lo definió la red europea de Excelencia EURopean RECcomendations Aligned (EURRECA) en el año 2005, tras la llamada de la Comisión Europea para establecer una red que consiguiera estandarizar los requerimientos de los micronutrientes en Europa. En ese momento, los micronutrientes se priorizaron en base a la importancia de su implicación para la salud, de la cantidad de estudios recientes, y de la heterogeneidad de las actuales recomendaciones en Europa para los mismos. Entre los micronutrientes seleccionados para establecer unas nuevas recomendaciones en Europa en adolescentes, entre otras poblaciones vulnerables, se incluyeron el ácido fólico o folato y la vitamina B12. Nuestro grupo de Investigación *Growth, Exercise, NUtrition and Development* (GENUD), como participante de esta red, fue encargado de revisar sistemáticamente la bibliografía existente sobre vitamina B12 para poblaciones jóvenes, y la falta de estudios de calidad suficientes, no nos permitieron el establecimiento de unos nuevos valores recomendados. Por estas razones, se desarrolló esta línea de investigación, pudiendo analizar los datos del estudio Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) realizado con adolescentes europeos.

Folato y vitamina B12, participan, junto con la vitamina B6, como coenzimas en el metabolismo de donantes de carbono (metilación), por lo que la deficiencia en una de ellas, puede provocar una alteración metabólica de la otra. La homocisteína (tHcy), es un aminoácido citotóxico no proteinogénico, resultante de esta ruta de la metilación. Su

eliminación del organismo, puede llevarse a cabo mediante dos vías: su re-conversión a metionina, que necesita del folato y de la vitamina B12; también puede ser re-convertida en cisteína, usando la vitamina B6, piridoxina o piridoxal-fosfato (PLP) como cofactor. Por la importancia de este ciclo, se consideró también la posibilidad de incluir la vitamina B6, junto con el folato y la vitamina B12, en esta muestra de adolescentes europeos.

El modelo DISH, que relaciona los alimentos, la nutrición y la salud, describe la relación entre los determinantes de los comportamientos alimentarios (D-*determinants*-), la ingesta de alimentos y nutrientes (I-*intake*-), el estado de los biomarcadores y su funcionalidad (S-*status*-) y los parámetros de salud relacionados (H-*health*-). Siguiendo este modelo, se establecieron los objetivos de la presente Tesis Doctoral; por una parte, centrados en estudiar los determinantes de la ingesta y estatus de las vitaminas B6, folato y B12; y por otra, en relacionar las ingestas y el estatus de las mismas, con otros indicadores de salud.

Para la investigación, se consideraron los adolescentes participantes en el estudio HELENA, estudio transversal y multicéntrico en el que se reclutaron 3,528 adolescentes (47 % varones) de edades comprendidas entre los 12.5 y los 17.5 años, procedentes de 10 ciudades europeas: Dortmund (Alemania), Viena (Austria), Gante (Bélgica), Lille (Francia), Atenas y Heraklion (Grecia), Pécs (Hungría), Roma (Italia), Estocolmo (Suecia), y Zaragoza (España). En él se evaluó un compendio de parámetros relacionados con la nutrición y los estilos de vida. De entre estos parámetros, se usaron: el Indice de Masa Corporal (IMC), factores sociodemográficos (educación y ocupación de los padres, nacionalidad, composición del hogar y riqueza familiar medida con el *family affluence scale*-FAS-), actividad física medida con el cuestionario *International Physical Activuty Questionnaire* (IPAQ), ingesta de energía obtenida mediante dos recuerdos de 24 horas con el software HELENA-DIAT, ingesta y biomarcadores de las vitaminas B6 (PLP), folato (folato en plasma y en células rojas) y

vitamina B12 (vitamina B12 en plasma y holotranscobalamina-HoloTC-), incluyendo también a la homocisteína.

Entre los determinantes analizados, los marcadores del nivel socioeconómico familar de los adolescentes europeos, han mostrado serlo de la ingesta y los biomarcadores de folato y vitamina B12. No se encontraron asociaciones con la riqueza familiar, mientras que la educación y ocupación de los padres fueron los marcadores más consistentemente asociados con la ingesta y el estatus de las vitaminas analizadas.

Las ingestas de vitamina B6, folato y B12 están asociadas con la ingesta de sus principales fuentes alimentarias, mientras que sus correspondientes biomarcadores, lo estuvieron más con la ingesta dietética global. La carne y los productos ricos en almidones determinaron, en mayor medida, la ingesta de vitamina B6, mientras que los cereales de desayuno y las margarinas y mantequillas determinaron su biomarcador PLP en chicas y chicos respectivamente; la ingesta de folato, estuvo determinada por la ingesta de frutas y verduras, mientras que los biomarcadores de folato, lo estuvieron más por los cereales de desayuno y las frutas en chicos y chicas, y también por margarinas y mantequillas en el caso de las chicas. Por último, la ingesta de vitamina B12, estuvo determinada por la ingesta de carne y lácteos, cuando sus biomarcadores lo estuvieron principalmente por los lácteos, para ambos sexos.

Una vez analizados los grupos de alimentos de una manera individualizada, se agruparon constituyendo patrones dietéticos que lograban explicar el mayor porcentaje posible de las ingestas y concentraciones de biomarcadores de las vitaminas analizadas. Los patrones obtenidos (uno por cada vitamina y sexo), explicaron entre un 23.7% y un 34.2% de la variabilidad de la ingesta de las vitaminas, mientras para sus biomarcadores, el rango explicado fue más bajo, oscilando entre un 7.0 % y un 17.2 %.

Se observaron asociaciones entre los biomarcadores de las vitaminas B6, folato y B12 y las concentraciones de ácidos grasos de cadena larga (AGCL), sobretodo con los de la serie ω3.

Por el contrario, el índice pro-inflamatorio ω6/ω3, los niveles de grasas trans y la relación oléico/esteárico estuvieron negativamente asociados con los biomarcadores de estas vitaminas.

Por otro lado, de entre las vitaminas B6, folato y B12, la vitamina B12 fue la más consistente y negativamente asociada con el IMC, IMG, y el índice de sensibilidad a la insulina (HOMA) en adolescentes europeos. Las diferencias halladas para la vitamina B12 en suero entre los grupos resultantes al combinar categorías de HOMA y de IMG, parecen no estar explicadas por las ingestas de vitamina B12, ya que no se observaron diferencias en la ingesta de B12 entre los grupos.

En conclusión, el gradiente de salud en base al nivel socioeconómico, se confirma para las ingestas y los biomarcadores de folato y vitamina B12 en los adolescentes europeos. Además, las ingestas de las vitaminas B6, folato y B12, se ven determinadas por sus principales fuentes alimentarias, mientras que los biomarcadores de las mismas no lo están tanto, porque otros mecanismos pudieran estar implicados. Observando los grupos de alimentos determinantes de los niveles de biomarcadores de las vitaminas, se podría sugerir, que los adolescentes que ingieren alimentos de baja densidad nutricional, como por ejemplo los snacks, con una variedad de la dieta también baja, podrían ver comprometidos sus concentraciones de vitaminas B6, folato y B12, y ello, a su vez, influenciar otros indicadores para la salud como podrían serlo los ácidos grasos circulantes o la adiposidad y la resistencia a la insulina.

Por lo tanto, mejorar la calidad de la dieta de los adolescents, debe de ser una prioridad desde el punto de vista de las deficiencias vitamínicas para el grupo B, y consecuentemente, para disminuir el riesgo de enfermedades crónicas futuras.

5. Abstract

Adolescence is a period in which nutritional requirements may be increased due to rapid growth and development. This could result into change of dietary habits with an increase of consumption of high energy density diets and/or low nutrient density diets. Also, some behaviours like skipping meals are usually settle during adolescence. For these reasons, adolescence is considered a vulnerable nutritional period, as it was defined also by the European Network of Excellence EURopean RECcomendations Aligned (EURRECA) in 2005. This network was created after the call from the European Commission to standardize the micronutrient requirement across Europe in vulnerable groups. Micronutrients were prioritized based on their relevance for health, the amount of recent studies and the heterogeneity of the current recommendations in European countries. Among the selected micronutrients and the vulnerable population groups, folate, vitamin B12 and adolescents were highlighted. Our Research Group, *Growth, Exercise, NUtrition and Development* (GENUD), was involved in this European Network and it was in charge of systematically reviewing the existing evidence about vitamin B12 in young population groups. However, a lack of dose-response studies of high quality did not allowed us to determine new recommendations of this micronutrient in young population groups. For this reason, data from the Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) study provided us a good opportunity to increase the evidence in this topic in European adolescents.

Folate and vitamin B12, participate, together with vitamin B6, as coenzymes in the methyl-donor metabolism (methylation) and, consequently, a deficiency in one of those can induce to metabolic alterations on the others. Homocysteine (tHcy), is a non-proteinogenic citotoxic aminoacid resulting from this metabolic path. Two different paths are followed to eliminate the tHcy from the organism: to convert it to methionine by the action of folate and vitamin B12 or to convert it

in cysteine using the vitamin B6 (piridoxine, PLP), as a cofactor. The study of the vitamin B6 and folate was also included in the objectives of this PhD, due to the importance in this cycle.

The DISH model, relating foods, nutrition and health, describes the relation between the determinants of the dietary behaviours (D-*determinants*-), intake of foods and nutrients (I-*intake*-), biomarkers and their functionality (S-*status*-), and related health indicators (H-*health*-). Therefore, and following this model, the objectives of this PhD Thesis were defined: firstly, looking for the determinants of the three B-vitamins intake and status; and secondly, to relate the intake and the status of the B-vitamins with some health indicators.

In the cross-sectional multicentre HELENA study, 3,528 adolescents were included (47 % males) from 12.5 y to 17.5 y, from 10 European cities: Dortmund (Germany), Viena (Austria), Ghent (Belgium), Lille (France), Athens and Heraklion (Greece), Pécs (Hungary), Rome (Italy), Stockholm (Sweden), and Zaragoza (Spain). A complete set of lifestyle and health related variables were evaluated in that adolescents, including: body mass index (BMI) socioeconomic factors (parental education and occupation, family affluence, household composition and migrant background), physical activity measured with the *International Physical Activity Questionnaire* (IPAQ), energy intake assessed by two computerized 24-hours dietary recalls using HELENA-DIAT software, and the intake and status of the vitamin B6 (PLP), folate (plasma folate and RBC-folate) and vitamin B12 (plasma vitamin B12 and holotranscobalamin-HoloTC-), including also homocysteine.

Among the analysed determinants, socioeconomic factors demonstrated to be associated with the intake and the status of the folate and vitamin B12 in European adolescents. Associations were found for parental education in detriment of those for family affluence.

Results from the present Phd showed that intake of vitamin B6, folate, and vitamin B12, were associated with the consumption of their main food sources, while for biomarkers, the whole diet quality was associated. Meat and starchy products determined the intakes of vitamin B6, while

cereal breakfast and lipids of mixed origin determined its biomarker PLP in females and in males, respectively. In addition, folate intake was determined by fruits and vegetables, while folate biomarkers were by breakfast cereals and fruits in males and females, and also by lipids of mixed origin for females. Lastly, intake of vitamin B12, were determined by meat and dairy products, while its biomarkers did it for dairy products in both sexes.

Then, foods were aggregated in dietary patterns and tested, to determine also the intake and the status of the three B-vitamins. The dietary patterns obtained (one for each B-vitamin, and sex) were able to explain between 23.7 % and 34.2 % of the variability in the intake of the vitamins, and between 7.0 % and 17.2 % of the variability regarding their corresponding biomarkers.

In terms of consequences, associations between the biomarkers of the three B-vitamins studied and the concentrations of the PUFAs were found, mainly with the ω3. In contrast, these associations were negative with the pro-inflammatory index ω6/ω3, the ratio oleic/stearic and the concentrations of the trans fatty acids with the biomarkers of the vitamin B6, folate, and B12.

Besides, plasma vitamin B12 were consistently and negatively associated with adiposity and insulin sensitivity. These differences might not be explained by the intake of vitamin B12. This evidenced that other mechanisms might be further investigated.

To conclude, the health gradient based on the socioeconomic factors, was confirmed by our results for B-vitamins intake and status for European adolescents. Besides, intakes of vitamin B6, folate, and vitamin B12, were determined by their main food sources while their biomarkers were more associated with the overall diet quality. These results show that those consuming lower nutrient density diets might be compromising their nutrient status, or even influence other health indicators such as the circulating fatty acids or the adiposity or insulin sensitivity. Therefore, efforts to improve diet quality from adolescence it´s a matter of importance to prevent B-vitamins deficiencies and subsequently, to decrease the risk of future chronic disease.

6. Introducción

La adolescencia es un período de la vida caracterizado por un ritmo de crecimiento especialmente rápido comparado con el de la niñez (1), que suele desarrollarse de un modo más paulatino. Además de los cambios físicos, esta etapa está ligada a cambios importantes a nivel hormonal, cognitivo y emocional (2). Ello se asocia a un importante incremento de la demanda energética y nutricional, incluyendo también la ingesta de micronutrientes (1).

Además de estos aspectos, se producen cambios en relación a los hábitos alimentarios, especialmente encaminados hacia el consumo de alimentos de mayor densidad energética y menor densidad nutricional (3). Ello supone un importante reto para los adolescentes, con el fin de prevenir enfermedades futuras (4, 5). En este sentido, y como un importante determinante de las mismas, caben destacar las deficiencias de micronutrientes, tal y como anunciaba la *Organización Mundial de la Salud (OMS)* en el año 2006 (6).

Además, en el año 2005, la *Dirección General (DG) de Investigación de la Comisión Europea (CE)*, incluía a los adolescentes como población vulnerable dentro de su llamada para la presentación de propuestas para la creación de una Red de Excelencia, *Network of Excellence (NoE)*, cuya misión fue establecer los requerimientos de algunos micronutrientes en poblaciones vulnerables, basada en la evidencia científica. En este proyecto, se puso de manifiesto la elevada variabilidad en las recomendaciones dietéticas de ingesta de micronutrientes entre los distintos países europeos. Así fue como nació la Red Europea de Excelencia "EURopean micronutrient RECommendations Aligned" (EURRECA), cuya misión fue la de armonizar las recomendaciones de algunos micronutrientes en poblaciones vulnerables de Europa (7).

Para la priorización de los micronutrientes en los que llevar a cabo la investigación, se tuvieron en cuenta los siguientes criterios (8):

(a) cantidad de nueva evidencia científica relevante disponible para cada micronutriente en las diferentes etapas de la vida;

(b) relevancia de un determinado micronutriente en términos de salud pública, para los grupos de población vulnerable definidos;

(c) heterogeneidad considerada como la variación en la definición de las actuales recomendaciones establecidas para el micronutriente en particular entre los diversos países europeos.

Entre los micronutrientes seleccionados para las poblaciones vulnerables definidas, entre las que se encontraba la de los adolescentes, figuraban las vitaminas hidrosolubles del grupo B, folato o vitamina B9 (ácido fólico en su forma sintética), y la cobalamina o vitamina B12.

Las interacciones metabólicas entre una y otra, así como su común asociación con la anemia megaloblástica, han sentado las bases de una historia que ha ido siempre de la mano (9). De hecho, junto con la vitamina B6, participan como coenzimas en el metabolismo de donantes de carbono (metilación), por lo que la deficiencia en una de ellas, puede provocar un metabolismo alterado de la otra (10, 11).

Metabolismo del folato, vitamina B12 y vitamina B6

Los folatos alimentarios se encuentran en un 90% en forma de poliglutamatos ligados a proteínas, mientras que la forma sintética (comúnmente conocida como ácido fólico), esta constituida por la estructura básica de todos los folatos, el ácido pteroilglutámico. En el intestino, estos poliglutamatos, son despojados de las proteínas, para ingresar a las células intestinales, en forma de monoglutamatos reducidos, pasando a ser biológicamente activos en su forma metilada 5-

metil-tetrahidrofolat0 (THF). Estas nuevas moléculas, son posteriormente transportadas al hígado, a través de las venas mesentéricas. Una vez allí, se almacenan para situaciones de ayuno o vuelven a la circulación principalmente para llegar a zonas de rápida división celular como la médula ósea o la mucosa gastrointestinal, ya que son los tejidos más demandantes de ácido fólico, para la sínTesis de ADN (9).

Una vez en los tejidos periféricos, el 5-metil-THF penetra en el interior de la célula, donde pierde su grupo metilo y se lo cede a la homocisteína, la cual, gracias a la metionina-sintasa y a la vitamina B12, se transformará en metionina.

La homocisteína (tHcy), es un aminoácido citotóxico no proteinogénico resultante del metabolismo de la metionina. Su eliminación del organismo, puede llevarse a cabo mediante dos vías: su re-conversión a metionina que necesita del folato y de la vitamina B12; también puede ser re-convertida en cisteína, usando otra vitamina hidrosoluble del grupo B, vitamina B6, piridoxina o piridoxal-fosfato como cofactor (9). De esta manera, una deficiencia en cualquiera de ellas, se traduce en un aumento de los niveles de homocisteína (12), que es un biomarcador clave de aumento del estrés oxidativo y daño endotelial, por lo que representa un factor de riesgo independiente de enfermedades cardiovasculares (13) y cerebrovasculares (14), en adultos.

Por otra parte, las cobalaminas (vitamina B12) son corrinoides constituidos por cuatro anillos pirrólicos, con el cobalto como núcleo central. La vitamina B12 la producen sólo los microorganismos, por lo que los vegetales no la contienen, salvo limitadas excepciones (9), por lo que la carne constituye su principal fuente alimentaria (15).

Las cobalaminas, unidas a las proteínas alimentarias, se liberan gracias al ácido clorhídrico gástrico y la pepsina, y se unen a proteínas haptocórricas procedentes de la saliva y del jugo gástrico. Posteriormente, se liberan de ellas, gracias a las proteasas pancreáticas y se unen al factor

intrínseco (FI), procedente de las células parietales gástricas. El complejo vitamina B12-FI, se absorbe a nivel del íleon, donde las cobalaminas, ya disgregadas, pasan al plasma sanguíneo gracias a las transcobalaminas (proteínas TCI, TCII, y TCIII). La cantidad de cobalamina almacenada en los tejidos de un adulto, oscila entre 2 y 3 mg y se divide entre el hígado y la circulación enterohepática. Sin embargo, el 95% de la cobalamina, pasa al espacio intracelular, donde su forma reducida, puede seguir dos vías: en la mitocondria se origina la desoxiadenosilcobalamina que se une a la metilmalonil-CoA-mutasa; y en el citoplasma se forma la metilcobalamina que actúa con la metionina sintasa (9) como se ha descrito anteriormente.

Por su parte, la vitamina B6 o piridoxina, es uno de los tres derivados piridínicos interconvertibles, y su derivado principal es la coenzima PLP. La vitamina B6 es absorbida en el yeyuno y de ahí pasa a la circulación sanguínea y llega al hígado, donde se forman a través de ella, las coenzimas activas. Allí se almacenan junto a las proteínas enzimáticas correspondientes, a las que se une el PLP. Éste colabora como coenzima en más de medio centenar de reacciones en el metabolismo de los aminoácidos, de entre las que ya se ha descrito su relación con la cisteína y la homocisteína.

En la figura 1, se puede observar el ciclo de la metilación en el que se conectan las tres vitaminas que nos ocupan.

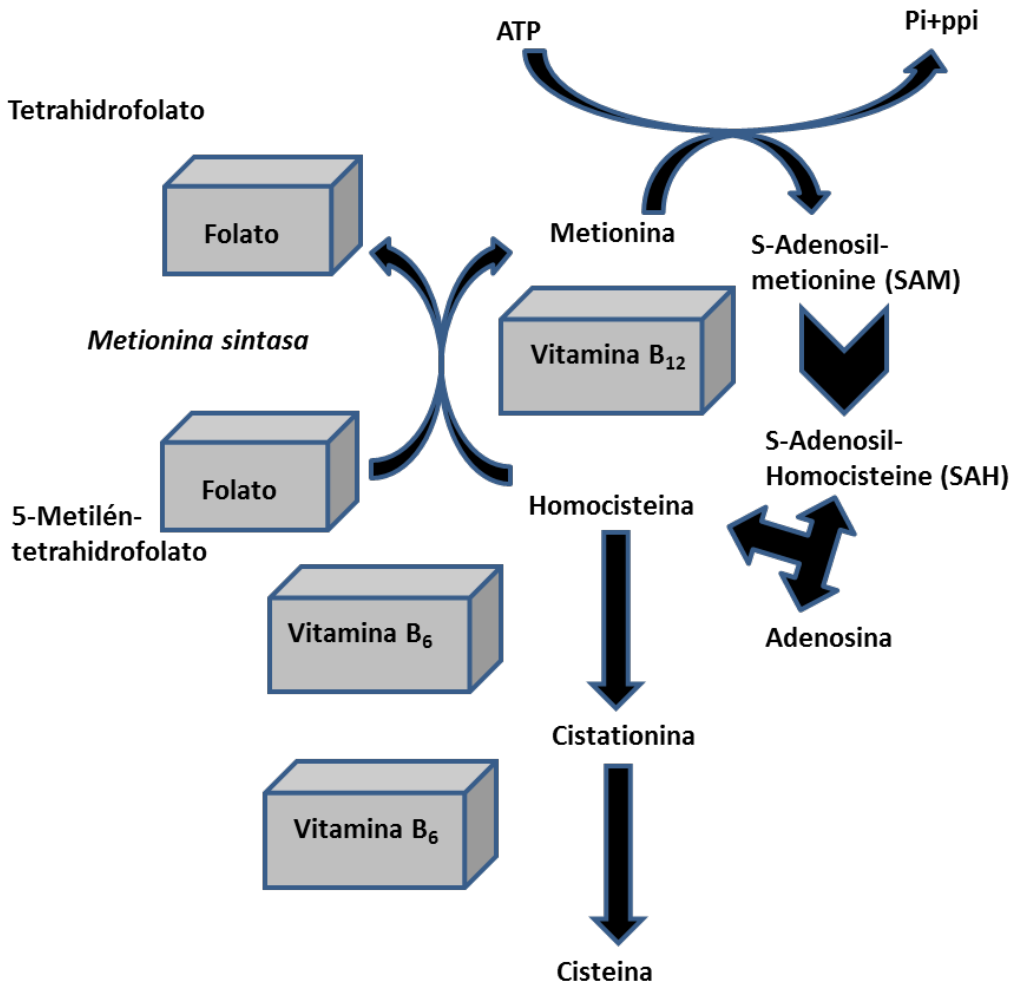


Figura 1. Ciclo de la metilación

Recomendaciones dietéticas de las vitaminas B6, folato y B12

El conocimiento científico de la influencia de la alimentación y la nutrición sobre la salud, lleva algunas décadas preocupando a los científicos en el sentido de establecer los valores de referencia de ingesta de nutrientes con el fín de que la población tenga un estado nutricional adecuado. Así, surgió en concepto de ingestas recomendadas, también conocidas en los países anglosajones como Recommended Dietary Allowances (RDA) (16) o Recommended Nutritional Intakes (RNI) (17) en Canadá y Reino Unido. Inicialmente, los esfuerzos se centraron en evitar carencias

nutricionales, pero gracias al avance de la ciencia en relación a la composición de los alimentos así como de los procesos metabólicos, el estudio ahora se centra en la prevención de enfermedades crónicas y degenerativas, y tienen como sentido final, la promoción de la salud (18). Es por ello, que han ido surgiendo nuevos conceptos en relación a las ingestas de referencia. Por ejemplo, en Estados Unidos y Canadá, a partir de 1997, los RDA fueron sustituidos y ampliados por las DRI (Dietary Reference Intakes- Ingestas dietéticas de referencia), entre las que destacan (19):

-EAR (Estimated Average Requirement- Requerimiento medio estimado), que es el nivel de ingesta diaria de un nutriente que se estima necesaria para cubrir los requerimientos de la mitad de los individuos sanos de un grupo de población en una etapa de la vida y sexo específico.

-RDA (Recommended Dietary Allowances- Aportes dietéticos recomendados), que es el nivel de ingesta media diaria de un nutriente que se considera suficiente para cubrir los requerimientos nutricionales del 97,5% de los sujetos sanos de un grupo poblacional en una etapa de la vida y sexo específicos.

-AI (Adequate Intakes-Ingestas adecuadas), que es el nivel de ingesta media de nutrientes de grupos de individuos sanos, determinados mediante estudios observacionales, experimentales o por extrapolación. Suponen los valores de referencia cuando no hay evidencia suficiente que permita establecer las EAR y calcular las RDA.

-UL (Tolerable upper intake level-Nivel de ingesta máxima tolerable), que es el nivel de ingesta diaria más alto de un nutriente que probablemente, no implicaría efectos negativos en la salud de un grupo de individuos de la población general.

Las ingestas dietéticas de referencia (IDR), han sido diseñadas en diferentes países por diferentes comités nacionales e internacionales de expertos. Los valores establecidos por el Comité Americano de Alimentación y Nutrición, del Instituto de Medicina de Estados Unidos, FNB-IOM en sus siglas en inglés (Food and Nutrition Board of the American Institute of Medicine), en

colaboración con científicos canadienses, quizá sean (20) los que tienen mayor relevancia a nivel mundial (20).

Por ejemplo, para las vitaminas B6, folato y B12, estas serías las ingestas dietéticas de referencia en Europa y Estados Unidos y Canadá, respectivamente:

	Europa			Institute of Medicine (IOM)		
	Grupos de edad	Chicos	Chicas	Grupos de edad	Chicos	Chicas
Vitamina B6 (mg/día)	11-14 años	1,3	1,1	9-13 años	1,0	1,0
	15-17 años	1,5	1,1	14-18 años	1,3	1,2
Folato (µg/día)	11-14 años	180	180	9-13 años	300	300
	15-17 años	200	200	14-18 años	400	400
Vitamina B12 (µg/día)	11-14 años	1.3	1,3	9-13 años	1,8	1,8
	15-17 años	1.4	1,4	14-18 años	2,4	2,4

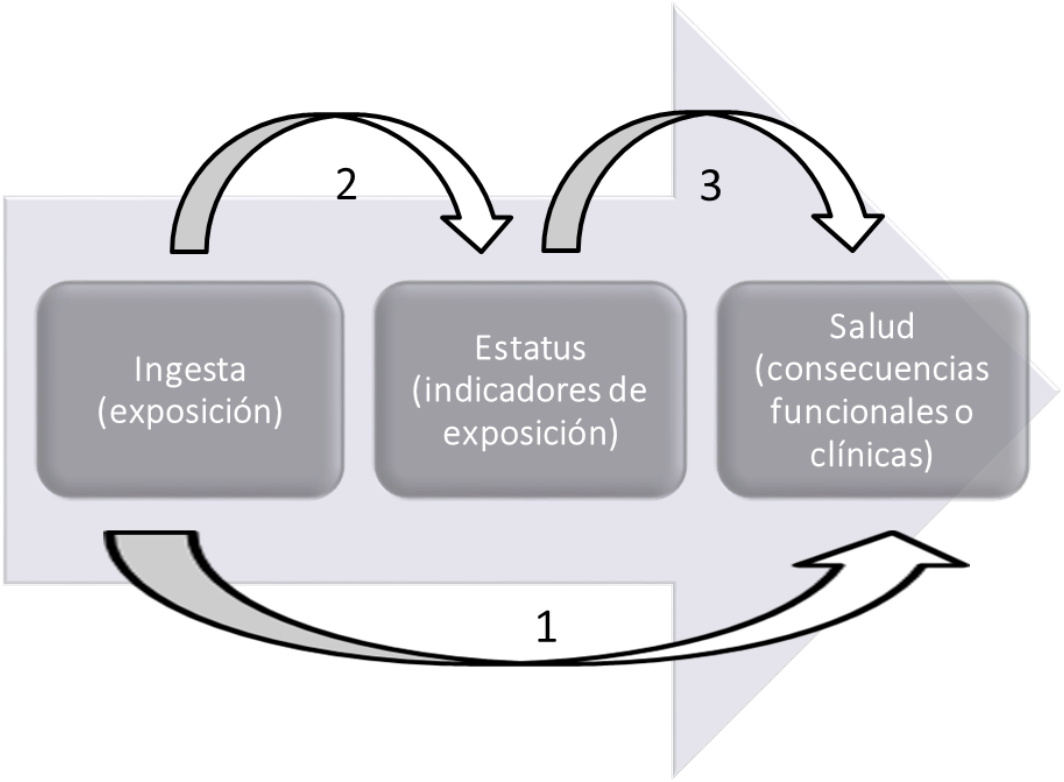
En estudios en niños y adolescentes en Europa (21-25), en Asia (26), y Norte América (27-29), se han observado concentraciones bajas de vitamina B6, folato y vitamina B12 en sujetos aparentemente sanos. Es por ello que algunos autores se plantean si se deberían revisar las actuales recomendaciones dietéticas de referencia propuestas por los diferentes organismos internacionales. Tal es el caso de los valores de referencia establecidos por el *D-A-CH* (siglas de

la agrupación de los países Alemania -Deutchland- (D), Austria (A) y Suiza (CH)), para el ácido fólico. En el año 2000, estos valores habían sido establecidos en 400 µg/día para los adultos, al igual que para otros organismos internacionales como el *Instituto de Medicina* americano *(IOM) (19)*, o la *OMS* y la *Organización para la Agricultura y los Alimentos* (en su nombre en inglés *Food and Agriculture Organization -FAO-*) (30). En ese momento, la prevención de las enfermedades cardiovasculares mediante la reducción de los niveles de homocisteína, habían sido considerados como el objetivo más relevante a la hora de definirlos (31). Sin embargo, a pesar de que en algunos estudios (32-34) se observó una asociación inversa entre dietas ricas en ácido fólico y las enfermedades crónicas, en algunos ensayos clínicos aleatorizados no se logró observar un efecto preventivo del riesgo tromboembólico ni de las enfermedades cardiovasculares en general, con la suplementación con ácido fólico y otras vitaminas del grupo B, incluyendo B6 y B12. Además, en otros estudios, ingestas de ácido fólico por debajo de los 400 µg/día, aseguraban unas concentraciones adecuadas en sangre (32-34). Es por ello, que, por ejemplo, las recomendaciones DACH para el folato han acabado por ajustarse a 300 µg/d para adultos (31).

Además de la citada controversia existente entre las recomendaciones dietéticas para las vitaminas, tampoco los métodos de valoración del estatus en sangre de las mismas están del todo establecidos. Tanto es así, que por ejemplo, recientemente se publicó (35) una revisión para intentar identificar cuáles serían las consideraciones a tener en cuenta a la hora de valorar el estatus de la vitamina B12 desde sus distintos parámetros bioquímicos. Las principales conclusiones de la misma, fueron que no existía un consenso generalizado sobre el uso de los valores de referencia y rangos y sus pricipales metabolitos; y su principal recomendación, la de que sería necesario que el sexo, la edad y el método analítico, fueran tenidos en cuenta a la hora de establecer los valores de referencia.

Es por todo ello, que siguiendo el modelo de análisis que se empleó en la Red EURRECA para la búsqueda de una adecuada armonización de los valores de referencia, ingesta-biomarcador-resultado para la salud (8), resulte de gran importancia, estudiar la ingesta y sus determinantes y las concentraciones de estas vitaminas en sangre, así como el resultado en algunas variables relacionadas con la salud (Figura 2).

Figura 2. Modelo de asociaciones posibles entre la ingesta, el estatus y la salud en relación con la dieta.



Metodos de valoracion de la ingesta de vitaminas

Existen varios métodos de valoración de la ingesta de nutrientes, entre los que se incluyen las vitaminas. Entre ellos, destacan el cuestionario de frecuencia de consumo (Food Frequency

Questionnaire-FFQ- en sus siglas en inglés), el recuerdo dietético de 24 horas y el registro dietético. Se sabe que todos estos métodos tienen sus ventajas e inconvenientes y ninguno de ellos resulta enteramente satisfactorio. El método normalmente aplicado en los largos estudios epidemiológicos, es el cuestionario de frecuencia de consumo debido a su bajo coste y a su fácil aplicación (es auto-referido), mientras que el recuerdo de 24 horas y el registro dietético, se usan como métodos de referencia junto con el uso de biomarcadores, por ser métodos de mayor precisión, pero que lógicamente requieren de un mayor esfuerzo para su puesta en práctica (36).

El método de registro dietético, requiere de mucho esfuerzo por parte del participante, ya que tienen que registrar todo lo que bebe y come en el momento de hacerlo, por lo que a mayor número de días registrados, suele acumularse el efecto fatiga para el participante, y la información recogida suele ser de menor precisión (37, 38).

El método de recuerdo dietético de 24 horas cuenta con varias ventajas. Un entrevistador entrenado se encarga habitualmente de registrar las respuestas del participante, por lo que no se requiere de su alfabetización. Además, la inmediación de la administración del cuestionario con respecto al día de ingesta, hace que no suela haber falta a la verdad de los alimentos ingeridos por falta de memoria (38).

Metodos de valoración del estatus de vitaminas

Vitamina B6

La vitamina B6 puede ser medida mediante biomarcadores directos o mediante biomarcadores funcionales. Entre los directos, que pueden medirse en plasma/suero, orina o eritrocitos, el piridoxal 5'-fosfato (PLP) es el más extendido y aceptado (12, 39). Entre los biomarcadores funcionales, figuran la actividad de las transaminasas eritrocitarias y, más recientemente, los niveles plasmáticos de los metabolitos envueltos en las reacciones dependientes del PLP, como la cistationina, la serina o la glicina. Sin embargo, la combinación del uso de varios

biomarcadores, sería lo más recomendado para eliminar la influencia de los principales confusores, como la inflamación (39),

Folato

Los biomarcadores disponibles más sensibles y precisos para determinar el estatus en folato, son el folato eritrocitorio (red blood cell folate -RBC-folate-, en sus siglas en inglés) para la valoración de las reservas de folato, y el folato en suero o plasma, para el folato ingerido recientemente. Adicionalmente, la homocisteína plasmática, puede considerarse un buen biomarcador funcional del folato, pero sin embargo, no es muy específico y puede verse influído por el estatus nutricional de la vitamina B6 o la B12, así como de otros factores no nutricionales como la edad, el sexo, la etnia, estado fisiológico, o los estilos de vida (40).

Vitamina B12

Si bien los biomarcadores más extendidos y aceptados para la vitamina B6 y para el folato, están bastante definidos, no lo es así para la vitamina B12. Hay un amplio consenso sobre que la vitamina B12 en suero no es un buen biomarcador para valorar el estatus de vitamina B12, pero es un biomarcador usado a menudo. Valores por encima de sus valores de referencia, no siempre indican un estatus adecuado de vitamina B12, porque esos valores pueden estar mantenidos a costa de las reservas en los tejidos. Por ello, la holotranscobalamina (HoloTC en sus siglas en inglés), se propone como mejor biomarcador por su capacidad de ligar a la vitamina B12 a la proteína responsable de mediar con el receptor de entrada celular (12).

El ácido metilmalónico se considera el "gold standard" para medir la deficiencia de vitamina B12. Este metabolito aumenta cuando existe deficiencia de vitamina B12. La enzima metilmalonil-

CoA mutasa requiere de vitamina B12 para convertir el ácido metilmalónico en succinil-CoA, importante para la producción de energía celular.

Finalmente, la homocisteína, vuelve a considerarse, como para el folato, un biomarcador no específico, que además puede verse influido como ya se ha comentado anteriormente, por factores nutricionales y por no nutricionales (41).

Determinantes de la ingesta y del estatus de la vitamina B6, folato y vitamina B12

De la ingesta de micronutrientes, la cantidad que utilizará el organismo está determinada por una serie de factores, entre los que destacan la *"biodisponibilidad"* desde una fuente o alimento específicos, el ritmo y *procesos metabólicos* individuales de cada individuo, y las *interacciones* que se puedan producir con otros micronutrientes (42).

Además de estos factores intrínsecos, que no vamos a poder modificar, existen otros factores, entre los que destaca la *ingesta dietética*, que ya fue considerada por la FAO, como la principal causa de malnutrición de micronutrientes (43), sobre los que sí vamos a poder influir y que abren una gran ventana a la investigación en el campo de la prevención y las políticas de salud pública.

Entre los determinantes de la ingesta dietética o la elección de alimentos, el principal factor determinante de la alimentación es la sensación de hambre. Sin embargo, lo que decidimos comer, no está determinado exclusivamente por factores fisiológicos. Los factores que influyen en la elección de los alimentos son (44):

- ✓ Determinantes biológicos como el hambre, el apetito y el sentido del gusto
- ✓ Determinantes económicos como el coste, los ingresos y la disponibilidad en el mercado

- ✓ Determinantes físicos como la accesibilidad, la educación, las capacidades personales (por ejemplo, para cocinar) y el tiempo disponible
- ✓ Determinantes sociales como la cultura, la familia, los compañeros de trabajo y los patrones de alimentación
- ✓ Determinantes psicológicos como el estado de ánimo, el estrés y la culpa
- ✓ Actitudes, creencias y conocimientos en materia de alimentación

Entre todos ellos, en la presente Tesis Doctoral, se consideran los determinantes sociales de los patrones de alimentación y por ello son los que se revisan a continuación.

Se ha descrito con frecuencia que el *nivel socioeconómico* influye en la *calidad de la dieta* (45). De hecho, en estudios epidemiológicos, las asociaciones entre calidad de la dieta e indicadores de salud, podrían haber estado mediadas por factores socioeconómicos no tenidos en cuenta. En los grandes estudios epidemiológicos se pueden usar distintos factores socioeconómicos, los cuales permiten obtener más información sobre su papel mediador en estas posibles asociaciones. La elección del indicador socioeconómico más adecuado está constantemente sujeto a debate (46). Por ejemplo, los factores sociales como el número de *miembros que cohabitan bajo un mismo techo*, o el hecho de *ser o no inmigrante*, han demostrado estar fuertemente asociados con los *comportamientos alimentarios* (47). Del mismo modo, los *factores económicos* como la *educación*, la *ocupación*, y los *ingresos*, se consideran relacionados con la salud, dada su influencia directa sobre los estilos de vida (45, 47).

La ingesta de los diferentes *alimentos o grupos de alimentos* podría influir en la ingesta y las concentraciones de las vitaminas B6, folato y vitamina B12. La valoración de los determinantes de la ingesta y el estatus de los diferentes nutrientes, y en este caso, de las vitaminas B6, folato y vitamina B12, supone la posibilidad de identificación de los grupos de riesgo de déficit de las mismas, los cuales podrían beneficiarse más de las intervenciones de salud pública (48). Esto es

importante, ya que, en numerosas ocasiones, no nos sirve conocer el contenido de un nutriente en un alimento y basarnos en sus contenidos brutos para saber cuáles son los principales alimentos que contribuyen a la ingesta y el estatus de un determinado nutriente, sino que hace falta, además, conocer esas cantidades, pero en relación a la ingesta total de los alimentos en la misma dieta. Como se ha dicho anteriormente, hay que tener en cuenta que, además de las principales fuentes de una vitamina, hay otros factores que juegan un importante papel, como lo son el acceso a esas fuentes alimentarias y su elección dentro de todo el espectro alimentario disponible en la actualidad, la *biodisponibilidad* de ese nutriente en el alimento, las *pérdidas por procesado*, y las posibles *interacciones* con otros alimentos, nutrientes o fármacos (49-51). La influencia de todos estos factores, implica que muchas veces, no nos sirve con saber la cantidad estimada de nutriente que ingiere un individuo o población para precisar certeramente unos adecuados niveles de vitamina o un estado deficitario, y es ahí donde entra en juego la inestimable ayuda de los *biomarcadores* sanguíneos. Los *biomarcadores* de ingesta y, en este caso, los relacionados con las tres vitaminas que nos ocupan, B6, folato y B12, suponen una herramienta precisa de valoración de la ingesta, si se compara con los métodos auto-referidos de valoración de la *ingesta dietética* (52). Las concentraciones sanguíneas de los *biomarcadores* de las vitaminas del grupo B, pueden considerarse como la consecuencia biológica de la *ingesta dietética* o de los *patrones dietéticos* (53).

