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Randomized Control Trials

Effect of two different sublingual dosages of vitamin B_{12} on cobalamin nutritional status in vegans and vegetarians with a marginal deficiency: A randomized controlled trial

Cristian Del Bo'*, Patrizia Riso, Claudio Gardana, Antonella Brusamolino, Alberto Battezzati, Salvatore Ciappellano

Department of Food, Environmental and Nutritional Sciences, Division of Human Nutrition, Università degli Studi di Milano, Milan, Italy

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Background & aims: Vegetarians and vegans are more vulnerable to vitamin B_{12} deficiency with severe risks of megaloblastic anemia, cognitive decline, neuropathy, and depression. An easy and simple method of supplementation consists of taking one weekly dosage of 2000 µg. However, single large oral doses of vitamin B_{12} are poorly absorbed. The present research evaluates the ability of two different sublingual dosages of vitamin B_{12} (350 µg/week vs. 2000 µg/week) in improving cyanocobalamin (vitamin B_{12}) nutritional status in vegans and vegetarians with a marginal deficiency.

Methods: A 12-week randomized, double-blind, controlled, parallel intervention trial was performed. Forty subjects with marginal vitamin B_{12} deficiency were enrolled and randomly divided into two groups: test group *Ld* (low dose, 350 µg/week) and control group *Hd* (high dose, 2000 µg/week) vitamin B_{12} supplementation. Blood samples were collected at baseline and after 15, 30, 60, and 90 days from the intervention for the determination of vitamin B_{12}, related metabolic markers, and blood cell counts.

Results: Two-way analysis of variance showed a significant effect of *time* ($P < 0.0001$) and of *time × treatment interaction* ($P = 0.012$) on serum concentration of vitamin B_{12} that increased after 90-day supplementation (*Ld* and *Hd*) compared to baseline. Both the supplements increased ($P < 0.0001$, *time* effect) the levels of holotranscobalamin, succinic acid, methionine and wellness parameter, while decreased ($P < 0.0001$, *time* effect) the levels of methylmalonic acid, homocysteine and folate compared to baseline. No difference was observed between groups (*Ld* vs. *Hd*). No effect was detected for vitamin B_6 and blood cell count.

Conclusions: In our experimental conditions, both supplements were able to restore adequate serum concentrations of vitamin B_{12} and to improve the levels of related metabolic markers in subjects with a marginal deficiency. The results support the use of a sublingual dosage of 50 µg/day (350 µg/week) of cobalamin, instead of 2000 µg/week (provided as a single dose), to reach a state of nutritional adequacy of vitamin B_{12} in this target population.

This study was registered at www.isrctn.org as ISRCTN75099618.

1. Introduction

Vitamin B_{12} (cyanocobalamin) represents an important and essential water-soluble nutrient involved in the formation of erythrocytes, in the maintenance of the central nervous system, and in cognitive performance [1]. Cyanocobalamin is present in large amounts in animal products such as meat, organ meats, shellfish, eggs, milk, and other dairy foods. Plant foods do not contain vitamin B_{12} unless they are fortified (e.g., some breakfast cereals); however, the body absorbs animal sources of vitamin B_{12} much better than plant sources [1,2]. The physiological absorption of vitamin B_{12} is mediated by the glycoprotein intrinsic factor (IF). For its absorption, the formation of the IF-B_{12} complex and the transport of vitamin B_{12} across the ileum is required [1,2]. Once

* Corresponding author. Department of Food, Environmental and Nutritional Sciences, Division of Human Nutrition, Università degli Studi di Milano, Via G. Celoria 2, 20133, Milano, Italy. Fax: +390250316721.
E-mail address: cristian.delbo@unimi.it (C. Del Bo').

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absorbed, vitamin B_{12} is mainly accumulated in the liver and stored for years before using [1,2].

The recommendations for B_{12} intakes vary significantly from country to country and individual to individual [3]. Normally, in healthy individuals with an ordinary omnivorous diet, a daily consumption of a few micrograms of vitamin B_{12} is enough to preserve adequate levels of the vitamin [3,4]. In Italy, the National Reference of Energy and Nutrient Intake Levels (LARN) identified an average requirement of 2.4 μg a day for adults and up to 2.6 μg and 2.8 μg in pregnancy and lactation, respectively [4]. A deficiency of vitamin B_{12} could be the result of gastrointestinal disorders, celiac disease, Crohn's disease, and genetic polymorphisms leading to malabsorption of the nutrient [1,2]. However, this condition is less frequent; elderly and vegetarians are more susceptible to the condition of vitamin B_{12} deficiency due to their limited intake of meat products [5,6]. On the contrary, vegans that exclude animal products from their diet frequently become deficient in vitamin B_{12}. In this regard, a recent systematic review evaluated the prevalence of vitamin B_{12} deficiency in individuals adhering to vegetarian and vegan diets [7]. The authors documented that adherence to a vegan diet was associated with an increased risk of vitamin B_{12} deficiency compared to a vegetarian diet [7]. These findings were in line with the observations reported by other authors [8–11].

