Vitamin B₁₂ and folate status in Spanish lacto-ovo vegetarians and vegans

Angélica Gallego-Narbón¹, Belén Zapatera¹, Laura Barrios² and M. Pilar Vaquero¹*
¹Department of Metabolism and Nutrition, Institute of Food Science, Technology and Nutrition (ICTAN-CSIC), José Antonio Novais 10, 28040 Madrid, Spain
²Statistics Department, Computing Center (SGAI-CSIC), Pinar 19, 28006 Madrid, Spain

(Received 10 December 2018 – Accepted 16 January 2019)

Abstract
Studies on the nutritional status of vegetarians in Spain are lacking. Prevention of vitamin B₁₂ deficiency is the main concern, as dietary sources are of animal origin. The present study aimed to evaluate vitamin B₁₂ and folate status of Spanish vegetarians using classical markers and functional markers. Participants were adult and healthy lacto-ovo vegetarians (forty-nine subjects) and vegans (fifty-four subjects) who underwent blood analyses and completed a FFQ. Serum vitamin B₁₂, homocysteine (Hcy), methylmalonic acid (MMA), erythrocyte folate and haematological parameters were determined. The effects of the type of plant-based diet, and the intake of supplements and foods were studied by a FFQ. Mean erythrocyte folate was 1704 (SD 609) nmol/l. Clinical or subclinical vitamin B₁₂ deficiency was detected in 11 % of the subjects (MMA>271 nmol/l) and 33 % of the participants showed hyperhomocysteinaemia (Hcy>15 µmol/l). Regarding plant-based diet type, significantly higher Hcy was observed in lacto-ovo vegetarians compared with vegans (P=0.019). Moreover, use of vitamin B₁₂ supplements involved an improvement of vitamin B₁₂ status but further increase in erythrocyte folate (P=0.024). Consumption of yoghurts was weakly associated with serum vitamin B₁₂ adequacy (P=0.049) and that of eggs with lower Hcy (P=0.030). In conclusion, Spanish vegetarians present high folate status but vitamin B₁₂ subclinical deficiency was demonstrated using functional markers. The lack of influence of dietary sources on functional markers and the strong effect of vitamin B₁₂ supplement intake emphasise the need of cobalamin supplementation in both lacto-ovo vegetarians and vegans.

Key words: Vitamin B₁₂; Folate; Vegetarians; Vegans; Supplementation

Plant-based diets have been reported to provide several health benefits, reducing the risk of atherosclerosis, CHD, type 2 diabetes and the metabolic syndrome⁵⁻⁶. However, these diets may also increase the risks of nutritional deficiencies³⁻⁵. Vitamin B₁₂, or cobalamin, acts as a cofactor of the enzymes methionine synthase, acting in the conversion of homocysteine (Hcy) to methionine, and methylmalonyl-CoA mutase, that produces succinyl-CoA from methylmalonyl-CoA, the active form of methylmalonic acid (MMA)⁶. These reactions are involved in the methionine cycle and the folate cycle, both essential for DNA and RNA synthesis, erythropoiesis and the production of neurotransmitters⁶⁻⁷. Therefore, deficiencies of vitamin B₁₂ and folate can result in neurological damage, due to an inhibition of the formation of the myelin sheath, and megaloblastic anaemia⁸.

Clinical deficiency of vitamin B₁₂ can cause megaloblastic anaemia, which is characterised by high mean corpuscular erythrocyte volume (MCV) and erythrocyte distribution width, and low erythrocyte count⁹⁻¹⁰. Nevertheless, an early detection of vitamin B₁₂ deficiency is a challenge, as there is not a single standard marker but several markers should be analysed for diagnosis¹¹. In this regard, although serum vitamin B₁₂ has been widely used to assess cobalamin deficiency, it can remain normal under functional deficiency conditions and

* Corresponding author: M. Pilar Vaquero, email mpvaquero@ictan.csic.es

Abbreviations: Hcy, homocysteine; HHcy, hyperhomocysteinaemia; MCH, mean corpuscular Hb; MCV, mean corpuscular erythrocyte volume; MMA, methylmalonic acid.