Los seres humanos consumen los alimentos de forma combinada, y por ello resulta imprescindible analizar los *patrones dietéticos* (54). Para establecer los patrones dietéticos, se usan técnicas estadísticas sofisticadas y, en función del tipo de patrones dietéticos que se quieran establecer, resulta más útil trabajar con unos u otros. Entre dichas técnicas, encontramos el análisis de componentes principales (o PCA en sus siglas en inglés: Principal Component Analysis), que consiste en agrupar los alimentos, buscando la combinación lineal de los mismos, y que además sean independientes; el análisis de conglomerados (o Cluster Analysis en inglés), que trata de

lograr la máxima homogeneidad en cada grupo y la mayor diferencia entre los grupos; y, por ultimo, el análisis de regression de rangos reducidos (o Reduced Rank Regression analysis en inglés -RRR-) que es más potente y flexible que el PCA y usa conocimiento previo sobre la materia, para agrupar los alimentos de un modo similar al PCA (55).

Vitamina B6, folato y B12 y enfermedades o condiciones clínicas asociadas

Como se ha descrito previamente, las tres vitaminas están estrechamente ligadas por compartir parte de su camino metabólico. A nivel funcional, déficits de las mismas o alteraciones de su metabolismo, pueden originar una serie de consecuencias para la salud, que podrían manifestarse como alteraciones en el crecimiento y desarrollo de los adolescentes, o con la presencia de niveles bajos de vitamina B6, folato, o vitamina B12, y subsecuentemente altos de homocisteína. Kerr y cols. (56), observaron que concentraciones bajas de estas vitaminas del grupo B y altos de homocisteína durante la niñez, podían estar asociadas con una mayor incidencia de enfermedades crónicas en la edad adulta. Algunas de estas asociaciones han quedado ampliamente demostradas en la literatura, como en el caso de las enfermedades cardiovasculares, los defectos del tubo neural en niños recién nacidos, algunos tipos de cáncer, con la función cognitiva y la salud ósea (57, 58). Estudios más recientes, han mostrado asociaciones con alteraciones en el control del metabolismo de la glucosa, el estrés oxidativo, y la inflamación (59, 60). Además, dado su rol en la replicación del ADN (Ácido Desoxiribonucleico), son importantes factores del crecimiento y el desarrollo y, por consiguiente, cruciales en la infancia y la adolescencia (19).

Clásicamente, la deficiencia en vitamina B6 se ha relacionado con anemia hipocrómica, dermatitis seborreica, glositis, depresión y convulsiones. El folato, con el cáncer, defectos del tubo neural, la anemia megaloblástica y alteraciones en la función cognitiva. Estas dos últimas, consecuencias compartidas con el déficit en vitamina B12 (9).

Sin embargo, existen asociaciones con otros marcadores de salud, que han sido menos estudiadas, o estudiadas sólo en poblaciones adultas o animales de experimentación, o bien obteniendo resultados de gran controversia, algunas de las cuales son objeto de estudio en esta Tesis Doctoral.

Vitamina B6, folato y B12 y salud cardiovascular

En las últimas décadas, la presencia de factores de riesgo de enfermedades cardiovasculares ha aumentado, incluso entre los más jóvenes (61); para lo que la lactancia materna, por ejemplo, ha demostrado tener un efecto protector en la muestra que nos ocupa de adolescentes europeos (62). Entre los principales factores de riesgo, se podrían enumerar las concentraciones elevadas de colesterol total o triglicéridos (TG), y las concentraciones elevadas de colesterol unido a lipoproteínas de baja densidad (LDL en sus siglas en inglés), o las concentraciones bajas de colesterol unido a lipoproteínas de alta densidad (HDL en sus siglas en inglés), que se asocian con un aumento de las concentraciones de homocisteína (tHcy), considerada un predictor independiente de mortalidad cardiovascular (63), como así lo sugieren grandes estudios prospectivos (64). Una revisión sistemática reciente, determinó la ausencia de disminución de eventos cardiovasculares al suplementar con vitaminas del grupo B, con el objetivo de conseguir una disminución de los niveles de tHcy (65). Este hecho, sugiere la posibilidad de que este efecto esté enmascarado por otros factores de riesgo cardiovascular (66) como por ejemplo, concentraciones bajas de ácidos grasos de cadena larga (AGLC) ω3. Esto podría explicarse por el papel de las vitaminas B6, folato y vitamina B12, como cofactores en la metilación de la fosfatidiletanolamina en fosfatidilcolina, que ayuda a transportar los AGLC ω3 en sangre. Sin embargo, los resultados de los estudios (49, 67-71) que han intentado confirmar esta teoría, son controvertidos.

La importancia de este hecho, radica, en que la dieta occidental se caracteriza por un aporte mayoritario de ácidos grasos ω6, siendo el de ω3 muy bajo, o casi inexistente en algunos casos, cuando paralelamente, tienen una gran importancia a nivel de salud del sistema nervioso, visual, y como ya se ha dicho anteriormente, cardiovascular (9).

Vitamina B6, folato, B12 y obesidad y resistencia a la insulina

Por otra parte, el sobrepeso y la obesidad infantil alcanzan cifras que van desde el 10% al 40% en Europa (72). La obesidad supone la quinta causa más importante de muerte en todo el mundo, debido a las morbilidades que conlleva (73). La obesidad está causada por un desequilibrio energético entre las calorías consumidas con la alimentación, y las gastadas mediante la actividad diaria en un contexto multifactorial (74). Paradójicamente, en niños con obesidad se pueden observar deficiencias en micronutrientes (75, 76). Sin embargo, en adolescentes, la literatura es escasa en relación con las vitaminas B6, folato y vitamina B12.

Las personas con obesidad, incluídos niños y adolescentes, tienen mayor riesgo de padecer otras enfermedades, como por ejemplo la diabetes de tipo 2 o las enfermedades cardiovasculares. Además, algunas enfermedades que clásicamente no se habían relacionado con la deficiencia de la vitamina B12, han empezado a hacerlo y así se ha observado en grandes estudios epidemiológicos. Ello, nos ha llevado a preguntarnos si la vitamina B12 podría ser considerado un factor de riesgo independiente o mera casualidad en estas asociaciones (77). En la literatura, los resultados obtenidos hasta ahora, no son muy consistentes (24, 59, 78). Por ejemplo, Pinhas-Hamiel y col. (24) hallaron estas asociaciones en una muestra de niños y adolescentes con sobrepeso y obesidad y Mandy Ho y col (59) más recientemente, también con la resistencia a la insulina. En el estudio de Pinhas-Hamiel (24), se observó cuatro veces mayor riesgo de niveles bajos de vitamina B12 en niños y adolescentes obesos en Israel en comparación con los

normopesos. En el estudio de MacFarlane (79) basado en la población canadiense de la encuesta de salud, se observó que los jóvenes obesos de 6 a 19 años tenían más posibilidades de tener niveles inadecuados de vitamina B12 en comparación con los sujetos con normopeso.

Justificación

La adolescencia es un período de vida crítico en lo que a estado nutricional se refiere: convergen las necesidades nutricionales aumentadas debidas al ritmo rápido de crecimiento y desarrollo, y el cambio en los hábitos alimentarios que suelen ser negativos, con respecto a la etapa anterior. Dada la escasez de literatura científica, la heterogeneidad en sus recomendaciones dietéticas en Europa, y su relevancia para la salud, destacando las enfermedades neurológicas y el cáncer a largo plazo, describir los determinantes y consecuencias de la ingesta y estatus de las vitaminas B6, folato y B12 en adolescentes europeos, son el objetivo principal de esta Tesis Doctoral.

Descubrir cuáles son los factores socioeconómicos más consistentemente asociados con las ingestas y el estatus de las citadas vitaminas (Artículo I), así como definir cuáles son los alimentos (Artículo II), y los patrones dietéticos (Artículo III), que los determinan, suponen un gran avance epidemiológico para el diseño y desarrollo de futuras estrategias preventivas efectivas focalizadas en grupos específicos de edad, sexo, y nivel socioeconómico, con la finalidad de asegurar unos niveles saludables de vitaminas en este grupo poblacional tan vulnerable.

Esto, cobra aún mayor importancia teniendo en cuenta la relevancia clínica, a corto o largo plazo, que puede tener el déficit en estas vitaminas. Es por ello, que se requiere de investigaciones sobre nuevas asociaciones que quizás no hayan quedado bien establecidas previamente, como son las asociaciones con el metabolismo de los ácidos grasos (Artículo IV), o con la composición corporal o la sensibilidad a la insulina (Artículo V).

De este modo, queda patente la unidad temática que comparten los artículos incluidos en la presente Tesis Doctoral y que, secuencialmente abarcan una parte epidemiológica y otra, clínica.

7. Objetivos

En esta sección, se presentarán los objetivos de la Tesis Doctoral. Para entender mejor su concepto general, se presenta previamente un modelo simple de cadena causal, teniendo en cuenta los determinantes y las consecuencias para la salud de la ingesta y el estatus de las vitaminas B6, folato y vitamina B12 (Figure 3).

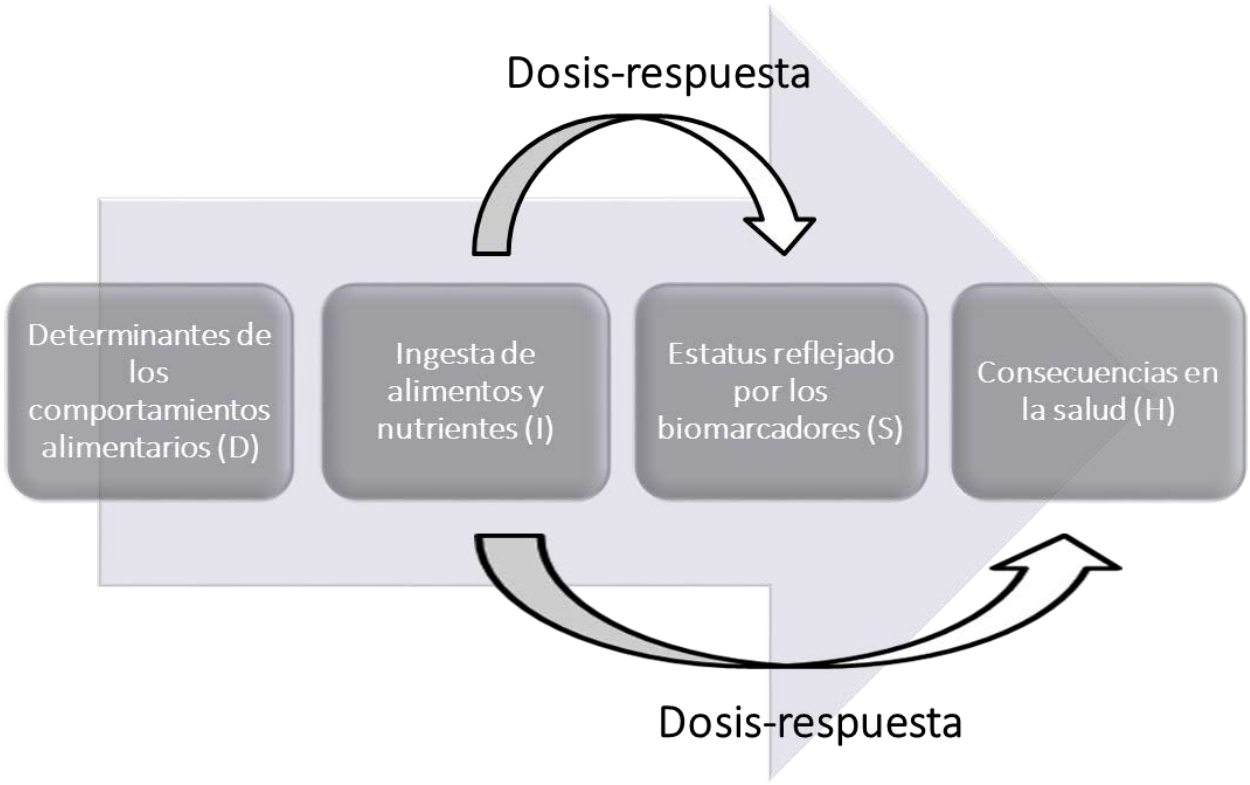


Figura 3. El modelo DISH, que relaciona los alimentos, la nutrición y la salud, describe la relación entre los determinantes de los comportamientos alimentarios (D-*determinants*-), la ingesta de alimentos y nutrientes (I-*intake*-), estado de los biomarcadores y funcionalidad (S-*status*-) y los parámetros de salud relacionados (H-*health*-) (80).

La presente Tesis Doctoral pretende evaluar, por un lado, los determinantes de la ingesta de la vitamina B6, folato y B12, así como los parámetros de salud en relación con la ingesta y los biomarcadores de las citadas vitaminas). No se incluye la valoración de la relación dosis-respuesta, ya que ello necesitaría la realización de un ensayo clínico aleatorio.

Los objetivos son:

Determinantes de la ingesta y estatus de las vitaminas B6, folato y vitamina B12.

Artículo I. Valorar la asociación entre distintos factores socioeconómicos y las ingestas y concentraciones de folato y vitamina B12 en adolescentes europeos.

Artículo II. Identificar los principales grupos de alimentos que determinan la ingesta y el estatus de las vitaminas B6, folato y B12 en adolescentes europeos.

Artículo III. Describir los patrones alimentarios que determinan la ingesta y el estatus de las vitaminas B6, folato y B12 en adolescentes europeos.

Consecuencias para la salud de la ingesta y estatus de las vitaminas B6, folato y vitamina B12

Artículo IV. Valorar la asociación entre los biomarcadores de las vitaminas B6, folato y vitamina B12 y los lípidos y ácidos grasos en los fosfolípidos en suero, en adolescentes europeos.

Artículo V. Evaluar si los adolescentes con mayor índice de masa corporal (IMC), o de masa grasa (IMG), en combinación con la resistencia a la insulina (medida con el Modelo de Valoración Homeostática -índice HOMA-), ingieren y tienen valores más bajos de vitamina B6, folato y vitamina B12.

8. Objectives

The objectives of this thesis are positioned along a simplified model of the causal chain (Figure 3), starting with the determinants of vitamins B6, folate, and B12 intakes and status and leading eventually to health outcomes (Figure 3).

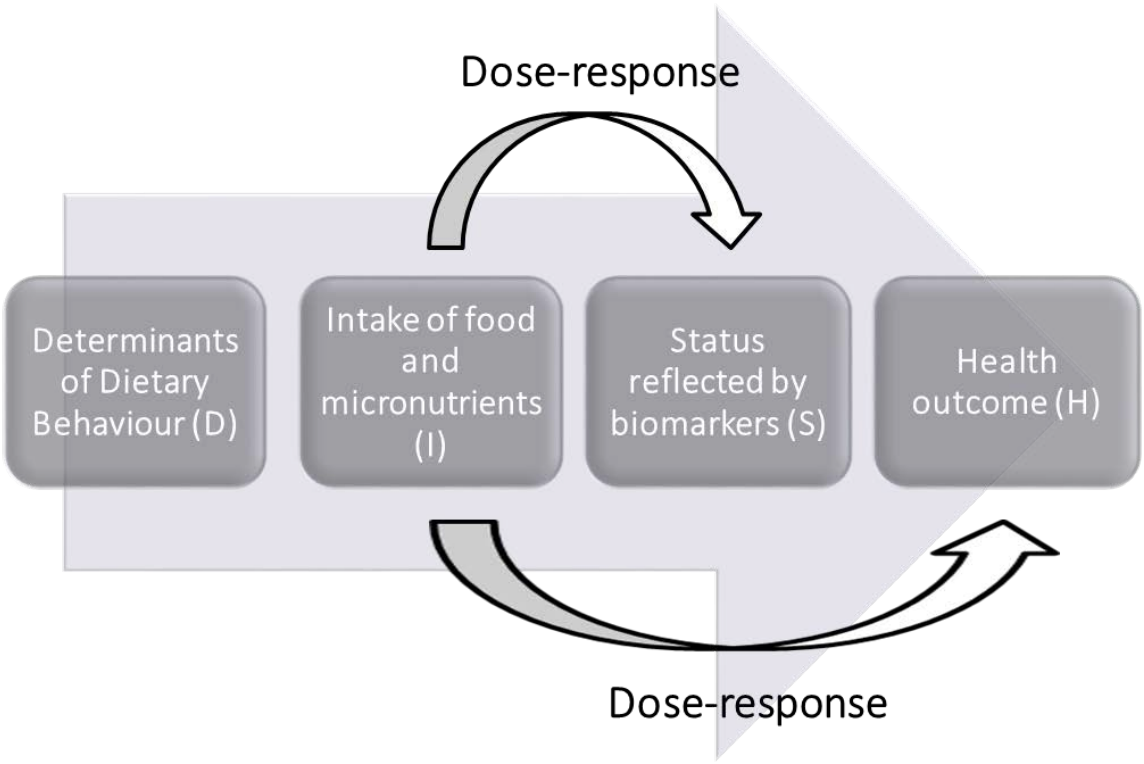


Figure 3. The DISH model for food, nutrition and health research describes the relationship between determinants of dietary behaviour (D), intake of foods and nutrients (I), biomarkers of status and function (S) and health outcome of interest (H) (80).

This PhD thesis evaluates the relation of the first pilar of the aforementioned model (determinants of vitamins B6, folate, and B12 intake and status) with the last pilar referred to as health consequences with regards to vitamin B6, folate, and B12 intakes and statuses. The study design

used for the purposes of undertaking this thesis does not allow analysis of the two central parts (analyses of dose-response) of the model as data form randomized control trials will be required and was outside the scope of the current research.

The objectives are:

Determinants of the intake and status of vitamins B6, folate, and B12

Article I. To examine the association between socioeconomic factors as determinants for intakes and statuses of folate and vitamin B12 to contribute to a better understanding of the DISH model for food, nutrition, and health in European adolescents.

Article II. To identify the main food groups contributing to the intake and status of vitamins B6, folate, and vitamin B12 in European adolescents.

Article III. To identify the dietary patterns which determine both the intake and status of vitamins B6, folate, and vitamin B12 in European adolescents.

Consequences of the intake and status of the vitamins B6, folate, and B12 in health related outcomes

Article IV. To examine the association between vitamin B6, folate, and vitamin B12 biomarkers, and lipids in serum and fatty acids in serum phospholipids in European adolescents.

Article V. To assess whether adolescents with high body mass index (BMI), or fat mass index (FMI), in combination with insulin resistance (assessed with the HOmeostatic Model Assessment –HOMA- index) had also lower blood vitamin B6, folate and vitamin B12 concentrations.

9. Material y métodos

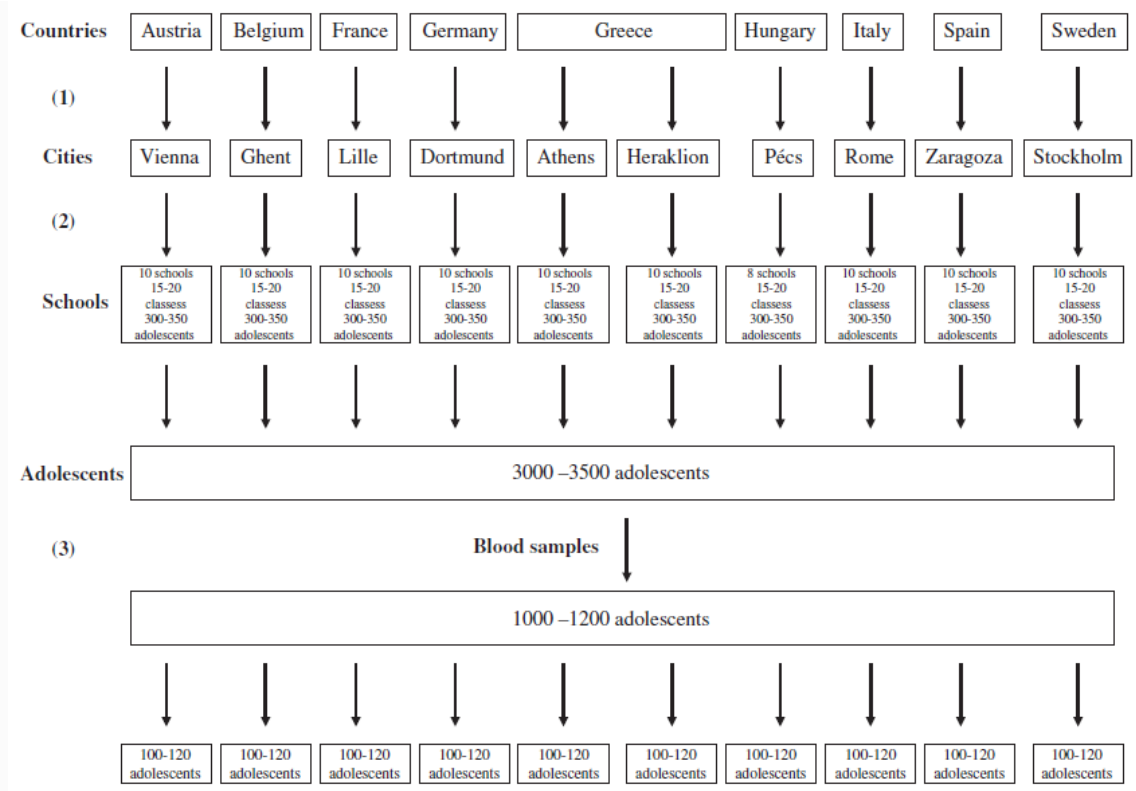
La presente Tesis Doctoral incluye, el estudio de los determinantes de la ingesta y estatus de las vitaminas B6, folato y B12 (Artículos II, III y IV), y de algunas de las posibles consecuencias que podrían tener en la salud (Artículos V y VI) en adolescentes europeos que participaron en el estudio HELENA (81).

Diseño y muestra del estudio

El estudio Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA study), es un estudio transversal y multicéntrico en el que se reclutaron 3,528 adolescentes (47 % varones) de edades comprendidas entre los 12.5 y los 17.5 años, procedentes de 10 ciudades europeas de más de 100,000 habitantes: Dortmund (Alemania), Viena (Austria), Gante (Bélgica), Lille (Francia), Atenas y Heraklion (Grecia), Pécs (Hungría), Roma (Italia), Estocolmo (Suecia), y Zaragoza (España).

Para el reclutamiento, se realizó un muestreo aleatorio por conglomerados, donde los adolescentes se estratificaban por localización geográfica, la edad y el nivel socioeconómico. Se invitó a participar a alumnos de entre una selección de clases y colegios de las citadas ciudades. Los criterios de inclusión del estudio HELENA, consistían en que los adolescentes no podían estar participando en ningún ensayo clínico, no haber estado enfermo la semana anterior a la realización de las pruebas, haber firmado el consentimiento informado. Adicionalmente, y con el fin de poder considerar a los adolescentes para los análisis estadísticos, deberían de tener registrados peso y talla, además del 75% de las pruebas de la batería del estudio HELENA. Esta información puede revisarse también en detalle en el capítulo 2 del Manual de Operaciones de HELENA (82).

Figura 3. Proceso de muestreo del estudio HELENA (83)



Comité de Ética

El protocolo del estudio, se desarrolló según la normativa española y siguiendo las consignas éticas establecidas por la Declaración de Helsinki en 1,964, posteriormente revisadas en el año 2,000 en Edimburgo (84). A continuación, el protocolo fue aprobado por el Comité de Ética de cada centro en el que se llevó a cabo el estudio (85). En Zaragoza, fue el Comité Ético de Investigación Clínica en Aragón (CEICA) (86). Padres o tutores y adolescentes debieron firmar sendos consentimientos informados antes de que éstos últimos realizaran las pruebas (85).

A continuación, se pasan a detallar materiales y métodos específicos de cada una de las pruebas cuyos resultados se han utilizado para la realización de esta Tesis Doctoral.

Factores sociodemográficos/socioeconómicos

La información sociodemográfica se obtuvo mediante un cuestionario completado por los adolescentes, que fue aprobado finalmente por todos los miembros del estudio HELENA (87), y que había demostrado ser una herramienta adecuada para tal fin (88). El cuestionario abarca un amplio rango de factores sociales que incluyen la riqueza familiar mediante unas preguntas que habían sido modificadas por Currie y col (89) llamadas Family Affluence Scale (FAS) (90), la educación de los padres, ocupación de los padres mediante el International Standard Classification of Occupations (ISCO) de 1988 (91), los antecedentes migratorios, y la composición del hogar. Puede encontrarse una descripción detallada de las preguntas realizadas para tal fin en el capítulo 6 del Manual de Operaciones de HELENA (82), y el cuestionario completo en el anexo 2.

Examen físico

Todas las mediciones antropométricas se realizaron siguiendo las pautas internacionales de referencia establecidas por la Sociedad Internacional para el Avance de la Cineantropometría (o en inglés: International Society for the Advancement of Kinanthropometry -ISAK-)(92). Se midió el peso del adolescente (kg) con una báscula electrónica (SECA 861), y la altura (cm) mediante un estadiómetro SECA 225. Posteriormente, se calcularon el índice de masa corporal (IMC) dividiendo el peso en kg por la altura en metros al cuadrado, y el índice de masa grasa (IMG) usando la ecuación de Slaughter (93) para el cálculo de la masa grasa a través de los pliegues tricipital y subescapular, y dividiendo la masa grasa por la altura en metros al cuadrado. La batería completa de medidas antropométricas realizadas, puede consultarse en el capítulo 15 del Manual de Operaciones de HELENA (82).

Biomarcadores en sangre

En los colegios, a primera hora de la mañana, tras un ayuno mínimo de 10 horas, se extrajeron 30 ml de sangre en un tercio de la muestra final de HELENA (1,089 adolescentes) por personal médico o de enfermería (en función de la normativa del país) cualificado, acreditado y experimentado (94). El procedimiento detallado puede consultarse en el capítulo 17 del Manual de Operaciones de HELENA (82).

Vitaminas B

Para la valoración del piridoxal 5´fosfato (PLP), biomarcador de la vitamina B6, la sangre contenida en el tubo con ácido tetraacético etileno diamina (EDTA) fue centrifugado a 3,500 g durante 15 minutos, el plasma fue alicuotado, transportado en frío, después congelado y almacenado a -80°C hasta ser analizado. El PLP se midió mediante cromatografía líquida de alta calidad (HPLC en sus siglas en inglés) (Varian Deutschland GmbH, Darmstadt, Alemania; coeficiente de variación (CV) = 1%) mediante el método modificado de Kimura y col (12, 95) en Laboratorio Central del proyecto HELENA de la Universidad de Bonn (IEL-Institut fuer Ernährungs- und Lebensmittelwissebschaften-, Alemania) (82).

Para la valoración de folato y de vitamina B12 en plasma, se utilizaron tubos con heparina que, tras la extracción, se colocaron en hielo directamente. En los 30 minutos siguientes, se centrifugaron a 3,500 g durante 15 min. A continuación, el plasma se alicuotó, se transportóa una temperatura constante de 4-7°C al Laboratorio Central de la Universidad de Bonn (IEL-Institut fuer Ernährungs- und Lebensmittelwissebschaften-, Alemania) y almacenó a -80°C hasta ser analizado. El tubo con EDTA, se usó también para analizar el folato en glóbulos rojos (red blood

cell folate en inglés). Para ello, la sangre contenida en el tubo con EDTA se diluyó en una ratio de 1:5 en un preparado de ácido ascórbico al 0.1% para la lisis celular e incubado durante 60 minutos en la oscuridad antes de almacenarlo a -80°C. El folato en plasma y en eritrocitos, así como la vitamina B12 en suero y la homocisteína, se analizaron por inmunoensayo competitivo usando Immunolite 2000 analyzer (DPC Biermann GmbH, Bad Nauheim, Alemania) (CV para el folato en plasma = 5.4%, para el folato en eritrocitos = 10.7%, vitamina B12 en suero = 5.0%) (94). El suero para medir la holotranscobalmina (HoloTC), el otro marcador de la vitamina B12, se obtuvo centrifugando la sangre colectada en tubos sin anticoagulante a 3,500 g durante 15 minutos. Una vez enviados al laboratorio central, la sangre fue alicuotada y almacenada a -80°C hasta ser transportada en hielo seco al Laboratorio de la Universidad Politécnica de Madrid para su análisis (Laboratorio número 242). La HoloTC fue medida mediante inmunoensayo de micropartícula enzimática (Active B12 Axis-Shield Ltd, Dundee, Escocia, UK) con el uso de AxSym (Abbot Diagnostics, Abbott Park, IL, USA) (CV = 5.1%) (82, 96).

Perfil lipídico y metabolismo de la glucosa

Las concentraciones de TG, HDL y LDL-colesterol se anlizaron por métodos enzimáticos de rutina mediante Dimension RxL clinical chemistry system (Dade Behring) (82).

Los ácidos grasos en fosfolípidos en suero, se determinaron mediante cromatografía de gas capilar (Model 3900, Varian GmbH, Darmstadt, Germany) tras la extracción de cromatografía de capa fina (82) usando el estándar 1,2 Dipentadecanoil-sn-glicero-3-fosfocolina (97).

La glucosa fue medida mediante métodos enzimáticos de rutina (Dade Behring, Schwalbach, Alemania). Los niveles de insulina se obtuvieron usando el Immulite 2000 analyser (DPC Bierman GmbH, Bad Nauheim, Alemania) (82). El modelo de valoración homeostático (HOMA) se calculó a través de la resistencia a la insulina (glicemia X insulina/22.5) (98).

Dieta

La dieta de los adolescentes, se evaluó mediante 2 recuerdos de 24 horas electrónicos y autoadministrados en un programa de ordenador específicamente diseñado para tal fin. En todo momento había personal entrenado para resolver cualquier duda que les pudiera surgir en su autocompletado. Esta plataforma se llamaba HELENA-Dietary Assessment Tool (HELENA-DIAT), basado en un software previo que había sido diseñado para adolescentes belgas y denominado YANA-C (99) y sus principales características pueden consultarse en el capítulo 11 del Manual de Operaciones de HELENA (82). Los dos recuerdos se realizaron en días no consecutivos y en un plazo máximo de dos semanas y siempre en día lectivo, por lo que no se dispone de información relativa a viernes ni sábados. Las tomas dietéticas se dividieron en 6: desayuno, almuerzo, comida, merienda, cena y recena. A posteriori, los datos fueron vinculados a las tablas de composición de alimentos alemanas (German Food Code and Nutrition Database) (100) para obtener las cantidades de calorías en kcal/d y las de nutrientes en g/d. mg o μg/d para el caso de las vitaminas.

Los datos relativos a las ingestas dietéticas de los adolescentes de Pécs y Heraklion tuvieron que ser excluidos debido a que no estaban completos.

Además, se calculó el consumo habitual de cada uno de los grupos de alimentos obtenidos con una corrección de variación intra-individuos, mediante el método de fuente múltiple, o en inglés *multiple source method* (MSM) (101).

El índice de calidad de la dieta (o *Diet Quality Index*, en sus siglas en inglés *DQI*) se obtuvo a través de los dos recuerdos de 24 horas mediante el programa ya mencionado *HELENA Dietary Assessment Tool (HELENA DIAT)* (99). Este *DQI*, inicialmente se había diseñado para su uso en niños (102), y fue posteriormente adaptado y validado para su uso en adolescentes (103). Para su

cálculo, el índice utiliza tres componentes: la calidad, la diversidad y el equilibrio de la dieta (103).

Actividad física

Para valorar los niveles de actividad física de los adolescentes, se usó el cuestionario internacional validado para los adolescentes, en inglés International Physical Activity Questionnaire for Adolescents (IPAQ-A), adaptado y validado para el estudio HELENA (104). El IPAQ-A cubrió cuatro dominios de la actividad física realizada durante la semana: la actividad física en el colegio o instituto (incluyendo la realizada durante las clases de actividad física, y la practicada de manera libre durante los recreos), el método de transporte, actividades en casa y actividades en el tiempo de ocio. Después, esas actividades, fueron clasificadas según su intensidad en bajas, moderadas y vigorosas siguiendo estándares internacionales (105). Para mayor información sobre las características e información recogida por este cuestionario, conviene consultar el capítulo 12 del Manual de Operaciones de HELENA (82), además del Anexo 3.

Análisis estadístico

Todos los análisis estadísticos presentados en los artículos que conforman esta Tesis Doctoral, se han realizado separando por sexo o género, dado que en relación con los estilos de vida y las variables que en esta Tesis nos ocupan, siempre se han hallado diferencias significativas entre chicas y chicos.

Como norma general, en todos los artículos se presentan tablas descriptivas que muestran las diferencias entre chicos y chicas para las variables de interés en el artículo en concreto, y a

continuación, análisis bivariado de las variables de interés separado por sexos, que darán paso a análisis estadísticos más complejos, como lo es, por ejemplo, la regresión lineal multivariada.

La tendencia en esta Tesis Doctoral, ha sido la de usar análisis estadísticos paramétricos por su mayor potencia estadística, cuando las variables de interés así lo han permitido. De no haber sido así porque las variables no cumplieran la hipóTesis de una distribución normal (test de Kolmogorov-Smirnof), el paso previo a los análisis, ha sido una transformación de las variables para intentar que cumpliera los requisitos de una variable de distribución normal mediante los métodos convencionales: transformación logarítmica o con la raíz cuadrada.

Las covariables empleadas en los análisis estadísticos han sido el valor z standard del IMC ajustado para la edad y el sexo, la educación de la madre, la actividad física, y la ingesta calórica de la dieta en Kcal/día.

Los análisis estadísticos se llevaron a cabo utilizando el paquete estadístico *Predictive Analytics Software* (PASW) en su versión para Windows SPSS 20.0 (SPSS Inc., Chicago, IL, USA), excepto para la regresión de rangos reducidos, para la que se usó el programa estadístico SAS 9.3 para Windows (SAS Institute Inc., Cary, NC).

Artículo I

La asociación entre las ingestas y los biomarcadores del folato y de la vitamina B12 (variables dependientes), y el nivel socioeconómico de los adolescentes valorado mediante 7 diferentes parámetros (pasado migratorio, composición del hogar, riqueza familiar, educación de padre y madre, y ocupación de padre y madre) (variables independientes), ajustadas por los covariables generales citadas anteriormente, se evaluaron mediante regresión lineal múltiple. Además, las diferencias entre medias de las ingestas y los biomarcadores de las vitaminas B entre las diferentes categorías de las variables en relación al nivel socioeconómico, fueron evaluadas mediante ANCOVA, usando las correcciones de Bonferroni para comparaciones múltiples.

Artículo II

Se calcularon los terciles de las ingestas y los biomarcadores de las vitaminas B6, folato y B12. Estas distribuciones se introdujeron en un modelo de análisis discriminante junto con los 31 grupos de alimentos en los que se habían agrupado los consumos procedentes del recuerdo de 24 horas (HELENA-DIAT). Con ello, se establecieron cuáles eran los grupos de alimentos que conseguían discriminar mejor entre el primer tercil y el tercer tercil de las ingestas y niveles de las vitaminas. Además, se realizó un modelo mixto lineal incluyendo los efectos aleatorios introducidos por el hecho de pertenecer a los distintos centros europeos en los que se llevó a cabo HELENA, con el fin de observar las asociaciones lineales entre el consumo de los 31 grupos de alimentos y las ingestas y niveles de las vitaminas.

Artículo III

En este artículo, se realize una correlación no paramétrica de Spearman entre los 31 grupos de alimentos y las ingestas y los niveles de las vitaminas B6, folato y B12. Después, se realizó una regression de rangos reducidos (*reduced rank regression* -o *RRR*- en sus siglas en inglés) para agrupar el consumo de alimentos (y establecer patrones) que fueran capaces de explicar el mayor porcentaje de varianza de nuestras variables respuesta (ingesta y niveles de vitaminas). Los grupos de alimentos podían *cargar* al patrón positiva o negativamente. Cargas positivas al patrón, implicaban que el grupo de alimentos estaba positivamente asociado al patrón, y cargas negativas, significaban que el alimento estaba negativamente asociado al patrón. También, cargas más altas indicaban una mayor asociación al patrón que cargas más bajas. Previamente, los consumos de los diferentes grupos de alimentos, fueron ajustados por las covariables habituales: puntuación típica del IMC, educación de la madre e ingesta de energía, usando modelos lineares mixtos para introducir el efecto del centro, y en el RRR se introdujeron los residuos de los grupos de alimentos.

Artículo IV

En este artículo, se examinaron las asociaciones entre el perfil lipídico y los ácidos grasos en los fosfolípidos en suero, y los niveles de vitaminas B6, folato, B12 y homocisteína. Estas asociaciones se observaron mediante modelos lineares de efecto mixto para controlar también por el efecto aleatorio introducido por el centro de los adolescentes, y usando las covariables habituales.

Artículo V

En este artículo, se usó la mediana de las puntuaciones típicas del IMC, índice de masa grasa (*fat mass index -FMI-* en inglés), y del índice HOMA (sensibilidad a la insulina) ajustadas por sexo y edad. Se usó el test Mann-Whitney para examinar las diferencias entre la ingesta y los niveles de las vitaminas B6, folato y B12 entre los adolescentes que quedaban por encima y por debajo de las medianas de las citadas variables (IMC, FMI, HOMA). Después, los grupos resultantes de cada variable se combinaron con los resultantes de las otras para ver si las diferencias observadas en las ingestas y los niveles de las vitaminas se mantenían al combinar las categorías "más saludables" (menores IMC y menores FMI o HOMA) y las "menos saludables" (mayores IMC y mayores FMI o HOMA). El test Kruskal-Wallis fue el que se aplicó para examinar las diferencias de las ingestas y los niveles de las vitaminas entre los 4 grupos resultantes al combinar las dos categorías procedentes de cada variable. Después se realizó una ANCOVA para introducir las variables habituales de ajuste en estas comparaciones de las ingestas y niveles de vitaminas entre las cuatro categorías.

10. Resultados (Results)

Los resultados de la presente Tesis Doctoral se muestran en forma de artículos científicos publicados en revistas de impacto según el Journals Citation Report (JCR). El formato en el que se incluyen, es en el que han quedado finalmente publicados en las revistas.

The results of this PhD Thesis are shown in the form of peer-reviewed scientific articles published in impact journals following the guides of the Journals Citation Report (JCR) and are presented in their original published form.