Vitamin B_{12} deficiency has been associated with several metabolic disorders such as macrocytic anemia, hyperhomocysteinemia, cardiovascular, cerebrovascular, and neurological disorders [6,12–14]. However, despite the high risk of developing vitamin B_{12} deficiency and related complications, numerous vegans consider supplementation unnecessary. The deficiency appears after a long period of depletion (can take years in some), due to the stocks of vitamin present in the liver [15]. Individuals with serum levels of B_{12} < 150 pmol/L are considered deficient [15,16], while subjects who have values between 150 and 221 pmol/L are considered marginally deficient [17,18]. In this specific situation, the integration of vitamin B_{12} by the parenteral route is required. However, this approach is poorly accepted because the results painful and expensive [19] as well as substituted by oral formulations. However, this is not effective in subjects suffering from vomiting or diarrhea or are not able to tolerate oral therapies [20]. Moreover, when high doses of vitamin B_{12} are given orally, only a small percentage seems to be absorbed. Recently, the administration of vitamin B_{12} in sublingual form has been developed [20]. Although sublingual vitamin B_{12} is often promoted for better absorption, inconsistent results have been obtained as to the effects of administration of low and high doses of vitamin B_{12}.

The aim of the present study was to evaluate the ability of two different doses (350 μg/week vs. 2000 μg/week) of sublingual supplements in improving the nutritional status of cyanocobalamin in a group of vegans and vegetarians with a marginal deficiency. The low dose (*Ld*) consisted of 7 sublingual tablets each providing 50 μg/day (350 μg/week) of vitamin B_{12}, while the high dose (*Hd*) consisted of 1 sublingual tablet (2000 μg) for the entire week. The latter represents the most common method of supplementation, even if it is administered by the oral or parenteral route. In this regard, several studies have shown low absorption following the intake of high doses [1,21]. In addition, this practice could be less tolerated in some subjects; for example, some authors found adverse effects (e.g., hyperhidrosis and blurred vision) following supplementation with 1 mg/day of vitamin B_{12} in individuals with mild and moderate Alzheimer disease [22]. Our hypothesis is that the sublingual administrations of low (350 μg/week) and high (2000 μg/week) doses of cyanocobalamin are both able to restore the nutritional adequacy of vitamin B_{12} within 90 days [23–25] in vegans and vegetarians affected by a marginal deficiency.

2. Materials and methods

2.1. Subject recruitment

The screening of the participants was performed between March 2015 and July 2016 through advertisements on bulletin boards, telephone, or e-mail. Subjects were visited for a routine medical examination by a physician to assess their eligibility to participate in the trial. The eligibility was assessed by a physician through an accurate examination and by means of a health/medical questionnaire to exclude subjects with diseases such as diabetes, renal insufficiency, allergies, chronic constipation, diarrhea, or any other gastrointestinal disorder. Moreover, a small aliquot of blood was collected to ascertain vitamin B_{12} nutritional status. Subjects were selected according to the following inclusion criteria: vegan and vegetarian subjects in a condition of marginal vitamin B_{12} deficiency (<220 pmol/L) or full-blown (<150 pmol/L), non-smokers or light smokers (maximum 5–6 cigarettes/day), and moderate alcohol consumption (up to 14 glasses of wine/beer per week). Subjects with cardiovascular, coronary, diabetes, hepatic, renal, or gastrointestinal diseases were excluded. Subjects were not included if using drugs, medications, and/or supplements at least one month before the beginning of the experiment. Moreover, subjects were excluded if taking vitamin B_{12} supplements at least one year before the experiment. The study was performed in accordance with the ethical standards established in the 2013 Declaration of Helsinki and approved by the Ethics Committee of the University of Milan (March 4, 2015, ref. 11/15). The study was registered at www.isrctn.org as ISRCTN75099618. All participants signed an informed consent form.

2.2. Experimental design

A researcher who was not involved in the study and in sample analysis was appointed to allocate patients to the different treatments according to a randomization list obtained through the center's database. The number of participants who were randomly assigned to different study groups, the rate of patients completing the study, and patients analyzed for the primary outcome are depicted in Fig. 1. Forty subjects were enrolled and randomly divided into two groups of 20 subjects each for a 12-week double-blind (participants and outcome assessors), randomized, controlled, parallel dietary intervention study. The study was performed between May 2015 and October 2016. One group received the supplement at a low dose (*Ld*; equivalent to 50 μg/day, 350 μg/week), while the other group (control) received the supplement at a high dose (*Hd*; equivalent to 2000 μg/week in a single dose). Vitamin B_{12} was provided to the volunteers in one stock at the beginning of the study. Each subject received 13 boxes containing the doses for a week in a blind condition. All tablets were packaged and numbered (from 1 to 7) in single-dose blisters. Subjects were instructed to follow the sequence of numbers and to swallow one tablet per day in the morning before breakfast. The *Ld* group ingested 7 sublingual tablets/week of cyanocobalamin (50 μg each, equivalent to 350 μg), while the *Hd* group took only 1 sublingual tablet of vitamin B_{12} (2000 μg) and 6 sublingual tablets of placebo. For both groups (*Ld* and *Hd*), the tablets of vitamin B_{12} consisted of mannitol, maize starch, vegetable stearate magnesium, beet juice, and sucralose. The placebo tablets matched the shape, size, color, flavor, and the composition of the vitamin B_{12} supplements. The sublingual vitamin B_{12} tablets were obtained from bacteria with a manufacturing process compatible with the strictly vegan dietary requirements. The crystalline form of cyanocobalamin was used for the preparation of the tablets.