© The Author(s) 2019. This is an Open Access article, distributed under the terms of the Creative Commons Attribution licence (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted re-use, distribution, and reproduction in any medium, provided the original work is properly cited.
false positives and negatives are common\(^{(12,13)}\). A more specific marker of functional vitamin B\(_{12}\) deficiency is Hcy, which increases under cellular vitamin B\(_{12}\) deficiency. Nevertheless, Hcy is also elevated under deficiencies of folate or vitamin B\(_{6}\) and by diets rich in methionine\(^{(6,14-16)}\). Hyperhomocysteinaemia (HHcy) promotes the formation of active oxygen species and the release of inflammatory mediators, and therefore it is considered a risk factor for CVD\(^{(10,16-18)}\). Finally, MMA represents the most specific marker for vitamin B\(_{12}\) deficiency, as it is independent of folate status and increases in cobalamin deficiency conditions before the appearance of clinical signs\(^{(6,19)}\).

Cobalamin is synthesised exclusively by bacteria and archaea, but it is accumulated along the food chain, and animal foods including meat, milk, eggs, fish and shellfish are considered the main dietary sources\(^{(1,20)}\). Other food sources are scarce and include several mushroom species like shiitake (Lentinula edodes), black trumpet (Craterellus cornucopioides) and golden chanterelle (Cantharellus cibarius), and certain algae and cyanobacteria such as Chlorella and spirulina (Arthrospira platensis). These are frequently used to produce tablets consumed as cobalamin supplements by vegetarians and particularly vegans\(^{(20)}\). Nevertheless, it has been observed that these supplements also contain high amounts of inactive analogues of vitamin B\(_{12}\)\(^{(21-23)}\). Therefore, vitamin B\(_{12}\) deficiency is considered an extended problem for vegetarians, especially for vegans, if nutritional cobalamin supplements are not consumed\(^{(6,24)}\).

Very limited information exists on the nutritional status of vegetarians in Spain\(^{(25)}\). It is estimated that they represent at least 1.5% of the Spanish population\(^{(26)}\). In the present study we evaluate the vitamin B\(_{12}\) and folate status of vegetarians and particularly vegans\(^{(26)}\). In the present study we evaluate the vitamin B\(_{12}\) and folate status of Spanish vegetarians using markers of clinical deficiency (serum vitamin B\(_{12}\) and haematological markers) and subclinical deficiency (Hcy, MMA and erythrocyte folate), constituting the first complete study on vitamin B\(_{12}\) and folate status in this population. The associations of the studied markers with the type of diet (lacto-ovo vegetarian or vegan), and the intake of vitamin B\(_{12}\)-rich products and supplements were also addressed.

**Materials and methods**

**Study design and participants**

The study followed a cross-sectional design. Healthy lacto-ovo vegetarian or vegan adults (age $\geq$18 years) were recruited through advertisements in web pages in the area of Madrid, Spain. Exclusion criteria were: occasional meat or fish consumption, diagnosed digestive, renal, haematological, endocrine or oncological diseases, eating disorders, pregnancy, lactation and menopause. Subjects who had donated blood in the 3 months prior to the study were also excluded. A total of 207 subjects were initially interested, of which forty-four declined to participate and fifty-eight did not meet the inclusion criteria. In all, 105 volunteers were selected, and finally 103 completed the study and underwent all the biochemical analyses. None of the selected volunteers reported any diagnosed illness and they did not consume any medication in the days prior to blood extraction.

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Clinical Research Ethics Committee of Hospital Puerta de Hierro (Majadahonda, Spain) under the reference number 06.17 and the Ethics Committee of the Spanish National Research Council (CSIC). Written informed consent was obtained from all the participants.