Artículo I/ Paper I published in *Nutrition Research*

IF: 2.472 (Q2 in NUTRITION & DIETETICS)

Socioeconomic factors are associated with folate and vitamin B12 intakes and related biomarkers concentrations in European adolescents: The HELENA Study

Artículo II/ Paper II published in *European Journal of Nutrition*

IF: 3.239 (Q2 in NUTRITION & DIETETICS)

Foods contributing to vitamin B6, folate, and vitamin B12 intakes and biomarkers status in European adolescents: The HELENA study

Artículo III/ Paper III Revision submitted to *Nutrition*

IF: 2.839 (Q2 in NUTRITION & DIETETICS)

Do dietary patterns determine levels of vitamin B6, folate, and vitamin B12 intakes and corresponding biomarkers in European adolescents? The HELENA study

Artículo IV/ Paper IV published in *British Journal of Nutrition*

IF: 3.311 (Q2 in NUTRITION & DIETETICS)

Folate and vitamin B12 concentrations are associated with plasma docosahexaenoic and eicosapentaenoic fatty acids in European adolescents: the HELENA study

Artículo V/ Paper V accepted for publication in *Nutrición Hospitalaria*

IF: 1.497 (Q4 in NUTRITION & DIETETICS)

Associations between insulin resistance and three B-vitamins in European adolescents: The HELENA study

Determinants

Socioeconomic factors are associated with folate and vitamin B12 intakes and related biomarkers concentrations in European adolescents: the Healthy Lifestyle in Europe by Nutrition in Adolescence study

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Socioeconomic factors are associated with folate and vitamin B_{12} intakes and related biomarkers concentrations in European adolescents: the Healthy Lifestyle in Europe by Nutrition in Adolescence study

Iris Iglesia[a,b], *Theodora Mouratidou*[a,b], *Marcela González-Gross*[c], *Romana Novakovic*[d], *Christina Breidenassel*[c,e], *David Jiménez-Pavón*[a,b], *Inge Huybrechts*[f,g], *Stefaan De Henauw*[f], *Anouk Geelen*[h], *Frédéric Gottrand*[i], *Anthony Kafatos*[j], *Lorenza Mistura*[k], *Fátima Pérez de Heredia*[l], *Kurt Widhalm*[m], *Yanis Manios*[n], *Denes Molnar*[o], *Peter Stehle*[e], *Mirjana Gurinovic*[d], *Adrienne E.J.M. Cavelaars*[h], *Pieter Van't Veer*[h], *Luis A. Moreno*[a,b,*]
On behalf of the HELENA study group

[a] *GENUD: "Growth, Exercise, Nutrition and Development" Research Group, University of Zaragoza, Zaragoza, Spain*
[b] *Faculty of Health Sciences, University of Zaragoza, Zaragoza, Spain*
[c] *ImFINE Research Group, Department of Health and Human Performance, Universidad Politécnica de Madrid, Madrid, Spain*
[d] *Institute for Medical Research, Centre of Research Excellence in Nutrition and Metabolism, University of Belgrade, Belgrade, Serbia*
[e] *Department of Nutrition and Food Science, University of Bonn, D-53115 Bonn, Germany*
[f] *Department of Public Health, Ghent University, Ghent, Belgium*
[g] *International Agency for Research on Cancer (IARC), 150 Cours Albert Thomas, 69372 Lyon Cedex 08, France*
[h] *Division of Human Nutrition, Wageningen University, Wageningen, The Netherlands*
[i] *Inserm U995, Faculté de Médecine, Université Lille 2, Lille, France*
[j] *University of Crete School of Medicine, GR-71033 Crete, Greece*
[k] *Human Nutrition Unit, INRAN, Rome 00100, Italy*
[l] *Immunonutrition Research Group, Department of Metabolism and Nutrition, Institute of Food Science, Technology and Nutrition (ICTAN), Spanish National Research Council (CSIC), Madrid, Spain*
[m] *Division of Clinical Nutrition and Prevention, Department of Pediatrics, Medical University of Vienna, Vienna, Austria*
[n] *Department of Nutrition and Dietetics, Harokopio University, Athens, Greece*
[o] *Department of Paediatrics, University of Pécs, Pécs, Hungary*

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ABSTRACT

Because socioeconomic factors (SEFs) may influence dietary quality and vitamin intakes, this study aimed to examine associations between socioeconomic factors and folate and vitamin B_{12} intakes as well as their related biomarkers in the Healthy Lifestyle in Europe by Nutrition in Adolescence study. Vitamin intakes were obtained from two 24-hour recalls in 2253 participants (47% males). Vitamin B biomarkers were assessed in a subsample of 977

Abbreviations: BMI, body mass index; CI, confidence intervals; EAR, Estimated Average Requirement; EURRECA, EURopean RECommendations Aligned; FAS, Family Affluence Scale; HELENA, Healthy Lifestyle in Europe by Nutrition in Adolescence; holoTC, holotranscobalamin; RBC, red blood cell; SEF, socioeconomic factor.

* Corresponding author at: Universidad de Zaragoza, E.U. Ciencias de la Salud, C/Domingo Miral s/n, 50009 Zaragoza, Spain. Tel.: +34 876554457; fax: +34 976761720.
E-mail address: lmoreno@unizar.es (L.A. Moreno).

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participants (46% males). Socioeconomic factors were assessed by questionnaire, and 1-way analysis of covariance and linear regression analysis were applied. For males and females, mean intakes of folate were 211.19 and 177.18 μg/d, and for vitamin B_{12}, 5.98 and 4.54 μg/d, respectively. Levels of plasma folate, red blood cell folate, serum B_{12}, and holotranscobalamin were 18.74, 807.19, 330.64, and 63.04 nmol/L in males, respectively, and 19.13, 770.16, 377.9, and 65.63 nmol/L in females, respectively. Lower folate intakes were associated with several SEFs, including maternal and paternal education in both sexes. Regarding folate biomarkers, lower plasma folate intakes were associated with single/shared care in males and with lower paternal occupation in females. Lower vitamin B_{12} intakes were associated with almost all the studied SEFs, except paternal occupation in both sexes. In females, when considering vitamin B_{12} biomarkers, lower plasma vitamin B_{12} was associated with lower maternal education and occupation, and lower holotranscobalamin was associated with lower maternal education and lower paternal occupation. In conclusion, from the set of socioeconomic determinants studied in a sample of European adolescents, maternal education and paternal occupation were more consistently associated with folate and vitamin B_{12} intakes and biomarkers concentrations.

1. Introduction

During critical periods of rapid growth and development, such as adolescence, adequate micronutrient status is essential [1]. Irregular patterns in meals and increased consumption of unhealthy products during this period may lead to nutritional deficits, especially when considering micronutrients [2]. Folate [3] and vitamin B_{12} [4] deficiencies during childhood and adolescence are not uncommon, at least at the subclinical level [5]. These vitamins are important contributors for healthy growth and development due to their significant role in cell formation [6].

Socioeconomic factors (SEFs) are known to influence dietary quality [7] and vitamin intakes [8]. The reported associations between diet quality and health, found in epidemiologic studies, may have been mediated by unreported SEF; however, available literature addressing such issues is limited. For instance, the results of the National Diet and Nutrition Survey of 4 to 18 years old in the UK [8] indicated that participants in lower socioeconomic positions (social class status of the head of the household, benefits received by the young person's household, household income and family composition) tended to have lower micronutrient intakes and corresponding biomarker concentrations. There is a positive association between folate intakes and maternal/paternal but not between folate biomarkers and maternal/paternal education in a sample of Swedish adolescents participating in The Nord-Trondelag Health Study [9]. The most appropriate SEF in epidemiological studies is subject to debate, and often, the final choice reflects data availability and study resources [10]. For instance, social factors such as household composition and migration background [11] have demonstrated linked strong correlation with eating behavior [12]. Economic factors such as education, occupation, and income [7] are also considered to be related to health outcomes due to their direct influence on lifestyle behaviors [13].

To develop effective strategies for health promotion, differences between SEF regarding health-related issues highlight the need to use parallel social and economic factors to obtain an in-depth picture of the influence that social inequalities play on dietary habits. These challenged proxies are mediated by the reporting bias in dietary assessment. For instance, it is well established that higher position socioeconomic groups tend to overreport healthy foods in dietary surveys [14]. Because of this, serum vitamin analyses may be essential to better understand the interrelations between SEF and micronutrient adequacy [15,16].

In terms of intakes and plasma concentrations in relation to SEF among adolescents in Europe, there is a lack of large studies addressing micronutrient status. The present study aims to examine socioeconomic factors as determinants for intakes and statuses and will contribute to a better understanding of the DISH model for food, nutrition, and health research [17] (see Fig., adapted from Romana Novakovic's thesis [18]). In this case of the Romana-Novakovic's thesis, the relationships are based on folate and vitamin B_{12} intakes and their related blood concentrations in a large sample of European adolescents aged 12.5 to 17.5 years.

2. Methods and materials

2.1. *Subjects, recruitment, and study design*

The Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) Cross-Sectional Study is a multicenter study of lifestyle and nutrition among adolescents from 10 European cities from 9 countries; Athens and Heraklion (Greece), Dortmund (Germany), Ghent (Belgium), Lille (France), Pecs (Hungary), Rome (Italy), Stockholm (Sweden), Vienna (Austria), and Zaragoza (Spain). The mean participation rate for adolescents in our study was 67%, which is considered acceptable for such a demanding epidemiological study [19]. Inclusion criteria were as follows: 12.5 to 17.5 years old, not simultaneously participating in another clinical trial, and free of any acute infection occurring less than 1 week before inclusion [20]. A total of 3528 adolescents (47% males) were recruited between October 2006 and December 2007. Data from Heraklion and Pecs were not included in the dietary intake analysis (7% of the total sample) because no nutrient

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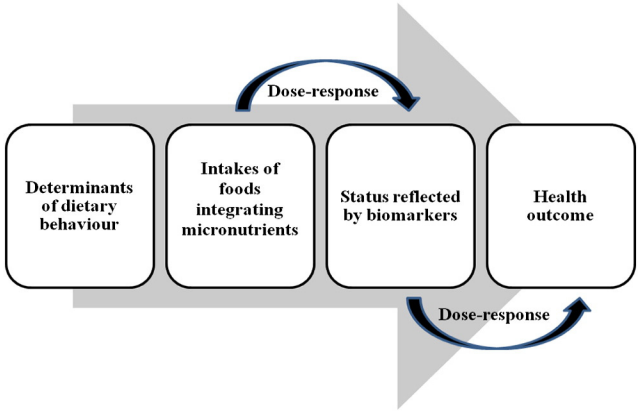

Fig. – The DISH model for food, nutrition, and health research describes the relationship between determinants of dietary behavior (D), intake of foods and nutrients (I), biomarkers of status and function (S), and health outcome of interest (H). Adapted from Romana Novakovic's thesis [18].

intake was calculated for these 2 cities. The final sample available for the dietary analysis included 2253 adolescents (46% males) with complete data on 2 nonconsecutive 24-hour recalls and requested SEF. A random subsample of 977 adolescents (46% males) were included in the blood parameters analysis that was related to vitamin status (data from Heraklion and Pecs were included in this instance). More details on the sampling procedures, pilot study, and reliability of the data are published elsewhere [20]. The protocol was approved by the Human Research Review Committees of the corresponding centers (cities) [21], and informed consent was obtained from all participants and/or their parents [21].

2.2. *Assessment of SEF*

Information on SEF was obtained via a self-administered questionnaire that was completed by the adolescents. The questionnaire addressed a wide range of social factors, including family affluence, paternal and maternal education, paternal and maternal occupation levels, migration background, and household composition. As some questions included in the socioeconomic status questionnaire produced difficulties, these questions were reformulated or deleted. The SES questionnaire was then modified, corrected, and finally approved by all partners involved in the HELENA [22]. The methodology used to assess SEF had been previously demonstrated to be an accurate proxy for parental socioeconomic status [23]. A modified version of the Family Affluence Scale (FAS), developed by Currie et al [24], was used. The FAS [25] included questions on (i) bedroom availability, (ii) number of family cars, (iii) number of personal computers at home, and (iv) Internet availability. Thereafter, for every possible answer, a numerical value was given and a final score was created for each individual, ranging from 0 to 8. Scores were grouped into 3 levels: low (0-2), medium (3-5), and high (6-8) [26].

Parental education (both paternal and maternal) was assessed using 4 levels (elementary, lower secondary, higher secondary, or tertiary education). In terms of parental occupation, reported information was classified into 1 of the 12 classification categories that were based on the International Standard Classification of Occupations from 1988 [27]. Subsequently, they were grouped into 4 categories: (i) unemployed, (ii) low occupational level, (iii) medium occupational level, and (iv) high occupation level. Mean intakes and biomarker levels were used in these 4 categories. However, both parental education and occupation were categorized into 2 categories (low and high, grouping the lowest and the highest categories together) to make the found associations easier to interpret. Information on parental migration backgrounds was also obtained by the following queries: (i) if both parents were born abroad, (ii) if only 1 parent was born abroad, and (iii) if both parents were born in the country where the study was performed. Household composition included (i) those living in single-parent families (either lone parent household or "shared-care" between parents) and (ii) those living with both parents (parents and/or step parents) [28]. The coding of the SEF (as indicated by ascending numbers) denoted gradual higher socioeconomic position: (i) low position, (ii) medium-low position, (iii) medium-high position, and (iv) high position. Subsequent comparisons considered the high position category as the reference group.

2.3. *Weight and height*

Following standard protocol procedures, weights were measured with participants in underwear and without shoes on an electronic scale (Type SECA 861) and rounded to the nearest 0.05 kg. Height was measured without shoes in the Frankfort plane with a telescopic height measuring instrument (Type SECA 225) and rounded to the nearest 0.1 cm. Body mass index (BMI) was calculated using the Quetelet formula and used as a covariate.

2.4. *Assessment of folate and vitamin B_{12} intakes*

The dietary intake analysis included 1029 males and 1224 females from 8 centers. Dietary intakes were assessed using the HELENA-Dietary Assessment Tool self-administered, computerized 24-hour recall that was based on the Young Adolescents' Nutrition Assessment software and adapted for European adolescents [29]. The HELENA-Dietary Assessment Tool is based on 6 "meal occasions" (breakfast, morning snack, lunch, afternoon snack, evening meal, and evening snack). Supported by trained staff that included dieticians, the adolescents completed the 24-hour recalls at school time, twice within a 2-week timeframe [29]. Data were linked to the German Food Code and Nutrient Data Base (BLS, Bundeslebensmittelschlüssel, version II.3.1, 2005), which contains approximately 12 000 coded foods, menus, and menu components and encompassing up to 158 nutrient data points for each product. Data from each country were linked to the database to ensure standardization of available measures. If a food item was missing in the German food composition table, then calculations were made via recipes or a local food composition table for the specific country [29]. The Multiple Source Method [30] is a new statistical method used for

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calculating usual dietary intake based on 2 or more dietary assessment methods, such as 24-hour dietary recalls, and it may include habitual use or nonuse of a food as a covariate in the model as well as a parameter for identifying consumers and nonconsumers. The result is a method that removes the effect of day-to-day within-person variability and random error in the recalls. The software is hosted on a Web site established at the German Institute of Human Nutrition and can be accessed at https://nugo.dife.de/msm.

2.5. Assessment of folate and vitamin B_{12} biomarkers concentrations

A blood sample was taken on the day of the first 24-hour recall [31]. At a school setting in the early morning and following an overnight fast, 30 mL of blood was drawn, according to a standardized blood collection protocol. More details on sample transport and quality assurance can be found elsewhere [32].

For the measurement of plasma folate and serum cobalamin, heparinized tubes were collected, placed immediately on ice, and centrifuged within 30 minutes (3500*g* for 15 minutes). The supernatant fluid was transported at a stable temperature of 4°C to 7°C to the central laboratory at the University of Bonn (Institut fuer Ernährungs und Lebensmittelschaften, Germany) and stored at –80°C until assayed. After measuring the hematocrit in situ, EDTA whole blood was used for the red blood cell (RBC) folate analysis. Before storage at 80°C, EDTA whole blood was diluted 1:5 with freshly prepared 0.1% ascorbic acid for cell lysis and incubated for 60 minutes in the dark. Plasma and RBC folate and serum cobalamin were measured by means of a competitive immunoassay, using the Immunolite 2000 analyzer (DPC Biermann GmbH, Bad Nauheim, Germany) (coefficient of variation for plasma folate, 5.4%; RBC folate, 10.7%; cobalamin, 5.0%) [32]. Serum for measuring holotranscobalamin (holoTC) was obtained by centrifuging blood collected in evacuated tubes without anticoagulant at 3500*g* for 15 minutes within 1 hour. Once sent to the laboratory, the sera were aliquoted and then stored at –80°C in dry ice until transport to the biochemical lab at the Universidad Politécnica de Madrid for analysis (Laboratory number 242 of the Laboratory Network of the Region of Madrid). Holotranscobalamin was measured by microparticle enzyme immunoassay (Active B_{12} Axis-Shield Ltd, Dundee, Scotland, UK) with the use of AxSym (Abbot Diagnostics, Abbott Park, IL) (coefficient of variation, 5.1%) [33].

2.6. Statistical analyses

The SPSS version 17.0 (SPSS, Inc, Chicago, IL) was used to analyze the data. All statistical tests and corresponding *P* values were 2 sided, and $P < .05$ was considered statistically significant. Statistical analysis was stratified by sex. Descriptive data are presented as means and SE, and confidence intervals (CI) were 95%. This study focused on city-based samples and tried to accurately represent each city. Statistically, the group is considered homogeneous without the variability caused by the center.

For this study, we used the reference values for vitamin intakes based on the American Estimated Average Requirement (EAR) [34] and used the reference values previously used in another publication based on the HELENA study for the biomarkers [35]. Variables were log transformed to improve their normality in distribution.

Initially, a generalized linear model with the inclusion of a random intercept for study center was used to examine the relationship between related intakes and biomarker concentrations and SEF. Age, BMI, and adjusted energy intakes (kilocalories) were entered as covariates, based on other similar studies such as the one in 2001 from Galobardes et al [36]. Because of the observed low variance component associated with the study center (<5%), the authors proceeded with models that did not include random effects for center. Therefore, the relationship between folate and vitamin B_{12} intakes and biomarkers concentrations (dependent variables) and SEF (independent variables) was examined using multiple linear regression analysis. Age, BMI, adjusted energy intakes (kilocalories), and the set of SEFs were included in the model as covariates to establish the associations between SEF and vitamins, both intakes and biomarkers, independently. Values are presented as adjusted β values (estimated unstandardized regression coefficient) and 95% CI. In addition, differences in folate and vitamin B_{12} mean intakes and respective biomarkers concentrations, according to SEF, were analyzed by 1-way analysis of covariance, adjusted for age, BMI, and Multiple Source Method adjusted energy intakes (kilocalories). Bonferroni corrections were used for post hoc multiple comparisons test, and the *P* for trend was provided based on the F test. This information is provided in the supplemental material.

3. Results

Subjects included in the dietary analysis were significantly older than those not included, and they also had lower BMI and lower energy (kilocalories) consumption. The ratio of males/females was significantly higher in subjects not included ($P < .05$). In addition, they differ significantly in terms of all SEF variables addressed ($P < .05$) apart from parental migration background. However, the found associations between SEF and vitamins intakes and statuses were the same when considering also excluded adolescents. The biomarkers concentrations analysis included 457 males and 520 females from all 10 centers. The characteristics of the subjects included in the biomarker analysis did not differ significantly from those not included. Table 1 presents the adolescents' characteristics by SEF categories, stratified by sex and dietary and biomarker groups.

3.1. Folate intakes and the related biomarkers between different SEF groups

Mean intakes of folate were 211.19 for males (3% meet the American EAR) and 177.18 for females (2% meet the American EAR). Levels of plasma folate and RBC folate for males were 18.74 and 807.19 nmol/L, and for females, 19.13 and 770.16 nmol/L, respectively. Among males, 18.4% and 2% did not meet plasma folate and RBC folate concentration recommendations. These percentages for females were 17.7% and 2%, respectively. Adolescents in categories that denoted

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Table 1 – Socioeconomic factors in the dietary and biomarker samples of European adolescents

Characteristic	Dietary intake sample		Biomarkers sample	
	Males, n (%)	Females, n (%)	Males, n (%)	Females, n (%)
Sex	1029 (46.0)	1224 (54.0)	457 (47.0)	520 (53.0)
Age (mean, SE, 95% CI)	14.8, 0.4, 14.7-14.9	14.7, 0.0, 14.7-14.8	14.8, 0.1, 14.7-14.9	14.7, 0.1, 14.6-14.8
BMI (mean, SE, 95% CI)	21.3, 0.1, 21.1-21.5	21.2, 0.1, 21.1-21.4	21.5, 0.2, 20.7-21.7	21.2, 0.1, 20.9-21.6
Energy in kilocalories (mean, SE, 95% CI)	2531.03, 26.8, 2478.5-2583.6	1941.06, 17.4, 1907.0-1975.2	2600.29, 51.8, 2498.4-2702.2	1950.90, 31.9, 1888.2-2013.6
FAS				
Low FAS	83 (8.1)	140 (11.4)	49 (10.7)	78 (15.0)
Medium FAS	589 (57.2)	664 (54.2)	267 (58.4)	291 (56.0)
High FAS	351 (34.1)	141 (33.8)	141 (30.9)	150 (28.8)
Migrant background				
Both parents born abroad	65 (6.3)	72 (5.9)	18 (3.9)	22 (4.2)
One parent born abroad	50 (4.9)	65 (5.3)	21 (4.6)	19 (3.7)
Both parents born in survey's country	887 (86.2)	1050 (85.8)	413 (90.4)	468 (90.0)
Household composition				
Single/shared care	297 (28.9)	355 (29.0)	116 (25.4)	13 (26.3)
Traditional family	732 (71.1)	869 (71.0)	341 (74.6)	383 (73.7)
Maternal education				
Low	62 (6.0)	80 (6.5)	32 (7.0)	52 (10.0)
Medium-low	269 (26.1)	300 (24.5)	113 (24.7)	124 (23.8)
Medium-high	286 (27.8)	368 (30.1)	142 (31.1)	174 (33.5)
High	356 (34.6)	416 (34.0)	150 (32.8)	155 (29.8)
Paternal education				
Low	40 (3.9)	84 (6.9)	34 (7.4)	42 (8.1)
Medium-low	284 (27.6)	330 (27.0)	123 (26.9)	146 (28.1)
Medium-high	258 (25.1)	297 (24.3)	130 (28.4)	156 (30.0)
High	365 (35.5)	403 (32.9)	145 (31.7)	156 (30.0)
Maternal occupation				
Low	208 (20.2)	246 (20.1)	95 (20.8)	110 (21.2)
Medium-low	196 (19.0)	204 (16.7)	88 (19.3)	77 (14.8)
Medium-high	390 (37.9)	494 (40.4)	186 (40.7)	196 (37.7)
High	142 (13.8)	180 (14.7)	62 (13.6)	99 (19.0)
Paternal occupation				
Low	63 (6.1)	62 (5.1)	31 (6.8)	31 (6.09
Medium-low	312 (30.3)	323 (26.4)	143 (31.3)	135 (26.0)
Medium-high	304 (29.5)	344 (28.1)	143 (31.3)	128 (24.6)
High	220 (21.4)	322 (26.3)	110 (24.1)	180 (34.6)
Center (cities)				
Athens	123 (12.0)	140 (11.4)	38 (8.1)	56 (10.8)
Dortmund	218 (21.2)	165 (13.5)	62 (13.6)	40 (7.7)
Gent	139 (13.5)	165 (13.5)	56 (12.3)	50 (9.6)
Lille	82 (8.0)	124 (10.1)	33 (7.2)	49 (9.4)
Heraklion	–	–	39 (8.5)	42 (8.1)
Rome	95 (9.2)	152 (12.4)	46 (10.1)	48 (9.2)
Stockholm	107 (10.4)	184 (15.0)	44 (9.6)	47 (9.0)
Vienna	157 (15.3)	177 (14.5)	42 (9.2)	59 (11.3)
Zaragoza	108 (10.5)	117 (9.6)	47 (10.3)	58 (11.2)
Pecs	–	–	49 (10.7)	71 (13.7)

higher socioeconomic positions had significantly higher intakes and biomarkers levels of folate compared with those in the rest of the socioeconomic positions (Supplementary Table 1). More specifically, in males, folate intakes were significantly different for household composition ($P < .05$) and paternal education (medium-low vs high position groups at $P < .05$ level). In females, folate intakes were significantly different for paternal education (medium-low vs medium-high and medium-low vs high, position groups, at $P < .05$ level) (Supplementary Table 1).

Regarding biomarkers for plasma folate concentrations, significant differences were observed in males based on household composition ($P < .05$), thus indicating that males living with both parents had higher plasma folate values (19.03 nmol/L) in comparison with those living in single-parent families (15.78 nmol/L) ($P < .05$). In females, no significant differences were observed.

Maternal education was the only SEF for which significant differences were found for RBC folate concentrations, both in males and females. In males, significant differences were observed between the low and high position groups, and in females, significant differences were noted between low and medium-high position groups ($P < .05$) (Supplementary Table 1).

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3.2. Vitamin B_{12} intakes and the related biomarkers between different SEF groups

Vitamin B_{12} intakes were 5.98 μg/d in males and 4.54 μg/d in females (91% and 98% meet the American EAR, respectively). For males and females, intakes for serum vitamin B_{12} were 330.64 and 377.9 nmol/L, and for holoTC, were 63.04 and 65.63 nmol/L, respectively. Among males, 3.7% and 9% did not meet serum vitamin B_{12} and holoTC concentration recommendations; these percentages for females were 6.3% and 8.5%, respectively. Adolescents in categories denoting higher socioeconomic positions had significantly higher intakes and biomarkers levels of vitamin B_{12}, compared with those in the rest of the socioeconomic positions (Supplementary Table 2). In males, vitamin B_{12} intakes were significantly different according to FAS (low vs medium and high position groups), maternal education (high vs medium-low and medium-high position groups), and paternal education (medium-low vs high position group), all at $P < .05$ level. In females, differences were observed between high and middle and low position groups of FAS, parental migration background (low position group vs middle and high), household composition, maternal education (high position group vs all other groups), paternal education (high position group vs medium-low and medium-high position groups, and low position group vs medium-high position group), and maternal occupation (high position group vs medium-low and low position groups) ($P < .05$) (Supplementary Table 2).

Moreover, serum cobalamin concentrations in females were significantly different between high and medium-high and low position groups of paternal education ($P < .05$). Significant differences in holoTC were observed in females by paternal education groups (low vs high position) (Supplementary Table 2).

3.3. Associations between SEF and folate intakes and biomarkers

Table 2 presents observed associations between folate intakes and biomarkers and SEF through unstandardized β coefficients. These unstandardized βs explain the change in units of measurements of the intakes and biomarkers when compared with any of the categories of the SEF with the category denoting higher socioeconomic level. In males, lower folate intakes were associated with single/shared care and lower maternal and paternal education levels ($P < .05$). In females, lower folate intakes were observed for lower maternal and paternal education levels and lower paternal occupation ($P < .05$). Concerning plasma folate, lower concentrations were associated with single/shared care and paternal occupation in males ($P < .05$); no associations were found for females. In regards to RBC folate concentrations, no associations were found for males or females.

3.4. Associations between SEF and vitamin B_{12} intakes and biomarkers

In Table 3, lower intakes of vitamin B12, both in males and females, were associated with lower FAS. Migrant background, lower levels of maternal education, lower maternal occupation, and single/shared care ($P < .05$) in females were also associated with lower vitamin B_{12} intakes. In males, parental education was associated with lower vitamin B_{12} levels ($P < .05$).

Table 2 – Linear regression analysis assessing the associations between folate intakes, plasma folate, and RBC folate and SEFs after adjusting by age, BMI, and total energy intake (kilocalories)

Indicators	Folate intakes (μg/d)				Plasma folate (nmol/L)				RBC folate (nmol/L)			
	Males		Females		Males		Females		Males		Females	
	B	95%CI	B	95%CI	B	95%CI	B	95%CI	B	95%CI	B	95%CI
FAS												
Low FAS	0.0	−0.0, 0.1	0.0	−0.0, 0.1	0.1	−0.2, 0.4	0.1	−0.0, 0.3	−0.0	−0.3, 0.2	0.0	−0.1, 0.2
Medium FAS	−0.0	−0.0, 0.0	0.0	−0.0, 0.0	0.0	−0.1, 0.2	−0.0	−0.1, 0.1	0.0	−0.1, 0.1	−0.0	−0.1, 0.1
Migrant background												
Both parents born abroad	0.0	−0.1, 0.1	0.0	−0.0, 0.1	0.0	−0.3, 0.4	0.2	−0.1, 0.4	−0.1	−0.4, 0.2	0.1	−0.1, 0.3
One parent born abroad	−0.0	−0.1, 0.0	0.0	−0.1, 0.1	−0.2	−0.5, 0.1	−0.0	−0.3, 0.2	−0.0	−0.3, 0.2	0.2	−0.0, 0.4
Household composition												
Single/shared care	−0.0a	−0.1, −0.0	−0.0	−0.0, 0.0	−0.2a	−0.3, −0.0	−0.0	−0.1, 0.1	−0.0	−0.2, 0.1	0.0	−0.1, 0.1
Maternal education												
Low	−0.0a	−0.1, −0.0	−0.1a	−0.1, −0.0	0.0	−0.1, 0.1	−0.1	−0.2, 0.0	0.0	−0.1, 0.1	−0.0	−0.1, 0.0
Paternal education												
Low	−0.0a	−0.1, −0.0	−0.1a	−0.1, −0.0	−0.1	−0.2, 0.0	−0.1	−0.2, 0.0	−0.1	−0.2, 0.0	−0.1	−0.2, 0.0
Maternal occupation												
Low	0.0	−0.0, 0.0	−0.0	−0.0, 0.0	−0.1	−0.2, 0.1	−0.1	−0.2, 0.0	−0.0	−0.2, 0.1	−0.1	−0.1, 0.0
Paternal occupation												
Low	−0.0	−0.1, 0.0	−0.0a	−0.1, −0.0	−0.2a	−0.3, −0.0	−0.1	−0.2, 0.1	−0.1	−0.2, 0.0	−0.1	−0.2, 0.0

Abbreviation: B, unstandardized B coefficients.
High position categories for each indicator were used as a reference.
Significant differences ($P < .05$) between groups are indicated by letters (always in comparison with the high position group).

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Table 3 – Linear regression analysis assessing the associations between cobalamin intakes, serum cobalamin, and holoTC and SEFs after adjusting by age, BMI, and total energy intake (kilocalories)

Indicators	Vitamin B_{12} intakes (μg/d)				Serum vitamin B_{12} (nmol/L)				holoTC (nmol/L)			
	Males		Females		Males		Females		Males		Females	
	B	95%CI	B	95%CI	B	95%CI	B	95%CI	B	95%CI	B	95%CI
FAS												
Low FAS	−0.2a	−0.3, −0.1	−0.1a	−0.2, −0.1	0.1	−0.2, 0.3	−0.1	−0.2, 0.2	−0.1	−0.3, 0.2	0.0	−0.1, 0.2
Medium FAS	−0.0	−0.1, 0.0	−0.0a	−0.1, −0.0	−0.0	−0.1, 0.1	−0.0	−0.1, 0.1	−0.0	−0.1, 0.1	−0.0	−0.1, 0.1
Migrant background												
Both parents born abroad	−0.1a	−0.2, −0.0	−0.2a	−0.3, −0.1	−0.1	−0.3, 0.2	0.0	−0.2, 0.2	−0.1	−0.4, 0.2	−0.2	−0.4, 0.1
One parent born abroad	−0.1a	−0.2, −0.0	−0.0	−0.1, 0.0	0.0	−0.2, 0.3	−0.1	−0.3, 0.1	−0.1	−0.4, 0.1	−0.1	−0.3, 0.2
Household composition												
Single/shared care	0.0	−0.0, 0.1	−0.1a	−0.1, −0.0	0.1	−0.0, 0.2	−0.0	−0.1, 0.1	0.1	−0.1, 0.2	−0.0	−0.1, 0.1
Maternal education												
Low	−0.1a	−0.1, −0.0	−0.1a	−0.1, −0.0	−0.1	−0.2, 0.0	−0.1a	−0.2, 0.0	−0.1	−0.2, 0.1	−0.1a	−0.2, −0.0
Paternal education												
Low	−0.1a	−0.1, −0.0	−0.0	−0.1, 0.0	−0.1	−0.2, 0.0	−0.1	−0.2, 0.0	−0.1	−0.2, 0.1	−0.1	−0.2, 0.0
Maternal occupation												
Low	−0.1a	−0.1, −0.0	−0.1a	−0.1, −0.0	−0.1	−0.1, 0.0	−0.1a	−0.2, −0.0	−0.0	−0.1, 0.1	−0.1	−0.2, 0.0
Paternal occupation												
Low	−0.0	−0.1, 0.0	−0.0	−0.1, 0.0	−0.0	−0.2, 0.1	−0.1	−0.2, 0.0	−0.0	−0.1, 0.1	−0.1a	−0.2, −0.0

High position categories for each indicator were used as a reference.
Significant differences ($P < .05$) between groups are indicated by letters (always in comparison with the high position group).

Table 3 also presents associations between lower serum cobalamin and holoTC and lower maternal education in females ($P < .05$). In addition, lower serum cobalamin concentrations were significantly associated with lower maternal occupation and lower holoTC concentrations with lower paternal occupation ($P < .05$) in females.

4. Discussion

The results showed that SEF are associated with folate and vitamin B_{12} intakes and biomarkers in both males and females. In this study, maternal education and paternal occupation seem to be the most related SEF to folate and vitamin B_{12} intakes and biomarkers. In general, the results of this study indicated that SEFs are more associated with intakes than with biomarkers and are more relevant for females than for males. Following the model proposed in the introduction, the main possible explanation is that SEF is an important determinant of dietary habits and, consequently, of vitamin intakes and nutritional biomarkers. However, these biomarkers could also be influenced by genetics, physiologic status, and interactions between other nutrients [37–39]. These differences in associations obtained between intakes and biomarkers emphasize the importance of measuring biomarkers in these kinds of studies to better understand how SEF affects health. Moreover, this highlights the need for further research focusing on the main food contributors to these vitamin biomarkers.

Our findings support the hypothesis that the socioeconomic gradient which affects a number of health outcomes could be apply to folate and vitamin B_{12} intakes and blood-related biomarkers in this population of European adolescents. To our knowledge, this is the first study with a large sample of adolescents in 8 European countries that examines the associations between this complete set of SEF and folate and vitamin B_{12} intakes and their related blood biomarkers concentrations.

Folate and the metabolically related B vitamins, such us vitamin B_{12}, are critical throughout childhood and adolescence. Optimal levels of folate and vitamin B_{12} must be prioritized due to folate's role in the prevention of neural tube defects and cardiovascular diseases [5] and vitamin B_{12}'s role in regards to cognitive functions, megaloblastic anemia, and growth [40]. In a recent review [6], folate is recognized as a possible risky micronutrient in healthy European adolescents in terms of intake and status adequacy. Nevertheless, vitamin B_{12} deficiency is uncommon in young populations unless they are vegan, live in a developing region, or have a congenital malabsorption syndrome [41]; however, the prevalence could be higher than formerly recognized [42]. The manifestations of both deficiencies are indistinguishable where hematological complications are concerned, so it is important to have reliable methods that discriminate between the 2 vitamin deficiencies and to monitor intervention programs designed to avoid their deficiencies (such as the use of fortification or supplements) [43].

Comparability with other studies is limited due to the lack of available studies during adolescence. At European level, a review of vitamin B intakes and status in European adolescents pointed out that nutritional status was closely related with SEF [6]. However, the findings of a recently conducted systematic review [44], developed within the EURopean RECommendations Aligned (EURRECA) frame, failed to identify high-quality studies showing a consistent association between folate or vitamin B_{12} intakes or biomarkers concentrations and SEF in Western European countries.

Associations between folate intakes were observed with maternal and paternal education for both males and females

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and also with household composition (for males) and paternal occupation (for females). For biomarkers concentrations, associations were only found in males in regards to household composition and paternal occupation (plasma folate). All the presented associations confirm that adolescents in lower socioeconomic positions have lower intakes and biomarkers concentrations. In addition, the issue that most of the sample have unhealthy values of folate intakes (<400 μg/d) [45] and healthy values of folate biomarkers (>13.6 nmol/L of plasma folate and >06 nmol/L of RBC folate) [31,35] could be due to the high dependency of the retention of folate, both on the food in question and the method of cooking [46]. It could also be explained by the usage of supplements, which was not controlled for in this study [47].

For vitamin B_{12} intakes, associations were observed with FAS, migrant background, maternal education, and maternal occupation, both in males and females. In males, associations with paternal education were also found, and in females, there were associations with household composition. In the case of biomarkers concentrations, associations were only found for females. It was also shown that maternal education is associated with both serum vitamin B_{12} and holoTC and with maternal occupation in the case of serum B_{12} as well as with paternal occupation in the case of holoTC.

In spite of the scarce literature available, previous findings on micronutrient intakes and SEF in young populations also show this socioeconomic gradient. Studies during adolescence suggested decreased vitamin B_{12} intakes with decreased wealth in regions of the developed and developing world [4,8,48]. The results reported by the UK National Diet and Nutrition Survey of young people (4-18 years old) showed that participants in households of the lower socioeconomic position had lower intakes of most vitamins and minerals studied, including folate and vitamin B_{12} [8]. The results of a Norwegian study suggested healthier food habits with higher levels of parental education [48], similar to those of US and Spanish studies [49,50] where higher parental education levels and higher income positions, respectively, were consistently associated with adequate levels of nutrient intakes (such as folate and vitamin B_{12}). Finally, a study of Brazilian adolescents indicated that participants in the lower income and parental educational positions were at highest risk of having inadequate intakes for vitamin B_{12} [51].

In young population groups, very few studies have addressed the relationship between folate and vitamin B_{12} intakes and biomarkers concentrations and SEF [52]. In our sample, higher concentrations of folate and vitamin B_{12} biomarkers were associated with higher parental socioeconomic status, contrary to the results of a recent Greek study [53] that showed no association with parental education. However, in that study, homocysteine was the biomarker used to relate with parental education, and it is possible that homocysteine is not the most appropriate biomarker in reflecting vitamin B_{12} status because it is influenced by complex interactions between B-group vitamins [54].

As socioeconomic variables may all be related to a minor or major degree, the effect of interactions between SEF was also studied based on 1-way analysis of covariance's models (results not shown). However, most of the time, the effect on the current dependent variables points in the same direction, and their isolated effect is more or less the same than when we checked the effect for all possible interactions between different socioeconomic variables. For this reason, the present results were finally presented.

An important strength of this study is the relatively large sample of adolescents representing various European regions and the application of tested and standardized procedures [20]. Similarly, the study offered the opportunity to examine relations using a large pool of accurate SEF [22,24,28,23,55], measured with reliable and validated questionnaires [22]. The clustered sample was representative for the participating cities but not for the countries [20]. Moreover, the use of self-reported data in questionnaires should be considered a limitation of this study [56].

The method of 2 dietary 24-hour recalls is the most used for population mean analysis of participants 10 years and older in different European countries [29] because it allows statistical adjustments for within-person variation. However, the influence of the method's limitations reflecting accurate assessment should be considered [57]. In absence of the frequency of consumption used as a covariate, the assessment of two 24-hours recalls within 2 weeks might misrepresent the food consumption over the 4 seasons [58]. Bias in the calculation of dietary estimates might have been introduced by the use of the German food composition table due to the absence of a European one. However, preliminary analyses of estimates obtained using both the BLS and national food composition tables indicated small and mostly negligible differences using the 2 approaches (unpublished results). Adolescents identified as underreporters were also included in the analysis as groups at risk for inadequate intakes and are slightly biased by true underreporters [59]. Although correlations between biomarkers and usual food intakes obtained from the recalls were low in this sample [31], the use of biochemical markers strengthened the investigation and provided a deeper understanding of observed associations by avoiding the typical measurement error in dietary assessment [60]. However, a dietary study limitation is that supplement use was not used as a covariable, which is a covariable between SEF and vitamin intakes [47,61] and could have explained some of the differences obtained in the results between intakes and biomarkers. Furthermore, blood biomarkers were all analyzed together in the same center, strengthening the reliability of the results.

Our study makes an important contribution in providing evidence on folate and vitamin B_{12} intakes and status of European adolescents. Intakes of folate and vitamin B_{12} were consistently more associated with SEF than their biomarkers, mainly because biomarkers are determined for other several factors, such as genetics, physiologic status, and interactions with other nutrients. Considering SEF, maternal education and paternal occupation were most associated with folate and vitamin B_{12}. However, the homogenizing effect regarding habits among adolescents, as a defining feature (such as school, peers, youth culture), can intercede in these associations, and thus, further investigations need to assess it.

As part of a strategy to prevent adult diseases, we should aim at initiatives to improve adolescents' dietary habits and, consequently, assure adequate levels of vitamins. Because dietary habits and vitamin intake are associated

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with various serious health problems, such as megaloblastic anemia, cardiovascular disease, or cognitive impairment, special considerations should be given to adolescents in lower socioeconomic positions, and future research focusing on the social pattern of adolescents' eating habits should be priority.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nutres.2014.01.006.

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Supplementary table 1. Estimates of folate intakes and plasma and RBC-folate concentrations by socioeconomic factors, adjusted for age, body mass index, and total energy intake (kcal) in European adolescents.