Subjects were instructed to maintain their dietary and lifestyle habits as declared before enrollment. Moreover, they were

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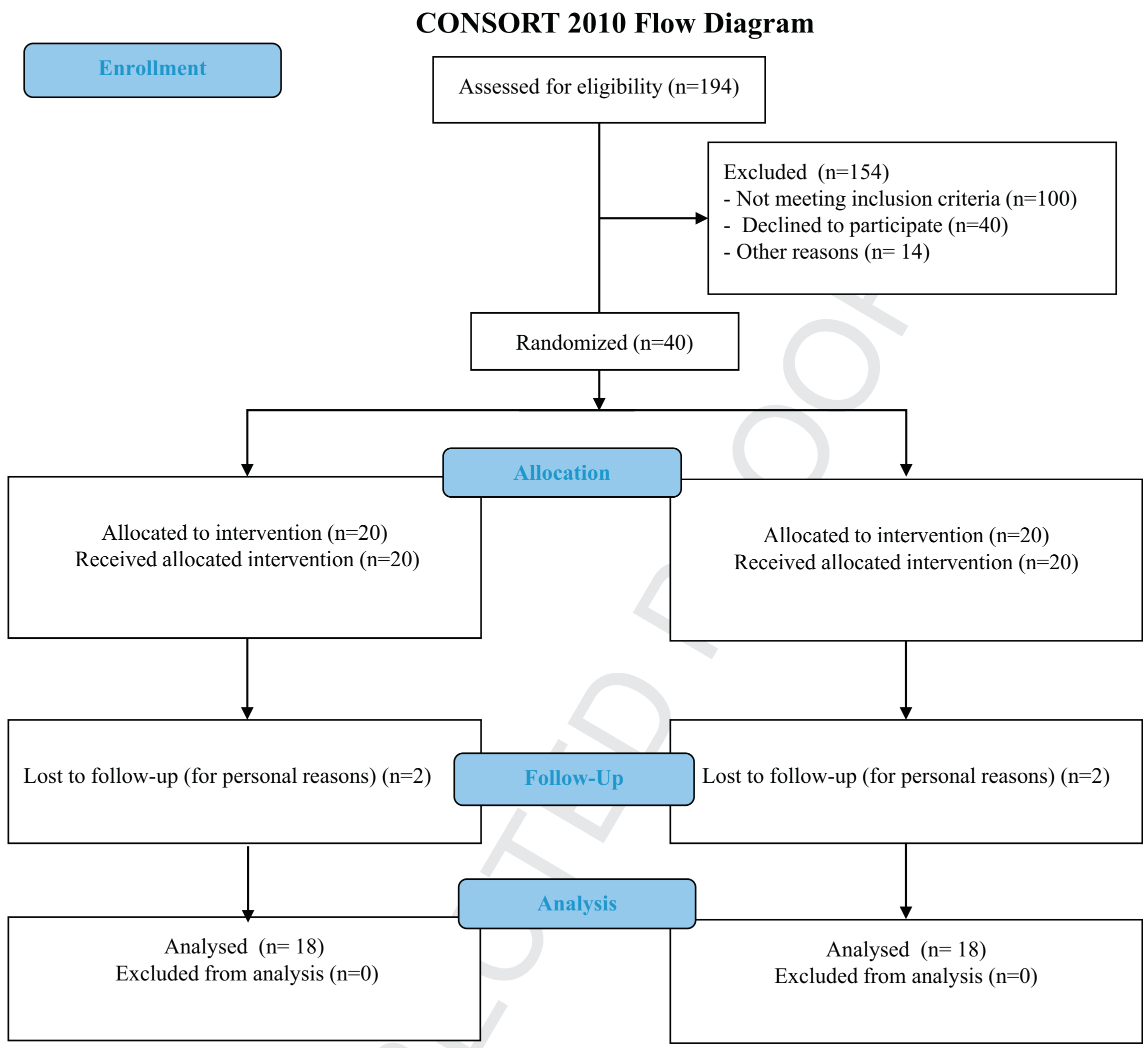

Fig. 1. Study flow-chart showing the process of patient selection and enrollment, allocation to the two study groups, and rate of patients completing the study. Ld: group treated with low dosage of vitamin B_{12} (350 μg/week); Hd: group treated with high dosage of vitamin B_{12} (2000 μg/week).

encouraged to abstain from consuming sources of vitamin B_{12} (e.g., spirulin, yeast, fortified foods). A 24-h record of food consumption was kept by each volunteer the day before blood collection to check compliance with the dietary instructions. Every 2 weeks, subjects returned the empty blisters (as evidence of the consumption of the tablets) and received the new supplements. A 3-day food record and a weekly direct interview were also scheduled randomly during the experimental period to check compliance with the dietary instructions and to assure the consumption of the tablets. The day of the experiment, after an overnight fast, subjects reported to the laboratories of the University of Milan. Blood samples were collected at baseline (time 0) and after 15, 30, 60, and 90 days of intervention.

2.3. Study variables

The improvement of serum levels of vitamin B_{12} was considered the primary endpoint. The other variables under study were as follows: holotranscobalamin, methylmalonic acid, succinic acid, methionine, homocysteine, vitamin B_6, folic acid, and complete blood count. Since the amount of cobalt provided through the supplement was negligible with respect to the circulating blood levels, this variable was not evaluated.

2.4. Sampling and analysis of biochemical parameters

Blood was collected in the morning by a phlebotomist. Samples were drawn into evacuated tubes with or without K_2EDTA. Serum was separated within 1 h, while plasma was separated within 30 min (min) after collection by centrifugation (15 min at 2300 × g at 4 °C). Plasma and serum were aliquoted and stored at −80 °C until analysis. All the samples were analyzed blind. Blood cell count was evaluated by routine laboratories assessment.

Vitamins B_{12} levels were measured by a competitive test principle using IF specific for this vitamin. Vitamin B_{12} was analyzed by electrochemiluminescence immunoassay (ECLIA) using Cobas immunoassay analyzers (Roche Diagnostics, North America). Also, the assessment of serum folate was performed with electrochemiluminescence immunoassay (ECLIA) using Cobas immunoassay analyzers (Roche Diagnostics, North America).