**Procedure, blood sampling and biochemical assays**

Volunteers attended the Human Nutrition Unit of ICTAN-CSIC between 08.00 and 08.30 hours after a 12 h fasting period. Height of the subjects was measured, and body weight, body composition and BMI were obtained using the body composition monitor Tanita BC-601 (Tanita Ltd). Blood samples were collected in Vacutec Z Serum Sep Clot Activator and K3 EDTA tubes (Greiner Bio-One GmbH). Serum was separated by centrifugation in a Jouan CR-312 centrifuge (Jouan Ltd) at 1000 $g$ for 15 min and stored at $–80^\circ$C. Vitamin B\(_{12}\) and Hcy were analysed in 100 $\mu$l and 20 $\mu$l of serum, respectively, by competitive immunoassay of direct chemiluminescent technology in an ADVIA Centaur XP autoanalyzer (Siemens Healthineers). Serum samples for MMA determination were prepared by solid-phase extraction using Bond Elute strong anion exchange cartridges (100 mg bed/1 ml) from Agilent. The analysis was performed using a liquid chromatography–tandem MS method previously optimised by our research group\(^{(25)}\).

Erythrocyte count, erythrocyte distribution width, packed cell volume, MCV, Hb and mean corpuscular Hb (MCH) were determined in whole blood using an ADVIA 2120 flow cytometer (Siemens Healthineers). Erythrocyte folate was analysed from whole-blood samples that were refrigerated, diluted 1:25 in folate lysing agent (Beckman Coulter, Inc.), mixed, protected from light for 90 min, and finally measured using the body composition monitor Tanita BC-601 (Tanita Ltd).

**Definitions**

The proportion of individuals that presented inadequate levels within each group was defined considering the following reference levels of normality: vitamin B\(_{12}\) (>150 pmol/l), Hcy (5–15 $\mu$mol/l), MMA (<271 nmol/l), erythrocyte folate...
 (>305 nmol/l), erythrocyte distribution width (<14 %), MCV (80–96 fl), MCH (>27 pg), Hb (>130 g/l for men and >120 g/l for women), erythrocyte count (>3.6 x 10^{12}/l for men and >4.2 x 10^{12}/l for women) and packed cell volume (>41 % for men and >36 % for women)\(^{(15,27,28)}\).

**FFQ**

A FFQ previously validated and applied for vegans\(^{(29)}\) was modified in order to estimate the composition of the diet of the Spanish vegetarian subjects. The questionnaire was completed by 103 participants. Volunteers were classified as lacto-ovo vegetarians or vegans and their consumption of supplements of vitamin B\(_12\) and folate acid was also recorded. The subjects were asked to indicate the frequency of consumption of each food item as consumed: never or rarely, two to four times a month, two to three times a week, four to six times a week, once per d and two or more times per d. The intake of supplements was classified according to the following frequency categories: never, one to twelve times a year, two to five times a month, two to six times a week, and daily consumed. Vitamin B\(_12\), folic acid and multivitamin supplements were considered. Subjects were considered supplement users if they consumed supplements at least two to five times a month. Food items included in the study of vitamin B\(_12\) status were: animal milk, cheese, eggs, yoghurts and vegetable milks. Mushrooms and vitamin B\(_12\)-rich algae were not considered due to their low rate of consumption (less than 10 % of the subjects reported an occasional intake). The consumption of yoghurts was assessed through three different items (natural or flavoured yoghurts, non-fat yoghurts, other yoghurts) and vegetable milks through six items (soya milk, almond milk, oat milk, coconut milk, rice milk and cashew milk).