INDICATORS	***FOLATE INTAKES (µg/d)***				***PLASMA FOLATE (nmol/L)***				***RBC FOLATE (nmol/L)***			
	Males		**Females**		**Males**		**Females**		**Males**		**Females**	
	Mean	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
FAS												
Low FAS	220.0	5.9	177.6	3.9	17.9	3.1	21.1	1.56	728.37	115.42	779.97	22.73
Medium FAS	209.8	2.2	177.3	1.8	18.7	0.8	17.5	0.74	827.71	29.08	738.72	22.73
High FAS	211.6	2.9	176.7	2.3	17.4	1.0	18.4	0.90	802.68	37.61	778.66	27.72
P for trend												
Migrant background												
Both parents born abroad	216.2	6.7	180.9	5.4	18.1	3.4	21.9	2.5	691.8	128.5	799.2	74.5
One parent born abroad	203.8	7.6	181.5	5.7	14.8	2.8	17.6	2.6	796.5	110.9	919.3	77.5
Both parents born in survey's country	212.2	1.8	176.1	1.4	18.3	0.6	18.1	0.6	820.5	23.8	749.6	17.4
P for trend												
Household composition												
Single/shared-care	204.8a	3.1	177.6	2.4	15.8a	1.2	18.5	1.1	779.8	44.5	786.6	32.3
Traditional family	213.8a	2.0	177.0	1.6	19.0a	0.7	18.2	0.6	827.1	26.2	748.0	19.0
P for trend	**0.02**				**0.01**							
Maternal education												
Low	218.6	6.8	177.3	5.1	14.3	2.5	15.6	1.9	639.1a	92.8	623.3a	56.3
Medium-low	206.6	3.3	170.5	2.7	19.3	1.3	18.3	1.2	882.3	48.4	770.9	34.9
Medium-high	212.0	3.2	177.9	2.4	17.5	1.1	19.2	1.0	786.0	41.3	813.2a	29.1
High	215.6	2.9	181.8	2.3	18.7	1.0	18.4	1.0	830.0a	38.1	736.4	29.7
P for trend									**0.02**		**0.03**	

Paternal education												
Low	221.5	8.6	173.9	5.1	15.9	3.0	15.0	2.0	689.6	112.6	640.2	60.4
Medium-low	205.8a	3.2	171.4a b	2.5	17.7	1.2	18.0	1.1	806.6	46.1	738.2	31.8
Medium-high	211.0	3.4	181.9a	2.7	17.9	1.2	18.9	1.0	836.9	43.6	801.9	30.9
High	216.9a	2.9	181.4b	2.3	19.5	1.0	18.9	1.0	841.5	38.3	768.5	29.4
P for trend	**0.01**		**0.00**									
Maternal occupation												
Low	217.7	3.8	178.0	2.9	18.1	1.4	17.5	1.2	770.9	50.7	740.7	34.5
Medium-low	209.4	4.5	173.2	3.4	15.6	1.9	17.8	1.2	785.2	70.8	708.5	36.9
Medium-high	210.6	2.7	176.5	2.0	18.8	0.9	18.1	0.9	808.6	34.1	755.3	25.3
High	213.7	3.9	177.3	3.2	18.2	1.3	20.0	1.4	903.3	49.3	803.1	40.2
P for trend												
Paternal occupation												
Low	213.1	6.9	170.1	5.8	16.0	2.4	19.3	2.1	732.3	91.1	676.6	66.5
Medium-low	207.2	3.7	174.5	2.6	16.9	1.4	17.4	1.0	787.8	52.6	743.2	30.1
Medium-high	209.8	3.1	180.1	2.5	19.0	1.0	17.5	1.0	839.5	37.8	743.6	31.0
High	216.9	3.1	180.8	2.6	18.9	1.1	19.6	1.0	837.6	39.5	801.8	31.7
P for trend			**0.05**									

Abbreviations: RBC-folate, red blood cell folate; SE, Standard Error; FAS, Family Affluence Scale

Low position categories denote lower well-being and subsequently lower position of inadequacy of both intakes and biomarkers levels.

Significant differences ($p<0.05$) between groups are indicated by the same superscripts letters.

P for trend based on F test are stated in bold only when significant (p-values$\leq$0.05).

Supplementary table 2. Estimates of vitamin B_{12} intakes and serum cobalamin and Holotranscobalamin by socioeconomic indicators, adjusted for age, body mass index, and total energy intake (kcal) in European adolescents.

INDICATORS	*VITAMIN B12 INTAKES (µg/d)*				*SERUM VITAMIN B12 (pmol/L)*				*HOLOTRANSCOBALAMIN (pmol/L)*			
	Males		**Females**		**Males**		**Females**		**Males**		**Females**	
	Mean	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
FAS												
Low FAS	5.4ab	0.2	4.2ab	0.1	342.7	40.2	378.5	25.5	57.2	11.1	72.1	6.1
Medium FAS	6.0a	0.1	4.5a	0.1	331.0	10.1	380.9	12.2	64.1	2.9	67.1	3.0
High FAS	6.1b	0.1	4.8b	0.1	338.9	13.0	395.0	14.9	69.4	3.6	64.3	3.6
P for trend	**0.00**		**0.00**									
Migrant background												
Both parents born abroad	5.6	0.3	3.9ab	0.2	320.3	44.7	403.7	40.4	57.1	12.4	53.7	9.5
One parent born abroad	5.4	0.3	4.6a	0.2	339.6	37.1	347.0	42.1	56.2	10.7	61.6	9.6
Both parents born in survey´s country	6.1	0.1	4.6b	0.1	335.6	8.2	386.4	9.4	66.9	2.3	66.7	2.1
P for trend	**0.02**		**0.00**									
Household composition												
Single/shared-care	6.0	0.1	4.4a	0.1	351.5	15.5	374.3	17.3	68.6	4.5	65.5	4.2
Traditional family	6.0	0.1	4.6a	0.1	328.4	90.4	389.6	10.3	64.9	2.6	67.1	2.5
P for trend			**0.02**									
Maternal education												
Low	6.3	0.3	4.6a	0.2	308.0	30.9	362.0	30.4	56.0	8.7	56.9	7.0
Medium-low	5.7a	0.1	4.3b	0.1	308.7	16.1	357.7	18.7	62.5	4.6	61.9	4.4
Medium-high	5.8b	0.1	4.3c	0.1	328.1	13.6	378.0	15.8	65.9	4.0	64.0	3.5
High	6.3ab	0.1	5.0abc	0.1	355.0	12.6	415.3	15.9	70.1	3.7	72.9	3.7

P for trend	**0.00**		**0.00**								**0.03**	
Paternal education												
Low	6.2	0.3	4.8a	0.2	344.8	38.8	318.0a	31.9	70.6	9.8	52.5a	7.8
Medium-low	5.8a	0.1	4.4b	0.1	308.7	15.8	385.3	17.0	60.5	4.1	68.6	4.3
Medium-high	5.9	0.1	4.3ac	0.1	337.7	15.0	356.0b	16.7	64.7	3.9	61.2	4.0
High	6.3a	0.1	4.9bc	0.1	347.3	13.1	427.3a b	15.6	69.4	3.4	74.2a	3.9
P for trend	**0.00**		**0.00**				**0.00**				**0.01**	
Maternal occupation												
Low	5.8	0.1	4.5a	0.1	330.2	17.4	363.0	19.2	64.4	5.1	64.0	4.9
Medium-low	5.8	0.2	4.4b	0.1	312.0	24.8	350.8	20.4	62.9	7.1	62.8	4.9
Medium-high	6.1	0.1	4.6	0.0	334.3	11.9	400.0	14.3	66.3	3.4	66.1	3.5
High	6.2	0.1	4.6ab	0.1	354.0	17.1	418.3	22.2	65.4	5.0	76.8	5.4
P for trend	0.04		0.00									
Paternal occupation												
Low	5.8	0.3	4.3	0.2	358.7	31.7	386.4	35.0	65.9	8.8	59.7	8.4
Medium-low	5.9	0.1	4.5	0.1	319.7	18.3	362.7	16.2	64.0	5.2	62.6	3.9
Medium-high	6.0	0.1	4.6	0.1	327.9	13.0	418.6	16.7	63.6	3.7	73.8	4.0
High	6.1	0.1	4.8	0.1	350.0	13.7	380.1	17.1	69.4	4.0	64.0	4.2
P for trend												

Abbreviations: SE, Standard Error; FAS, Family Affluence Scale.

Low position categories denote lower well-being and subsequently lower position of inadequacy of both intakes and biomarkers levels.

Significant differences ($p<0.05$) between groups are indicated by the same superscripts letters.

P for trend based on F test are stated in bold only when significant (p-values$\leq$0.05).

Determinants

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

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ORIGINAL CONTRIBUTION

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

Iris Iglesia[1,2] · Theodora Mouratidou[1] · Marcela González-Gross[3] · Inge Huybrechts[4,5] · Christina Breidenassel[3,6] · Javier Santabárbara[7] · Ligia-Esperanza Díaz[8] · Lena Hällström[9,10] · Stefaan De Henauw[4] · Frédéric Gottrand[11] · Anthony Kafatos[12] · Kurt Widhalm[13] · Yannis Manios[14] · Denes Molnar[15] · Peter Stehle[6] · Luis A. Moreno[1,16] · on the behalf of HELENA study group

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Abstract
Purpose To examine the association between food groups consumption and vitamin B_6, folate and B_{12} intakes and biomarkers in adolescents.
Methods In total 2189 individuals participating in the cross-sectional Healthy Lifestyle in Europe by Nutrition in Adolescence study met the eligibility criteria for analysis of dietary intakes (46 % males) and 632 for biomarker analysis (47 % males). Food intakes were assessed by two non-consecutive 24-h recalls. Biomarkers were measured by chromatography and immunoassay. Food groups which best discriminated participants in the extreme tertiles of the distribution of vitamins were identified by discriminant analyses. Food groups with standardised canonical coefficients higher or equal to 0.3 were selected as valid *discriminators* of vitamins intake and biomarkers extreme tertiles. Linear mixed model elucidated the association between food groups and vitamins intakes and biomarkers.
Results Vitamin B_6 intakes and biomarkers were best discriminated by meat (males and females), margarine and mixed origin lipids only in males and breakfast cereals (females). Breakfast cereals (males), and fruits, margarine and mixed origin lipids, vegetables excluding potatoes, breakfast cereals, and soups/bouillon (females) determined the most folate intakes and biomarkers. Considering vitamin B_{12} intakes and biomarkers, meat, and white and butter

✉ Iris Iglesia
iglesia@unizar.es

1 GENUD (Growth, Exercise, Nutrition and Development) Research group, Instituto Agroalimentario de Aragón (IA2), Instituto de Investigación Sanitaria Aragón (IIS Aragón), Universidad de Zaragoza, C/Pedro Cerbuna, 12, SAI Building (Servicio de Apoyo a la Investigación), 2nd floor, 50009 Zaragoza, Spain

2 Red de Salud Materno-infantil y del Desarrollo (SAMID), Madrid, Spain

3 ImFINE Research Group, Department of Health and Human Performance, Universidad Politécnica de Madrid, Madrid, Spain

4 Department of Public Health, Ghent University, Ghent, Belgium

5 International Agency for Research on Cancer (IARC), 150 Cours Albert Thomas, 69372 Lyon Cedex 08, France

6 Department of Nutrition and Food Science, University of Bonn, 53115 Bonn, Germany

7 Department of Preventive Medicine and Public Health, Universidad de Zaragoza, Zaragoza, Spain

8 Immunonutrition Research Group, Department of Metabolism and Nutrition, Institute of Food Science, Technology and Nutrition (ICTAN), Spanish National Research Council (CSIC), Madrid, Spain

9 Unit for Preventive Nutrition, Department of Biosciences and Nutrition, Karolinska Institute, Stockholm (Huddinge), Sweden

10 Sweden and School of Health, Care and Social Welfare Mälardalens University, Västerås, Sweden

11 Inserm U995, Faculté de Médecine, Université Lille 2, Lille, France

12 University of Crete School of Medicine, 71033 Crete, Greece

13 Division of Clinical Nutrition and Prevention, Department of Pediatrics, Medical University of Vienna, Vienna, Austria

14 Department of Nutrition- and Dietetics, School of Health Science and Education, Harokopio University, Athens, Greece

15 Department of Paediatrics, University of Pécs, Pécs, Hungary

16 Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y Nutrición (CIBERObn), Madrid, Spain

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milk (males and females), snacks (males), and dairy products (females) best discriminated individual in the extremes of the distribution. Fewer associations were obtained with mixed model for biomarkers than for vitamins intakes with food groups.
Conclusions Whereas B-vitamin intakes were associated with their food sources, biomarkers did with overall food consumption. Low-nutrient-density foods may compromise adolescents' vitamin status.

Keywords Foods contributors · B-vitamins · Adolescents

Abbreviations

HELENA	Healthy Lifestyle in Europe by Nutrition in Adolescence
Cbl	Cobalamin
MSM	Multiple-source method
PLP	Pyridoxal 5′-phosphate
EDTA	Ethylenediaminetetraacetic acid
HPLC	High-performance liquid chromatography
CV	Coefficient of variation
RBC-folate	Red blood cell folate
HoloTC	Holotranscobalamin
SD	Standard deviations
BMI	Body mass index
FCDB	Food composition databases

Introduction

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

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

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

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

Methods

The Healthy Lifestyle in Europe by Nutrition in Adolescence Cross-Sectional Study, (HELENA-CSS), is a multicentre study of lifestyle and nutrition among adolescents from 10 European cities from nine countries. A random cluster sampling (all adolescents from a selection of classes from all schools in the selected cities) of 3000 adolescents aged 12.5–17.5 years, stratified by geographical location, age and socio-economic status, was carried out in Athens and Heraklion (Greece), Dortmund (Germany), Ghent (Belgium), Lille (France), Pécs (Hungary), Rome (Italy), Stockholm (Sweden), Vienna (Austria), and Zaragoza (Spain). Inclusion criteria were: not participating simultaneously in another clinical trial and being free of any acute infection occurring <1 week before inclusion [12]. The initial number of HELENA participants was 3528 (47 % males). The average participation rate in the study was 67 %, which is acceptable for this demanding epidemiological study [13]. Due to logistical reasons, participants from Heraklion and Pécs (7 % of the total sample) did not provide dietary data. For the purposes of this analysis, 2189 adolescents (46 %

males) were included having complete data on two non-consecutive 24-h recalls, including Sundays, and valid data on maternal education, among others. From a random sub-sample of 941 adolescents (46 % males) with available blood parameters (including adolescents from Heraklion and Pécs), a sample of 632 adolescents (47 % males) was included in the current analysis having met the inclusion criteria, e.g. having complete data on B-vitamins biomarkers, and data on two 24 h dietary recalls. Further details on the sampling procedures, pilot study and reliability of the data have been published elsewhere [12]. Informed consent was obtained from all participants and their parents, and the protocol was approved by the Human Research Review Committees of the corresponding centres [14].

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

Dietary intakes were examined using the computerised 24-h recall, self-administered HELENA-Dietary Assessment Tool (HELENA-DIAT), adapted for European adolescents from the Young Adolescents' Nutrition Assessment on Computer (YANA-C) software [15]. The adolescents completed the 24-h recalls two times in a 2-week period. Trained staff was present during this assessment [15]. More information about this tool can be found elsewhere [15, 16]. Difficulties in obtaining comparable measures of energy density of each food across countries precluded the use of country-specific food composition tables. For this reason, data were linked to the German Food Code and Nutrient Data Base (BLS—Bundeslebensmittelschlüssel, version II.3.1, 2005), which includes 12,000 coded foods, and with up to 158 nutrient data points available for each product. The Multiple-source method (MSM) [17] was used to calculate usual nutrient intake removing the effect of day-to-day within-person variability and random error in the recalls. For that purpose, the default model was used, and consequently, all the surveyed adolescents were considered as habitual consumers assuming indeed that most nutrients are consumed on a daily basis in contrast as happens with foods that might be consumed from time to time only. The lack of food frequency information determined that step 2 was not included in the model.

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

All reported 4179 foods and beverages, as part of recipes or as individual food, were aggregated in initial 29 food groups based on the European Food Groups classification system [15, 19]. As part of the general HELENA analysis, these foods were disaggregated into 43 food groups. For the purposes of the current analysis and based on their nutritional composition some of these food groups were further aggregated. Among those aggregated were alcoholic drinks (beer, wine, others…), complex carbohydrates (pasta, rice, flour…), sugar products (honey, and other sugar products…), oily fruits (nuts and seeds, avocado and olives…), milk products (yogurt and white cheese and milk and yogurt beverages), and other milk products (desserts and puddings milk based, creams…). In "Appendix", there is an explanation of all the milk-based related categories. Four food groups were eliminated from the current analysis namely 'products for special nutrition use', 'soya beverages', 'miscellaneous', and 'meat substitutes'). This was done on the basis of very low consumption (0 median and mode and more than the 85 % of the sample did not report consumption). So, in the end, the final number of food groups for this analysis was 31 food groups which are fully presented in Table 4. In any case, for the estimation of vitamin intakes, all the food groups were included so as not to underestimate them even when the vitamins provided by the eliminated food groups were almost insignificant.

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

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

At school, early in the morning, and following an overnight fast, 30 ml of blood was drawn according to a standardised blood collection protocol by a certified phlebotomist. More details on sample transport and quality assurance can be found elsewhere [20]. For the measurement of pyridoxal 5′phosphate (PLP), biomarker of vitamin B_6, ethylenediaminetetraacetic acid (EDTA) whole blood was centrifuged at $3500 \times g$ for 15 min. The supernatant fluid was transported at a stable temperature of 4–7 °C to the central laboratory at the University of Bonn (IEL-Institut fuer Ernährungs und Lebensmittelschaften-, Germany) and stored at −80 °C until analysed. PLP was measured by high-performance liquid chromatography (HPLC) (Varian Deutschland GmbH, Darmstadt, Germany; CV = 1 %) with a modified method of Kimura et al. [6, 21].

For the measurement of plasma folate and Cbl, heparinised tubes were collected, placed immediately on ice, and centrifuged within 30 min (3500 g for 15 min). The supernatant fluid was transported at a stable temperature of 4–7 °C to the central laboratory at the University of Bonn

Table 1 Adolescents' characteristics belonging to the HELENA study with complete information on dietary intake

Characteristics	Males (1004)						Females (1185)					
	% PII	Mean ± SD	Median	T1	T2	T3	% PII	Mean ± SD	Median	T1	T2	T3
Age	–	14.8 ± 1.3	14.8	–	–	–	–	14.7 ± 1.2	14.7	–	–	–
BMI (kg/m^2)	–	21.3 ± 3.9	20.5	–	–	–	–	21.2 ± 3.5	20.0	–	–	–
Energy intake (kcal/d)	–	2493.5 ± 828.1	2371.0	–	–	–	–	1894.4 ± 583.2	1830.8	–	–	–
Vitamin B_6 intake (µg/d)	0.0	1797.5 ± 649.0	1700.3	412.8–1450.6	1450.6–1994.2	1994.2–5035.5	0.0	1432.9 ± 482.3	1379.1	439.2–1195.5	1195.5–1562.1	1562.1–4516.5
Folate intake (µg/d)	98.0	212.0 ± 75.5	199.6	44.0–171.8	171.8–236.1	236.1–487.0	100.0	177.5 ± 59.4	170.1	50.2–147.0	147.0–195.1	195.1–491.7
B_{12} intake (µg/d)	2.9	6.0 ± 2.4	5.7	0.6–4.8	4.8–6.7	6.7–15.6	6.0	4.6 ± 1.8	4.2	0.9–3.7	3.69–5.0	5.0–14.9

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

(IEL-Institut fuer Ernährungs und Lebensmittelschaften-, Germany) and stored at −80 °C until assayed. After measuring the hematocrit in situ, EDTA whole blood was used for the red blood cell folate (RBC-folate) analysis. EDTA whole blood was diluted 1:5 with freshly prepared 0.1 % ascorbic acid for cell lysis and incubated for 60 min in the dark before storage at −80 °C. Plasma and RBC-folate and plasma cobalamin were measured by means of a competitive immunoassay using the Immunolite 2000 analyser (DPC Biermann GmbH, Bad Nauheim, Germany) (CV for plasma folate = 5.4 %, RBC-folate = 10.7 %, Cobalamin = 5.0 %) [20]. Serum for measuring holotranscobalamin (HoloTC) were obtained by centrifuging blood collected in evacuated tubes without anticoagulant at 3500 g for 15 min within 1 h. Once send to IEL, the sera were aliquoted and stored at −80 °C until transport in dry ice to the biochemical lab at the Universidad Politécnica de Madrid for analysis (Laboratory number 242 of the Laboratory Network of the Region of Madrid). HoloTC was measured by microparticle enzyme immunoassay (Active B_{12} Axis-Shield Ltd, Dundee, Scotland, UK) with the use of AxSym (Abbot Diagnostics, Abbott Park, IL, USA) (CV = 5.1 %) [22].

Confounders

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

Statistical analysis

The Statistical Package for Social Sciences version 17.0 (SPSS Inc., Chicago, IL, USA) was used for analyses. All statistical tests and corresponding p values were two-sided, and $p < 0.05$ was considered statistically significant. All analyses were sex-specific. Descriptive data are presented as medians, means and standard deviations (SD), and also tertiles in case of B-vitamins intake and biomarkers concentrations. Tertile distribution of B-vitamins intake and biomarkers was used to examine major food sources

based on discriminant function analyses. This method tests whether cases (B-vitamins intakes and biomarkers of the adolescents) are well classified from a set of discriminating variables (all 31 food groups) into predefined groups of a criterion variable (the B-vitamins tertiles). Ideally, data to run discriminant analyses should be normally distributed. However, it is recognised as a robust method and can be used also with nonparametric data as in this analysis [24]. Moreover, it has been already used for similar purposes in the literature [25].

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

Results

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

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

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

Table 4 presents changes in B-vitamins values (both intakes and statuses) per 10-g increases in the

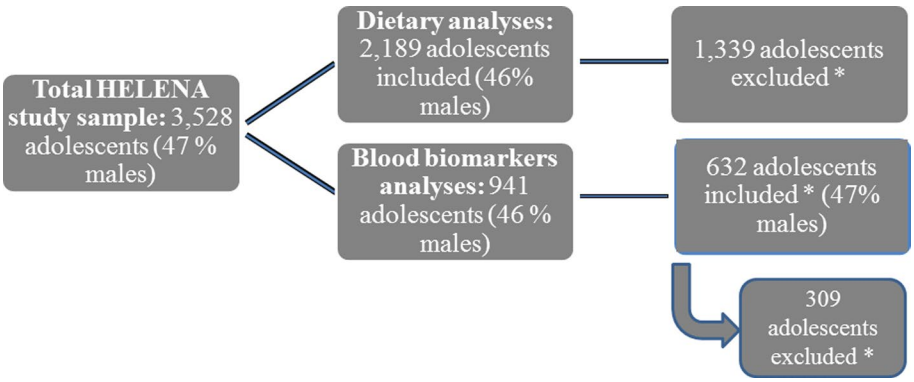

Fig. 1 Sampling selection. *Based on the exclusion criteria and the data availability in the covariates of the analysis and the two 24-h recalls and biomarkers, respectively

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

Characteristics	Males (295)						Females (337)					
	% PII	Mean ± SD	Median	T1	T2	T3	% PII	Mean ± SD	Median	T1	T2	T3
Age		14.84 ± 1.3	14.8	–	–	–		14.8 ± 1.18	14.8	–	–	–
BMI (kg/m^2)		21.1 ± 3.8	20.4	–	–	–		21.16 ± 3.40	20.6	–	–	–
Energy intake (kcal/d)		2551.1 ± 876.9	2418.6	–	–	–		1896.0 ± 555.5	1852.5	–	–	–
Vitamin B_6 intake (µg/d)	0.0	1839.0 ± 652.9	1795.1	495.5–1469.9	1469.9–2075.8	2075.8–5035.2	0.0	1453.2 ± 508.2	1402.1	439.2–1225.1	1225.1–1584.0	1584.0–4516.5
Folate intake (µg/d)	98.3	215.1 ± 73.74	205.7	54.7–174.1	174.1–244.6	244.6–472.6	100.0	180.7 ± 58.0	175.3	50.2–154.1	154.1–201.2	201.2–491.7
B_{12} intake (µg/d)	1.4	6.2 ± 2.5	5.9	0.7–4.7	4.7–6.7	6.74–15.6	4.2	4.6 ± 1.7	4.3	0.9–3.8	3.8–5.0	5.0–13.0
Serum vitamin B_6 (nmol/L)(264, 317)		68.7 ± 45.3	55.9	13.2–44.8	44.8–69.7	69.3–315.7		62.5 ± 64.5	47.1	5.8–39.1	39.1–61.7	61.7–899.1
Plasma folate (nmol/L) (295, 337)		18.2 ± 10.3	15.3	4.9–12.8	12.8–19.0	19.0–82.9		18.3 ± 9.7	15.9	4.8–13.3	13.3–20.1	20.1–70.5
RBC-folate (nmol/L) (293, 332)		819.0 ± 378.5	742.4	228.32–630.4	630.4–868.0	868.0–3673.3		766.7 ± 305.8	727.9	241.3–622.5	622.5–855.4	855.4–2361.9
Serum vitamin B_{12} (pmol/L) (295, 336)		333.6 ± 130.5	306.0	114.0–247.0	247.0–375.3	375.3–725.0		378.0 ± 158.3	348.0	110.0–282.3	282.3–432.0	432.0–1036.0
HoloTC (pmol/L) (272, 325)		65.0 ± 31.8	61.1	16.23–50.9	50.9–67.3	67.3–265.0		64.5 ± 33.9	59.1	18.4–50.0	50.0–67.5	67.5–325.5

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

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

	Males		Females	
	Univariate F ratio[a]	Standardised canonical coefficients[b]	Univariate F ratio[a]	Standardised canonical coefficients[b]
Vitamin B_6 intakes ($+3^{\infty}$, $+3^{\infty}$)				
Fish products (g/day)	28.25	0.34	41.70	0.34
Fruits (g/day)	100.72	0.45	92.53	0.38
Fruit and vegetable juices (g/day)	31.25	0.31	40.37	0.31
Meat (g/day)	320.76	0.80	280.80	0.65
Starch roots and potatoes (g/day)	117.52	0.45	152.84	0.45
Vegetables (excluding potatoes) (g/day)			105.92	0.36
White and butter milk (g/day)	70.99	0.31		
Serum B_6 (-3^{∞}, -3^{∞})				
Breakfast cereals (g/day)			10.11	−0.58
Cereals (g/day)	6.97	0.79		
Margarina and mixed lipids (g/day)	4.36	−0.63		
Oily fruits (g/day)			4.52	0.44
Starch roots and potatoes (g/day)			7.89	0.58
Vegetable oils (g/day)			3.44	0.43
Folate intakes ($+3^{\infty}$, $+3^{\infty}$)				
Bread and rolls (g/day)			131.61	0.40
Cheese excluding quark (g/day)	105.97	0.35		
Fruits (g/day)	32.20	0.39	143.76	0.46
Fruit and vegetable juices (g/day)	65.63	0.43	56.74	0.36
Pulses (g/day)			29.14	0.34
Vegetables (excluding potatoes) (g/day)	204.04	0.41	273.93	0.54
White and butter milk (g/day)	30.56	0.31		
Plasma folate (-3^{∞}, $+3^{\infty}$)				
Bread and rolls (g/day)			2.37	−0.43
Breakfast cereals (g/day)	10.87	−0.55	12.60	0.51
Cereals (g/day)	13.86	0.76		
Carbonated/soft drinks (g/day)	3.63	0.49		
Chocolate (g/day)			2.27	0.37
Fish products (g/day)			2.68	0.36
Fruits (g/day)			9.80	0.49
Margarina and mixed lipids (g/day)			6.22	0.47
Savoury snacks (g/day)			2.94	−0.39

Table 3 continued

	Males		Females	
	Univariate F ratio[a]	Standardised canonical coefficients[b]	Univariate F ratio[a]	Standardised canonical coefficients[b]
RBC-folate (-3^{∞}, -3^{∞})				
Breakfast cereals (g/day)	8.15	−0.52		
Carbonated/Soft drinks (g/day)	4.67	0.49		
Cereals (g/day)	7.92	0.58		
Dairy products (g/day)	1.62	0.46		
Fruits (g/day)	6.70	−0.40	8.60	−0.56
Margarina and mixed lipids (g/day)			7.24	−0.58
Savoury snacks (g/day)			2.25	0.39
Soups/bouillon (g/day)			6.42	0.53
Sugar products (g/day)			3.33	0.44
Vitamin B_{12} intake ($+3^{\infty}$, $+3^{\infty}$)				
Cheese excluding quark (g/day)			48.88	0.46
Dairy products (g/day)	48.22	0.40	53.81	0.37
Fish products (g/day)	49.87	0.37	53.55	0.48
Meat (g/day)	280.48	0.77	234.63	0.70
White and butter milk (g/day)	126.65	0.50	147.65	0.53
Plasma vitamin B_{12} ($+3^{\infty}$, $+3^{\infty}$)				
Cereals (g/day)			3.74	0.41
Dairy products (g/day)			7.08	0.59
Savoury snacks (g/day)	5.66	0.68		
White and butter milk (g/day)	8.57	0.81	12.43	0.66
Other milk products			5.76	0.41
Holotranscobalamin ($+3^{\infty}$, $+3^{\infty}$)				
Cakes (g/day)	3.15	−0.44		
Carbonated/Soft drinks (g/day)	8.47	−0.53		
Dairy products (g/day)			9.91	0.74
Savoury snacks (g/day)	0.01	0.41		
White and butter milk (g/day)	26.87	0.80	9.37	0.73

The number 3 between parenthesis next to the vitamin intake or biomarker represent the sign of the canonical coefficient in relation to the position of centroids in the discriminant function for the third tertile, for males and females, respectively. The +3 in Holotranscobalamin in males, together with a negative standardised canonical coefficient for cakes, means that those subjects with higher intake of cakes are more probably settled down in the first tertile of the biomarker

[a] Univariate *F* ratio: ratio of between-groups variability to the within-groups variability. Large *F* values indicate greater discriminating power

[b] Standardised canonical coefficients: partial contribution (discriminating power) of each variable to the discriminate function controlling for all other variables in the equation

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

Food groups	Vitamin B_6				Folate						Vitamin B_{12}					
(1 unit = 10 g/day)	Intakes (µg/d)		Serum vitamin B_6 (nmol/L)		Intakes (µg/d)		Plasma folate (nmol/L)		RBC-folate (nmol/L)		Intakes (µg/d)		Plasma vitamin B_{12} (pmol/L)		HoloTC (pmol/L)	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Alcoholic drinks	0.010	−0.008	−0.011	−0.085	0.007	0.002	0.003	0.023	−0.029	−0.036	0.015	−0.012	0.000	0.003	−0.008	0.002
Bread and rolls	−0.005	−0.015	−0.149[a]	−0.171[a]	0.074[b]	0.094[b]	−0.110[a]	−0.055	−1.019	−0.052	0.000[b]	0.029	−0.050	−0.073	−0.027	0.008
Breakfast cereals	0.041	0.048	0.091	0.740[b]	0.049	0.112[a]	0.409[b]	0.786[b]	4.196	0.444	0.094[a]	0.032	0.056	0.250	0.116	0.237
Butter and animal fats	−0.135[a]	−0.192[a]	0.145	−0.084	−0.008	−0.045	0.033	−0.097	0.282	−0.195	−0.092	−0.033	0.030	0.295	0.004	0.477
Cakes	−0.080[b]	−0.103[b]	0.089	−0.017	−0.055[b]	−0.129[b]	−0.004	−0.018	0.067	0.003	−0.052	−0.110[b]	−0.028	−0.045	−0.063	−0.019
Carbonated/soft drinks	−0.013	−0.010[a]	0.011	0.029	−0.022[b]	−0.024[b]	−0.019[a]	−0.026	−0.023	−0.027	−0.015[b]	−0.015[b]	−0.005	−0.015	−0.025[b]	−0.019
Cereals products	−0.071[b]	−0.076[b]	−0.081	−0.019	−0.003	−0.018	−0.084[a]	−0.069	−0.043	−0.025	−0.061[b]	−0.043[a]	0.017	0.021	−0.012	0.031
Cheese excluding quark	−0.142[b]	−0.123[a]	−0.120	−0.125	0.195[b]	0.134[b]	0.069	0.001	0.046	0.063	0.061	0.170[b]	−0.038	−0.158	0.053	0.050
Chocolate	−0.168[b]	−0.202[b]	−0.223[a]	−1.645	−0.113[b]	−0.179[b]	−0.016	0.034	−0.052	0.018	−0.159[b]	−0.118[b]	−0.158[a]	0.040	−0.076	−0.226[a]
Coffee/tea	−0.001	−0.002	0.049	0.007	0.017[a]	0.024[b]	−0.020	0.078[b]	0.020	0.055	−0.008	−0.005	0.010	0.026	−0.006	0.055[a]
Confectionary products	−0.186	−0.157[b]	0.024	0.415	−0.117[a]	−0.238[b]	0.199	−0.254	0.187	0.046	−0.362[b]	−1.170	0.069	0.341	−0.007	−0.131
Dairy products	0.004	0.000	0.003	0.069[a]	0.013	0.011	−0.006	0.034	−0.021	0.027	0.034[b]	0.050[b]	0.008	0.047[a]	−0.028	0.085[b]
Eggs	0.026	−0.052	−0.295	−0.120	0.321[b]	0.327[b]	0.223	−0.092	0.155	−0.158	0.311[b]	0.222[b]	0.012	0.207	−0.128	0.074
Fish products	0.145[b]	0.223[b]	0.273	0.033	0.079[a]	0.099[b]	0.153	0.222[a]	0.085	0.169	0.295[b]	0.305[b]	0.012	0.047	−0.036	0.006
Fruits	0.068[b]	0.078[b]	0.027	0.074	0.090[b]	0.101[b]	0.052[a]	0.064[a]	0.050	0.046	−0.018	−0.012	0.002	0.014	0.009	0.016
Fruit and vegetable juices	0.018[b]	0.018[b]	−0.008	0.018	0.042[b]	0.036[b]	0.055[b]	0.019	0.022	−0.001	−0.028[b]	−0.026[b]	−0.005	−0.015	0.025	−0.034
Margarine and mixed origin lipids	−0.075	−0.252[a]	0.596	−0.012	−0.038	−0.032	0.464[a]	0.493	0.231	0.546	0.058	0.080	0.321	0.353	0.305	0.476
Meat	0.163[b]	0.143[b]	0.018	−0.023	−0.041[b]	−0.059[b]	−0.062	−0.050	−0.068	−0.008	0.170[b]	0.144[b]	0.060[a]	0.005	0.045	0.034
Oily fruits	−0.122	0.160b	−0.047	−0.407	0.028[b]	0.336[b]	−0.186	0.087	−0.172	−0.041	−0.269[a]	−0.079	−0.253	0.236	−0.823[a]	−0.135
Other milk products	−0.026	−0.070[a]	−0.098	0.041	−0.038	−0.073[a]	0.118	0.046	0.111	−0.025	0.067	−0.023	0.108	0.212[a]	0.004	0.095
Pulses	0.071[b]	0.037	0.081	−0.040	0.170[b]	0.199[b]	−0.035	−0.131	−0.058	−0.003	−0.040	−0.029	−0.005	−0.113	−0.082	0.068
Sauces	−0.097[b]	−0.031	−0.231	0.171	−0.084[b]	−0.069	−0.069	−0.133	−0.029	0.024	0.058	0.046	−0.031	−0.205	−0.086	−1.660
Savoury snacks	0.004	0.057	−0.199	−0.216	−0.199[b]	−0.135[a]	−14.922	−0.521[b]	−0.087	−0.430	−0.143[a]	−2.826[b]	0.172	−0.200	−0.008	−0.325
Soups/bouillon	0.046[b]	0.016	−0.048	−0.026	0.026[a]	−0.018	0.024	−0.047	0.035	−0.060	0.055[b]	0.024	0.029	0.014	0.075[a]	−0.013
Starch roots and potatoes	0.204[b]	0.229[b]	0.022	−0.196[a]	0.066[b]	0.046[a]	0.000	−0.109	−0.051	−0.082	0.049	0.045	−0.002	0.019	−0.051	0.008
Sugar products	−0.041	−0.049	0.047	−0.269	−0.051	−0.004	−0.016	−0.269	0.088	−0.338	−0.065	−0.103	0.135	−0.299	0.331[b]	−0.237
Vegetable oils	−0.078	0.253[a]	0.038	−0.700	0.666[b]	0.624[b]	−0.328	−0.514	0.098	−0.078	−0.210	−0.178	−0.039	0.280	−0.265	0.223
Vegetables (excluding potatoes)	0.067[b]	0.133[b]	−0.027	0.063	0.283[b]	0.308[b]	0.071	0.130	0.071	0.075	0.031	0.023	0.009	0.021	0.012	0.014
Vegetarian products	0.065	−0.019	0.267	−0.270	0.208[a]	0.209[b]	0.057	−0.106	−0.160	−0.174	0.077	−0.255[b]	−0.001	−0.021	−0.098	−0.056

Table 4 continued

Food groups	Vitamin B_6				Folate						Vitamin B_{12}					
(1 unit = 10 g/day)	Intakes (μg/d)		Serum vitamin B_6 (nmol/L)		Intakes (μg/d)		Plasma folate (nmol/L)		RBC-folate (nmol/L)		Intakes (μg/d)		Plasma vitamin B_{12} (pmol/L)		HoloTC (pmol/L)	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Water	0.003	−0.004	0.003	−0.008	0.003	0.005[b]	0.005	−0.005	0.003	0.002	0.004[a]	0.004[a]	0.002	−0.004	0.005	−0.001
White and butter milk	0.019[b]	0.016[a]	0.003	0.044	0.039[b]	0.044[b]	0.024	0.053	0.039	0.015	0.053[b]	0.055[b]	0.015	0.055[b]	0.032[b]	0.052[b]
Yogurt and white cheese	0.031[a]	0.024[b]	−0.011	0.066	0.050[b]	0.071[b]	0.051	0.066	0.038	0.011	0.065[b]	0.062[b]	−0.009	0.069	−0.005	0.116[b]

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

[a] Statistically significant at the 0.05 level

[b] Statistically significant at the 0.01 level

corresponding food group. In summary, fewer significant changes were observed for biomarkers than for vitamin intakes. The distribution and strength of changes in B-vitamins derivate from the increase in 10 g of the food groups' consumption were very similar in males and females.

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

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

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

Discussion

The results showed that intakes and biomarkers levels of B-vitamins are associated with overall dietary patterns and not only with their main dietary sources. Such observations provide useful information for the development of public health interventions aiming to increase B-vitamin intakes and levels in adolescents, mainly in the case of folate for which more than 90 % of both males and females did not

meet the current recommendations. However, in a previous report [6] resulted from the HELENA study, only 35 % of the adolescents had PF values (recent intakes) under the recommendations, and 27 % did it for RBC-folate (stores). Comparing with the same report, differences between vitamin B_6 and B_{12} intakes in relation with their respective biomarkers were not such pronounced. The proportion of adolescents who did not meet the recommended values for PLP were 20 % (compared with our 0 % regarding intakes of vitamin B_6), and between 2 and 5 % of our sample of adolescents did not meet the recommended values of vitamin B_{12} biomarkers accordingly as what we have obtained in our study for this vitamin. This is not surprising as in this sample, correlation between micronutrient intakes and concentrations in blood were not very high [28]. These differences found between intakes and status among the B-vitamins we are concerning about in this study may be given by the differences in the bioavailability [29–31] of all of them. For instance, for folate bioavailability ranges from around 30–50 % [29]; for vitamin B_{12} between 42–66 %, considering that its bioavailability significantly decreases with increasing intake of vitamin B_{12} per meal [31]; and for vitamin B_6, 75 % on average [30]. Besides, folate fortification of foods might be more common than for the other B-vitamins, or even underestimated [32].

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

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

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

A higher number of significant changes were observed for intakes than for blood concentrations. This is no surprising as blood concentrations could be influenced by other mechanisms such as cooking method, metabolism, interference with other nutrients, and physiologic status [42–44], among others. B-vitamins concentrations are not only related with foods containing these vitamins but more with dietary patterns [11], as also reported Vandevijvere et al. [28]. They reported that correlations found were better between food frequency consumption and concentration biomarkers than between food intakes (and concentration biomarker, also for folate and vitamin B_{12} biomarkers. This is most likely because food frequency consumption represents usual intake, while 24-h recalls represent current intake, in particular for foods that are generally not consumed daily. Another plausible explanation for these differences between intakes and biomarkers can be the fact that nutrients and food components can vary considerably

for the same food depending on where or how the food was grown or how it was processed [11]. Besides, the biomarkers used in our study do not always reflect the recent anecdotic intakes, but rather the usual intake and therefore do not necessarily match with the results from the 24-h recalls that were used for this study. For instance, PLP, RBC-folate, and HoloTC are not the most suitable biomarkers to detect changes in day-to-day variation intakes [3]. We should also consider that B-vitamins supplement use was not assessed in the HELENA study and fortification of the products has not been taking into account in the Food Composition DataBases (FCDBs) and these can also explain these differences [28]. In this sense, it is important to mention that the consumption of breakfast cereals (often fortified product) [37] was more strongly associated with B-vitamin biomarkers than with their intakes. This finding suggests that the quantification of vitamins used in the food composition table could underestimate the real amount of these vitamins because of the fortifications as occurs with folate and white and butter milk, or for vitamin B_{12} and fruit and vegetable juices, etc., or that the BLS FCDB (the Bundeslebensmittelschlüssel, the German food composition database used to assess all the dietary intake sample with the 24-h recalls) gives lower nutrient values than other national databases as was already shown by our colleagues Julián-Almárcegui et al. [45].