Holotranscobalamin concentration were determined in serum by immunoenzymatic assay kit (BIOHIT HealthCare, Helsinki, Finland). Briefly, the microtiter plate wells were coated with a highly specific monoclonal antibody for BIOHIT Active B_{12} (holoTC). During the first incubation, holoTC specifically bound to the surface coated with the antibody. Successively, the conjugate was added for the binding of holoTC; the wells were then washed to remove

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unbound components and holoTC was detected following the incubation with the substrate. Before the analysis, a stop solution was added and the absorbance was read at 405 nm (mod. F200 Infinite, TECAN Milan, Italy).

Serum vitamin B_6 concentrations were evaluated by high performance liquid chromatography method using the relevant commercial kit (Chromsystems Instruments & Chemicals, Munich, Germany) [26]. Homocysteine (HCy), methionine (Met), methylmalonic acid (MMA), succinic acid (SA), tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl), methanol, and formic acid were obtained from Sigma–Aldrich (St. Louis, MO, USA). Water was obtained from the Milli-Q apparatus (Millipore, Milford, MA). The determination of HCy, Met, MMA, and SA was performed according to Fu et al. [27], with slight modifications. Briefly, 200 μL of heparinized plasma was added to 100 μL of water and 100 μL of TCEP-HCl (0.1 M). The mixture was vortexed for 10 s (s), incubated for 15 min at room temperature, and transferred to an Amicon 10 K Da filter. The filter was centrifuged at 9000 g for 30 min, the filtrate was transferred to a microvial, and 5 μL injected into the Ultra Performance Liquid Chromatography (UPLC)-high resolution (HR)-mass spectrometers (MS). The analysis was carried out on an UHPLC model Acquity (Waters) coupled with a High-Resolution Fourier Transform mass spectrometer (Orbitrap) model Exactive (Thermo Scientific) equipped with an HESI-II probe for electrospray ionization and a collision cell (HCD). The column was a 1.8 μm HSS T3 C_{18} (150 × 2.1 mm, Waters), flow rate was 0.45 mL/min, and the eluents were 0.1% formic acid in water (A) and acetonitrile (B). The column and sample were kept at 60 °C and 15 °C, respectively. The UHPLC separation was performed by the following linear elution gradient: 100% of A for 5 min, 0–100% B in 1 s, 100% B for 2 min, from 100% to 0% B in 1 min, and then isocratic for 2 min.

For HCy and Met (0–3.2 min), the operative conditions were spray voltage +3.0 kV, sheath gas flow rate 55, auxiliary gas flow rate 20, capillary temperature 320 °C, capillary +47.5 V, tube lens +110 V, skimmer +20 V, and heather temperature 120 °C. The acquisition was performed in full-scan mode in the range $(m/z)^+$ 60–180 u.

For MMA and SA (3.2–5 min) the operative conditions were spray voltage −3.0 kV, sheath gas flow rate 55, auxiliary gas flow rate 20, capillary temperature 320 °C, capillary −35 V, tube lens −70 V, skimmer −16 V, and heather temperature 120 °C. The acquisition was performed in full-scan mode in the range $(m/z)^-$ 60–130 u and the ions with *m/z* 91.0038, corresponding to the formic acid dimer $[2M\text{-}H]^-$ that was used as the lock mass. The isolation window, automatic gain control target, injection time, mass resolution, energy, and gas in the collision cell were ±2 ppm, 1×10^6, 100 ms, 50 K, 20 V, and N_2, respectively. The MS data were processed using Xcalibur software (Thermo Scientific). The peak identity was ascertained, evaluating the accurate mass and the fragments obtained in the collision cell. Calibration curves were in the range 0.15–14.8, 0.13–33.5, 0.17–42.5, and 0.25–44 μMolar for HCy, Met, MMA, and SA, respectively. Finally, the wellness parameter was calculated according to the Fedosov formula [28]: "wellness parameter": $w = \log_{10}(holoTC_n) + \log_{10}(B_{12n}) - \log_{10}(MMA_n) - \log_{10}(HCy_n)$, where concentrations are normalized (e.g., $MMA_n = MMA/MMA_n$ normal).

2.5. Statistical analysis

Sample size was estimated, based on previous studies, in order to detect significant differences in the serum vitamin B_{12} levels [23–25]. Sixteen subjects per group were considered sufficient to demonstrate at least a 70% improvement in the levels of vitamin B_{12} after supplementation with a p value of 0.05 and a power of 80%. The calculation was based on the assumptions that the mean ± standard deviation (SD) baseline vitamin B_{12} concentration was 140 ± 40 μmol/L and that the treatment would increase the levels of cyanocobalamin up to 240 μmol/L. This value represents the mean found in an Italian blood donor population [4].

All analyses were performed using STATISTICA software (StatSoft Inc., Tulsa, OK, USA). Results are expressed as mean ± SD or standard error of the mean (SEM). Data were tested for normality of distribution by the Shapiro–Wilk test. Variables normally distributed were analyzed by two-way analysis of variance (ANOVA) considering the treatment (350 μg/week vs. 2000 μg/week) and the time (0, 15, 30, 60, and 90 days) as dependent variables. Data that were not normally distributed were logarithmically transformed. Log-transformed data were subjected to analysis by the non-parametric Friedman test. Differences were considered significant for $p < 0.05$; the least significant difference test was applied, as well as post hoc analysis, to show differences between treatments. The level of statistical significance was fixed at $p < 0.05$.