**Statistical analysis**

**Sample size justification.** Sample size was calculated for the main variable, serum vitamin B\(_12\). Previous reports indicate mean values of 209 (SD 47) and 172 (SD 59) pmol/l for lacto-ovo vegetarians and vegans, respectively\(^{(30)}\). Therefore, to detect as significant a difference of 15 % between the two diet option groups, an allocation ratio of 1, a two-sided \(P = 0.05\) and statistical power of 0.80, a total of eighty-eight volunteers was needed. Calculations were performed using G\(^*\) Power 3\(^{(31)}\). This number was increased by 15 % to ensure a sufficient sample size in case several participants decided not to complete the study. Results are presented for 103 volunteers, with forty-nine lacto-ovo vegetarians and fifty-four vegans.

**Data analysis.** Mean and standard deviation were calculated for each of the markers. Normality was studied by the Kolmogorov–Smirnov test and non-normal variables were transformed according to their distribution. Vitamin B\(_12\), folate, MCV and MCH were log-transformed, while MMA and erythrocyte distribution width were transformed using the inverse function. The effect of supplementation was only studied for vitamin B\(_12\) supplements, as only one volunteer reported the intake of folate supplements and multivitamins were consumed occasionally for only ten individuals. Differences owed to vitamin B\(_12\) supplementation and diet option were studied (fixed factors) by general linear models of the transformed variables including sex and age (random factors). The age effect was not significant and therefore this variable was not included in further analyses. According to cut-off levels for each parameter, binomial variables classifying each individual as adequate or inadequate were calculated. These results are presented within diet (lacto-ovo vegetarian or vegan) and supplementation (vitamin B\(_12\) supplement user or non-user) groups. Furthermore, we analysed the differences in the frequency of consumption of the studied food items between subjects with adequate levels and subjects with inadequate levels of serum vitamin B\(_12\), Hcy and MMA through contingency tables. When independent food items were analysed (animal milk, cheese, eggs), Monte Carlo two-sided tests with 10,000 simulations and a CI of 99 % were used to provide reliability to the analysis. Yoghurt items, as well as vegetable milk items, were converted in multiple response sets and considered as food groups for the analysis, which was performed using Pearson’s \(\chi^2\) tests. Significance was set at \(P < 0.05\) and all the statistical analyses were performed in SPSS 24 (SPSS Inc.).

**Results**

**Population characteristics**

The participating volunteers were young adults, with a mean age of 30.3 (SD 7.7) years, and only two subjects were older than 46 years. Most of the volunteers were women, 78 % of the total (Table 1). In relation to diet option, 47.6 % of the participants were lacto-ovo vegetarians and 52.4 % were vegans. They were normoweight with slightly higher BMI in men than women. Hcy, erythrocyte count, erythrocyte distribution width, packed cell volume and Hb were significantly different between men and women (\(P < 0.001\) in all cases). Vitamin B\(_12\) supplements were consumed by 72.8 % of the participants.

**Diet and supplementation effects**

Significant differences were found between vitamin B\(_{12}\) users and non-users, with higher levels of vitamin B\(_{12}\) (\(P < 0.001\)) and erythrocyte folate (\(P = 0.024\)), and lower MMA (\(P = 0.012\)) and Hcy (\(P = 0.015\)) in volunteers consuming vitamin B\(_{12}\) supplements (Table 2). Vegans presented significantly lower erythrocyte count (\(P = 0.032\)) and erythrocyte distribution width (\(P = 0.003\)) and higher MCV (\(P = 0.036\)) and MCH (\(P = 0.023\)) compared with lacto-ovo vegetarians. A significant interaction diet option x supplementation was found for Hb (\(P = 0.034\)). Means of all these haematological variables were in the normal range.