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

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

Strengths and limitations

The main strength of this study is the use of harmonised and standardised procedures in a large sample of adolescents from various European cities [12]. Questionnaires used in the study were previously validated [48]. Another important strength of the study is the correction procedures used to avoid the limitations of the 24-h recalls; the use of the MSM method to correct the crude intake data values for within-person variation [49], and the use of the correspondent B-vitamins biomarkers. Blood biomarkers were analysed in a centralised laboratory, strengthening the reliability of the results [20]. However, correlations between biomarkers and usual food intakes obtained from the recalls were low in this sample [28]. This could be due to the fact that dietary intakes correlate better with biomarkers when the number of days covered by the reference method increases [50]. On the other hand, due to standardization reasons, the use of the German food composition table, provided differences in B-vitamins composition when compared with national food composition tables. These differences were small, and for most nutrients negligible, which implies a reliable estimation [45]. Moreover, local adaptations to foods and recipes were done based on a protocol which was developed to make locally culture-specific food pictures. Each centre contributed to the upgrade of the tool to a European level by making inventories of country-specific food lists and by providing pictures of typical recipes [15]. An additional limitation is that food fortification is not included the German food composition database.

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

Conclusions

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

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

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

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HELENA Study Group

Co-ordinator Luis A. Moreno.

Core Group members Luis A. Moreno, Fréderic Gottrand, Stefaan De Henauw, Marcela González-Gross, Chantal Gilbert.

Steering Committee Anthony Kafatos (President), Luis A. Moreno, Christian Libersa, Stefaan De Henauw, Sara Castelló, Fréderic Gottrand, Mathilde Kersting, Michael Sjöstrom, Dénes Molnár, Marcela González-Gross, Jean Dallongeville, Chantal Gilbert, Gunnar Hall, Lea Maes, Luca Scalfi.

Project Manager Pilar Meléndez.

Universidad de Zaragoza (Spain) Luis A. Moreno, Jesús Fleta, José A. Casajús, Gerardo Rodríguez, Concepción Tomás, María I. Mesana, Germán Vicente-Rodríguez, Adoración Villarroya, Carlos M. Gil, Ignacio Ara, Juan Fernández Alvira, Gloria Bueno, Aurora Lázaro, Olga Bueno, Juan F. León, Jesús Mª Garagorri, Manuel Bueno, Idoia Labayen, Iris Iglesia, Silvia Bel, Luis A. Gracia Marco, Theodora Mouratidou, Alba Santaliestra-Pasías, Iris Iglesia, Esther González-Gil, Pilar De Miguel-Etayo, Cristina Julián Almárcegui, Mary Miguel-Berges, Isabel Iguacel.

Consejo Superior de Investigaciones Científicas (Spain) Ascensión Marcos, Julia Wärnberg, Esther Nova, Sonia Gómez, Ligia Esperanza Díaz, Javier Romeo, Ana Veses, Belén Zapatera, Tamara Pozo, David Martínez.

Université de Lille 2 (France) Laurent Beghin, Christian Libersa, Frédéric Gottrand, Catalina Iliescu, Juliana Von Berlepsch.

Research Institute of Child Nutrition Dortmund, Rheinische Friedrich-Wilhelms-Universität Bonn (Germany) Mathilde Kersting, Wolfgang Sichert-Hellert, Ellen Koeppen.

Pécsi Tudományegyetem (University of Pécs) (Hungary) Dénes Molnar, Eva Erhardt, Katalin Csernus, Katalin Török, Szilvia Bokor, Mrs. Angster, Enikö Nagy, Orsolya Kovács, Judit Répasi.

University of Crete School of Medicine (Greece) Anthony Kafatos, Caroline Codrington, María Plada, Angeliki Papadaki, Katerina Sarri, Anna Viskadourou, Christos Hatzis, Michael Kiriakakis, George Tsibinos, Constantine Vardavas, Manolis Sbokos, Eva Protoyeraki, Maria Fasoulaki.

Institut für Ernährungs- und Lebensmittelwissenschaften – Ernährungphysiologie. Rheinische Friedrich Wilhelms Universität (Germany) Peter Stehle, Klaus Pietrzik, Marcela González-Gross, Christina Breidenassel, Andre Spinneker, Jasmin Al-Tahan, Miriam Segoviano, Anke Berchtold, Christine Bierschbach, Erika Blatzheim, Adelheid Schuch, Petra Pickert.

University of Granada (Spain) Manuel J. Castillo, Ángel Gutiérrez, Francisco B Ortega, Jonatan R Ruiz, Enrique G Artero, Vanesa España, David Jiménez-Pavón, Palma Chillón, Cristóbal Sánchez-Muñoz, Magdalena Cuenca.

Istituto Nazionalen di Ricerca per gli Alimenti e la Nutrizione (Italy) Davide Arcella, Elena Azzini, Emma Barrison, Noemi Bevilacqua, Pasquale Buonocore, Giovina Catasta, Laura Censi, Donatella Ciarapica, Paola D'Acapito, Marika Ferrari, Myriam Galfo, Cinzia Le Donne, Catherine Leclercq, Giuseppe Maiani, Beatrice Mauro, Lorenza Mistura, Antonella Pasquali, Raffaela Piccinelli, Angela Polito, Romana Roccaldo, Raffaella Spada, Stefania Sette, Maria Zaccaria.

University of Napoli "Federico II" Dept of Food Science (Italy) Luca Scalfi, Paola Vitaglione, Concetta Montagnese.

Ghent University (Belgium) Ilse De Bourdeaudhuij, Stefaan De Henauw, Tineke De Vriendt, Lea Maes, Christophe Matthys, Carine Vereecken, Mieke de Maeyer, Charlene Ottevaere, Inge Huybrechts.

Medical University of Vienna (Austria) Kurt Widhalm, Katharina Phillipp, Sabine Dietrich, Birgit Kubelka, Marion Boriss-Riedl.

Harokopio University (Greece) Yannis Manios, Eva Grammatikaki, Zoi Bouloubasi, Tina Louisa Cook, Sofia Eleutheriou, Orsalia Consta, George Moschonis, Ioanna Katsaroli, George Kraniou, Stalo Papoutsou, Despoina Keke, Ioanna Petraki, Elena Bellou, Sofia Tanagra, Kostalenia Kallianoti, Dionysia Argyropoulou, Stamatoula Tsikrika, Christos Karaiskos.

Institut Pasteur de Lille (France) Jean Dallongeville, Aline Meirhaeghe.

Karolinska Institutet (Sweden) Michael Sjöstrom, Jonatan R Ruiz, Francisco B. Ortega, María Hagströmer, Anita Hurtig Wennlöf, Lena Hallström, Emma Patterson, Lydia Kwak, Julia Wärnberg, Nico Rizzo.

Asociación de Investigación de la Industria Agroalimentaria (Spain) Jackie Sánchez-Molero, Sara Castelló, Elena Picó, Maite Navarro, Blanca Viadel, José Enrique Carreres, Gema Merino, Rosa Sanjuán, María Lorente, María José Sánchez.

Campden BRI (United Kingdom) Chantal Gilbert, Sarah Thomas, Elaine Allchurch, Peter Burgess.

SIK - Institutet foer Livsmedel och Bioteknik (Sweden) Gunnar Hall, Annika Astrom, Anna Sverkén, Agneta Broberg.

Meurice Recherche & Development asbl (Belgium) Annick Masson, Claire Lehoux, Pascal Brabant, Philippe Pate, Laurence Fontaine.

Campden & Chorleywood Food Development Institute (Hungary) Andras Sebok, Tunde Kuti, Adrienn Hegyi.

Productos Aditivos SA (Spain) Cristina Maldonado, Ana Llorente.

Cárnicas Serrano SL (Spain) Emilio García.

Cederroth International AB (Sweden) Holger von Fircks, Marianne Lilja Hallberg, Maria Messerer.

Lantmännen Food R&D (Sweden) Mats Larsson, Helena Fredriksson, Viola Adamsson, Ingmar Börjesson.

European Food Information Council (Belgium) Laura Fernández, Laura Smillie, Josephine Wills.

Universidad Politécnica de Madrid (Spain) Marcela González-Gross, Raquel Pedrero-Chamizo, Agustín Meléndez, Jara Valtueña, David Jiménez-Pavón, Ulrike Albers, Pedro J. Benito, Juan José Gómez Lorente, David Cañada, Alejandro Urzanqui, Rosa María Torres, Paloma Navarro.

Compliance with ethical standards

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

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

Appendix

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

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Determinants

Do dietary patterns determine levels of vitamin B6, folate, and vitamin B12 intakes and corresponding biomarkers in European adolescents? The HELENA study.

Do dietary patterns determine levels of vitamin B_6, folate, and vitamin B_{12} intakes and corresponding biomarkers in European adolescents? The HELENA study.

Iris Iglesia, [1,2,3]*, Huybrechts, I.[4,5], Mouratidou, T.[1], Santabárbara, J.[6], Fernández-Alvira, J.M.[1,7], Santaliestra-Pasías, A.M.[1,2,8], Manios, Y.[9], De la O Puerta, A.[10], Kafatos, A.[11], Gottrand, F.[12], Marcos, A.[13], Sette, S.[14], Plada, M.[11], Stehle, P.[15], Molnár, D.[16], Widhalm, K.[17], Kersting, M.[18], De Henauw, S.[4], Moreno, L.A.[1,2,8], González-Gross, M.[19], on behalf of the HELENA study group*.

Affiliations

[1] GENUD (Growth, Exercise, NUtrition and Development) Research group, Universidad de Zaragoza
[2] Instituto Agroalimentario de Aragón (IA2), Instituto de Investigación Sanitaria Aragón (IIS Aragón),
[3] Red de Salud Materno-infantil y del Desarrollo (SAMID)
[4] Department of Public Health, Ghent University, Ghent, Belgium
[5] International Agency for Research on Cancer (IARC), 150 Cours Albert Thomas, 69372 Lyon Cedex 08, France
[6] Department of Preventive Medicine and Public Health, Universidad de Zaragoza,
Zaragoza, Spain
[7] Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III, Madrid, Spain
[8] Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y Nutrición (CIBERObn)
[9] Department of Nutrition and Dietetics, School of Health Science and Education, Harokopio University, Athens, Greece
[10] Department of Physiology, School of Medicine, University of Granada, Avenida de Madrid 11, 18012 Granada, Spain.
[11] University of Crete School of Medicine, GR-71033 Crete, Greece
[12] Univ. Lille, CHU Lille, LIRIC UMR 995 Inserm, Clinical Investigation Centre, CIC-1403–Inserm–CHU, F-59000 Lille, France
[13] Immunonutrition Research Group, Department of Metabolism and Nutrition, Institute of Food Science, Technology and Nutrition (ICTAN), Spanish National Research Council (CSIC), Madrid, Spain
[14] Council for Agricultural Research and Economics, Research Centre on Food and Nutrition, Via Ardeatina 546, 00178 Rome, Italy
[15] Department of Nutrition and Food Science, University of Bonn, D-53115 Bonn, Germany
[16] Department of Paediatrics, University of Pécs, Hungary
[17] Division of Clinical Nutrition and Prevention, Department of Pediatrics, Medical University of Vienna, Vienna, Austria
[18] Research Institute of Child Nutrition, Pediatric University Clinic, Ruhr-University Bochum, Germany
[19] ImFINE Research Group. Department of Health and Human Performance, Universidad Politécnica de Madrid, Madrid, Spain

Correspondence: Iris Iglesia*, 'Growth, Exercise, Nutrition and Development' (GENUD) Research group, Faculty of Health Sciences, University of Zaragoza, Spain.

C/Pedro Cerbuna, 12, Edif. SAI (Servicio de Apoyo a la Investigación), 2nd floor, 50009, Zaragoza, Spain.

Telephone: 0034 876 553 756

Email: iglesia@unizar.es

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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.

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 % males) and 586 did it for biomarkers analysis (47 % males). 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 females, 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 B_6, and vitamin B_{12} 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 B_6), 50 % insufficient values of folate, and 5 % of vitamin B_{12}. Deficiency of these vitamins can manifest in different population groups at different life stages when requirements are increased, such as during growth in children and adolescence (4). Deficiency of these three vitamins at early stages of life has been related to developmental delay, feeding problems, failure to thrive, irreversible neurological damage (5), severe or recurrent headache or migraine (6), or potential implications for an altered risk for chronic disease in later life (7).

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 (8) 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 (8).

A previous analysis based on the HELENA study (9), 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 (10) investigating the associations between PCA-derived dietary patterns (DPs) and the intake of some nutrients including vitamin B_6, folate, and vitamin B_{12}, there is no study addressing also B-vitamins concentrations in parallel in relation to dietary patterns obtained through RRR analyses among European adolescents. Therefore, the aim of this study is to determine dietary patterns which explain higher variability in intakes and statuses of B-vitamins in a large sample of European adolescents aged 12.5 to 17.5 years through RRR analysis.

Material and methods

Sampling

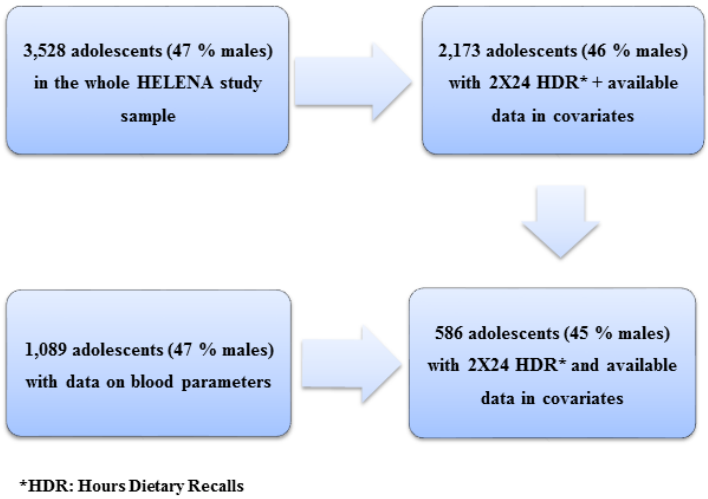
3,528 adolescents (47% males) from 10 European cities in 9 countries were recruited to participate in The Healthy Lifestyle in Europe by Nutrition in Adolescence Cross-Sectional Study (HELENA-CSS). The cities participating in this multi-centre study were Athens and Heraklion (Greece), Dortmund (Germany), 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 (11). 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 (12). 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 (13). 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 (14, 15). 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) (13).

From the initial number of 3,528 adolescents, 2,173 (46% males) 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% males) (12) of the initial sample of 3,528 adolescents did blood drawings. of these, only 586 adolescents (45% males) 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 (16).

Sampling selection procedures are shown in figure 1. Further study details were published elsewhere (12).

Figure 1. Sampling selection process.



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

Dietary food intake was estimated by means of self-administered computerized 24 HDR using the HELENA-Dietary Assessment Tool (HELENA-DIAT), adapted for European adolescents (16) 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 (16, 17). Finally, the Multiple Source Method (MSM) (18) was used to calculate usual nutrient intake removing the effect of day-to-day within-person variability and random error in the recalls.

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 (16, 19). 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', 'miscellaneous', and 'meat substitutes' 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 quantity of B-vitamins intake resulted from the combination of all the original food groups.

Assessment of vitamin B_6, folate and vitamin B_{12} biomarkers 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 (20). 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). The time span between sampling and processing was up to 24 h. However, a novel handling and transport system following the Good Clinical Practices (21), 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 (20).

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 haematology) (22).

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, 23). Plasma and RBC-folate and plasma vitamin 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) (20). Holotranscobalamin (HoloTC) was analysed in the biochemical lab at the Universidad Politécnica de Madrid by microparticle enzyme immunoassay (Active B_{12} Axis-Shield Ltd, Dundee, Scotland, UK) with the use of AxSym (Abbot Diagnostics, Abbott Park, IL, USA) (24).

Covariates

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 (25). 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 (26), 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 tes*t. 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 males and females in Europe (27). 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 (8). 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 (28). 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).

Results

Sensitivity analyses were performed comparing participants included vs. not included in the study (data not shown). The proportion of males in relation to females 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 males. For the dietary analyses, included males 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 females were significantly younger and presented higher BMI than those excluded ($p<0.05$). Regarding the biomarkers analyses, included males had significantly lower BMI ($p<0.05$). Included males

and females, 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 males than in females. 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 ($r_s= 0.06$) to moderate ($r_s=0.57$). Food and beverage intakes were mainly correlated with B-vitamins intake. For vitamin B_6 intake, both in males and in females, 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.

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*

†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.

	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)	
Food groups	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
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†

	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)	
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, potatoes	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
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

	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)	
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

	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)	
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

* $p<0.05$; † $p<0.01$

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.

	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		

	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)	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
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				
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	

	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)	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
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		

	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)	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
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	

RRR:Reduced-Rank regression analyses; HoloTC: Holotranscobalamin

In case of B-vitamins biomarkers, the foods and beverages which correlated higher, significantly and positively with them were: margarine with PLP both in males and females and breakfast cereals also in females; breakfast cereals both in males and females with plasma folate, and in females 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 males, and yogurt & milk, breakfast cereals, and water in females; plasma vitamin B_{12} correlated in males with butter & animal fats, breakfast cereals, white & buttermilk, and savoury snacks, and in females, with chocolate, yogurt and milk, and with white & buttermilk; and finally, with HoloTC, pulses and white & buttermilk correlated in males, and pasta, rice and flavour, yogurt and milk, and white & buttermilk correlated in females.

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 were able to explain between 34.2 % of the variance for folate in females to 23.7 % of the variance for vitamin B_{12} also in females. For B-vitamins biomarkers, the variance explained by the different dietary patterns ranged from17.2% for PLP in males and 7.0 % for plasma vitamin B_{12} in females. 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 males and females.

For biomarkers, results were less consistent in terms of foods loading direction, even loading oppositely for males and females in some cases. For instance, alcoholic drinks showed positive loadings for males and negative for females; sugars, and soft drinks both showed positive loadings for females and negative for males. 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 males and females, but negatively for biomarkers, also in both sexes.

Discussion

To our knowledge, there are no previous studies investigating the dietary patterns determining both the intake and the status of vitamin B_6, folate and vitamin B_{12} 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 B_6, folate, and vitamin B_{12} intake and status in a large sample of European adolescents.

The RRR-derived dietary patterns are able to 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 (9) 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 (29). 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. (10), in a similar population group, dietary patterns were obtained using Principal Components Analyses (PCA) and, in males, 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 (10). This "healthy pattern" consisted of high consumption frequencies of fruits, vegetables, legumes, mushrooms, chicken, rice, vegetable oil, soup, and grains in males; and of rice, vegetable oil, soup, chicken, legumes, vegetables, fruits, vegetarian dishes, eggs, fish, water, warm sauces and mushrooms in females. In our study, males' patterns presented some similarities, at least regarding vitamin B_6 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 B_6; 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 B_{12} intake density in males (10), consisted of processed meat, potatoes, white bread, margarine, meat (except chicken), eggs, cheese, and fish, while in our study, vitamin B_{12} intake was related to white and buttermilk, cheese and eggs but negatively associated with meat and starch roots and potatoes. In females, vitamin B_{12} intake density was associated with the so-called 'traditional and western' pattern in the cited German study by Ritcher et al. (10), 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 B_{12} intake in females was determined by a different pattern, characterized by the consumption of bread and rolls, yogurt and milk, eggs, white and buttermilk, and cheese, in detriment of meat and starch roots and potatoes. 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 B_{12} intake both in males and females. 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 males, B-vitamins intakes were determined by similar patterns in both studies, we found larger differences in females. 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 (30).

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. (31), a lacto-vegetarian dietary pattern was related to folate status and negatively related to vitamin B_{12} status. In USA Health-Professionals, folate status was negatively correlated with a 'Western' dietary pattern (32). 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 males and for females, apart the 'breakfast pattern' which explained a variance of 17.2 % and 10.7 % for males and females, respectively for PLP.

For instance, a dietary pattern that could be considered as "unhealthy" might explain between 12.8 % and 9.0 % of the variance in plasma folate concentrations in males and females, respectively. In males, RBC-folate was determined by a dietary pattern that could be considered as "healthy", while in females it was determined by a 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_{12} in males and females, respectively. A dietary pattern characterized by food items served at breakfast explained the variance for HoloTC in both sexes. Around 16 % of the females, of the 584 adolescents which had blood drawings, (33) used contraceptives, and B-vitamins status might be affected from them as was suggested for the literature, at least for vitamin B_6 (PLP). However, it is precisely for this vitamin for which we have obtained more similar patterns between males and females.

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 (9). 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 (9). In addition, two types of biomarkers were used in our study: those which reflect the recent intakes (such us plasma folate and plasma vitamin B_{12}), but also PLP, RBC-folate, and HoloTC which are more focused on detecting the corresponding vitamins storages (5). 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 *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 *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 (34, 35); however, *a priori* methods do not consider the correlated aspects of some food groups (36). New hybrid methods use a combination of *a priori* knowledge on nutrient intake or risk factors for disease and the underlying dietary data to derive dietary patterns (37), and RRR is among them (8).

Apart from that, the use of a large and culturally diverse sample of European adolescents from 9 European countries is also another important strength (12). Moreover, the questionnaires used to assess maternal education, were previously validated (38). 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 (39), 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

(29). 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 (40). 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 (41). Blood biomarkers were analysed in the same centre, strengthening the reliability of the lab-results (20).

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. Such differences might be due to the time lag between consumption and blood drawings. Besides, studies in this population group or using reduced rank regression analyses as a statistical technique have been performed to compare with our results are very scarce. In consequence, there is an urgent need for investigating what are the dietary patterns which determine B-vitamins intake and status in adolescents worldwide and to elucidate what are the main determinants of these patterns.

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Consequences

Folate and vitamin B_{12} concentrations are associated with plasma DHA and EPA fatty acids in European adolescents: the Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) study

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Folate and vitamin B_{12} concentrations are associated with plasma DHA and EPA fatty acids in European adolescents: the Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) study

I. Iglesia[1,2]*, I. Huybrechts[3,4], M. González-Gross[5,6], T. Mouratidou[1], J. Santabárbara[7], V. Chajès[4], E. M. González-Gil[1,6], J. Y. Park[4], S. Bel-Serrat[1], M. Cuenca-García[8,9], M. Castillo[8], M. Kersting[10], K. Widhalm[11], S. De Henauw[3], M. Sjöström[12,13,14], F. Gottrand[15], D. Molnár[16], Y. Manios[17], A. Kafatos[18], M. Ferrari[19], P. Stehle[20], A. Marcos[21], F. J. Sánchez-Muniz[22] and L. A. Moreno[1,2,6,] on behalf of the HELENA Study Group†

[1]*Growth Exercise, Nutrition and Development (GENUD) Research Group, Universidad de Zaragoza, Instituto Agroalimentario de Aragón (IA2), Instituto de Investigación Sanitaria de Aragón (IIS), 50009 Zaragoza, Spain*
[2]*Red de Salud Materno-infantil y del Desarrollo (SAMID), Instituto de Salud Carlos III, C/ Monforte de Lemos 3-5, Pabellón 11, Planta 0, 28029 Madrid, Spain*
[3]*Department of Public Health, Ghent University, University Hospital, De Pintelaan 185, entrance 42 (building K3), 4th floor, B-9000 Ghent, Belgium*
[4]*International Agency for Research on Cancer (IARC), 150 Cours Albert Thomas, 69372 Lyon Cedex 08, France*
[5]*ImFINE Research Group, Department of Health and Human Performance, Universidad Politécnica de Madrid, C/ Martín Fierro, 7, 28040 Madrid, Spain*
[6]*Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y Nutrición (CIBERObn), Instituto de Salud Carlos III, C/ Monforte de Lemos 3-5, Pabellón 11, Planta 0, 28029 Madrid, Spain*
[7]*Department of Preventive Medicine and Public Health, Universidad de Zaragoza, 50009 Zaragoza, Spain*
[8]*Department of Physiology, School of Medicine, University of Granada, Avenida de Madrid 11, 18012 Granada, Spain*
[9]*Department of Physical Education, Faculty of Education Science, University of Cádiz, Avenida República Saharaui sn 11519 Cádiz, Spain*
[10]*Research Institute of Child Nutrition Dortmund, Pediatric University Clinic, Ruhr-University Bochum, Heinstück 11, D-44225 Dortmund, Germany*
[11]*Department of Pediatrics, Division of Clinical Nutrition and Prevention, Medical University of Vienna, 1090 Vienna, Austria*
[12]*Department of Public Health Sciences, Division of Social Medicine, Karolinska Institutet, Norrbacka, level 3, 17176 Stockholm, Sweden*
[13]*Unit for Preventive Nutrition, Department of Biosciences and Nutrition, Karolinska Institute, Novum, SE-141 83 Huddinge, Sweden*
[14]*School of Health, Care and Social Welfare, Mälardalens University, Box 883, 721 23 Västerås, Sweden*
[15]*Inserm U995, Faculté de Médecine, Université Lille 2, F-59045 Lille Cedex, France*
[16]*Department of Paediatrics, University of Pécs, Szigeti str 12, H-7624 Pécs, Hungary*
[17]*Department of Nutrition and Dietetics, Harokopio University, E. Venizelou 70, 17671 Kallithea, reece, Kallithea-Athens, Greece*
[18]*School of Medicine, University of Crete, GR-71033 Crete, Greece*
[19]*CREA – Council for Agricultural Research and Economics – Research Center for Food and Nutrition, Via Ardeatina 546 - 00178 Roma, Italy*
[20]*Department of Nutrition and Food Science, University of Bonn, D-53115 Bonn, Germany*
[21]*Immunonutrition Research Group, Department of Metabolism and Nutrition, Institute of Food Science, Technology and Nutrition (ICTAN), Spanish National Research Council (CSIC), E-28040 Madrid, Spain*
[22]*Departamento de Nutrición, Facultad de Farmacia, Universidad Complutense de Madrid, 28040 Madrid, Spain*

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

* **Corresponding author:** I. Iglesia, email iglesia@unizar.es

† See online Supplementary material for the HELENA study group members.

Abstract

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

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

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

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

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

A recent systematic review examining the impact of homocysteine-lowering interventions via increased vitamins B_6, folate or B_{12} supplement uptake on cardiovascular events failed to identify any relationship[10]. This fact set out the possibility that the positive associations between serum homocysteine and CVD risk may be confounded by other cardiovascular risk factors[11] such as low plasma (*n*-3) long-chain PUFA levels. The main (*n*-3) long-chain PUFA such as EPA and DHA are suggested to act as cardioprotective elements by lowering blood pressure and heart rate, reducing serum TAG, thrombotic tendency, inflammation and arrhythmias and by improving endothelial function, insulin sensitivity, paraoxonase concentrations and plaque stability[12]. Previous animal[13–15] and human[16–18] studies have reported that some changes in plasma fatty acid (FA) concentrations might be explained by B-vitamin biomarkers because of their role as cofactors in the methylation of phosphatidylethanolamine (PE) to phosphatidylcholine (PC), crucial in the transport of PUFA in the blood. The latter could explain the controversial results observed regarding the association between tHcy and CVD risk. However, results obtained in such studies were also inconsistent among them[13–18].

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

Methods

Study design

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

Subjects

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

The present study was performed following the ethical guidelines of the Declaration of Helsinki, the Good Clinical Practice rules and the legislation about clinical research in

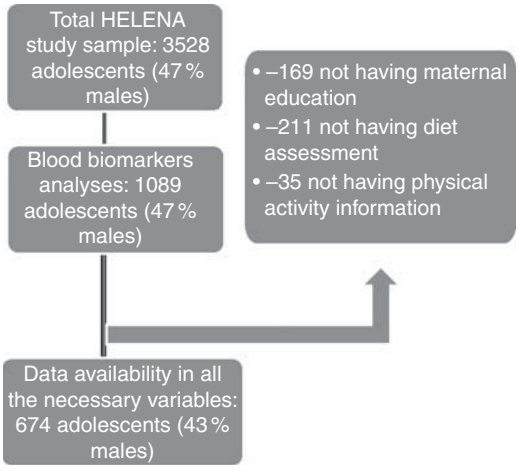

Fig. 1. Flow chart of the selection process from the Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) study initial sample.

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

Assessment of vitamin B_6, folate and vitamin B_{12} biomarker concentrations as well as serum phospholipid fatty acids

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

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

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

Diet Quality Index for adolescents

DQI was computed from data obtained from two non-consecutive, 24-h recalls performed via a computer-based, self-reported tool called the HELENA Dietary Assessment Tool[25]. This tool was based on a previous version developed for Flemish adolescents, the Young Adolescents' Nutrition Assessment on Computer valid for twenty-nine food groups aggregated according to the European Food Groups classification system[26] and for several nutrients apart from energy, including macronutrients, fibre, Ca, Fe and vitamin C[25]. Total daily food intake was distributed into six meal occasions. For each occasion, the user was invited to select all the consumed food items and beverages from a standardised food list. If any food or beverage was not included in the list, they could be added manually at any time. Household measurements or pictures of portion sizes were used to obtain information on quantities. The DQI used in this study was originally developed for preschool-aged children[27], and was later adapted and validated for use in adolescents[28] and consists of three components: dietary quality, dietary diversity and dietary equilibrium. This index is used as an indicator of compliance with the Flemish Food Based Dietary Guidelines, which are very similar to dietary guidelines in other countries including the WHO CINDI (Countrywide Integrated Non-communicable Disease Intervention programme) pyramid, making it applicable for a European population[28].

Physical examination

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

Socio-economic status and self-reported physical activity

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

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

Statistical analyses

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

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

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

Results

Several significant differences in terms of descriptive characteristics were observed between included and excluded participants (data not shown). In the whole HELENA study sample, males represented 48 %, whereas for the sample included in this analysis males represent 43 %. For instance, included males had significantly lower BMI and higher DQI scores, and there were significant differences in terms of maternal education ($P<0·001$) as compared with those excluded. No differences were observed in females. Included males had lower levels of DHA ($P<0·05$), whereas included females had higher levels of palmitoleic acid and palmitoleic:palmitic ratio ($P<0·05$). Both male and female participants had higher levels of α-linolenic fatty acid compared with adolescents excluded from the analysis. No differences were found between included and excluded adolescents regarding B-vitamin biomarkers.

Table 1 presents adolescents' characteristics stratified by sex. Females had significantly higher DQI scores and lipid parameters as opposed to males. Males had significantly higher weekly moderate-to-vigorous physical activity, lower levels of serum vitamin B_{12}, and higher PLP and tHcy levels ($P<0·01$). The mean palmitoleic:palmitic acid ratio was identical for both sexes.

Associations presented in Table 2 reflect changes in 10 units of the independent variables (B-vitamin biomarkers). A coefficient lesser than 10 implies a negative association. Overall, more significant associations were found between folate and vitamin B_{12} biomarkers and fasting lipid profile and serum PL FA status as compared with PLP or tHcy concentrations. All significant associations found with tHcy were negative, except for the oleic:stearic ratio where a positive significant association was observed in males with an increase of 10 µmol in tHcy concentrations, resulting in a decrease in the PL FA in serum. The associations found between either fasting lipid profile or serum PL FA and B-vitamin biomarkers were always in the opposite direction as those found with tHcy. Moreover, the association found between TAG and serum vitamin B_{12} was positive for males and negative for females. Most of the significant associations were observed between B-vitamin biomarkers and DHA, *n*-3 FA and *n*-3:*n*-6 ratio as the dependent variables.

Discussion

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

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

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

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

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

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

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

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

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

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

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

	PLP (nmol/l)		PF (nmol/l)		RBC-folate (nmol/l)		tHcy (μmol/l)		Serum vitamin B_{12} (pmol/l)		HoloTC (pmol/l)	
	β	SE	*β*	SE	*β*	SE	*β*	SE	*β*	SE	*β*	SE
Fasting lipid profiles: males												
TAG (mmol/l)	9·93	11·07	10·02	11·27	11·75	11·40	11·22	11·46	13·18*	11·56	12·02	11·53
Total cholesterol (mmol/l)	10·23	10·42	10·72	10·52	10·47	10·57	9·12	10·57	11·22*	10·62	11·75*	10·59
HDL-cholesterol (mmol/l)	11·22*	10·45	10·23	10·52	10·23	10·59	9·12	10·59	10·23*	10·64	10·72	10·62
LDL-cholesterol (mmol/l)	9·55	10·69	10·96	10·81	10·47	10·91	9·55	10·96	11·75*	11·04	11·48	10·96
Serum PL fatty acids												
Pentadecanoic (μmol/l)	10·47	11·02	9·95	11·25	11·75	11·40	8·32	11·40	17·78*	11·46	18·62*	11·43
Palmitic (μmol/l)	10·23	10·40	9·55	10·50	10·00	10·54	10·00	10·54	10·96*	10·59	10·47	10·57
Palmitoleic (μmol/l)	10·47	10·84	8·71	11·07	10·00	11·17	10·47	11·19	11·22	11·27	10·00	11·22
Stearic (μmol/l)	10·47	10·42	10·00	10·54	10·23	10·62	8·71*	10·64	11·48*	10·67	11·22*	10·64
Oleic (μmol/l)	9·55	10·64	7·94*	10·79	9·33	10·89	11·22	10·89	9·55	10·96	8·71*	10·94
Linoleic (μmol/l)	10·47	10·47	9·55	10·59	9·77	10·64	9·33	10·67	10·96*	10·69	10·47	10·67
γ-Linolenic (μmol/l)	9·95	11·07	7·76*	11·35	10·00	11·51	10·72	11·56	10·72	11·67	14·45	11·59
ALA (μmol/l)	10·47	11·14	8·51	11·40	11·75	11·56	8·51	11·56	12·30*	11·67	9·77	11·61
Eicosanoic (μmol/l)	10·05	10·76	8·91	10·94	9·33	11·04	9·77	11·04	8·71*	11·12	9·55	11·12
EPA (μmol/l)	12·30	11·27	10·47	11·59	14·79*	11·75	8·91	11·83	14·45*	11·89	14·79*	11·83
DHA (μmol/l)	12·30*	10·79	12·88*	10·96	14·13*	11·07	7·94*	11·09	13·18*	11·17	13·18*	11·14
Trans (μmol/l)	9·33	10·84	8·13*	11·02	9·12	11·14	11·22	11·14	8·32*	11·22	7·59*	11·17
SFA (μmol/l)	10·17	10·30	9·86	10·40	10·16	10·45	9·66	10·45	10·96*	10·47	10·57	10·47
MUFA (μmol/l)	9·77	10·54	8·32*	10·72	9·55	10·79	10·47	10·76	10·23	10·86	9·55	10·84
PUFA (μmol/l)	10·47	10·42	9·93	10·52	10·12	10·59	9·33	10·59	11·22*	10·64	10·96*	10·62
n-3 PUFA (μmol/l)	12·30*	10·81	12·30*	10·99	15·85*	11·09	7·94*	11·12	13·18*	11·19	13·49*	11·14
n-6 PUFA (μmol/l)	10·47	10·42	9·77	10·52	9·77	10·59	9·33	10·59	11·22*	10·64	10·72	10·62
Ratio *n*-6:*n*-3 PUFA	8·51*	10·62	7·94*	10·74	7·08*	10·81	11·48	10·84	8·32*	10·89	8·13*	10·86
Ratio oleic:stearic (oleoyl-CoA activity)	9·12	10·57	7·76*	10·69	8·91	10·76	12·88*	10·76	8·32*	10·81	7·76*	10·81
Ratio palmitoleic:palmitic (stearoyl-CoA activity)	9·12	10·72	7·76	10·89	8·91	10·99	12·88	10·99	8·32	11·07	7·76	11·02
Fasting lipid profile: females												
TAG (mmol/l)	10·09	10·84	10·23	11·14	12·02	11·40	8·32	11·61	7·41*	11·27	9·33	11·30
Total cholesterol (mmol/l)	10·05	10·33	10·47	10·42	11·48*	10·52	10·05	10·59	10·72	10·47	12·02*	10·47
HDL-cholesterol (mmol/l)	10·72	10·38	9·77	10·50	10·14	10·59	10·72	10·69	11·22	10·54	11·48*	10·54
LDL-cholesterol (mmol/l)	9·55	10·52	11·22	10·69	12·59*	10·84	9·77	10·99	10·72	10·79	12·30*	10·76
Serum PL fatty acids												
Pentadecanoic (μmol/l)	10·96	10·84	10·96	11·14	13·49*	11·38	6·76*	11·59	12·59	11·25	15·49*	11·25
Palmitic (μmol/l)	10·00	10·33	9·77	10·45	11·22*	10·52	10·00	10·62	9·33	10·50	10·47	10·50
Palmitoleic (μmol/l)	10·23	10·76	11·22	11·04	11·48	11·25	9·55	11·43	9·55	11·14	10·96	11·14
Stearic (μmol/l)	10·23	10·35	9·77	10·47	10·47	10·57	9·12	10·67	9·77	10·52	10·96*	10·52
Oleic (μmol/l)	10·23	10·52	9·12	10·72	10·96	10·86	9·77	10·99	8·71	10·76	10·23	10·76
Linoleic (μmol/l)	10·47	10·38	9·33	10·52	10·96	10·62	10·00	10·72	9·12	10·57	10·23	10·57
γ-Linolenic (μmol/l)	9·77	10·76	8·91	11·04	10·47	11·27	7·59*	11·46	9·33	11·17	11·75	11·17
ALA (μmol/l)	11·48	10·91	9·91	11·25	12·59*	11·51	7·08*	11·72	10·96	11·35	10·47	11·32
Eicosanoic (μmol/l)	8·71	10·59	8·91	10·81	10·72	10·99	7·94*	11·14	9·77	10·89	10·96	10·86
EPA (μmol/l)	11·48	11·04	10·72	11·46	15·85*	11·75	7·94	12·05	12·88	11·59	15·85*	11·56
DHA (μmol/l)	10·23	10·64	12·88*	10·84	15·14*	11·02	8·32	11·17	10·23	10·94	13·49*	10·91
Trans (μmol/l)	9·33	10·69	8·13*	10·91	10·96	11·12	11·48	11·30	9·12	11·04	9·12	11·02
SFA (μmol/l)	10·06	10·26	9·89	10·35	10·76*	10·42	9·84	10·47	9·51	10·40	10·62	10·40
MUFA (μmol/l)	10·09	10·45	9·55	10·62	10·96	10·74	9·77	10·84	9·12	10·67	10·72	10·67
PUFA (μmol/l)	10·23	10·35	9·77	10·47	11·22*	10·54	9·42	10·64	11·22	10·52	10·72	10·52
n-3 PUFA (μmol/l)	10·47	10·67	12·5*	10·89	15·85*	10·96	7·94	11·25	10·47	10·99	14·13*	10·96
n-6 PUFA (μmol/l)	10·23	10·35	9·55	10·47	10·96*	10·57	9·55	10·64	9·33	10·52	10·47	10·52
Ratio *n*-6:*n*-3 PUFA	9·55	10·54	7·76*	10·72	7·24*	10·86	11·48	10·99	8·91	10·79	7·94*	10·79
Ratio oleic:stearic (oleoyl-CoA activity)	9·93	10·42	9·33	10·57	10·23	10·69	10·72	10·79	9·12	10·62	9·33	10·62
Ratio palmitoleic:palmitic (stearoyl-CoA activity)	9·93	10·62	9·33	10·86	10·23	11·04	10·72	11·19	9·12	10·94	9·33	10·94

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

arachidonic acid (20 : 4 *n*-6) and DHA (22 : 6 *n*-3). This was also observed in one *in vitro* study with cultured cells[48] in which a reduction in vitamin B_6 levels led to changes in the *n*-3 and *n*-6 long-chain PUFA profile in parallel with reductions in the rate of the desaturation processes. They also found that concentrations of total FA and PL species were higher in vitamin B_6-deficient cells, as it was also observed in some studies in rats[49,50]. This might be because of the reduction in FA oxidation rates and

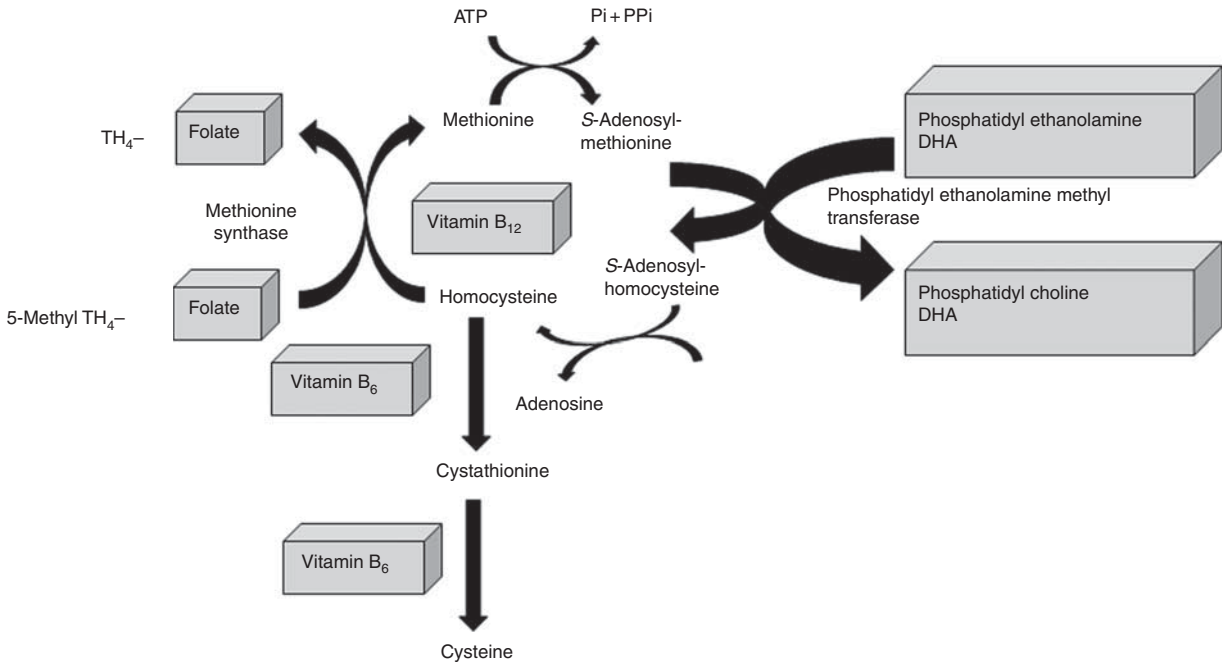

Fig. 2. One-carbon cycle: interactions between B-vitamins and DHA (adapted from Kulkarni *et al.*[46]).