3. Results

3.1. Baseline characteristic of the study population

Baseline characteristics of the subjects enrolled in each group are reported in Table 1. Four subjects (2 for each group) were lost during the follow-up period due to personal reasons. All subjects (n = 36) showed a marginal deficiency of vitamin B_{12} (<220 pmol/L) [3]. Regarding the other biomarkers of cobalamin status: 27 out of 36 subjects had serum levels of MMA above 750 nmol/L (cut-off above which cobalamin deficiency is diagnosed), while14 out of 36 subjects documented moderate hyperhomocysteinemia (range 17.6–33.8 μmol/L) with plasma total homocysteine (HCy-pt) value ≥ 15 μmol/L [3]. Moreover, six subjects had folate levels (range 7–9 nmol/L) below 10 nmol/L, suggesting a folate deficiency [29]. Two subjects showed low vitamin B_6 levels (<21.3 nmol/L) and one also had low holotranscobalamin levels (<21 pmol/L) [3]. No abnormalities in blood cell count were observed. The age, sex, hemoglobin level, platelet and white blood cell counts, mean corpuscular volume, and serum cobalamin levels were not significantly different between groups (Table 1).

3.2. Compliance

Subjects were highly motivated to participate in the intervention and confirmed the consumption of the tablets. The compliance was verified during a weekly direct interview, as previously reported, and confirmed by returning the empty blisters (100%

Table 1
Subjects characteristics at the beginning of the study.[a]

	Ld group	*Hd* group	P value[b]
Number of volunteers	18	18	–
Male/Female	9/9	9/9	–
Age (years)	43 ± 12	42 ± 13	0.98
Weight (kg)	63.9 ± 11.5	68.7 ± 17.9	0.36
Body mass index (kg/m^2)	21.6 ± 2.6	23.2 ± 5.4	0.29
Total Vitamin B_{12} (pmol/L)	146 ± 36	131 ± 56	0.29
Erythrocytes (10ˆ6/μL)	4.6 ± 0.4	4.4 ± 0.3	0.06
Mean corpuscular volume (*f*L)	88.5 ± 3.6	89.5 ± 4.6	0.32
White blood cells (10ˆ3/μL)	5.3 ± 1.8	4.7 ± 0.8	0.17
Hemoglobin (g/dL)	13.7 ± 1.1	13.1 ± 1.0	0.07
Hematocrit (%)	40.9 ± 3.4	39.1 ± 2.7	0.06
Platelets (10ˆ3/μL)	219.4 ± 41.0	249.5 ± 56.7	0.09

[a] Data are expressed as mean ± SD. Subjects were randomly assigned to 1 of the 2 groups (Ld *vs* Hd) and supplemented for 90 days. Ld = low dosage; Hd = high dosage.
[b] P value derived by one way ANOVA.

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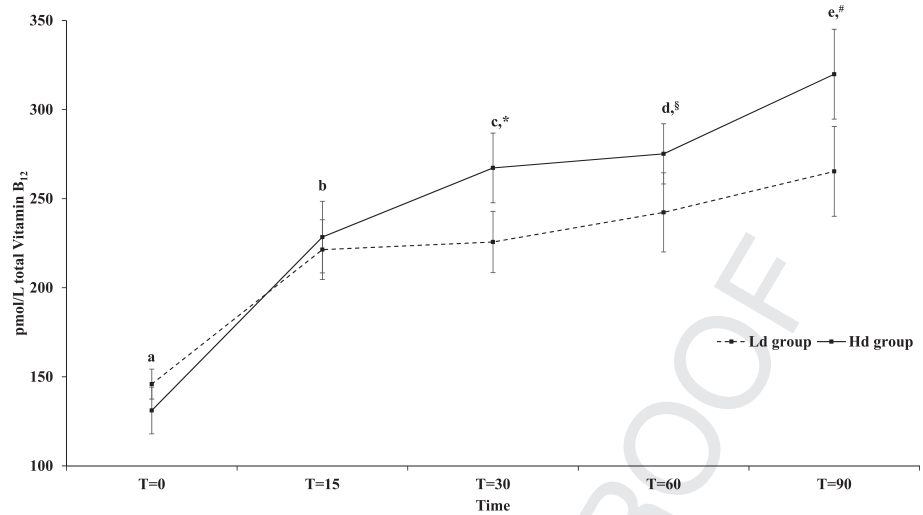

Fig. 2. Effect of supplementation on serum circulating levels of total vitamin B_{12} in the two intervention groups (Ld *vs* Hd). The concentrations were measured at baseline (T0) and after 15, 30, 60 and 90 days. N=18 for each group. Data are expressed as mean ± SEM. [a,b,c,d,e]Data with different letters are significantly different within the same treatment (time effect; P < 0.05). [*,§,#]Data with different symbols are significantly different between treatment (treatment effect; P < 0.05).

compliance). Not one participant declared adverse effects following the supplementation.