Two participants presented vitamin B\(_{12}\) levels below the cut-off value of 150 pmol/l that indicates clinical deficiency (Fig. 1), while eleven subjects presented elevated MMA. HHe was detected in thirty-three individuals. The prevalence
of HHcy in vitamin B12 non-users (twelve subjects, 43 % of the group) was higher than in vitamin B12 users (twenty-one subjects, 28 % of the group). Erythrocyte count was low in sixteen subjects (15.5 % of the volunteers) and erythrocyte distribution width in seventeen subjects (16.5 % of the volunteers). Erythrocyte count low values were more frequent in vegans than lacto-ovo vegetarians (fourteen subjects, 25.9 % of vegans; r. two subjects, 4.1 % of lacto-ovo vegetarians). MCV was elevated in sixteen subjects, most of them vegans (twelve subjects, 22.2 % of vegans; r. three subjects, 6.1 % of vegetarians). Two of these subjects also presented elevated MMA and Hcy. High erythrocyte distribution width was observed mainly in vitamin B12 users (sixteen subjects, 21.3 % of users; r. one subject, 3.6 % of non-users).

The number of subjects with inadequate levels in association with the frequency of consumption of dairy products and eggs is presented in Table 3. Serum vitamin B12 sufficiency was associated with the consumption of yoghurts (P = 0.049), and Hcy was associated with egg consumption (P = 0.030). MMA did not present an association with any of the considered food items.

**Discussion**

This study represents the first complete report on vitamin B12 and folate status in Spanish vegetarians. Results reveal sufficient serum vitamin B12 levels in the studied subjects independently of their diet option, and the prevalence of clinical vitamin B12 deficiency was very low. However, by measuring MMA we detected subclinical deficiency, particularly in the non-users of vitamin B12 supplements. Regarding haematological results, the differences between vegans and vegetarians did not reach significance, and macrocytosis was observed in several participants but a clear correspondence with the biochemical biomarkers of vitamin B12 was not found.

Elevated MMA was detected in more than 10 % of the population, emphasising the utility of this marker to detect subclinical deficiencies. HHcy was found in more than 30 % of the subjects. The metabolic reactions leading to the production of MMA and Hcy require different cobalamin forms (with methylcobalamin acting in the Hcy pathway and adenosylcobalamin in the formation of succinyl-CoA) and occur in different cell compartments (as the methionine and folate cycles occur in the cytoplasm and the synthesis of succinyl-CoA in the mitochondria). In this context, previous research has stated that serum vitamin B12 might be a misleading marker, as the latency period to detect clinical deficiencies is long. Therefore, we encourage the use of both MMA and Hcy together with serum vitamin B12, as each of these biomarkers informs about the action of vitamin B12 in different metabolic pathways.

The use of several functional markers is even more important if we consider recent relevant research revealing a relationship between MMA levels and certain genotypes involved in valine catabolism that might affect MMA levels independently of cobalamin status. Therefore the use of Hcy to detect subclinical vitamin B12 deficiency through a different pathway is recommended. Consistently, when cobalamin status was assessed considering serum vitamin B12, MMA and Hcy simultaneously, the proportion of subjects with values out of range of at least one of the biomarkers was remarkably higher than the proportion detected by using only serum vitamin B12. The observed results underline the limitations of serum vitamin B12 to detect vitamin B12 deficiency and the need of using several functional markers to detect subclinical deficiencies accurately.

Most of the hyperhomocysteinaemic individuals presented mild HHcy (16–30 µmol/l) while only three volunteers had moderate HHcy (31–100 µmol/l). There is a great variability in the values obtained in European vegetarians; the volunteers in the present study had Hcy levels higher than German