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

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

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

It is worth noticing that we observed an inverse association between PF concentrations and *trans*-FA. *Trans*-FA can inhibit the activity of Δ6 desaturase in the liver and subsequently decrease the efficacy of HUFA interconversion[18] into PUFA. *Trans*-FA are a special type of unsaturated fatty acids with one double bond in the trans position and they can be naturally or industrially present in food products. Their presence in food products is highly variable (from 1 to 50 % industrially, to 2 to 9 % naturally)[53]. Because of the established association between *trans*-FA and CVD[54], the food industry has drastically decreased the content of these *trans*-FA in their products in the last decade, but there are still some European countries, especially in Eastern Europe, where levels are still elevated[53]. Our study was performed in 2006; at the time, the vast majority of *trans*-FA intakes came from industrial products and their level in blood was already low. The levels of these FA would be even lower, and it is very likely that such an association would not have been found if our study was reproduced today.

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

Considerations of the study

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

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

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

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

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

Conclusion

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

To conclude, our findings suggest that higher B-vitamin biomarker concentrations might be related to higher PUFA concentrations (mainly *n*-3 series). Besides, the pro-inflammation marker *n*-6:*n*-3 PUFA ratio decreased with increasing values of B-vitamin biomarkers, as with *trans*-FA (probably because of different dietary sources in this case) and the oleic:stearic acid ratio. Consequently, assuring a good status of B-vitamin biomarkers is very important as it might influence cardiovascular health by lowering homocysteine levels, but also by increasing circulating *n*-3 PUFA and decreasing the pro-inflammatory marker *n*-6:*n*-3 PUFA ratio.

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The authors declare that there are no conflicts of interest.

Supplementary material

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

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Consequences

Associations between insulin resistance and three B-vitamins in European adolescents: The HELENA study

Associations between insulin resistance and three B-vitamins in European adolescents: The HELENA study

Iglesia, I.[1,2], González-Gross, M.[3,4], Huybrechts, I.[5,6], De Miguel-Etayo, P.[1,4], Molnár, D.[7], Manios, Y.[8], Widhalm, K.[9], Gottrand, F.[10], Kafatos, A.[11], Marcos, A.[12], de la O Puerta, A.[13], Leclercq, C.[14], De Henauw, S.[5], Stehle, P.[15], Kersting, M.[16], Mouratidou, T.[1], Moreno, L.A.[1,4] on behalf of the HELENA study group.

[1] GENUD (Growth, Exercise, Nutrition and Development) Research group, Instituto Agroalimentario de Aragón (IA2), Instituto de Investigación Sanitaria Aragón (IIS Aragón), Universidad de Zaragoza
[2] Red de Salud Materno-infantil y del Desarrollo (SAMID)
[3] ImFINE Research Group. Department of Health and Human Performance, Universidad Politécnica de Madrid, Madrid, Spain
[4] Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y Nutrición (CIBERObn)
[5] Department of Public Health, Ghent University, Ghent, Belgium
[6] International Agency for Research on Cancer (IARC), 150 Cours Albert Thomas, 69372 Lyon Cedex 08, France
[7] Department of Paediatrics, University of Pécs, Hungary
[8] Department of Nutrition and Dietetics, Harokopio University, Athens, Greece
[9] Division of Clinical Nutrition and Prevention, Department of Pediatrics, Medical University of Vienna, Vienna, Austria
[10] Inserm U995, Faculté de Médecine, Université Lille 2, Lille, France
[11] University of Crete School of Medicine, GR-71033 Crete, Greece
[12] Immunonutrition Research Group, Department of Metabolism and Nutrition, Institute of Food Science, Technology and Nutrition (ICTAN), Spanish National Research Council (CSIC), Madrid, Spain
[13] Department of Physiology, School of Medicine, University of Granada, Avenida de Madrid 11, 18012 Granada, Spain.
[14] National Research Institute on Food and Nutrition, Rome, Italy
[15] Department of Nutrition and Food Science, University of Bonn, D-53115 Bonn, Germany
[16] Research Institute of Child Nutrition Dortmund, Pediatric University Clinic, Ruhr-University Bochum, Germany

Correspondence author: Iris Iglesia*, "Growth, Exercise, Nutrition and Development (GENUD) Research group, Faculty of Health Sciences, University of Zaragoza, Spain.
C/Pedro Cerbuna, 12, Edif. SAI (Servicio de Apoyo a la Investigación), 2nd floor, 50009, Zaragoza, Spain.
Telephone: 0034 876 553 756
Email: iglesia@unizar.es

ABSTRACT

OBJECTIVE: To assess whether adolescents with high body mass index (BMI), or fat mass index (FMI), in combination with insulin resistance (assessed with the HOmeostatic Model Assessment –HOMA- index) had also lower blood vitamin B_6, folate and vitamin B_{12} concentrations.

METHODS AND MATERIALS: 615 adolescents from the Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) study with data on B-vitamins (both intakes and status), and BMI, FMI, HOMA, were selected. Intakes were assessed by two non-consecutive 24-h recalls. B-vitamins biomarkers were measured by chromatography and immunoassay. Analysis of covariance was applied to elucidate the differences in B-vitamins between combinations of groups defined according to the median of the z-scores of markers of body composition and insulin sensitivity.

RESULTS: When considering energy intakes and education of the mother in the model, in females, vitamin B_6 intakes were higher in the high BMI/high HOMA group than in the high BMI-low HOMA group. Similarly, high FMI/high HOMA group than in the low FMI/low HOMA group. and Plasma vitamin B_{12} in males, were significantly lower in the high FMI/high HOMA than in the low FMI/low HOMA group keeping also significant their trends throughout the groups, what can be observed also for females ($p<0.05$).

CONCLUSION: Adolescents with combined higher adiposity and higher HOMA insulin sensitivity showed lower vitamin B_{12} plasmaconcentrations. These differences do not seem to be explained by dietary vitamin B_{12} intake.

Keywords: Vitamin B6, folic acid, vitamin B12, adolescent, body composition, insulin sensitivity

Abbreviations:

BMI: Body Mass Index

FMI: Fat Mass Index

DNA: Deoxyribonucleic acid

HoloTC: Holotranscobalamin

PF: Plasma Folate

RBC: Red blood cell

PLP: Pyridoxal-Phosphate

HELENA: Healthy Lifestyle in Europe by Nutrition in Adolescence

HOMA: HOmeostatic Model Assessment

HELENA-DIAT: HELENA-Dietary Assessment Tool

YANA-C: Young Adolescents' Nutrition Assessment on Computer

BLS: Bundeslebensmittelschlüssel

MSM: Multiple Source Method

EDTA: Ethylene Diamine Tetraacetic Acid

HPLC: High Performance Liquid Chromatography

CV: Coefficient of Variation

IEL: Institut fuer Ernährungs- und Lebensmittelwissebschaften

ANCOVA: Analysis of Covariance

HBSC: Health Behaviour in School-aged Children

1. Introduction

Prevalence of overweight and obesity in European children range between 10 and 40 percent among European adolescents (1) while obesity is currently considered the fifth leading risk for global deaths (2). Aside genetics, inadequate lifestyle factors like unhealthy dietary habits and/or insufficient physical activity are the main attributable causes of both overweight and obesity (3). Childhood obesity has been shown to be accompanied by low micronutrient intake and micronutrient deficiencies (4). For instance, obesity has been related to low iron intake in children and adolescents in a study developed in Israel (5), where obese children and adolescents showed a higher prevalence of iron deficiency or even iron deficiency anaemia than non-obese.

The body mass index (BMI) is the most widely used height-normalized index for the screening of excess body fat, also in adolescents, but it can be criticized since it does not discriminate between lean- and fat-mass (6). Measurement of skinfolds thickness allows an estimation of subcutaneous adipose tissue deposition and thus, the use of the fat mass index (FMI=kg fat mass/m^2) instead of the BMI for classifying obesity status in children (6). Obesity is often associated with hyperinsulinism and insulin resistance, which over time can develop into in glucose intolerance, impaired β-cell function and diabetes mellitus type 2 (7).

Vitamin B_{12}, is a crucial nutrient present in animal products (8). Its main roles are linked with cognitive function, bone health, and DNA-replication during periods of rapid growth and development like childhood and adolescence (9, 10). An optimal vitamin B_{12} status during early life stages is essential in preventing future health risks like anaemia (9). Besides, vitamin B_{12} deficiency contributes to hyperhomocysteinemia, which is an independent risk factor for atherosclerotic disease (11). Sub-clinical deficiencies of vitamin B_{12} status are not uncommon

during adolescence and in high risk population groups like vegans or vegetarians, elderly or low-resource people (12).

Low vitamin B_{12} status can be due to gut malabsorption syndromes, pernicious anaemia (13), or secondary malabsorption produced by metformin therapy, an insulin sensitizer used for the treatment of type 2 diabetes and insulin resistance in adolescents (14, 15).

A recent paper based on the HELENA study reported levels of B-related vitamins such as B_6, folate and B_{12} (16), in which 2% of studied adolescents had low plasma vitamin B_{12} and 5% had low holotranscobalamin (HoloTC) concentrations. Besides, low concentrations of both plasma folate (PF) and red blood cell (RBC-folate) were identified in 10% of the HELENA adolescents and low pyridoxal-phosphate (PLP) concentrations were also identified in 5% of them.

A recent Australian study with obese adolescents (13) called for investigation of associations between vitamin B_{12} status and insulin sensitivity including also dietary intakes. Consequently, *this study aims to assess whether adolescents with higher body mass index (BMI) or fat mass index (FMI), in combination with higher insulin sensitivity (high HOmeostatic Model Assessment (HOMA) index) had also lower B-vitamins concentrations.*

To our knowledge, this is the first study in European adolescents assessing the association between B-vitamins intake and concentrations and insulin sensitivity, considering indicators of body composition like BMI or FMI.

2. Methods and materials

The multicentre and cross-sectional study Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA-CSS) recruited adolescents aged 12.5-17.5 years, from 10 cities from 9 European countries: Athens and Heraklion in Greece, Dortmund in Germany, Ghent in Belgium, Lille in France, Pecs in Hungary, Rome in Italy, Stockholm in Sweden, Vienna in Austria, and Zaragoza in Spain. The purpose of the study was to provide complete and reliable information about the nutritional status of European adolescents (17). Inclusion criteria were: not participating simultaneously in another clinical trial and being free of any acute infection occurring < 1 week before inclusion (18). The total number of participants was 3,528 with an average participation rate of 67%, which can be considered acceptable for such a demanding epidemiological study (19). In one third of the sample in each study centre (1,076 adolescents), blood drawing was obtained. Participants from Heraklion and Pecs (7% of the total sample) did not provide comparable dietary data. For the purposes of this analysis, 615 adolescents were included having complete data on BMI, skinfold thickness to calculate FMI, maternal education, vitamin B_6, folate and vitamin B_{12}intakes and biomarkers and having the HOMA index for insulin sensitivity or resistance excluding also outliers (for biochemical measurements, outliers were considered when values were ±4 standard deviations from the mean). Further details on the HELENA sampling procedures, pilot study and reliability of the data have been published elsewhere (19). 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 (20).

2.1. Assessment of vitamin B_6, folate, vitamin B_{12} and energy intakes

Vitamin and energy intakes were assessed using the computerized 24-hour recall, self-administered HELENA-Dietary Assessment Tool (HELENA-DIAT), adapted for European adolescents from the Young Adolescents' Nutrition Assessment on Computer (YANA-C)

software (21). The adolescents completed the 24-hour recalls twice in a fortnight period. Trained staff were present during completion (21). Obtained data was linked to the German Food Code and Nutrient Data Base (BLS -Bundeslebensmittelschlüssel-, version II.3.1, 2005), with 12,000 coded foods, and with up to 158 nutrient data points available for each food item (21). When traditional or local foods were not available in the BLS table, recipes were composed using the foods from the BLS as ingredients. The Multiple Source Method (MSM) (22) was applied to calculate usual nutrient intakes removing the effect of day-to-day within-person variation and random error in the 2 recalls. B-vitamins diet densities were calculated as follows: (amount of B-vitamin intake per 1000 Kcal of diet/recommendation of the corresponding B-vitamin intake based on the Institute of Medicine recommendations) *100. Recommendations for vitamin B_6 intakes are 1,300µg for males and 1,200 µg for females, 400 µg for folate in males and females; and 2.4 µg for vitamin B_{12} in both sexes (9).

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

In schools, early in the morning, and in fasting status, 30 ml of blood was drawn according to a standardized blood collection protocol by a certified phlebotomist. More details on sample transport and quality assurance can be found elsewhere (23). For the measurement of pyridoxal 5´phosphate (PLP), biomarker of vitamin B_6, ethylene diamine tetra-acetic acid (EDTA) whole blood was centrifuged at 3,500 g for 15 min. The supernatants were stored at -80°C until analysed. PLP was measured by high performance liquid chromatography (HPLC) (Varian Deutschland GmbH, Darmstadt, Germany; CV = 1%) with a modified method of Kimura et al (16, 24).

For the measurement of plasma folate and plasma vitamin B_{12}, heparinised tubes were collected, placed immediately on ice, and centrifuged within 30 min (3,500 g for 15 min). The

supernatant fluid was transported at a stable temperature of 4-7°C to the central laboratory at the University of Bonn (IEL-Institut fuer Ernährungs- und Lebensmittelwissebschaften-, Germany) and stored at -80°C until assayed. After measuring hematocrit in situ, EDTA whole blood was used for the red blood cell folate (RBC-folate) analysis. EDTA whole blood was diluted 1:5 with freshly prepared 0.1% ascorbic acid for cell lysis and incubated for 60 min in the dark before storage at -80°C. Plasma folate, RBC-folate and plasma vitamin B_{12} were measured by means of a competitive immunoassay using the Immunolite 2000 analyzer (DPC Biermann GmbH, Bad Nauheim, Germany) (CV for plasma folate = 5.4%, RBC folate = 10.7%, Cobalamin = 5.0%) (23). Serum for measuring holotranscobalamin (HoloTC) were obtained by centrifuging blood collected in evacuated tubes without anticoagulant at 3,500 g for 15 min within 1 hour. Once send to the central laboratory, sera were aliquoted and stored at -80°C until transport in dry ice to the biochemical lab at the Universidad Politécnica de Madrid for analysis (Laboratory number 242 of the Laboratory Network of the Region of Madrid). HoloTC was measured by microparticle enzyme immunoassay (Active B_{12} Axis-Shield Ltd, Dundee, Scotland, UK) with the use of AxSym (Abbot Diagnostics, Abbott Park, IL, USA) (CV = 5.1%) (25).

Glucose was measured using enzymatic methods (Dade Behring, Schwalbach, Germany). Insulin levels were measured using an Immulite 200 analyser (DPC Bierman GmbH, Bad Nauheim, Germany). The homeostasis model assessment (HOMA) calculation was used as a measurement of insulin resistance (glycaemia X insulin/22.5) (26).

2.3. Confounders

Maternal education was used as proxy of socioeconomic status, obtained via self-administered questionnaire completed by the adolescents and expressed as: elementary, lower secondary,

higher secondary or tertiary education. This variable was one of the most related socioeconomic factors associated with the studied vitamins (27). Total energy intake in kcal/d assessed with the 24 hours' dietary recalls software HELENA-DIAT was also used as a covariate in the analyses.

2.4. Anthropometry

Anthropometry battery measurements were assessed following standardized and strictly controlled procedures previously described (28). Weight was measured in underwear and without shoes with an electronic scale (Type SECA 861) to the nearest 0.05 kg, and height was measured barefoot in the Frankfort plane with a telescopic height measuring instrument (Type SECA 225) to the nearest 0.1 cm. Body weight and height, together with subscapular and tricipital skinfold thicknesses, were measured in triplicate. BMI was calculated using the Quetelet formula (kg/m^2). The body fat percentage was calculated using Slaughter's equation (29), and thereafter the fat mass index (FMI) was calculated by dividing fat mass by height squared (m^2).

2.5. Statistical analysis

The Statistical Package for Social Sciences version 20.0 (SPSS Inc., Chicago, IL, USA) was used to analyse the data. All analyses were sex-specific. Descriptive data are presented as means and standard deviations (SD). Z-scores of BMI and FMI, and HOMA considering age and sex were used in the analyses. Mann-Whitney test for non-parametric variables was applied to examine differences between two predefined groups i.e. above or under the median of the z-scores for BMI, FMI, and HOMA. Thereafter, obtained categories were combined to create 4 subsequent groups to analyse intakes and statuses of the vitamins: Low BMI (or FMI)

– Low HOMA (the most favourable group in terms of body composition and insulin sensitivity); Low BMI (or FMI) – High HOMA, High BMI (or FMI) – Low HOMA, and finally, High BMI (or FMI) – High HOMA (the less favourable group). Kruskal-Wallis test were applied to look at the differences in B-vitamin intakes and biomarkers, among groups; with a Mann-Whitney test approach to contrast values 2x2. Finally, maternal education, and total energy intakes were included in the analysis of covariance (ANCOVA) as covariates. All statistical tests and corresponding p values were two-sided, and $p<0.05$ was the cut-off to consider a result statistically significant.

3. Results

In the whole HELENA sample, males represented 45% and this percentage is significantly lower than in our analysis in which males represented the 47% ($p=0.004$). In terms of maternal education, included males and females showed significantly higher maternal education levels than those who were excluded (data not shown) ($p<0.001$). Males included in the dietary analysis had significantly lower energy intake ($p=0.001$) than those excluded; no significant differences were observed in females. There was no statistical significant difference in biomarkers of all three vitamins between the samples included and excluded (based on the inclusion criteria and required data availability).

Table 1 presents the main characteristics of the sample, by sex. FMI and HOMA were significantly higher in females than in males whilst total energy intake was lower ($p<0.05$).

Table 1. Characteristics of the participants by sex.

Characteristics	Males (n=281), mean ± SD	Females (n=334), mean ± SD	p-values
Age*	14.8 ± 1.3	14.8 ±1.2	0.71
BMI (Kg/m^2)	20.9 ±3.6	21.2 ± 3.3	0.12
FMI (Kg fat mass/m^2)	12.6 ±9.2	15.0 ± 6.8	**0.03**
HOMA	2.0 ±1.2	2.2 ± 1.2	**< 0.00**
Energy intake (Kcal/d)*	2,523 ± 816	1,892 ± 544	**< 0.00**
Vitamin B_6 diet density (%)*	57.2 ± 14.8	64.8 ± 16.3	**< 0.00**
Folate diet density (%)*	21.8 ± 5.8	24.4 ± 6.5	**< 0.00**
Vitamin B_{12} diet density (%)*	104.4± 33.5	104.0 ± 38.0	0.43
ME: Low education (n, %)	17 (6)	29 (9)	0.17
ME: Medium-Low education (n, %)	60 (21)	83 (25)	
ME: Medium-High education (n, %)	92 (33)	115 (34)	
ME: High education (n, %)	112 (40)	107 (32)	

BMI: Body Mass Index; FMI: Fat Mass Index; HOMA: Homeostasis Model Assessment; ME: Maternal Education

B-vitamins diet densities were calculated as follows: (amount of B-vitamin intake per 1000 Kcal of diet/recommendation of the corresponding B-vitamin intake based on the Institute of Medicine recommendations) *100. Recommendations for vitamin B_6 intakes are 1,300µg for males and 1,200 µg for females, 400 µg for folate in males and females; and 2.4 µg for vitamin B_{12} in both sexes.

P-values in bold are the only significant ones, based either in Mann-Whitney test or t-test* at 0.05 level two-sided.

Table 2 presents adolescents' B-vitamins intakes and biomarkers by the corresponding groups based on the median z-scores of variables of interest (BMI, FMI, and HOMA). Adolescents with low BMI, HOMA and FMI z-scores consumed more B-vitamins than those with high BMI, HOMA and FMI z-scores, except in the case of males with low HOMA, who had lower folate intakes. Considering biomarkers, adolescents with lower BMI, FMI and HOMA, showed higher B-vitamins levels, except in the case of males belonging to the Low HOMA group, who had lower RBC-folate concentrations than their counterparts in the higher HOMA group ($p<0.05$).

Table 2. B-vitamin intakes and concentrations and the groups established by the medians of the sex-specific z-scores of body mass index, fat mass index and Homeostasis Model Assessment index stratified by sex.

B-vitamins	**Males**			**Females**		
	<median of BMI Zscore^ (n=140) Mean±SD	**>median of BMI Zscore^(n=141) Mean±SD**	**p-value**	**<median of BMI Zscore^ (n=167) Mean±SD**	**>median of BMI Zscore^(n=167) Mean±SD**	**p-value**
INTAKES						
Vitamin B_6 (µg/d)	1,925 ± 608	1,725±577	**0.01**	1,508 ± 511	1,385 ± 446	**0.02**
Vitamin B_6 diet density (%)	56.0 ± 13.5	58.4 ±16.0	0.25	61.8 ± 15.3	67.9 ± 16.8	**0.00**
Folate(µg/d)	222 ±73.2	206 ±69.5	0.11	189 ± 57.2	172 ± 56.2	**0.01**
Folate diet density (%)	21.0 ± 4.8	22.7 ± 6.6	**0.04**	23.5 ± 5.6	25.4 ± 7.2	**0.01**
Vitamin B_{12}(µg/d)	6.6 ± 2.4	5.8 ±2.5	**0.00**	4.8 ± 1.6	4.4 ± 1.7	**0.00**
Vitamin B_{12} diet density (%)	104.5 ± 32.6	104.4 ± 34.5	0.92	99.9 ±30.9	108.1 ± 43.7	0.13
STATUS						
PLP(pmol/L)	67.3 ±42.0	69.3 ±47.4	0.75	63.7 ± 80.3	62.0 ± 44.4	0.58

PF(nmol/L)	18.6 ±9.4	18.2 ±11.4	0.29	18.8 ± 9.3	17.8 ± 10.1	0.09
RBC-folate (nmol/L)	818±328	827±433	0.47	772 ± 305	761± 311	0.67
Plasma B_{12}(pmol/L)	358 ±143	318±115	**0.04**	403 ± 154	355 ± 159	**0.00**
HoloTC (pmol/L)	66.1 ± 36.9	64.5 ±27.4	0.64	64.8 ± 36.1	65.7 ± 36.6	0.82
	<median of FMI Zscore (n=140)	**>median of FMI Zscore (n=141)**		**<median of FMI Zscore (n=167)**	**>median of FMI Zscore (n=167)**	
INTAKES						
Vitamin B_6 (µg/d)	1,910 ± 606	1,740 ± 585	**0.01**	1,466 ± 458	1,426 ± 506	0.31
Vitamin B_6 diet density (%)	54.8 ± 12.9	59.6 ±16.3	**0.03**	62.1 ± 15.7	67.6 ± 16.5	**0.02**
Folate(µg/d)	222 ± 72.8	206 ± 70.0	0.07	185 ± 56.0	176 ± 57.7	0.10
Folate diet density (%)	20.8 ± 4.9	22.9 ± 6.4	**0.01**	23.6 ± 4.9	25.3 ± 7.1	**0.02**
Vitamin B_{12}(µg/d)	6.7 ± 2.5	5.7 ± 2.4	**<0.00**	4.7 ± 1.5	4.5 ± 1.8	0.15
Vitamin B_{12} diet density (%)	104.4 ± 33.6	104.4 ± 33.6	0.95	104.4 ±33.6	10.4.4 ± 33.6	0.13
STATUS						
PLP(pmol/L)	65.5 ± 41.7	71.1 ± 47.3	0.24	67.1 ± 81.4	58.4 ± 41.0	0.60
PF(nmol/L)	18.5 ± 9.5	18.3 ± 11.2	0.49	19.1 ± 10.1	17.5 ± 9.3	**0.07**
RBC-folate (nmol/L)	825 ± 335	820 ± 428	0.40	787 ± 311	746 ± 304	0.14
Plasma B_{12}(pmol/L)	362 ± 144	313 ± 113	**0.01**	403 ± 165	355 ± 147	**0.01**
HoloTC (pmol/L)	67.6 ± 38.4	63.0 ± 24.7	0.76	66.2 ± 38.3	64.4 ± 34.3	0.99
	<median of HOMA Zscore (n=140)	**>median of HOMA Zscore (n=141)**		**<median of HOMA Zscore (n=167)**	**>median of HOMA Zscore (n=167)**	
INTAKES						
Vitamin B_6 (µg/d)	1,868 ± 620	1,781± 579	0.20	1,431 ± 459	1,461 ± 506	0.88
Vitamin B_6 diet density (%)	56.2 ± 14.2	58.1 ± 15.4	0.29	62.5 ± 15.7	67.1 ± 16.6	**0.01**

Folate(µg/d)	212 ± 70.4	216 ±73.2	0.84	184 ± 57.4	178 ± 57.0	0.41
Folate diet density (%)	20.8 ± 5.1	22.9 ± 6.3	**0.00**	24.2 ± 6.6	24.7 ± 6.5	0.42
Vitamin B_{12}(µg/d)	6.5 ± 2.7	5.9 ±2.2	**0.04**	4.6 ± 1.7	4.5 ± 1.7	0.65
Vitamin B_{12} diet density (%)	105.5 ± 35.0	103.3 ± 32.1	064	101.5 ± 37.4	106.4 ± 38.6	0.18
STATUS						
PLP(pmol/L)	67.4 ± 42.0	69.1 ±47.3	1.0	67.5 ± 79.1	58.2 ± 46.3	0.24
PF(nmol/L)	18.9 ± 10.7	17.9 ±10.1	0.43	19.6 ± 10.4	17.0 ± 8.9	**0.00**
RBC-folate (nmol/L)	815 ± 395	829 ± 373	0.74	766 ± 285	766 ± 329	0.70
Plasma B_{12}(pmol/L)	354 ± 132	322 ± 129	**0.03**	397 ± 160	361 ± 154	**0.03**
HoloTC (pmol/L)	70.1 ± 40.4	60.5 ± 20.6	0.17	67.8 ± 40.1	62.8 ± 32.1	0.33

BMI: Body Mass Index; FMI: Fat Mass Index; HOMA: Homeostasis Model Assessment; PLP: Pyridoxal Phosphate; PF: Plasma Folate; RBC-folate: Red Blood Cell Folate; HoloTC: Holotranscobalamin; SD: Standard Deviation

B-vitamins diet densities were calculated as follows: (amount of B-vitamin intake per 100 Kcal of diet/recommendation of the corresponding B-vitamin intake based on the Institute of Medicine recommendations) *100. Recommendations for vitamin B_6 intakes are 1,300µg for males and 1,200 µg for females, 400 µg for folate in males and females; and 2.4 µg for vitamin B_{12} in both sexes.

P-value based on Mann-Whitney non-parametric test.

Statistical significance at 0.05 two-sided level.

^Sex-specific Z-scores of BMI, FMI and HOMA were adjusted by age.

Supplementary table 1 shows the differences in B-vitamins both in intakes and concentrations among the groups defined by the subsequent categories of <median and >median of BMI, FMI, and HOMA z-scores considered in combination, without any additional adjustment. All in all, results showed that adolescents belonging to the less favourable groups (high BMI-or FMI-/high HOMA), had lower B-vitamins intake and concentrations, and also lower total energy intakes. However, when an analysis of the covariance was performed to introduce in the model the covariates of education of the mother and total energy intake, most of the statistically significant differences among groups disappeared.

Supplementary table 1. Differences of B-vitamin intakes and biomarkers concentrations by resulting combination groups between z-scores of body mass index (BMI) and fat mass index (FMI) against z-scores of insulin sensitivity (HOMA) index.

Males	**energy intake (mean, SD)**	**Vitamin B_6 (mean, SD)**	**Folate (mean, SD)**	**Vitamin B_{12} (mean, SD)**	**PLP (mean, SD)**	**PF (mean, SD)**	**RBC-folate (mean, SD)**	**Plasma B_{12} (mean, SD)**	**HoloTC (mean, SD)**
BMI by HOMA (n)									
Low BMI-Low HOMA (91)	2,659 ±783**a**	1,910 ± 618**a**	215 ± 71	6.7 ±2.4**ab**	65.7 ± 40.8	18.3 ± 8.7	782 ± 300	366 ± 142**a**	70.3 ± 43.5
Low BMI-High HOMA (49)	2,763 ± 893	1,953 ± 596	236 ± 77.0	6.3 ± 2.4	70.3 ± 44.4	19.1 ± 10.5	882 ± 370	342 ± 147	58.5 ± 17.6
High BMI-Low HOMA (49)	2,502 ± 800	1,791 ± 552	208 ± 70.4	6.1 ± 3.1**a**	70.8 ± 44.6	20.0 ± 13.7	877 ± 525	330 ± 111	69.7 ± 34.6
High BMI-High HOMA (92)	2,273 ± 757**a**	1,689 ± 552**a**	206 ± 69.5	5.6 ± 2.1**b**	68.5 ± 49.0	17.3 ± 9.9	801 ± 373	311 ± 117**a**	61.6 ± 22.1
p-value	**<0.00**	**0.03**	0.19	**0.01**	0.97	0.64	0.55	0.08	0.44
FMI by HOMA (n)									
Low-FMI-Low HOMA (86)	2,660 ± 770**a**	1,903 ± 628**a**	215 ± 69.1	6.8 ± 2.5**ab**	65.5 ± 41.1	18.5 ± 9.1	778 ± 296	366 ± 141**a**	71.5 ± 44.7

Low FMI-High HOMA (54)	2,843 ± 952	1,921 ± 569	235 ± 78.1	6.4 ± 2.4	65.6 ± 43.5	18.6 ± 10.4	908 ± 385	357 ± 149.0	60.4 ± 20.7
High FMI-Low HOMA (54)	2,504 ± 822	1,805 ± 605	208 ± 73.1	6.0 ± 2.9**a**	71.1 ± 44.0	19.7 ± 13.2	882 ± 525	332 ± 113	67.5 ± 30.7
High FMI-High HOMA (87)	2,223 ± 677**a**	1,704 ± 574**a**	206 ± 68.6	5.6 ± 2.0**b**	71.0 ± 49.3	17.5 ± 9.9	785 ± 361	302 ± 112**a**	60.6 ± 20.7
p-value	**<0.00**	0.06	0.17	**0.00**	0.68	0.83	0.21	**0.02**	0.57
Females									
BMI by HOMA (n)									
Low BMI-Low HOMA (100)	2,045 ± 483**ab**	1,511 ± 488**a**	194 ± 52.4**ab**	4.8 ± 1.6**ab**	64.7 ± 94.4**a**	19.7 ± 9.3**ab**	779 ± 290	418 ± 149**ab**	67.2 ± 33.7
Low BMI-High HOMA (67)	2,043 ± 544	1,502 ± 546	181 ± 63.4	4.8 ± 1.5	62.2 ± 54.4	17.5 ± 9.2**a**	760 ± 329	380 ± 159	61.3 ± 39.5
High BMI-Low HOMA (67)	1,762 ± 449**a**	1,312 ± 386**a**	168 ± 61.5**a**	4.3 ± 1.7**a**	71.6 ± 49.1**a**	19.4 ± 11.9	747 ± 280	366 ± 172**a**	68.8 ± 48.3
High BMI-High HOMA (100)	1,727 ± 595**b**	1,433 ± 478	175 ± 52.6**b**	4.4 ± 1.7**b**	55.5 ± 39.9	16.7 ± 8.7**b**	770 ± 331	348 ± 151**b**	63.7 ± 26.6
p-value	**<0.00**	**0.05**	**0.01**	**0.02**	**0.04**	**0.02**	0.95	**0.00**	0.47
FMI by HOMA (n)									
Low FMI-Low HOMA (102)	1,995 ± 437**ab**	1,466 ± 417	189 ± 51.1	4.7 ± 1.5	70.0 ± 94.7	19.9 ± 10.4**a**	790.7 ± 294	421 ± 159**abc**	67.6 ± 35.4

Low FMI-High HOMA (65)	1,995 ± 554	1,467 ±520	180 ± 64.1	4.7 ± 1.5	63.4 ± 56.1	17.8 ± 9.7	780 ± 339	374 ± 157**a**	63.9 ± 42.6
High FMI-Low HOMA (65)	1,832 ± 550**a**	1,377 ± 517	175.4 ± 65.7	4.5 ± 1.9	64.2 ± 44.6	19.0 ± 10.4	728 ± 268	359 ± 156**b**	68.1 ± 46.8
High FMI-High HOMA (102)	1,764 ± 604**b**	1,457 ± 499	176 ± 52.3	4.5 ± 1.7	54.8 ± 38.3	16.6 ± 8.4**a**	757 ± 324	353 ± 142**c**	62.0 ± 23.4
p-value	**<0.00**	0.64	0.24	0.54	0.35	**0.02**	0.51	**0.01**	0.57

BMI: Body Mass Index; HOMA: Homeostasis Model Assessment (índice de resistencia a la insulina); FMI: Fat Mass Index; PLP: Pyridoxal Phosphate; PF: Plasma Folate; RBC-folate: Red Blood Cell Folate; HoloTC: Holotranscobalamin; SD: Standard Deviation

P-value based on Kruskal-Wallis non-parametric test.

Statistical significance was established at 0.05 two-sided level.

Superscripts letter in bold represent those groups with statistical significant differences.

Post hoc comparisons between groups have been established based on Mann-Whitney test. Statistical significance critical value established at 0.0167 two-sided level (resulting from dividing 0.05/3 comparisons with the reference category of "Low BMI (or FMI)-Low HOMA" which represents the more favorable option).

Table 3 shows similarly, the adjusted results of the table 2 (by energy intakes, and maternal education). In females, vitamin B_6 intakes were higher in the high BMI/high HOMA group than in the high BMI/low HOMA group ($p<0.05$). Similarly, high FMI/high HOMA group than in the low FMI/low HOMA group. Plasma vitamin B_{12} in males, were significantly lower in the high FMI/high HOMA than in the low FMI/low HOMA group keeping also significant their trends throughout the groups, what can be observed also for females ($p<0.05$). These trends can be followed in the figure 1.

Table 3. Adjusted estimates of B-vitamin intakes and biomarker status by combination of body mass index, fat mass index and Homeostasis Model Assessment index.