3.3. *Effect of supplementation on serum levels of total, active, and inactive form of vitamin B_{12}*

The serum levels of total vitamin B_{12}, measured at baseline (time 0 day) and after 15, 30, 60, and 90 days from the start of supplementation, are reported in Fig. 1. Subjects increased the serum concentrations of total vitamin B_{12} to above 240 pmol/L according to our hypothesis. On the whole, repeated measures of ANOVA did not show a significant effect of *treatment,* but revealed a significant effect of *time* ($P = 0.008$) and of *time* × *treatment* interaction ($P = 0.012$) for circulating levels of total vitamin B_{12} that increased following the treatments. In particular, post-hoc analysis showed a significant enhancement after 15 days from the start of the intake of the supplements (+51.7% in *Ld* group vs. +74.2% in *Hd* group; $P < 0.0001$). The values increased over time and appeared significantly different between groups after 30 days until the end of the experimental period ($P < 0.01$). Fig. 2A and B shows the levels of active (holotranscobalamin, HoloTC) (2A) and inactive forms (2B) of vitamin B_{12} measured at baseline and after 15 and 90 days from the start of supplementation. The analysis at 15 and 90 days was performed based on the prominent absorption observed in vitamin B_{12}. On the whole, ANOVA did not show a significant effect of *treatment* and of *time* × *treatment interaction*, but revealed an effect of *time* ($P < 0.0001$) for serum circulating levels of active and inactive vitamin B_{12} that increased during the treatments.

3.4. *Effect of supplementation on serum levels of methylmalonic acid and homocysteine*

The serum levels of MMA and HCy were measured at baseline (time 0 day) and after 15, 30, 60, and 90 days from the start of

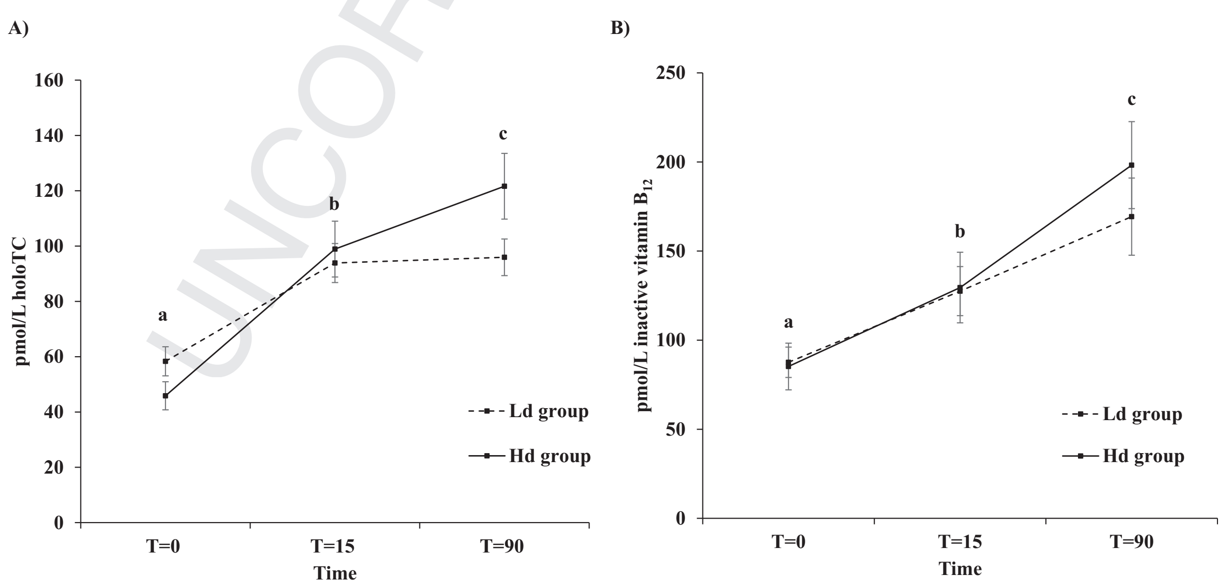

Fig. 3. Effect of supplementation on serum circulating levels of active (A) and inactive (B) form of vitamin B_{12} in the two intervention groups (Ld*vs*Hd). The concentrations were measured at baseline (T0) and after 15 and 90 days from the supplementation. Data are expressed as mean ± SEM. N=18 for each group.