---

**Table 1. Characteristics of the studied subjects**

<table>
<thead>
<tr>
<th></th>
<th>Women (n 80)</th>
<th>Men (n 23)</th>
<th>All (n 103)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Mean</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>±sd</td>
<td>±sd</td>
<td>±sd</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>29.6 ± 7.5</td>
<td>32.8 ± 8.0</td>
<td>30.3 ± 7.7</td>
</tr>
<tr>
<td>Vegans</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n (%)</td>
<td>42</td>
<td>12</td>
<td>54</td>
</tr>
<tr>
<td>Vitamin B12 users (%)</td>
<td>52.5 ± 7.1</td>
<td>52.2 ± 7.8</td>
<td>52.4 ± 7.2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.3 ± 3.2</td>
<td>24.0 ± 3.9</td>
<td>22.6 ± 3.4</td>
</tr>
<tr>
<td>Vitamin B12 (pmol/l)</td>
<td>344.7 ± 199.7</td>
<td>280.8 ± 74.9</td>
<td>330.4 ± 181.2</td>
</tr>
<tr>
<td>Hcy (µmol/l)</td>
<td>12.8 ± 3.1</td>
<td>14.9 ± 8.2</td>
<td>13.9 ± 5.1</td>
</tr>
<tr>
<td>MMA (nmol/l)</td>
<td>157.0 ± 79.8</td>
<td>222.3 ± 236.4</td>
<td>171.6 ± 133.2</td>
</tr>
<tr>
<td>Erythrocyte folate (nmol/l)</td>
<td>1713.5 ± 609.8</td>
<td>1670.8 ± 617.7</td>
<td>1704.0 ± 608.8</td>
</tr>
<tr>
<td>Erythrocyte count (10¹²/l)</td>
<td>4.4 ± 0.3</td>
<td>4.9 ± 0.4</td>
<td>4.6 ± 0.4</td>
</tr>
<tr>
<td>Erythrocyte distribution width (%)</td>
<td>13.5 ± 1.3</td>
<td>12.8 ± 0.5</td>
<td>13.4 ± 1.2</td>
</tr>
<tr>
<td>Packed cell volume (%)</td>
<td>40.9 ± 3.0</td>
<td>45.5 ± 2.6</td>
<td>41.9 ± 3.5</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>91.9 ± 5.4</td>
<td>92.3 ± 4.7</td>
<td>92.0 ± 5.2</td>
</tr>
<tr>
<td>Hb (g/l)</td>
<td>135 ± 11</td>
<td>154 ± 9</td>
<td>139 ± 13</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>30.3 ± 2.1</td>
<td>31.3 ± 1.6</td>
<td>30.5 ± 2.1</td>
</tr>
</tbody>
</table>

Hcy, homocysteine; MMA, methylmalonic acid; MCV, mean corpuscular erythrocyte volume; MCH, mean corpuscular Hb.

* Cut-off for men.
† Cut-off for women.
Table 2. Levels of the studied markers according to diet option and vitamin B12 supplementation (Mean values and standard deviations)

<table>
<thead>
<tr>
<th>Diet option</th>
<th>Vitamin B12 users (n = 38)</th>
<th>Vitamin B12 non-users (n = 12)</th>
<th>All (n = 50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (μmol/l)</td>
<td>313.3</td>
<td>138.6</td>
<td>256.0</td>
</tr>
<tr>
<td>SD</td>
<td>92.5</td>
<td>58.2</td>
<td>69.1</td>
</tr>
<tr>
<td>Vitamin B12 (pmol/l)</td>
<td>160.0</td>
<td>110.0</td>
<td>140.0</td>
</tr>
<tr>
<td>SD</td>
<td>35.2</td>
<td>22.0</td>
<td>32.1</td>
</tr>
<tr>
<td>Hcy (μmol/l)</td>
<td>0.106</td>
<td>0.53</td>
<td>0.33</td>
</tr>
<tr>
<td>SD</td>
<td>0.106</td>
<td>0.05</td>
<td>0.03</td>
</tr>
<tr>
<td>MMA (μmol/l)</td>
<td>7.3</td>
<td>5.6</td>
<td>6.6</td>
</tr>
<tr>
<td>SD</td>
<td>1.8</td>
<td>0.8</td>
<td>1.2</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>88.0</td>
<td>70.5</td>
<td>81.0</td>
</tr>
<tr>
<td>SD</td>
<td>4.5</td>
<td>3.1</td>
<td>4.3</td>
</tr>
<tr>
<td>Hb (g/l)</td>
<td>14.0</td>
<td>13.0</td>
<td>13.5</td>
</tr>
<tr>
<td>SD</td>
<td>0.9</td>
<td>0.6</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* Differences were analysed through general linear models. The diet option x vitamin B12 supplementation interaction was only significant for Hb (P = 0.034).