INDICATORS	Vitamin B_{12} intakes*(µg/d)*				Plasma B_{12} *(pmol/L)*				Holotranscobalamin*(pmol/L)*			
	Males		Females		Males		Females		Males		Females	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
BMI by HOMA (n)												
Low BMI-Low HOMA (90, 100)	6.5	0.2	4.5	0.1	364	13.7	416	15.7	70.1	3.5	65.1	3.8
Low BMI- High HOMA (50, 67)	5.9	0.3	4.6	0.2	339	18.6	373	19.2	57.9	4.8	60.2	4.6
High BMI-Low HOMA (50, 67)	6.1	0.3	4.4	0.2	330	18.6	352	19.1	69.2	4.7	69.0	4.5
High BMI-High HOMA (91, 100)	6.1	0.2	4.7	0.1	315	13.7	362	15.9	62.5	3.6	66.3	3.7
F and p-values	1.01	0.39	0.66	0.58	2.17	0.09	1.78	0.15	1.85	0.14	0.64	0.59
FMI by HOMA (n)												
Low-FMI-Low HOMA (86, 101)	6.5	0.2	4.5	0.1	361**a**	13.8	426	15.3	70.5	3.5	66.5	3.7
Low FMI-High HOMA (54, 66)	5.9	0.3	4.5	0.2	355	8.5	352	19.0	59.3	4.8	63.5	4.6
High FMI-Low HOMA (54, 66)	6.1	0.3	4.5	0.2	338	18.4	361	19.1	68.4	4.9	67.2	4.6
High FMI-High HOMA (87, 101)	6.1	0.2	4.8	0.1	306**a**	13.8	360	15.5	61.7	3.6	64.0	3.7
F and p-values	1.18	0.32	0.71	0.55	2.89	**0.04**	2.94	**0.03**	1.73	0.16	0.18	0.91

INDICATORS	Vitamin B_6 intakes *(µg/d)*				Pyridoxal phosphate *(pmol/L)*				Folate intakes (µg/d)				Plasma folate (pmol/L)				RBC-folate (pmol/L)			
	Males		Females		Males		Females		Males		Females		Males		Females		Males		Females	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
BMI by HOMA (n)																				
Low BMI-Low HOMA (90, 100)	1,832	46.1	1,409	36.5	65.9	4.9	63.6	6.9	207	5.5	183	4.6	18.7	1.1	19.6	1.0	792	41.0	776	31.7
Low BMI-High HOMA (50, 67)	1,834	62.6	1,416	44.3	69.7	6.8	60.8	8.2	221	7.4	171	5.5	19.2	1.5	17.3	1.2	882	55.2	753	38.4
High BMI-Low HOMA (50, 67)	1,803	62.4	1,385**a**	44.0	71.3	7.0	72.3	8.3	208	7.4	176	5.5	19.7	1.5	19.4	1.2	867	55.1	750	38.3
High BMI-High HOMA (91, 100)	1,823	46.1	1,544**a**	36.8	68.3	5.1	57.1	6.9	222	5.5	188	4.6	17.0	1.1	16.9	1.0	796	41.2	777	31.9
F and p-values	0.06	0.98	3.57	**0.02**	0.16	0.93	0.70	0.55	1.79	0.15	2.18	0.09	0.88	0.45	1.69	0.17	0.93	0.43	0.17	0.92
FMI by HOMA (n)																				
Low-FMI-Low HOMA (86, 101)	1,817	46.4	1,393**a**	35.8	65.8	5.0	68.9	6.7	205	5.5	181	4.5	18.7	1.1	19.8	1.0	786	41.2	788	30.9
Low FMI-High HOMA (54, 66)	1,759	62.2	1,408	44.9	64.7	7.0	62.4	8.3	215	7.4	173	5.6	18.6	1.5	17.8	1.2	904	54.9	780	39.7

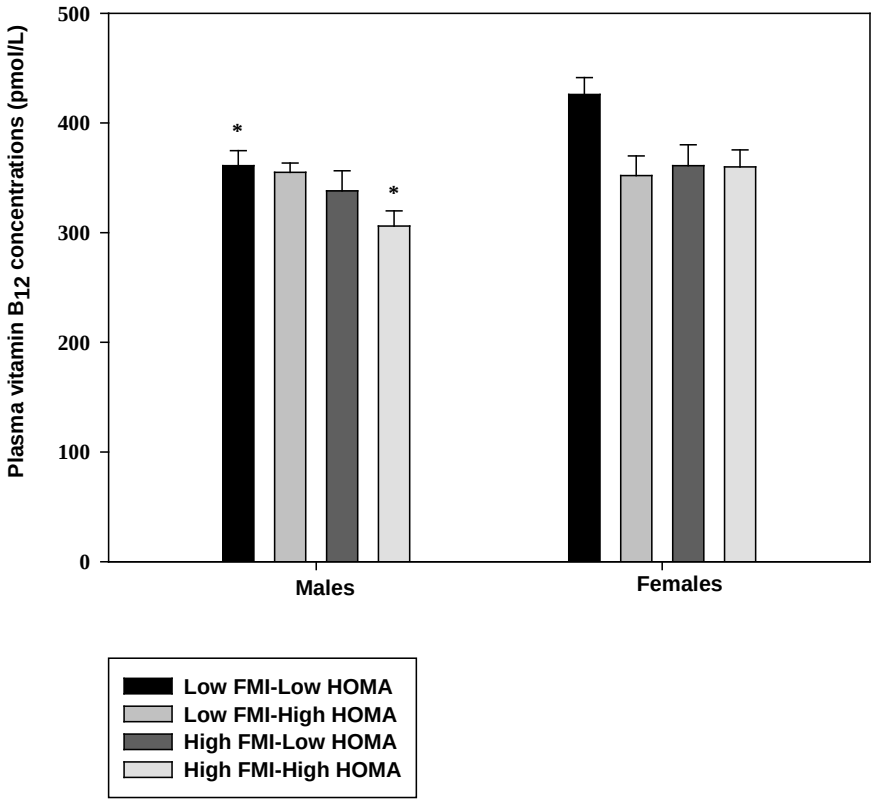
High FMI-Low HOMA (54, 66)	1,827	61.8	1,409	44.7	71.2	6.9	64.8	8.5	211	7.3	178	5.6	19.5	1.5	19.0	1.2	878	54.5	730	39.0
High FMI-High HOMA (87, 101)	1,866	46.5	1,547**a**	36.3	71.1	5.1	55.9	6.9	225	5.5	186	4.5	17.3	1.1	16.6	1.0	782	41.5	759	31.1
F and p-values	0.61	0.61	3.68	**0.01**	0.31	0.82	0.59	0.62	2.30	0.08	1.22	0.30	0.53	0.66	1.91	0.13	1.63	0.18	0.50	0.68

Abbreviations:RBC-folate, red blood cell folate; SE, Standard Error; n.s, not significant.

Significant differences ($p<0.05$) between groups are indicated by the same superscripts letters.

F tests the effect of the model.

Figure 1. Plasma vitamin B_{12} concentrations (pmol/L) according to the combination between categories of FMI and HOMA by sex.



4. Discussion

To the authors' knowledge, this is the first study to examine the association between B-vitamin intakes and corresponding biomarker concentrations and insulin resistance according to markers of body composition such as body mass index and fat mass index in European adolescents. The results suggest an association between higher adiposity together with higher insulin sensitivity and plasma vitamin B_{12} concentrations, showing the lowest vitamin B_{12} plasma concentrations in those adolescents with higher levels of adiposity combined with higher HOMA insulin sensitivity. Three B-vitamins intake and status corresponding to vitamin B_6, folate and vitamin B_{12} have been investigated in this study and we have obtained several

differences in them in different groups constituted by the combinations between BMI, FMI and HOMA categories. However, intake of vitamin B_6 and plasma vitamin B_{12} resulted to be the only for which a difference was found for categories of mentioned groups, when education of the mother and energy intake was taking into account.

The prevalence of insufficient vitamin B_{12} biomarker levels in this sample of European adolescents is low: 5% based on HoloTC and 2% based on plasma B_{12} (30), and corresponds approximately to the prevalence of inadequate vitamin B_{12} intakes reported previously (2.9% in males and 6.0% in females) (8) in a larger sample of the same adolescents. In other studies performed in Australia and Canada, the percentage of adolescents identified with low or borderline B_{12} status was higher to the one reported in our European adolescents (respectively, 32.1% in obese adolescents in the study from Australia (13), or 13.7% in all children and adolescents and 20.4% only in obese children and adolescents reported in the Canadian survey (31) using the same cut-points as in the Australian study). However, these percentages of low vitamin B_{12} status refer exclusively to overweight/obese adolescents and this could be the cause of such big differences with ours.

In our sample, 23.3% (32) of the adolescents were classified as overweight or obese. Even with these differences in rates of overweight/obesity and deficiency or not of vitamin B_{12} between our study and the Australian study (13), our results confirm the association between lower levels of vitamin B_{12} and higher HOMA and FMI. Besides, it is likely that this negative association between vitamin B_{12} plasma levels and values of insulin sensitivity and body composition markers are reproducible for other micronutrients (total carotenoids, alpha-carotene, beta-carotene, beta-cryptoxanthin, lutein/zeaxanthin, lycopene, vitamin E, vitamin C, selenium, vitamin A, vitamin D, folate, vitamin B_{12}, and RBC-folate) as reported in a study of US adults (33).

There are no previous studies investigating the same associations in similar population group. Nevertheless, previous studies (4, 13, 31) focused on obese adolescents or wide general population group, have also found that higher BMI *z*-scores were associated with lower vitamin B_{12} concentrations, with no significant sex effect.

There are several mechanisms which might explain this observation. For instance, adolescents have increased nutrient requirements secondary to increased growth and body size (34). Another reason is that adolescents with high BMI are thought to have diets with low micronutrient density. In our study, we found lower B-vitamins intake in adolescents with higher BMI while they have higher B-vitamins density diets. This can be explained by the fact that in HELENA study, adolescents with higher BMI reported lower total energy diets likely affected by underreporting (35). Another reason could be that obese adolescents may have repeated short-term restrictive diets (34); this could be the case in our study (35), because adolescents with high BMI and high FMI were those with lower total energy intakes.

There were several differences between groups constituted by the combination of body composition markers and HOMA insulin sensitivity in terms of B-vitamins biomarkers concentrations. However, only adolescents belonging to the most favorable group in terms of combination between FMI and HOMA (lower FMI combined with lower HOMA) have higher plasma vitamin B_{12} concentrations as it had been already shown by another study (13). However, the previous study did not include the analyses of B-vitamins dietary intake, as it is being shown in our study. This fact cannot be explained by higher vitamin B_{12} intakes but it might be due to lower vitamin B_{12} density diets in comparison to those adolescents belonging to the less favorable combination groups as has been shown in table 2, likely resulted from the restrictive diets or underreporting behaviors previously mentioned. Besides, while we have obtained statistically significant differences for vitamin B_6 intake regarding BMI and FMI based on the median categories combined with the categories of HOMA, was not the case for

vitamin B_{12}. This is of lots of interest owing to plasma vitamin B_{12} is supposed to reflect changes in day-to-day diet rather than HoloTC which is more efficient in predicting long-term diet changes (9), for which no difference among categories has been found.

Given that there seems not to be a plausible effect of adiposity and insulin resistance on plasma vitamin B_{12} concentrations, linked to vitamin B_{12} intake, we should consider the hypothesis that low vitamin B_{12} concentrations could influence insulin resistance. A recent genome-wide analysis (36) suggested that increased DNA methylation is associated with increased BMI in adults, and vitamin B_{12} is a determinant for DNA methylation.

Also, in a recent review about the transmission of obesity-adiposity and related disorders from the mother to the newborns (37), it is mentioned that in rural areas from India, with mothers consuming mainly vegetarian diets, the most insulin-resistant children were born to mothers who had low vitamin B_{12} but high folate levels, suggesting that a balance between these two vitamins is essential. This might be consequence from the fact that folate together with vitamins B_{12}, and B_6 among others, regulate maternal 1-carbon metabolism which influences cellular growth and differentiation by helping synthesis of nucleic acids. However, this explanation is not helpful to understand our results, owing to the differences in the characteristics of the population group and because our sample showed higher prevalence of folate deficiency than the vitamin B_{12} one (30).

Other study (38), showed the importance of levels of homocysteine in insulin resistance with an improvement of it with a lowering homocysteine by folate + vitamin B_{12} treatment and a correlation of 0.60 between homocysteine levels and insulin resistance as was also shown by similar studies.

Our findings might be of particular concern in case of adolescents diagnosed as obese patients because they will be prone to a further decrease in vitamin B_{12} concentrations if metformin therapy is recommended for the treatment of type 2 diabetes (15). For instance, in adults, vitamin B_{12} malabsorption was observed in approximately 20% of patients using metformin and this was associated with a 4%–24% decrease of vitamin B_{12} concentrations (39).

4.1. Limitations and strengths

The cross-sectional design of the study represents a limitation owing to causality cannot be established. The clinical interpretation of our findings is unclear because a very low percentage of adolescents presented inadequate intakes or very low plasma concentrations of vitamin B_{12}. However, the observed association between vitamin B_{12} plasma level and body composition and insulin resistance warn us about the importance of having a healthy nutritional status and elucidate the fact that overweight or obese people cannot get nutritional deficiencies. The use of harmonized and standardized procedures in a large sample of adolescents from Europe (18) should be considered as the main strength of the study, as well as the use of previously validated questionnaires and procedures (18, 19). Besides, the calculation of the usual intake values based on the MSM method to prevent limitations of the 24-h recalls (22) together with the use of widely accepted micronutrient biomarkers strengthen the reliability of the observations with any other marker or symptom (40).

4.2. Conclusion

Obesity is not only associated with cardiovascular conditions, cancer, dyslipidaemias, etc., but also with micronutrient deficiencies which can lead to serious health problems. Among vitamins B_6, folate, and B_{12}, it seems that vitamin B_{12} is the one most consistently and

negatively associated with BMI, FMI, and insulin sensitivity or resistance (HOMA) without discriminating between sexes of European adolescents.

In male and female adolescents with combined higher adiposity measured with fat mass index and higher HOMA insulin sensitivity, low vitamin B_{12} plasma concentrations were observed. These differences do not seem explained by dietary vitamin B_{12} intake. Further studies are necessary to elucidate the potential role of low vitamin B_{12} concentrations in the development of insulin resistance in adolescents in order to identify a plausible biological mechanism.

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11. Discusión

En este apartado, se discutirá de manera conjunta los distintos aspectos que se han considerado en los artículos publicados, así como algunas consideraciones metodológicas relevantes para la comprensión de los mismos. También se destaca la relevancia de los resultados obtenidos para la salud del grupo de población implicado.

Determinantes de la ingesta y el estatus de las vitaminas B6, folato y B12. En esta Tesis Doctoral se han evaluado diferentes determinantes de la ingesta y del estatus de vitamina B6, folato y vitamina B12, entre los que figuran el nivel socioeconómico, la ingesta de diferentes grupos de alimentos, y los patrones dietéticos.

En general, se han encontrado menos asociaciones entre los determinantes de las vitaminas B6, folato y B12 y los biomarcadores de las mismas, en comparación con las halladas para las ingestas. Varios mecanismos se han considerado para explicar este hecho. empezaren primer lugar, la biodisponibilidad de las vitaminas no se ha tenido en cuenta, y algunos estudios las sitúan entre el 30% para el folato, hasta el 75% para la vitamina B12 (106-108). Además, los biomarcadores considerados en el estudio HELENA para estas vitaminas, no siempre reflejan las ingestas puntuales, que es precisamente lo que hace nuestro método de valoración de la dieta, el recuerdo de 24 horas. De hecho, el PLP, el RBC-folato, y la HoloTC no son los mejores biomarcadores para reflejar la variación diaria en lo que a estos nutrientes se refiere (109). Por último, y en cuanto a los biomarcadores, éstos están influenciados por factores genéticos, fisiológicos, y por interacciones con otros nutrientes y medicamentos (49-51). Por otra parte, por lo que a la propia ingesta se refiere, en el estudio HELENA, no se consideró la fortificación de los alimentos en la base de datos de composición de alimentos que fue utilizada (110).

Nivel socioeconómico

En relación al nivel socioeconómico como determinante de la ingesta y los niveles de vitaminas B6, folato y B12, son pocos los estudios que existen en la literatura científica, pero la mayor parte, apuntan en la misma dirección a la observada en nuestro análisis. Por ejemplo, los resultados obtenidos en la encuesta nacional de dieta y nutrición en Reino Unido en poblaciones jóvenes (4-18 años), mostraron que los participantes procedentes de familias de nivel socioeconómico inferior, tuvieron ingestas menores de la mayoría de las vitaminas y minerales estudiados, entre los que figuraban el folato y la vitamina B12 (111). En poblaciones jóvenes, existen todavía menos estudios en los que se hayan analizado las concentraciones de las vitaminas en sangre. En nuestro estudio, los niveles de vitaminas en sangre, seguían el mismo patrón que el observado con las ingestas: los adolescentes de niveles socioeconómicos más bajos, mostraron menores concentraciones de folato y de vitamina B12. Sin embargo, en un estudio (112) realizado en Grecia, no se hallaron asociaciones entre el nivel socioeconómico y los niveles de folato y de vitamina B12, aunque en este estudio, se usó únicamente la homocisteina como biomarcador de las mismas, no siendo éste un buen marcador del folato o de la B12, por estar relacionado con ambas. Por otra parte, en nuestro estudio, la educación de los padres resultó ser el factor socioeconómico más relacionado con la ingesta y los niveles de folato y B12. Otro estudio (113), realizado con niños de 18 meses, mostró que la calidad de la dieta de niños con madres de niveles más altos de educación, resultaba ser más adecuada y por lo tanto, con unos niveles de ingesta de vitaminas y minerales superior. Además, otro estudio (114) realizado en una muestra representativa de niños y adolescentes franceses, observó recientemente que la educación de los padres o cuidadores, es el factor socioeconómico que más se asocia con la dieta de los jóvenes.

Contribución de los grupos de alimentos

En relación a los grupos de alimentos que contribuyen más a la ingesta y estatus de las vitaminas B6, folato, y B12, hay varios estudios que se centran en las ingestas (115-117), otros en los biomarcadores (48, 118, 119), y otros en adultos y ancianos (120, 121). En un estudio brasileño realizado en adolescentes (116), el arroz, el pollo y la ternera fueron los grupos que contribuyeron más a la ingesta de vitamina B6 mientras que en el nuestro fueron la carne, los cereales, y las patatas; para el folato, contribuyeron el pan, la pasta, y las judías, mientras que en HELENA, fueron las frutas y verduras, los cereales, el pescado, y las margarinas; y finalmente, en el estudio brasileño, la ternera y la leche entera contribuyeron en mayor medida a la ingesta de vitamin B12, mientras que en el estudio HELENA, fueron la carne, el pescado, la leche y los productos lácteos, y los aceites vegetales. Se pueden apreciar ciertas similitudes, pero prevalecen las diferencias derivadas principalmente de la diferente disponibilidad de alimentos y hábitos alimentarios en dos zonas geográficas tan dispares.

En nuestro estudio, muchos de los grupos de alimentos que fueron capaces de discriminar entre el primer y tercer tercil de las ingestas y los niveles de vitamina B6, folato y B12, mostraron asociación también con las citadas vitaminas, incluso si no se consideran fuentes principales de las mismas, tal y como ocurre por ejemplo con los productos lácteos, zumos de frutas y cereales. Una explicación viable para este hecho, es que suelen ser precisamente estos productos, vehículos frecuentes de fortificaciones (6).

Patrones dietéticos

Más escasos aún fueron los estudios similares encontrados en la bibliografía en relación a los patrones alimentarios y las vitaminas del grupo B en adolescentes. De hecho, solo hemos encontrado uno en niños alemanes (122), pero el método estadístico usado para obtenerlos fue el análisis de componentes principales o PCA (*principal component analyses*) en sus siglas en

inglés, cuando el nuestro fue la regresión de rangos reducidos o RRR (*reduced rank regression*) en sus siglas en inglés. Resulta pues difícil comparar los resultados, ya que uno y otro método estadístico no tratan de agrupar los alimentos para constituir un patrón dietético de la misma forma. El PCA, trata de agrupar los alimentos que suelen consumirse conjuntamente; sin embargo, el RRR trata de agrupar el consumo de diferentes grupos de alimentos en este caso, para explicar el mayor porcentaje de varianza de unas variables respuesta previamente asignadas (en nuestro caso ingesta y biomarcadores de vitaminas) (55).

Aún así, los patrones hallados por el estudio alemán (122) para la vitamin B6 y el folato, tuvieron ciertas similitudes con los que resultaron determinantes en nuestro caso, de un perfil más saludable. En nuestro estudio, las ingestas de vitamina B6 fueron mayormente determinadas por un patrón *tradicional* y *mediterráneo* para chicos y chicas, respectivamente. En el caso del PLP, para chicos, fue determinado por un patrón caracterizado por alimentos típicos de *desayuno*, y un patrón *tradicional* para chicas. Las ingestas de folato, se determinaron por un patrón *saludable* para chicos, y para chicas por un patrón *proteico.* Para los biomarcadores del folato en chicas, un patrón *no saludable* fue el más determinante. Para los chicos, un patrón caracterizado por productos de *snack* determinó el folato en plasma, y un patrón *saludable*, el folato en eritrocitos. Sin embargo, para la vitamina B12, los patrones observados fueron bastante diferentes a los del estudio alemán. Ellos observaron un patrón más occidental y tradicional, mientras que en el proyecto HELENA, no entraron en el patrón, las patatas y la carne, alimentos que caracterizan el patrón o*ccidental* y *tradicional*. En chicos, la ingesta de vitamina B12, fue determinada por un patrón *proteico*, mientras que, en chicas, por un patrón caracterizado por productos típicos del *desayuno*. En cuanto a la vitamina B12 en plasma, los chicos se caracterizaron por un patrón de *restaurante italiano*, mientras que las chicas, por uno relacionado con *fast-food*. En relación a la HoloTC, en ambos chicos y chicas, se caracterizaron por un patrón de *desayuno*.

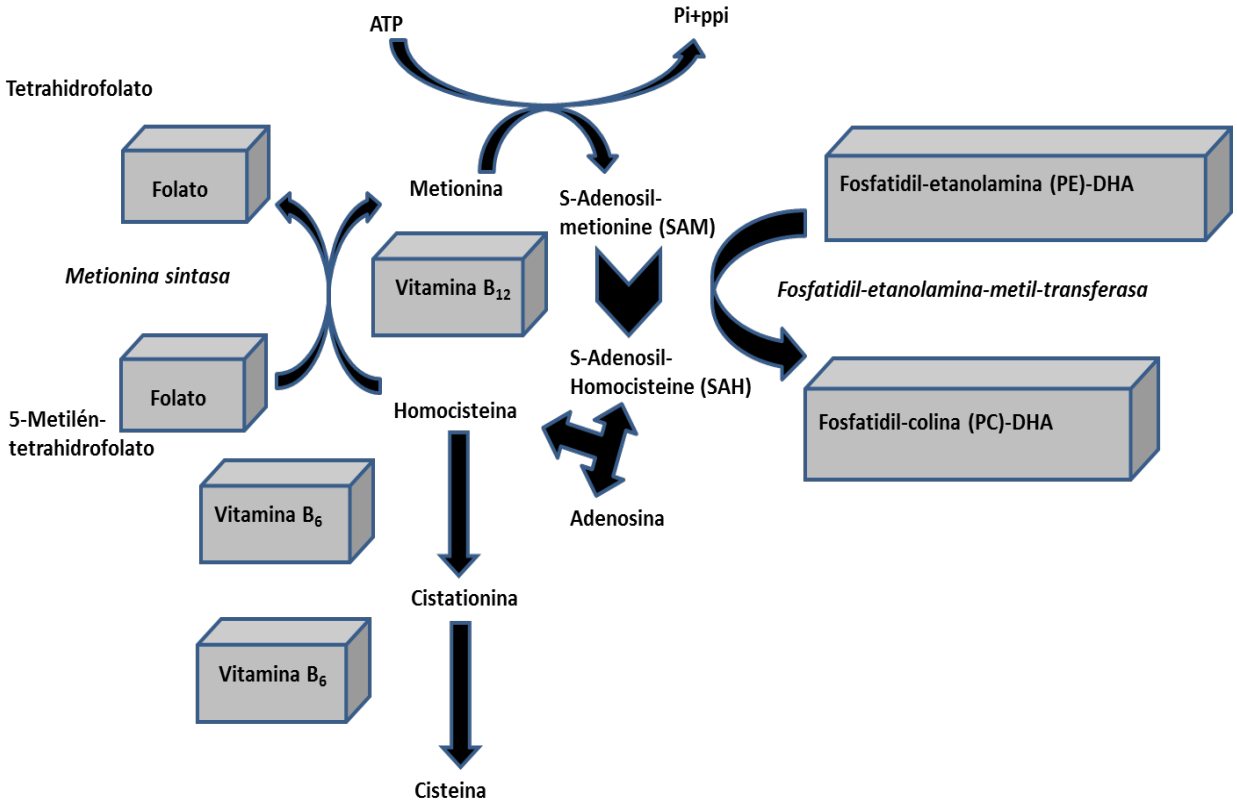
Relación de las vitaminas B6, folato y B12 con otros indicadores de salud

Actualmente, y como ya se ha comentado anteriormente, tampoco son abundantes los estudios que relacionen los niveles de vitaminas con otros aspectos de la salud, característicos de cada vitamina. Estos incluirían: las anemias en cualquier población para los déficits de vitamina B6, folato o vitamina B12; la dermatitis y la depresión en el caso de déficit de vitamina B6; los defectos del tubo neural de los recién nacidos en caso de mujeres embarazadas con déficit de folato, además de la depresión; y por último, defectos cognitivos en el caso de ancianos y déficit de vitamina B12 (9). En ese sentido, es la primera vez que se analizan en una muestra tan amplia europea de adolescentes, las asociaciones entre los biomarcadores de las vitaminas B6, folato y B12 con los ácidos grasos de cadena larga de la serie omega 3. Los estudios existentes que había hasta ahora, se realizaron enpersonas mayores o embarazadas, o en animales. Además, sus resultados habían sido bastante controvertidos, pues algunos habían hallado dichas asociaciones en animales (123) así como en adultos (69, 71), mientras que otros, no lo habían hecho en ancianos (49). En el estudio de Crowe et al. (49), en ancianos, los autores no encontraron efecto del tratamiento con vitaminas del grupo-B en los niveles plasmáticos de S-adenosil-homocisteina (SAH), lo que nos hace pensar que la suplementación con estas vitaminas no fue efectiva al no estar aumentados los niveles de este metabolito. Nuestros resultados están ajustados por el índice de calidad de la dieta de los adolescentes (Diet Quality Index-DQI en sus siglas en inglés-) que ha demostrado dar buenos resultados y tener una correlación bastante alta con los nutrientes de la misma, entre los que estaban las vitaminas que nos ocupan (103). Este hecho resulta importante, ya que la principal limitación de haber encontrado estas asociaciones, podría haber sido el que unos nutrientes y otros, procedieran de los mismos grupos de alimentos. Este hecho parece descartado con el ajuste realizado en nuestros análisis.

Además, de entre las posibles rutas metabólicas que explicarían estos hallazgos, al descartar las fuentes de alimentos comunes a los ácidos grasos y las vitaminas, nos hemos quedado con una,

que, dada la bibliografía existente (69, 71), nos parece la más plausible y queda resumida en la siguiente figura. En ella, puede apreciarse el ciclo de la metilación que ya habíamos visto previamente, y su unión metabólica con el ciclo de metilación de la fosfatidil-etanolamina en fosfatidil-colina mediante la fosfatidiletanolamina metiltransferasa.

Figura 4. Ciclo de la metilación ampliado.



En el último artículo incluido en esta Tesis Doctoral, observamos que aquellos adolescentes con composición corporal (IMC, IMG) o marcadores de riesgo metabólico (HOMA) en niveles menos saludables (más altos), tenían también menores ingestas y concentraciones en sangre de vitaminas B6, folato y B12. Esta tendencia también se mantuvo al combinar el índice HOMA con el IMC y con el IMG por separado para las tres vitaminas, tanto para su ingesta como para sus concentraciones. Sin embargo, estos resultados, no se mantuvieron, cuando ajustamos los análisis

por las covariables habituales: educación de la madre e ingesta de energía, a excepción de la vitamina B12 en plasma para los chicos cuando se combinaban el índice HOMA con el IMG. Como no se observaron diferencias significativas para la ingesta de vitamina B12, los resultados obtenidos para dicha vitamina en plasma, no parecen explicarse por la vitamina B12 dietética, por lo que más estudios deberían de profundizar en este hecho. Al parecer, las concentraciones de vitamina B12 podrían influenciar la sensibilidad a la insulina, tal y como sugiere una revisión reciente sobre la transmision de la obesidad-adiposidad y desórdenes asociados de madres a recién-nacidos (124); en la que, por ejemplo, madres que consumían dietas vegetarianas y tenían niveles bajos de vitamin B12, tenían hijos más insulino-resistentes. Del mismo modo, en un estudio australiano (59), adolescentes obesos con resistencia a la insulina, mostraron concentraciones bajas o al límite de vitamina B12.

Implicaciones para la salud pública

En los países desarrollados, la inversión científica y económica para el estudio de los déficits nutricionales de micronutrientes, sus causas y consecuencias, se ha limitado en las últimas décadas. En paralelo, ha aumentado la incidencia de sobrepeso y obesidad a nivel mundial. Las comorbilidades asociadas al sobrepeso y la obesidad son numerosas y sus complicaciones pueden ir desde leves hasta graves, pudiendo provocar la muerte en el caso de las enfermedades cerebrovasculares o distintos tipos de cáncer. Sin embargo, resulta paradójico, que una enfermedad como la obesidad, cuya causa es un desequilibrio entre la ingesta energética y el gasto energético, a favor de la primera, pueda acompañarse de déficits nutricionales, tal como se observa actualmente, incluso ya desde la adolescencia. Los motivos están relacionados con el consumo de dietas de elevada densidad energética en detrimento de dietas de elevada densidad nutricional, incluyendo cantidades significativas de micronutrientes. Además, en la adolescencia, no es infrecuente el consumo de dietas deficitarias en energía y nutrientes, seguidas de períodos

de elevada ingesta de alimentos ricos en energía, pero pobres en nutrientes, que también favorecerían este hecho.

En nuestra muestra del estudio HELENA, entre el 2 y el 5% de los adolescentes sanos presentaron concentraciones compatibles con deficiencias subclínicasde vitamina B12, el 20 % de vitamina B6, y entre el 27 y el 35% de folato, en función del biomarcador usado (12). Porcentajes nada despreciables para tratarse de países de Europa que no deberían de tener ninguna limitación para acceder a una dieta saludable. Por esta razón, resulta crucial tener un mayor conocimiento sobre los factores que se relacionan con la ingesta y estatus de las mismas, así como entender cuáles podrían ser sus principales consecuencias, además de las ya conocidas por la literatura clásica, en el estudio de los déficits nutricionales.

En este sentido, las implicaciones para la salud pública de los resultados obtenidos en esta Tesis Doctoral son notorias. En primer lugar, se halló escasa información de calidad, en la revisión sistemática sobre la vitamina B12 en poblaciones jóvenes, que se llevó a cabo antes de comenzar el desarrollo de esta Tesis Doctoral. Esto sentó las bases para obtener nuevos resultados en relación a estas vitaminas del grupo B, tan inter-relacionadas entre sí. A posteriori, destaca la importancia que juega el nivel socioeconómico en el riesgo de padecer una deficiencia subclínica en estas vitaminas del grupo B, al confirmar que el gradiente de nivel socioeconómico, se asocia también al de ingestas y niveles de las vitaminas analizadas. A nivel clínico, este hecho, nos debe hacer más prudentes y vigilar los patrones alimentarios de las personas que tengan menos recursos o menor nivel social. A nivel epidemiológico, los resultados obtenidos, nos ayudan a optimizar el uso de variables de ajuste en futuros análisis que potencialmente puedan estar confundidos o mediados por el nivel socioeconómico, realizados con adolescentes. Entre los resultados obtenidos, se evidencia cómo la educación de los padres parece ser la variable socioeconómica, que más se relaciona con la ingesta y el estatus de folato y vitamina B12.

Por otra parte, se pueden considerar como determinantes de la ingesta y el estatus de las vitaminas B6, folato, y B12, tanto los grupos de alimentos individuales, como la combinación de los mismos, a través de los patrones dietéticos. Es por ello que la determinación de estos patrones dietéticos, es también de gran relevancia para la salud pública, con el fin de fomentar en la población de adolescentes el consumo de alimentos incluidos en los patrones más saludables. Además, resulta de gran interés, observar como no siempre son las principales fuentes de alimentos de las citadas vitaminas, las que contribuyen más a su ingesta y estatus, y como esos grupos, además, pueden variar según regiones del mundo. Se necesitan más estudios para poder comparar y replicar estos resultados. Del mismo modo, los patrones dietéticos determinantes de las ingestas y concentraciones de las vitaminas, suponen una herramienta muy importante de caracterización de los adolescentes. En un futuro, aquellos que se alejen más de lospatrones más determinantes, se les podrá considerar con riesgo de deficiencias nutricionales subclínicas y ser con ellos más prudentes y vigilantes. Además, los patrones identificados, describen pautas de conductas alimentarias poblacionales que nos pueden ayudar a incidir en aspectos de las mismas que sean menos beneficiosas y más susceptibles de cambio.

Según los resultados obtenidos, se ha observado por primera vez en adolescentes, la asociación entre las vitaminas B6, folato y B12 y los ácidos grasos de cadena larga de la serie 3. Estos resultados, además de arrojar algo más de luz sobre una evidencia hasta ahora controvertida en la literatura, nos hacen pensar en procesos metabólicos que hasta ahora habían pasado desapercibidos, pero que pueden suponer nuevos retos y vías de estudio para contribuir a frenar, entre otras, las enfermedades de origen cardiovascular.

Finalmente, en cuanto a las consecuencias para la salud asociadas a las concentraciones de vitaminas del grupo B, los adolescentes que tienen sobrepeso u obesidad, pueden mostrar también concentraciones bajas de vitaminas de este grupo. Dada la elevada prevalencia de sobrepeso y obesidad en todo el mundo, resulta relevante confirmar este hecho, también entre adolescente

europeos. Los resultados observados, se pueden explicar por la frecuencia con que habitualmente presentan periodos de dietas restrictivas seguidos de periodos de *ingesta compulsiva* de alimentos de baja densidad nutricional y elevada densidad energética. También podría debersea que aquellos adolescentes con mayor IMC e índice de adiposidad, son aquellos cuyas ingestas autoreferidas, se ven más afectadas por la infradeclaración de las mismas. Los resultados obtenidos en este sentido, suponen un importante reto para la salud pública: convencer a la población, y más concretamente, a la población de adolescentes, de la importante que es comer de un modo saludable para evitar deficiencias nutricionales, como podría ser la de vitamina B12, asociadas al sobrepeso y obesidad.

Consideraciones metodológicas

El estudio HELENA, incluyó a una muestra importante de adolescentes europeos, que no fue representativa de los países de procedencia, pero sí de las ciudades seleccionadas, donde los adolescentes se estratificaron por sexo, edad y nivel socioeconómico. Además, las áreas rurales no se vieron representadas.

Su diseño transversal nos ha permitido establecer asociaciones entre las variables de interés para cada análisis e hipóTesis, pero no así establecer relaciones de causalidad. Es por ello, que para dar un paso más en el estudio de las relaciones descritas, sería conveniente replicar estos resultados en muestras europeas de igual o superior tamaño, y de un modo prospectivo longitudinal. De hecho, en 2011, la "Communication Star" de la Comisión Europea, premió al estudio HELENA dentro de la categoría de "proyectos pequeños" entre 25 candidatos, por ser el proyecto enmarcado dentro del sexto Programa Marco que más decisivamente había contribuído al sector alimentario y por difundir sus resultados a audiencias más amplias de un modo más efectivo (125). Por ello, en la actualidad, se está llevando a cabo en algunos centros, incluyendo

Zaragoza, una re-evaluaciónde los adolescentes que hace algo más de 10 años, participaron en el estudio HELENA. Se pretende valorar las mismas variables que se midieron en el estudio transversal.

La principal fortaleza del estudio HELENA, es el uso de herramientas y procedimientos estandarizados y validados (83, 87). En este sentido, para la valoración de la ingesta de vitaminas, el uso de dos recuerdos de 24 horas electrónicos, en el que podían observarse las porciones del alimento seleccionado, supuso un beneficio añadido, así como usar el método MSM para ajustar las ingestas por la variabilidad intra-individuo (126). Sin embargo, existen dos limitaciones importantes en el método utilizado para valorar la dieta, que han sido, la ausencia de datos en cuanto a la posible suplementación nutricional de los adolescentes, a la par que la base de datos de composición de alimentos alemana (BLS FGDB), que no tiene en cuenta la fortificación de los alimentos, por lo que las cantidades de ingesta de micronutrientes, entre las que se incluyen las vitaminas B6, folato y B12, podrían estar infra-valoradas (127).

En los artículos incluidos en esta memoria, no sólo se valoraron las ingestas de las vitaminas B6, folato y B12, sino también sus correspondientes biomarcadores, aceptados en la literatura (PLP, PF, RBC-folate, HoloTC, tHcy) o bien, ampliamente usados (plasma B12) (12). La mayor parte de los estudios referenciados, únicamente valoraron la ingesta de las mismas, y muy pocos las oncentraciones de las mismas, lo que aporta una mayor fortaleza a nuestros resultados. Además, tratándose de un estudio multicéntrico, todos los biomarcadores fueron analizados en el mismo laboratorio (94).

12. Conclusiones

Artículo I.

El gradiente clásico de salud en base al nivel socioeconómico, se ha confirmado para la ingesta y el estatus del folato y la vitamina B12 en adolescentes europeos. Además, se ha demostrado la falta de asociación de la ingesta y del estatus del folato y la vitamina B12 con la riqueza familiar, a favor la educación y ocupación de los padres.

Artículo II.

Las ingestas de vitaminas B6, folato y B12 están asociadas con la ingesta de sus principales fuentes alimentarias mientras que sus correspondientes biomarcadores, lo estuvieron más con ingesta dietética global.

En el futuro, deberemos de prestar más atención al estatus de las vitaminas B6, folato y B12 de los adolescentes, quienes tienden a consumir alimentos menos variados y de menor densidad en micronutrientes, por lo que están más expuestos a no cumplir las recomendaciones dietéticas de las citadas vitaminas.

Artículo III.

Los patrones dietéticos obtenidos para esta muestra de adolescentes europeos, logran explicar entre un 23.7% y un 34.2% de la variabilidad de la ingesta de vitaminas B6, folato y B12, mientras que, para sus biomarcadores, el rango oscila entre un 7.0 % and 17.2. En consecuencia, las investigaciones futuras deberán ir encaminadas a dilucidar cuales son los determinantes de estos patrones.

Artículo IV.

Mayores concentraciones de biomarcadores de las vitaminas B6, folato y B12 están asociadas con también mayores concentraciones de ácidos grasos de cadena larga (AGLC), sobretodo con los de serie ω3. Además, el marcador pro-inflamatorio ω6/ω3, así como la relación oléico/esteárico y las concentracionesde grasa trans, estuvieron negativamente asociados con las concentraciones de las vitaminas. En consecuencia, asegurar un adecuado estado de vitamina B6, folato y B12, puede suponer una importante disminución del riesgo de enfermedad cardiovascular, al aumentar los niveles de AGLC ω3 circulantes y al estar asociados a una disminución de las ratios ω6/ω3, oléico/esteárico y los niveles de ácidos grasos trans.

Artículo V.

La vitamina B12 es la más consistente y negativamente asociada con el BMI, el FMI, y el índice de sensibilidad a la insulina (HOMA), sin discriminar entre sexos de adolescentes europeos. Estas diferencias parecen no estar explicadas por las ingestas de vitamina B12, para las que no se observaron diferencias entre los grupos. Son necesarios más estudios para hallar mecanismos biológicos plausibles a este hecho.

13. Conclusions

Article I.

The classical health gradient based on the socioeconomic status, is also true for B-vitamins intake and status of European adolescents. This study highlights the lack of associations found between family income, and folate and vitamin B12 intakes and their related biomarkers concentrations, in favour of the importance of the parental education and occupation.

Article II.

As expected, B-vitamins intakes were associated with intakes of their main food sources. However, B-vitamins biomarkers were associated with the overall food consumption pattern.

In the future, special attention considering B-vitamins status should be put in adolescents who are used to consume food groups with low micronutrient density like savoury snacks, and not consuming a healthy varied food items more prone to meet the recommendations of vitamins B6, folate and B12, like fruit and vegetables.

Article III.

Dietary patterns obtained for this sample of European adolescents could explain between the 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. In consequence, there is an urgent need for investigating what are the dietary patterns which determine B-vitamins intake and status in adolescents worldwide and to elucidate what are the main determinants of these patterns.

Artile IV.

Our findings suggest that higher B-vitamin biomarkers concentrations might be related to higher PUFAs concentrations (mainly ω3 series). Besides, the pro-inflammation marker ω6/ω3 PUFA ratio decreased with increasing values of B-vitamin biomarkers, as well as happens with trans-fatty acids and oleic/stearic acid ratio. Consequently, assuring a good status of B-vitamin biomarkers is very important as it might influence cardiovascular health by lowering homocysteine levels, but also, by increasing circulating ω3 PUFA and decreasing the pro-inflammatory marker ω6/ω3 PUFA ratio.

Article V.

Vitamin B12 was consistently and negatively associated with BMI, FMI, and insulin sensitivity (HOMA) without discriminating between sexes of European adolescents. These differences did not seem to be explained by dietary vitamin B12 intakes. To identify a plausible biological mechanism, further studies are necessary to elucidate the potential role of low vitamin B12 concentrations in the development of insulin resistance in adolescents.

14. Principales aportaciones

Artículo I.

Ha proporcionado, por primera vez en la literatura, información sobre las asociaciones encontradas entre la ingesta y los biomarcadores de folato y de vitamina B12, con una batería muy completa de marcadores socioeconómicos en una muestra de adolescentes procedentes de nueve países europeos. Además, hemos podido comprobar, que la educación de la madre y del padre, parecen ser los aspectos socioeconómicos que mejor se relacionan con la ingesta y las concentraciones de los biomarcadores de las citadas vitaminas. Esto nos ha permitido utilizar la variable de la educación de la madre para el ajuste en posteriores análisis y publicaciones, ayudándonos a entender mejor la asociación de la ingesta y los biomarcadores del folato y la vitamina B12, con otros indicadores de salud.

Artículo II.

Este artículo nos proporciona información relevante en relación al consumo de grupos de alimentos, capaz de diferenciar entre los adolescentes que ingieren mayores y menores cantidades de vitamina B6, folato y B12, así como también discriminar entre los que tienen mayores y menores concentraciones de las citadas vitaminas. En relación a los grupos de alimentos, este artículo nos ayuda a entender que, si bien las ingestas dietéticas de las vitaminas están mayormente determinandas por sus fuentes alimentarias principales, las concentraciones de los correspondientes biomarcadores, se podrían ver influenciados por otros factores, dada la discrepancia observada entre los resultados para las ingestas y los biomarcadores. Además, ayuda a entender la importancia de consumir una dieta variada, que nos asegure unas concentraciones adecuadas de vitaminas en sangre.