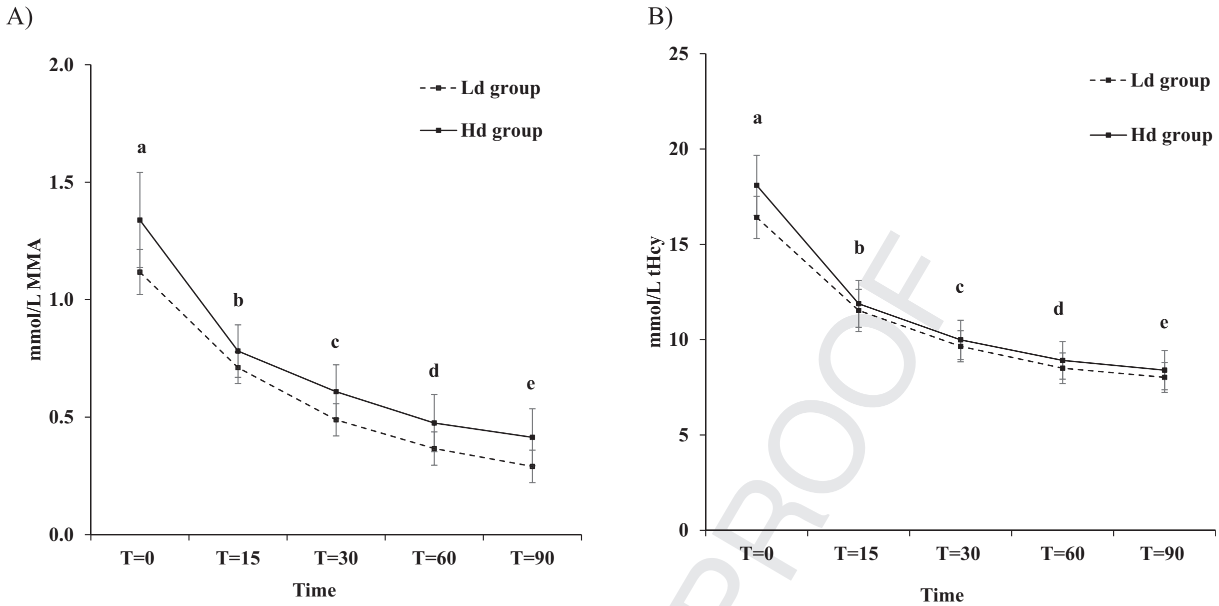

Fig. 4. Effect of supplementation on serum circulating levels of MMA (A) and tHcy (B) in the two intervention groups (Ld *vs* Hd). The concentrations were measured at baseline (T0) and after 15 and 90 days from the supplementation. N=18 for each group. Data are expressed as mean ± SEM. MMA, methylmalonic acid; tHcy, total homocysteine.

supplementation, are reported in Fig. 3A and B. ANOVA revealed only a significant effect of *time* ($P < 0.0001$) for serum circulating levels of MMA and HCy that decreased over time following both treatments (see Fig. 4).

3.5. *Effect of supplementation on serum concentrations of methionine, succinic acid, vitamin B_6 and folate, blood cell count, and wellness parameter*

The serum levels of Met, SA, vitamin B_6, and folate, measured at baseline (time 0 day) and after 15, 30, 60, and 90 days from the start of supplementation, are reported in Table 2. ANOVA revealed only a significant effect of *time* for serum circulating levels of folate ($P < 0.0001$), Met ($P < 0.0001$) and SA ($P < 0.0001$). In particular, folate showed a significant decrease over time, while Met and SA has significant increases.

In Table 2 are reported the values of the wellness parameter measured at baseline (time 0 day) and after 15 and 90 days from the start of supplementation are reported in Table 2. Since the index derives from a formula that also takes into consideration the levels of holoTC, this parameter was measured only at times for which the levels of holoTC were detected. On the whole, repeated measures ANOVA did not show a significant effect of *treatment,* but revealed a significant effect of *time* ($P < 0.0001$) and *time* × *treatment* interaction ($P = 0.046$). In particular, post-hoc analysis documented a significant improvement over time following the intake of both the supplements, with a difference between groups only at specific and independent time points.

No effect was documented for serum circulating levels of vitamin B_6 and blood cell count (data not shown).

4. Discussion

In the present study, we documented that as a little as 350 µg per week of vitamin B_{12} supplementation was enough to correct a marginal deficiency of cobalamin and to improve holoTC, MMA, and HCy (biomarkers of cobalamin status) in a group of vegans and vegetarians. The results obtained support the use of a sublingual supplement at low doses as an effective and non-invasive method to improve the cobalamin status in this target population.

It has been reported that the absorption of vitamin B_{12} from supplements does not depend only on the dose and frequency of the intake but also on the health status of the subjects. In particular, it is widely recognized that subjects suffering from gastric or small

Table 2
Effect of Vitamin B_{12} supplementation (low dosage versus high dosage) on serum levels of vitamin B_6, folates, methionine, succinic acid and Wellness parameter ($n = 18$)[1].

Variables	Treatments	T = 0	T = 15	T = 30	T = 60	T = 90	*P treatment*	*P time*	*P interaction*
Vitamin B_6 nmol/	*Ld*	55.2 ± 19.2	67.4 ± 37.8	72.3 ± 42.3	69.6 ± 41.2	73.3 ± 42.1	0.76	0.07	0.65
	Hd	61.6 ± 53.4	66.7 ± 38.2	62.6 ± 36.1	70.3 ± 45.4	60.2 ± 47.4			
Folates nmol/L	*Ld*	22.5 ± 8.8	20.8 ± 9.5	19.3 ± 10.0	18.3 ± 8.3	17.4 ± 10.1	0.23	<0.0001	0.43
	Hd	19.6 ± 9.2	17.2 ± 6.7	18.5 ± 8.0	17.5 ± 7.9	16.1 ± 7.5			
Methionine µmol/L	*Ld*	17.7 ± 6.5	18.3 ± 6.9	18.0 ± 6.5	17.2 ± 5.2	17.8 ± 5.6	0.31	<0.0001	0.55
	Hd	13.6 ± 3.9	15.6 ± 4.9	15.6 ± 4.8	15.5 ± 4.7	15.8 ± 4.6			
Succinic acid µmol/L	*Ld*	5.8 ± 3.6	6.2 ± 3.8	6.4 ± 3.9	6.7 ± 3.9	6.3 ± 3.6	0.43	<0.0001	0.11
	Hd	4.3 ± 3.5	5.3 ± 3.4	5.6 ± 3.6	5.7 ± 3.5	5.9 ± 3.4			
Wellness parameter	*Ld*	−1.0 ± 0.4[a]	−0.3 ± 0.6[b]	–	–	0.2 ± 0.6[c]	0.88	<0.0001	0.046
	Hd	−1.3 ± 0.7[a]	−0.2 ± 0.7[b]	–	–	0.3 ± 0.7[c]			

The variables were measured at baseline (time 0) and after 15, 30, 60, 90 days from the supplementation. Ld, low dosage; Hd, high dosage.
P values correspond to the treatment, the time and the interaction between treatment and time in the overall two way ANOVA.
[a,b,c] Data with different letters are significantly different between and within treatments.
[1] Data are expressed as mean ± SD.