Erythrocyte folate was always well above the cut-off value, which is reasonable considering that vegetarian diets are rich in folate(15). Interestingly, erythrocyte folate of more than 50% of the volunteers surpassed the suggested cut-off for high erythrocyte folate (1360 nmol/l)(36). This is in agreement with previous research that found higher folic acid intakes in lacto-ovo vegetarians and vegans than in omnivores(33,37). Furthermore, the studied subjects consumed fortified products such as vegetable milks that might also contribute to the high folate status. The high levels of folic acid observed discard folate deficiency as the cause of HHcy(14). Although the adverse effects of folic acid excess are unknown, an excessive intake of folate might mask cobalamin deficiency symptoms, preventing the increase of Hcy in vegetarian diets and the appearance of haematological symptoms, which may occur in the studied participants(15,24,38).

These results are consistent with previous literature considering that the recommended intake of vitamin B12 is different to achieve by vegetarians if supplements are not consumed(5,43), partly because the intake of fortified food items is not enough to provide the required doses of the vitamin(34).

In the present study it was clearly shown that vitamin B12 supplement users exhibited higher serum vitamin B12 and erythrocyte folate, as well as lower MMA and Hcy than non-users; thus the obtained results remark the importance of supplementation both for vegans and lacto-ovo vegetarians. These results are consistent with previous literature considering that the recommended intake of vitamin B12 is difficult to achieve by vegetarians if supplements are not consumed(5,43), partly because the intake of fortified food items is not enough to provide the required doses of the vitamin(34).

Considering all the analysed markers, the studied Spanish vegetarians presented sufficient folate and vitamin B12 status due to their general supplementation habit. These results
contrast with the findings of recent studies on vegetarian
Indians, where a prevalence of vitamin B₁₂ deficiency of 70
% has been estimated, and more than 50 % of the subjects
presented deficient levels of serum vitamin B₁₂ and
HHcy[44,45]. This may be explained by the differences in vege-
tarian diet composition and socio-economic status between
these two countries[46]. However, comparing our results with
those obtained in other European countries, such as
Germany, the Netherlands and UK, the differences remain[15,33,43]. We consider that the sufficient vitamin B₁₂ sta-
tus of our subjects might be mainly caused by the extended use
of cobalamin supplements in the Spanish participants, while the
proportion of vitamin B₁₂ consumers found in previous
research was smaller. Nevertheless, even the non-users of vita-
mín B₁₂ supplements presented a better status than the
lacto-ovo vegetarian and vegan participants of the above-
mentioned reports. The better cobalamin status detected in
our study might be explained by a higher consumption of vita-
min B₁₂-rich and vitamin B₁₂-fortified food products, and also
the age of the volunteers, as vitamin B₁₂ status is usually lower
in old individuals[1] (Fig. 1).

In this regard, the association of vitamin B₁₂-rich food with
cobalamin status was studied. The results suggest an improve-
ment of serum vitamin B₁₂ levels and a tendency to improve
Hcy and MMA, associated with the consumption of yoghurts
(including yoghurts of animal and vegetable origin, soya
yoghurts). Dairy products constitute a source of vitamin B₁₂
intake, but they have low concentrations of this vitamin and
their positive effects on cobalamin status are due to their ele-
vated consumption[20]. In the studied population, however, it
can be appreciated that most of the subjects (eighty-seven
volunteers) never consumed animal milk, which could explain
the absence of an effect on vitamin B₁₂ markers. It is remark-
able that thirty-three of these subjects were lacto-ovo vegetar-
ians. Interestingly, it was also observed that twenty-eight of the
hyperhomocysteinaemic individuals (thirty-three subjects, thir-
teen vegetarians) never consumed animal milk, suggesting a
relationship between milk consumption and normal Hcy
levels. This tendency was also observed for MMA levels, as
ten of the eleven deficient individuals belonged to the group
of animal milk non-consumers. Regarding the intake of vege-
table milks, it was observed that the consumption of these
food items was more frequent than that of animal milk.
However, vegetable milks did not have any influence on the
analysed biomarkers in this research, probably because these
milks were not vitamin B₁₂-fortified. Furthermore, an associ-
ation was detected between a higher concentration of eggs
and normal Hcy levels. Although the concentration of cobala-
min in eggs is high, its absorption is limited, presenting a low
bioavailability, which could explain the lack of association with
MMA, a specific marker of vitamin B₁₂ status[20,21]. It was
observed that sixty-three subjects never consumed eggs, repre-
senting most of the population, and only nine of these indivi-
duals were lacto-ovo vegetarians.