Artículo III.

Tradicionalmente, la investigación sobre dieta y salud, tenía un carácter reduccionista, centrado en el consumo de alimentos y la aparición de la enfermedad. Sin embargo, el ser humano, consume los alimentos en combinación. Por ello, resulta de gran relevancia el estudio de los patrones dietéticos. En este artículo, se han estudiado las relaciones entre los patrones dietéticos de los adolescentes europeos y la ingesta de vitaminas B6, folato y B12 y sus concentraciones en sangre. Ello nos ha permitido caracterizar patrones que pueden estar relacionados con los niveles de vitaminas y así, prestar más atención a aquellos adolescentes que se alejen de los patrones dietéticos que han resultado ser más determinantes para obtener unos niveles de vitaminas más altos.

Artículo IV.

En numerosos estudios, las concentraciones elevadas de homocisteína se han relacionado con mayor riesgo de enfermedades cardiovasculares. Sin embargo, una reciente revisión sistemática, no encontró disminución de mortalidad por enfermedad cardiovascular en ensayos clínicos que pretendían disminuir los niveles de homocisteina mediante suplementación con vitaminas B6, folato y B12. En varios estudios en ratas y en alguno en ancianos, se postuló que este efecto podría estar mediado por la influencia de estas vitaminas, en la conversión de la fosfatidiletanolamina en fosfatidilcolina, que está encargada de transportar los ácidos grasos de cadena larga ω3, que producen beneficio cardiovascular. Sin embargo, los resultados entre estudios fueron controvertidos. Nuestro artículo aporta evidencia adicional a favor de esta asociación, y además en una población para la que hasta ahora no existían resultados.

Artículo V.

Las asociaciones obtenidas entre las ingestas y biomarcadores de las vitaminas B6, folato y B12 y los diferentes parámetros de la composición corporal, como son el índice de masa corporal (IMC), índice de masa grasa (IMG) y la sensibilidad a la insulina (HOMA), y la combinación entre ellos, son de gran actualidad y novedad. Los resultados obtenidos han sido especialmente significativos para la vitamina B12 en plasma y ponen en evidencia, que los adolescentes con mayores IMC, IMG o índice HOMA, pueden tener más riego de deficiencia de vitamina B12. Estos resultados tienen una gran relevancia clínica, ya que el hecho de tener sobrepeso u obesidad y comorbilidades asociadas, no tiene que hacernos descartar déficits vitamínicos, consitituyendo así, la gran paradoja del siglo XXI, que sigue a la pandemia de obesidad, la doble carga de enfermedad.

15. Main Contributions

Article I.

This original contribution provides for the first time, information on the found associations between the intake and the biomarkers of vitamin B6, folate, and vitamin B12 and such a large battery of socioeconomic factors in adolescents from nine European countries. Besides, we habe observed that maternal and paternal education are the socioeconomic factors most associated with the intake and the biomarkers of vitamin B6, folate, and vitamin B12. This finding gave us the opportunity of using the variable of the maternal education as variable of adjustment in further analysis and publications, and allowing us to better understand the relationship of these vitamins with other parameters.

Article II.

This original contribution provides relevant information in relation to the consumption of different food groups and the discrimination of European adolescents who had higher and lower intakes and biomarkers concentrations of vitamins B6, folate, and vitamin B12. In relation to the obtained food groups which discriminates better, the intakes of the B-vitamins seem to be explained by the difference in the consumption of the main food sources, while the biomarkers of the B-vitamins might be influeced also by other factors given the disparity found in the results. Besides, it helps us to understand the importance of having a varied diet so as to assure adequate vitamin levels in blood.

Article III.

Traditionally, dietetic research investigating the relationship between diet and health, had a reductionist approach based on the consumption of foods and the aparience of disease. However, humans eat foods in combination. That is why it is suggested to study in depth the dietary patterns instead. In this original research, dietary patterns of European adolescents in relation with e intakes and biomarkers of vitamins B6, folate, and vitamin B12 have been investigated. The identification of patterns determining the levels of the B-vitamins allow us to warn about those adolescents at higher risk of inadequate B-vitamins levels.

Article IV.

Higher homocysteine levels had been associated with higher risk of cardiovascular diseases. However, in a recent systematic review, it was found that supplementation with B-vitamins in order to decrease levels of homocysteine did not affect the risk of mortality because of cardiovascular diseases. It was suggested in several studies in rats and some in elderly people, that this association may be mediated by the fact that vitamins B6, folate and B12, influence the conversión of phosphatidyl-ethanolamine in phosphatidyl-choline, in charge of transporting the ω PUFAs, which is another beneficial cardiovascular risk factor. Nevertheless, the obtained results across the studies were controversial, and our study was the first investigating this topic in European adolescents and adolescents in general.

Article V.

In this article, associations between the intake and biomarkers concentrations of vitamin B6, folate, and vitamin B12, and different body composition parameters such as BMI, FMI and insulin

sensitivity (HOMA) alone or in combination, were observed. The obtained results were especially significant for vitamin B12 in blood and showed that adolescents with higher BMI, FMI and HOMA, might have more risk of having inadequate levels of B-vitamins. This finding has rather clinic relevance, as overweight or obese patients might have also vitamins déficits, constituing the big paradox of the XXI century that follows the obesity pandemy (the doble burden of disease).

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No me puedo olvidar tampoco de las *young glories,* pero *glories* ya, a fin de cuentas, de pleno derecho*,* con las que da gusto trabajar y compartir el día a día, además de ver como perpetúan excepcionalmente la especie genudiana*.* Gracias Mary, por tu disposición para la ayuda, por ser una persona que hace mejor la vida de los demás, incluso sin que te lo pidan, y por tu admirable eficiencia. Gracias Cris, por tu compañerismo, por tu sentido de la justicia y por la pasión que le pones a nuestra profesión, porque la contagias. Gracias Isa, por toda tu ayuda y comprensión con esas tareas secundarias, pero también necesarias que han favorecido la progresión de esta Tesis Doctoral. Y muchas gracias a las tres, por vuestra *chispa* y vuestra ilusión.

También me gustaría agradecer a la parte de Actividad Física del grupo, liderada por José Antonio Casajús, su implicación y dedicación para hacer que todo haya funcionado tan bien. A Germán, quisiera agradecerle, además, el consejo que me dio hace 10 años, cuando después de hacer el período de prácticas en GENUD, yo quería estudiar Fisioterapia. Él, entonces, me dijo que, si ya me había decantado con la Nutrición, que era mejor que apostara por ella. Y te hice caso Germán, y la verdad, es que te estoy muy agradecida por ello. Y, en fin, a todos los que con cariño, decimos *inefillos,* por contribuir a la motivación, emprendimiento y buen rollo de esta familia GENUD: los Alexs, Alba, Ángel, Gabri, Jorge, Borja y David.

Y si bien he nombrado y agradecido a los actores principales de GENUD, son muchas las coenzimas que han pasado por GENUD. Si bien han contribuido colaborando en los proyectos del momento, haciendo trabajo de campo, pasando datos o escribiendo artículos; también han

conseguido difundir la semilla genudiana allá donde luego han ido, y lo que es más importante, han conseguido que una parte de ellos siempre se quede aquí en la sede principal, y que las distancias se queden cortas para esta gran familia GENUD. Por ello, quiero también agradecer un pedacito de esta Tesis Doctoral a Vanesa, Diego, Carol, Fer, Maria Luisa, David, Elena (la G-len), el grupo de brasimaños (Augusto, Tati, Marilia, Maria Paula, María da Luz, Camila, Elsie, Naiara, Milena), Sara, Laura, Luisa, Bea, Konstantina, Marcus, Liz, Paloma, Sandra, Alelí, y Alberto, principalmente. Igualmente, a las nuevas incorporaciones, Azahara y Natalia, que ya apuntan maneras, y que, seguro que van a dejar también su huella en GENUD, y en nuestras vidas.

También ha habido algún colaborador externo, que, si bien no ha estado tan relacionado con la realización de esta Tesis Doctoral, nuestros caminos se han cruzado en algún momento y también han contribuido a mi crecimiento como investigadora. Thank you Rosalie for leading me during my staying in Wageningen when I was really young and unexperienced, for your patience and kindness. Thank you Maike, Antje, Timm, Barbara, Hannah, and Ina for all your support, kindness and friendship during my staying in Bremen. Y gracias a Carolina y Leyre, por aquellas sesiones pre-estadísticas tan fructíferas, por el tiempo compartido durante nuestro proyecto común, y por todo el apoyo recibido. Y también, gracias a Estrella, Sara y Ana.

Y ya terminando, quisiera agradecer a mis vitaminas B12, mi familia y amigos que reciben las a veces, no tan buenas consecuencias del tiempo y esfuerzo que conlleva hacer una Tesis Doctoral.

En primer lugar, quiero agradecer a las personas que empezaron este camino de la Nutrición conmigo, mis compañeras de carrera, amigas con las que comparto pasión por la Nutrición, que se refleja en la creación de nuestra Asociación Gastronómico-Cultural DEBODIO. Gracias por vuestras palabras de apoyo y por vuestra comprensión cuando me he tenido que bajar del carro de algún plan.

Gracias también, y especialmente a Miriam, porque además de ser una amiga de toda la vida y para toda la vida, ha sido comprensiva con mi falta de tiempo.

Gracias a mi familia. A mi prima María, mis tíos, mis abuelos, y a Cristina, que siempre se han interesado por mí, por lo que estaba haciendo y porque siempre me han trasmitido todo su ánimo y su cariño.

Y, por último, gracias a la luz de mi vida. Gracias a mis padres, Emi y Juan, por preocuparos tanto por mí y por ayudarme siempre en todo lo que he necesitado. Por haberme facilitado todas las oportunidades para poder llegar hasta aquí y por tanto amor. Gracias Irene, por tu brillo en los ojos y por tu sonrisa cuando pasamos tiempo juntas y también por tu comprensión cuando a mí me ha costado sacar algún ratillo para vernos. Y, para terminar, gracias Jorge. Gracias por apoyarme siempre. Gracias por hacerme sentir que estás orgulloso de mí, porque eso, si son vitaminas que me dan fuerza para seguir haciendo las cosas bien. Gracias también por tu paciencia en estos 10 años al tener que compartirme con tantas otras actividades. Desde luego, parte del esfuerzo que yo he hecho, también es mérito tuyo, y de ahí, que este documento, sea más bien, *nuestra Tesis*. Y, sobre todo, gracias por tus abrazos después de un mal día, y por los de después de un día bueno.

18. About the author

2006	*Bsc Human Nutrition and Dietetics*
2011	MSc *Genetics, Nutrition and Environmental determinants of growth and development*
2006-2016	Junior Researcher at *Growth, Exercise, NUtrition and Development (GENUD) Research Group, Faculty of Health Sciences, University of Zaragoza, Spain.*
2017-	*Researcher at the Fundación Instituto de Investigación Sanitaria de Aragón (IIS)*

Publications

1. Iglesia I, González-Gross M, Huybrechts I, De Miguel-Etayo P, Molnar D, Manios Y, et al. Associations between insulin resistance and three B-vitamins in European adolescents: The HELENA study. Nutr Hosp2017.

2. Iglesia I, Huybrechts I, Gonzalez-Gross M, Mouratidou T, Santabarbara J, Chajes V, et al. Folate and vitamin B12 concentrations are associated with plasma DHA and EPA fatty acids in European adolescents: the Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) study. Br J Nutr2017 Jan 18:1-10.

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19. Material suplementario

Anexo 1. Errata

Errata en:

"Ingesta y estatus de vitamina B6, folato y vitamina B12 en adolescentes europeos. Determinantes y consecuencias"

En varios artículos del compendio presentado en esta memoria, aparece "serum vitamin B12" en vez de "plasma vitamin B12", lo cual es erróneo. Este error, no modifica los resultados ni la interpretación de los mismos. Los artículos afectados son:

'Socioeconomic factors are associated with folate and vitamin B12 intakes and related biomarkers concentrations in European adolescents: The HELENA Study' (Nutr Res)

'Folate and vitamin B12 concentrations are associated with plasma docosahexaenoic and eicosapentaenoic fatty acids in European adolescents: the HELENA study' (Brit J Nutr)

Erratum to:

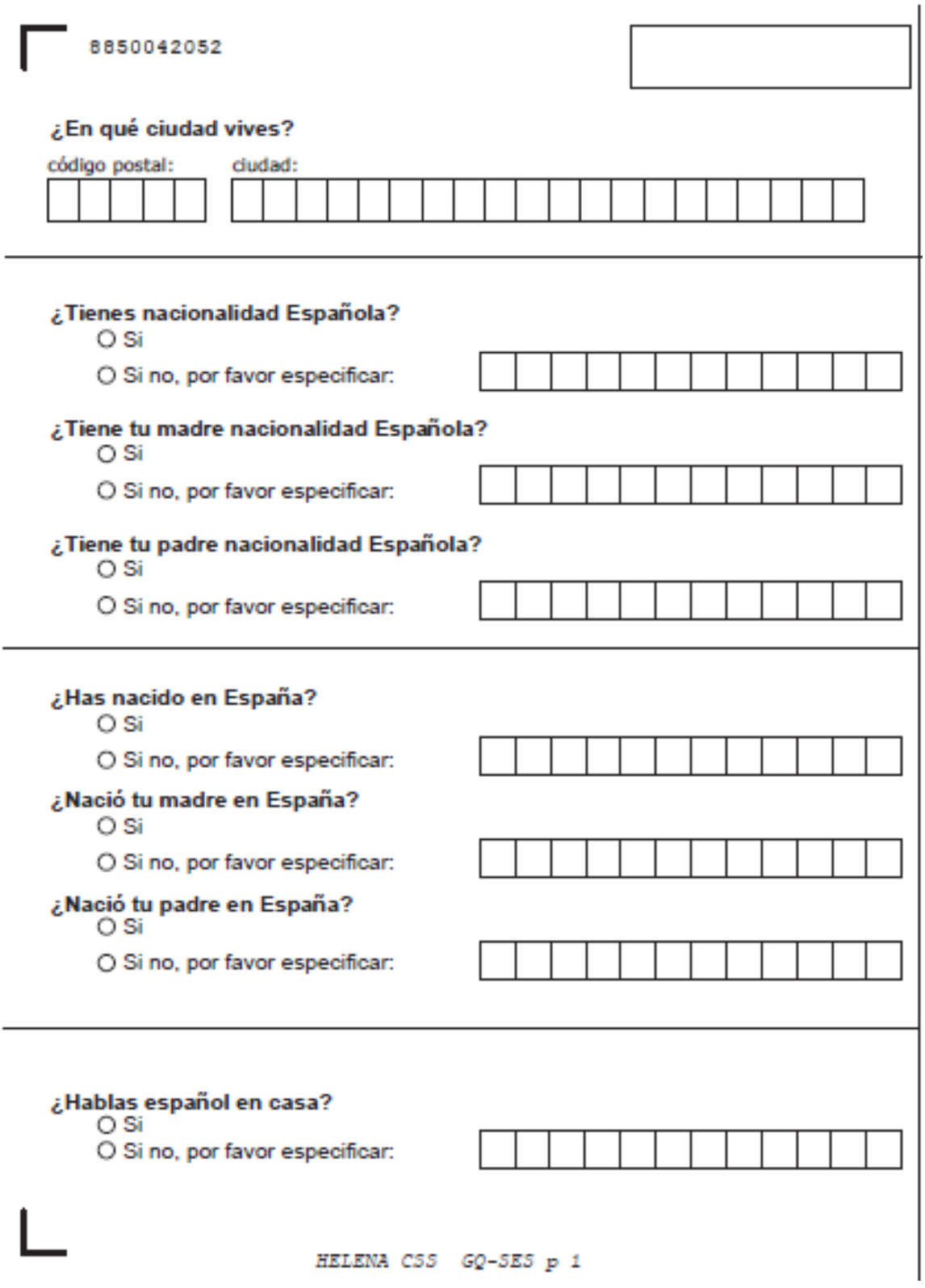
'Vitamin B6, folate and vitamin B12 intake and status in European adolescents. Determinants and consequences'

Plasma vitamin B12 must be placed instead of serum vitamin B12 throughout the manuscripts included in this PhD. This mistake does not interfere neither in the results or the interpretation of them. The affected manuscripts with this erratum are:

'Socioeconomic factors are associated with folate and vitamin B12 intakes and related biomarkers concentrations in European adolescents: The HELENA Study' (Nutr Res)

'Folate and vitamin B12 concentrations are associated with plasma docosahexaenoic and eicosapentaenoic fatty acids in European adolescents: the HELENA study' (Brit J Nutr)

Anexo 2. Cuestionario sociodemográfico utilizado en el estudio HELENA.



6850042052

¿En qué ciudad vives?

código postal: ciudad:

¿Tienes nacionalidad Española?

○ Si

○ Si no, por favor especificar:

¿Tiene tu madre nacionalidad Española?

○ Si

○ Si no, por favor especificar:

¿Tiene tu padre nacionalidad Española?

○ Si

○ Si no, por favor especificar:

¿Has nacido en España?

○ Si

○ Si no, por favor especificar:

¿Nació tu madre en España?

○ Si

○ Si no, por favor especificar:

¿Nació tu padre en España?

○ Si

○ Si no, por favor especificar:

¿Hablas español en casa?

○ Si

○ Si no, por favor especificar:

HELENA CSS GQ-SES p 1

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¿Has fumado tabaco alguna vez?

- ○ Sí
- ○ No

¿Con qué frecuencia fumas tabaco actualmente?

- ○ Cada día
- ○ Al menos una vez a la semana, pero no cada día
- ○ Menos de una vez a la semana
- ○ No fumo

¿Cuántos cigarros fumas por semana?

- ○ Ninguno
- ○ Menos de 5
- ○ Entre 5 y 10
- ○ Entre 11 y 20
- ○ Más de 20

¿Cuánto mides descalzo? 1 **m** ☐☐ **cm**

¿Cuánto pesas sin ropa? ☐☐☐,☐

Por favor, indícanos la afirmación más apropiada para tu madre:

- ○ tiene sobrepeso/obesidad
- ○ tiene peso normal
- ○ esta delgada/muy delgada
- ○ no lo sé

Por favor, indicanos la afirmación más apropiada para tu padre:

- ○ tiene sobrepeso/obesidad
- ○ tiene peso normal
- ○ esta delgado/muy delgado
- ○ no lo sé

El término familia se refiere a miembros viviendo juntos en la misma casa: padre, madre, hermanos
Para aquellos que vivan en dos familias, contesta acerca de la familia con la que vivas la mayor parte del tiempo.

¿Con quien vives la mayoría del tiempo?

- ○ con tus dos padres
- ○ con tu madre sóla
- ○ con tu madre y su nuevo compañero
- ○ con tu padre sólo
- ○ con tu padre y su nueva compañera
- ○ con tu madre la mitad del tiempo y tu padre la otra mitad
- ○ con tus abuelos
- ○ con padres adoptivos
- ○ en un centro de acogida
- ○ algún otro lugar

¿Cuantos de tus hermanas y/o hermanos incluyendo hermanastras y/o hermanastros viven en casa (excluyéndote a ti)?

○ 0 ○ 1 ○ 2 ○ 3 ○ 4 ○ más de 4

¿Dispones de tu propia habitación para ti sólo?

○ no ○ si

¿Cuantos coches posee tu familia? (por "familia" queremos decir miembros que viven juntos: padre, madre y hermanos)

○ 0 ○ 1 ○ 2 ○ más de 2

¿Tienes conexión a internet en casa?

○ no ○ si

¿Cómo le va a tu familia económicamente?

- ○ Fantásticamente bien
- ○ Muy bien
- ○ Normal
- ○ No muy bien
- ○ Mal

8965042059

Las siguientes preguntas son respecto a tu madre y a tu padre.
Si tienes madre y madrastra o padre y padrastro, contesta en relación a la persona más importante en tu educación.

Por favor indicar el nivel educativo más alto de tu madre y tu padre :

	PADRE	MADRE
educación elemental	○	○
terminó la ESO (BUP)	○	○
terminó Bachiller (COU)	○	○
termino educación superior (universitaria)	○	○

¿Cuál es la ocupación de tu padre ?

- ○ trabaja jornada completa
- ○ trabaja media jornada
- ○ amo de casa
- ○ retirado o enfermo
- ○ aprendiz/estudiante
- ○ en paro
- ○ temporalmente sin trabajar (ej: baja paternal)
- ○ nunca le veo
- ○ falleció
- ○ no lo sé

¿Cuál es la ocupación de tu madre ?

- ○ trabaja jornada completa
- ○ trabaja media jornada
- ○ ama de casa
- ○ retirada o enferma
- ○ aprendiz/estudiante
- ○ en paro
- ○ temporalmente sin trabajar (ej: baja maternal)
- ○ nunca la veo
- ○ falleció
- ○ no lo sé

Por favor, anota el tipo de trabajo que tienen tus padres. Si tienen más de un trabajo, indícalos

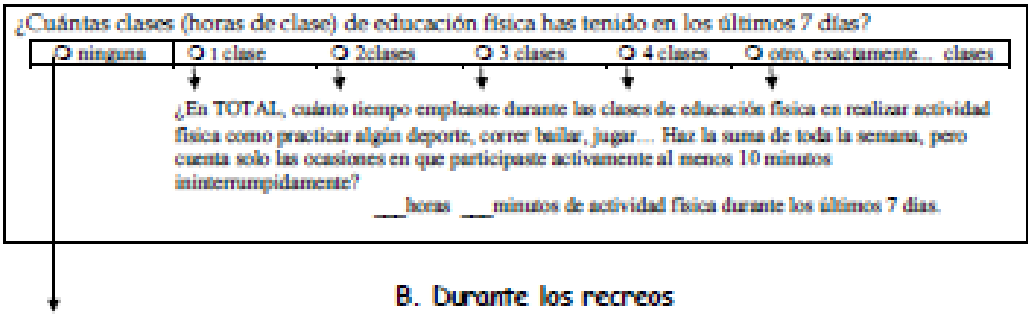
	PADRE	MADRE
1. Personal administrativo Presidente, Director de administración pública, Consejo de Administración (Jefe de Departamento o equivalente)	○	○
2. profesiones intelectuales y científicas Personal cualificado (matemático o especialista en ciencias y salud, especialistas técnicos :arquitectos, ingenieros, informáticos, biólogos, famrmacéuticos, médicos, abogados, profesor universitario, psicólogo, sociólogo, etc.)	○	○
3 Profesiones intermedias: Técnicos o peritos y otros trabajos intermedios (electricista, mecánico, enfermero/a, dietista, empleado de oficina, maestro, representante comercial y profesionales asociados)	○	○
4. Administración/Oficinas Banca, contabilidad, seguros, bibliotecarios, etc.	○	○
5. Empresas de negocios Ventas, marketing, publicidad, comunicaciones, etc.	○	○
6. Trabajadores cualificados de agricultura y pesca Granjeros, pescadores, guardabosques, etc.	○	○
7. Artesanos, manufactura y oficios relacionados Peluquero/a, mecánico, operario, artesano, mecánico, operario en industria textil, calzado, etc.	○	○
8. Operarios de maquinaria y montadores Trabajadores industriales y operarios de máquinas, conductores de grúas, etc.	○	○
9. Trabajos y ocupaciones elementales vendedores, empleados del hogar, albañiles, vigilantes de seguridad, limpiadores, etc.	○	○
10. fuerzas armadas	○	○
11. otro nombre (descríbelo detalladamente)	○	○
12. no trabaja	○	○

Anexo 3. Cuestionario International Physical Activity Questionnaire (IPAQ)

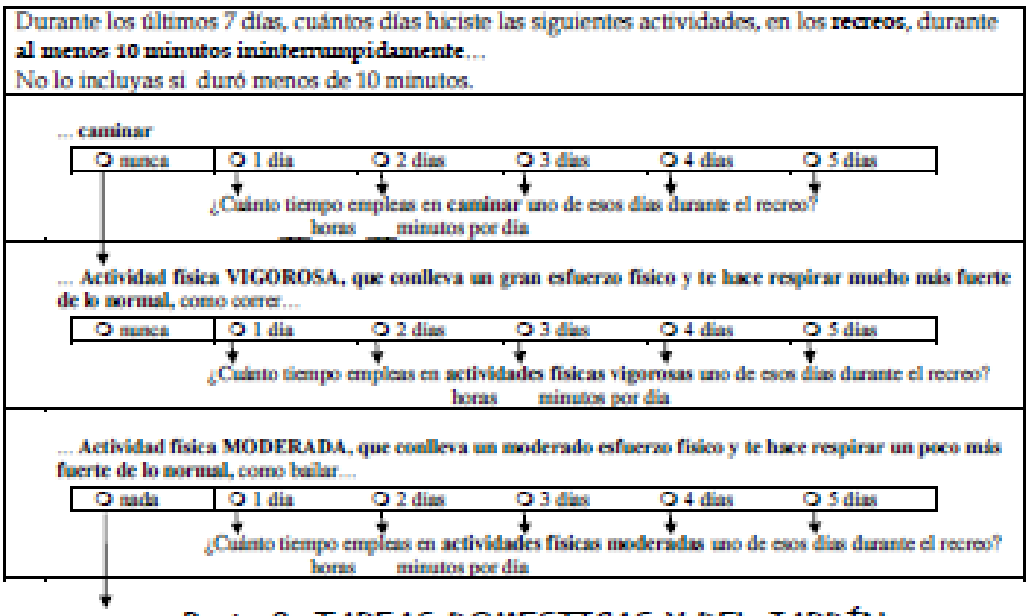
Parte 1: ACTIVIDAD FÍSICA EN EL COLEGIO

La parte 1 es sobre la actividad física que has hecho en los últimos 7 días durante las horas de colegio (clases y recreos). El transporte o forma de ir al colegio NO se incluye aquí.

A. Durante las clases de Educación Física

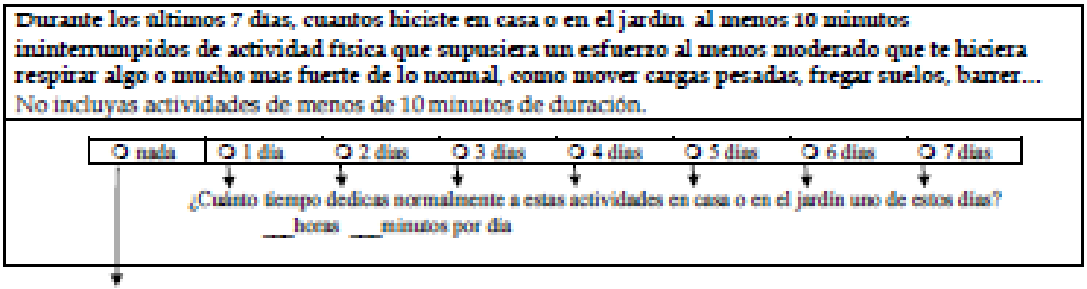


B. Durante los recreos



Parte 2: TAREAS DOMESTICAS Y DEL JARDÍN

Esta segunda parte es sobre la actividad física que hiciste en los últimos 7 días en casa o en el jardín.



Parte 3: TRANSPORTE Y ACTIVIDAD FISICA

Estas preguntas son sobre como te desplazaste de un lugar a otro, incluyendo lugares como el colegio, las tiendas, los cines, etc. durante los últimos 7 días.

Durante los últimos 7 días, cuantos viajaste al menos 10 minutos ininterrumpidamente...
No incluyas actividades que duraran menos de 10 minutos ininterrumpidos.

... **EN UN VEHICULO A MOTOR** como tren, coche, autobús, moto, metro,...?

O nada | O 1 día | O 2 días | O 3 días | O 4 días | O 5 días | O 6 días | O 7 días

¿Cuánto tiempo empleas normalmente en viajar en vehículos a motor uno de estos días?
___horas ___minutos por día

... **EN BICICLETA?**

O nada | O 1 día | O 2 días | O 3 días | O 4 días | O 5 días | O 6 días | O 7 días

¿Cuánto tiempo empleas uno de esos días en ir en bicicleta de un lugar a otro?
___horas ___minutos por día

... **ANDANDO?**

O nada | O 1 día | O 2 días | O 3 días | O 4 días | O 5 días | O 6 días | O 7 días

¿Cuánto tiempo empleas normalmente uno de esos días en ir andando de un lugar a otro?
___horas ___minutos por día

Parte 4: ACTIVIDAD FÍSICA DURANTE EL TIEMPO DE OCIO, DEPORTE Y TIEMPO LIBRE

Esta sección es sobre toda la actividad física que has hecho en los últimos 7 días, únicamente respecto al tiempo de ocio, de práctica deportiva, entrenamiento o placer. Por favor, no incluyas actividades que ya has mencionado.

Durante los últimos 7 días, cuántos hiciste una de las siguientes actividades por al menos 10 minutos sin parar, durante tu tiempo libre...

No incluyas actividades que duraran menos de 10 minutos ininterrumpidos.

... **CAMINAR**

O nada | O 1 día | O 2 días | O 3 días | O 4 días | O 5 días | O 6 días | O 7 días

¿Cuánto tiempo empleas normalmente uno de esos días en **caminar** en tu tiempo libre?
___horas ___minutos por día

... **Actividad física VIGOROSA, que conlleva un gran esfuerzo físico y te hace respirar mucho más fuerte de lo normal,** como ejercicio aeróbico, correr, andar en bici o nadar rápido...

O nada | O 1 día | O 2 días | O 3 días | O 4 días | O 5 días | O 6 días | O 7 días

¿Cuánto tiempo empleas normalmente uno de esos días en **actividad física vigorosa** en tu tiempo libre?
horas minutos por día

... **Actividad física MODERADA, que conlleva un moderado esfuerzo físico y te hace respirar un poco más fuerte de lo normal,** como bailar, nadar o montar en bicicleta despacio ...

O nada | O 1 día | O 2 días | O 3 días | O 4 días | O 5 días | O 6 días | O 7 días

¿Cuánto tiempo empleas normalmente uno de esos días en **actividad física moderada** en tu tiempo libre?
___horas ___minutos por día

Anexo 4. Decisión del editor de la revista *Nutrición Hospitalaria*

[NH] Decisión del editor/a

Asunto: [NH] Decisión del editor/a
De: "Elena Muñoz Grande" <nutricion1@grupoaran.com>
Fecha: 30/12/2016 19:23
Para: "Iris Iglesia Altaba" <iglesia@unizar.es>

El siguiente mensaje se está enviando a nombre de Nutrición Hospitalaria.

Estimado/a autor/a:
Iris Iglesia Altaba

El Comité Editorial de nuestra revista ha resuelto aceptar su trabajo "Associations between insulin resistance and three B-vitamins in European adolescents: The HELENA study" para su publicación en la revista Nutrición Hospitalaria.

Antes de que el artículo sea publicado, deberá abonar la cantidad establecida según aparece en las normas de publicación de la revista. Para el pago rogamos contacte con nutricion@grupoaran.com

Muchas gracias por su colaboración y reciba un cordial saludo.

Dr José Manuel Moreno Villares
josemanuel.moreno@salud.madrid.org
José Manuel Moreno Villares
Director revista Nutrición Hospitalaria
josemanuel.moreno@salud.madrid.org

Nutrición Hospitalaria
http://www.nutricionhospitalaria.org/

Anexo 5. Estado del artículo III en la revista *Nutrition*

Your Submission NUT-D-16-00774

Asunto: Your Submission NUT-D-16-00774
De: "Maria Isabel Correia" <eesserver@eesmail.elsevier.com>
Fecha: 08/03/2017 14:06
Para: iglesia@unizar.es

Ms. Ref. No.: NUT-D-16-00774
Title: Do dietary patterns determine levels of vitamin B6, folate, and vitamin B12 intakes and corresponding biomarkers in European adolescents? The HELENA study.
Nutrition

Dear Miss Iglesia,

Your article Do dietary patterns determine levels of vitamin B6, folate, and vitamin B12 intakes and corresponding biomarkers in European adolescents? The HELENA study. has been returned by our reviewers. In its present form, it cannot be accepted for publication in Nutrition.

However, Nutrition would be willing to reconsider it for possible publication, if you feel you can fully address the reviewers' comments. Please remember that we would have to send your revised manuscript back to the original referees and keep this time frame in mind when you respond to the critiques.

If you wish to have your manuscript reconsidered for publication, please revise it and return it to this office by 2017-04-07 00:00:00.

For your guidance, reviewers' comments are appended below.

Please provide along with your cover letter a list of your responses to the reviewers' critiques and the changes you have made in your manuscript. If you disagree with a referee's comments and choose to make no revision on certain points, please clearly support your view.

To submit a revision, please go to https://ees.elsevier.com/nut/ and login as an Author. On your Main Menu page is a folder entitled "Submissions Needing Revision". You will find your submission record there.

We look forward to hearing from you.

Yours sincerely,

Janicke Visser
Co-Regional Editor
Nutrition

Anexo 6. Lista de miembros del estudio HELENA

*HELENA Study Group

Co-ordinator: Luis A. Moreno.

Core Group members: Luis A. Moreno, Fréderic Gottrand, Stefaan De Henauw, Marcela González-Gross, Chantal Gilbert.

Steering Committee: Anthony Kafatos (President), Luis A. Moreno, Christian Libersa, Stefaan De Henauw, Sara Castelló, Fréderic Gottrand, Mathilde Kersting, Michael Sjöstrom, Dénes Molnár, Marcela González-Gross, Jean Dallongeville, Chantal Gilbert, Gunnar Hall, Lea Maes, Luca Scalfi.

Project Manager: Pilar Meléndez.

1. **Universidad de Zaragoza (Spain)**

   Luis A. Moreno, Jesús Fleta, José A. Casajús, Gerardo Rodríguez, Concepción Tomás, María I. Mesana, Germán Vicente-Rodríguez, Adoración Villarroya, Carlos M. Gil, Ignacio Ara, Juan Fernández Alvira, Gloria Bueno, Aurora Lázaro, Olga Bueno, Juan F. León, Jesús Mª Garagorri, Manuel Bueno, Idoia Labayen, Iris Iglesia, Silvia Bel, Luis A. Gracia Marco, Theodora Mouratidou, Alba Santaliestra-Pasías, Iris Iglesia, Esther González-Gil, Pilar De Miguel-Etayo, Cristina Julián Almárcegui, Mary Miguel-Berges, Isabel Iguacel.

2. **Consejo Superior de Investigaciones Científicas (Spain)**

Ascensión Marcos, Julia Wärnberg, Esther Nova, Sonia Gómez, Ligia Esperanza Díaz, Javier Romeo, Ana Veses, Belén Zapatera, Tamara Pozo, David Martínez.

3. **Université de Lille 2 (France)**

   Laurent Beghin, Christian Libersa, Frédéric Gottrand, Catalina Iliescu, Juliana Von Berlepsch.

4. **Research Institute of Child Nutrition Dortmund, Rheinische Friedrich-Wilhelms-Universität Bonn (Germany)**

   Mathilde Kersting, Wolfgang Sichert-Hellert, Ellen Koeppen.

5. **Pécsi Tudományegyetem (University of Pécs) (Hungary)**

   Dénes Molnar, Eva Erhardt, Katalin Csernus, Katalin Török, Szilvia Bokor, Mrs. Angster, Enikö Nagy, Orsolya Kovács, Judit Répasi.

6. **University of Crete School of Medicine (Greece)**

   Anthony Kafatos, Caroline Codrington, María Plada, Angeliki Papadaki, Katerina Sarri, Anna Viskadourou, Christos Hatzis, Michael Kiriakakis, George Tsibinos, Constantine Vardavas, Manolis Sbokos, Eva Protoyeraki, Maria Fasoulaki.

7. **Institut für Ernährungs- und Lebensmittelwissenschaften – Ernährungphysiologie. Rheinische Friedrich Wilhelms Universität (Germany)**

   Peter Stehle, Klaus Pietrzik, Marcela González-Gross, Christina Breidenassel, Andre Spinneker, Jasmin Al-Tahan, Miriam Segoviano, Anke Berchtold, Christine Bierschbach, Erika Blatzheim, Adelheid Schuch, Petra Pickert.

8. **University of Granada (Spain)**

Manuel J. Castillo, Ángel Gutiérrez, Francisco B Ortega, Jonatan R Ruiz, Enrique G Artero, Vanesa España, David Jiménez-Pavón, Palma Chillón, Cristóbal Sánchez-Muñoz, Magdalena Cuenca

9. **Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione (Italy)**

Davide Arcella, Elena Azzini, Emma Barison, Noemi Bevilacqua, Pasquale Buonocore, Giovina Catasta, Laura Censi, Donatella Ciarapica, Paola D'Acapito, Marika Ferrari, Myriam Galfo, Cinzia Le Donne, Catherine Leclercq, Giuseppe Maiani, Beatrice Mauro, Lorenza Mistura, Antonella Pasquali, Raffaela Piccinelli, Angela Polito, Romana Roccaldo, Raffaella Spada, Stefania Sette, Maria Zaccaria.

10. **University of Napoli "Federico II" Dept of Food Science (Italy)**

Luca Scalfi, Paola Vitaglione, Concetta Montagnese.

11. **Ghent University (Belgium)**

Ilse De Bourdeaudhuij, Stefaan De Henauw, Tineke De Vriendt, Lea Maes, Christophe Matthys, Carine Vereecken, Mieke de Maeyer, Charlene Ottevaere, Inge Huybrechts.

12. **Medical University of Vienna (Austria)**

Kurt Widhalm, Katharina Phillipp, Sabine Dietrich.

13. **Harokopio University (Greece)**

Yannis Manios, Eva Grammatikaki, Zoi Bouloubasi, Tina Louisa Cook, Sofia Eleutheriou, Orsalia Consta, George Moschonis, Ioanna Katsaroli, George Kraniou, Stalo

Papoutsou, Despoina Keke, Ioanna Petraki, Elena Bellou, Sofia Tanagra, Kostalenia Kallianoti, Dionysia Argyropoulou, Stamatoula Tsikrika, Christos Karaiskos.

14. Institut Pasteur de Lille (France)

Jean Dallongeville, Aline Meirhaeghe.

15. Karolinska Institutet (Sweden)

Michael Sjöstrom, Jonatan R Ruiz, Francisco B. Ortega, María Hagströmer, Anita Hurtig Wennlöf, Lena Hallström, Emma Patterson, Lydia Kwak, Julia Wärnberg, Nico Rizzo.

16. Asociación de Investigación de la Industria Agroalimentaria (Spain)

Jackie Sánchez-Molero, Sara Castelló, Elena Picó, Maite Navarro, Blanca Viadel, José Enrique Carreres, Gema Merino, Rosa Sanjuán, María Lorente, María José Sánchez.

17. Campden BRI (United Kingdom)

Chantal Gilbert, Sarah Thomas, Elaine Allchurch, Peter Burgess.

18. SIK - Institutet foer Livsmedel och Bioteknik (Sweden)

Gunnar Hall, Annika Astrom, Anna Sverkén, Agneta Broberg.

19. Meurice Recherche & Development asbl (Belgium)

Annick Masson, Claire Lehoux, Pascal Brabant, Philippe Pate, Laurence Fontaine.

20. Campden & Chorleywood Food Development Institute (Hungary)

Andras Sebok, Tunde Kuti, Adrienn Hegyi.

21. Productos Aditivos SA (Spain)

Cristina Maldonado, Ana Llorente.

22. Cárnicas Serrano SL (Spain)

Emilio García.

23. Cederroth International AB (Sweden)

Holger von Fircks, Marianne Lilja Hallberg, Maria Messerer

24. Lantmännen Food R&D (Sweden)

Mats Larsson, Helena Fredriksson, Viola Adamsson, Ingmar Börjesson.

25. European Food Information Council (Belgium)

Laura Fernández, Laura Smillie, Josephine Wills.

26. Universidad Politécnica de Madrid (Spain)

Marcela González-Gross, Raquel Pedrero-Chamizo, Agustín Meléndez, Jara Valtueña, David Jiménez-Pavón, Ulrike Albers, Pedro J. Benito, Juan José Gómez Lorente, David Cañada, Alejandro Urzanqui, Rosa María Torres, Paloma Navarro.