The categorical character of the collected dietary data and
the unbalanced proportion of vitamin B₁₂ users and non-users
represent limitations of the study. Further research on the
topic considering quantitative dietary data must be done to
assess the vitamin B₁₂ intake through dietary sources, clarify
the possible differences in methionine consumption between
lacto-ovo vegetarians and vegans, and discover dissimilarities
in diet composition with studies developed in other countries.
Participants in the present study were not representative of the
Spanish vegetarian population. In addition, the influence of
polymorphisms in genes involved in the vitamin B₁₂ and folate
routes was not analysed. However, this research also presents
several remarkable strengths, including a sufficient number of
volunteers recruited, a balanced distribution of lacto-ovo vege-
tarians and vegans, and the use of both markers of circulating
levels and functional status of cobalamin as previous studies
| Table 3. Adequacy of cobalamin markers regarding food frequency consumption  
(Number of answers recorded for each food item or food group) |
<table>
<thead>
<tr>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal milk (n 103)</td>
</tr>
<tr>
<td>Frequency of consumption</td>
</tr>
<tr>
<td>Never</td>
</tr>
<tr>
<td>2–4 times per month</td>
</tr>
<tr>
<td>2–3 times per week</td>
</tr>
<tr>
<td>4–6 times per week</td>
</tr>
<tr>
<td>Once per d</td>
</tr>
<tr>
<td>Two or more times per d</td>
</tr>
</tbody>
</table>

B12, vitamin B12; Hcy, homocysteine; MMA, methylmalonic acid; A, adequate levels; I, inadequate levels (see the Definitions section and Table 1).

* Differences in the frequencies of consumption between subjects with A levels and subjects with I levels of serum vitamin B12, Hcy and MMA (Pearson's χ² tests).
† 'Vegetable milks' is the total of answers for the following food items: soya milk, almond milk, oat milk, coconut milk, rice milk and cashew milk.
‡ 'Yoghurts' is the total of the answers for the following food items: natural or flavoured yoghurts, non-fat yoghurts and other yoghurts.

Conclusions

The study was supported by a research project financed by Laboratorios Zandín (Vegetgunn study). A. G.-N. wrote the original draft; B. Z. and A. G.-N., critical analysis; A. G.-N., writing of the original draft; L. B. and M. P. V., critical analysis; M. P. V., interpretation of results; A. G.-N., K. E. and M. P. V., collection and laboratory analyses; A. G.-N. and L. B., statistical analysis; A. G.-N. and M. P. V., interpretation of results; A. G.-N., writing of the original draft; L. B. and M. P. V., critical analysis; A. G.-N., M. P. V., and A. L. R., writing of the manuscript. The authors declare that there are no conflicts of interest.

Acknowledgments

Ana Salvador is acknowledged for critical review of the manuscript. The study was supported by the European Social Fund (ESF). The funders had no role in the design, analysis or writing of the article.

References

3. Pawlak R, Lester SE, Babatunde T (2014) The prevalence of vegetarian dietary habits in non-users of vitamin B12 supplements was higher, emphasising the need of vitamin B12 supplementation in vegetarian diets.