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intestine resections, inflammatory bowel disease, and other complications related to intestinal absorption may become deficient [30]. Moreover, the capacity of absorption is strictly dependent on saturable active transport and on the efficiency of the aspecific route. In this regard, different studies have shown that the absorptive capacity of vitamin B_{12} is high when the amount introduced is low. For example, the oral administration of different doses (1 μg, 10 μg, 50 μg, 500 μg, and 1000 μg) of vitamin B_{12} are absorbed with an efficiency of 56%, 16%, 3%, 2%, and 1.3%, respectively [31]. A plethora of studies investigated the effect of a supplementation on the levels of vitamin B_{12} and related cardiovascular markers; however, most of them where performed in the elderly [6], those with hyperhomocysteinemia [32,33], and undernourished children [34,35], while very few are involving vegetarians and/or vegans. A recent 12-week randomized, placebo-controlled trial performed in vegans documented that the use of a vitamin B_{12}-fortified toothpaste (about 100 μg/g depending on the number of brush sessions) improved serum and plasma concentrations of cobalamin and related associated markers [36]. Yajnik et al. [25] found that supplementation of vitamin B_{12} (500 μg/day), over a 6-week period, significantly increased plasma vitamin B_{12} concentration (from 125 to 215 pmol/L) in a group of healthy, lacto-vegetarian women. The improvement was observed within the first 2 weeks of intervention, and the levels maintained stability up to 4 weeks. Sharabi and coworkers documented similar findings following sublingual and oral administration of 500 μg of cobalamin in subjects with a deficiency [37].

In our experimental conditions, supplementation with low and high doses (350 μg/week vs. 2000 μg/week) of cobalamin significantly improved circulating serum levels of vitamin $B_{12,}$ suggesting the efficiency and efficacy of both supplements in restoring the levels of the vitamin (>240 pmol/L) [3]. However, serum levels of vitamin B_{12} above the cut-off point does not necessarily indicate an adequate nutritional status. In fact, there is inconsistency among the scientific community regarding the identification of reference values for cyanocobalamin. Future studies should be performed in order to identify the cut-offs according to individual variability (i.e., age, sex, etc.) and lifestyle habits (i.e., vegans, vegetarians). Holotranscobalamin represents the metabolically active form of vitamin B_{12} that delivers cobalamin to the target cells. Recently, it has been recognized as an early and reliable marker to discriminate an impaired cobalamin status [38]. However, discrepancies remain about mode of application and assignment of these cut-off values to diagnose a deficiency. Based on different populations and criteria, cut-off values from 21 to 45 pmol/L have been proposed as "sub-optimal" [3]. In our study, subjects have shown levels of holoTC within the range of normality. This is in line with the characteristics of our population that included only individuals with a marginal cobalamin deficiency. The supplementation with both dosages significantly increased the levels of holoTC. The improvement was comparable between groups, since only an effect of time, but not of treatments, was observed. The impact of vitamin B_{12} supplementation on levels of holoTC has been evaluated in different studies [39,40]. In a double-blind, placebo-controlled trial, 12 and 24 weeks of supplementation with 1000 μg vitamin B_{12} or 1000 μg vitamin B_{12} + 400 μg folic acid significantly increased the levels of cobalamin as well as those of holoTC in elderly subjects [39]. Brito et al. [40], reported that a single intramuscular injection of 10 mg vitamin B_{12} (providing 100 mg pyridoxine and 100 mg thiamine) significantly increased, after 4 months, serum vitamin B_{12} and holotranscobalamin levels in a group of 27 community-dwelling elderly Chileans.

Other biomarkers of cobalamin status include hematological changes and the metabolites MMA and HCy. These variables can add valuable information in conjunction with serum holoTC and/or cobalamin for assessment of B_{12}status. MMA is considered a biomarker of cobalamin function with regard to its role in the functioning of methylmalonyl-CoA mutase. Serum MMA concentration increases following an insufficient supply of cobalamin. As previously reported values above 750 nmol/L are used to discriminate a cobalamin deficiency [3].

Plasma HCy is not a specific marker of cobalamin status since it is affected also by dietary factors, such as folate, choline and betaine, as well as renal insufficiency, lifestyle factors (e.g. alcohol consumption) and age [41,42]. However, elevated plasma HCy concentration is commonly observed in subjects with a cobalamin deficiency. In our experimental conditions, most of the subjects showed baseline levels of MMA and HCy above the cut-off values, while only few subjects showed low levels of folate. For these reasons, those biomarkers, together with the levels of folate, vitamin B_6, Met and SA, can be considered a valid support for the assessment of the nutritional status of cobalamin in vegans and vegetarians. In fact, we were able to document a statistically significant decrease in the levels of MMA and HCy, and a significant increase in the levels of Met and SA. These results were in line with those obtained by other authors showing a general improvement after cobalamin supplementation [25,35,39,41]. An improvement in cobalamin nutritional status and a reduction of HCy and MMA may be also effective in the prevention of cardiovascular risk and neurological disorders. However, some studies failed to observe a significant modulation in HCy levels. For example, Sharabi and colleagues [37] did not document a decrease in HCy and MMA following 8 weeks of intervention with 500 μg/day of sublingual and oral B_{12} administration in subjects with a cobalamin deficiency.

As previously reported, there is an interrelationship between vitamin B_{12} and folate; in particular, vitamin B_{12} deficiency can lead to lowered levels of methionine synthetase, which results in folate deficiency and an increased proportion of the 5-methyl derivative. In our experimental conditions, we did not quantify the levels of the 5-methyl derivative, but only folate that significantly reduced following cobalamin supplementation. These results are complex to explain; we may hypothesize that the improvement in B_{12} status, also in terms of MMA and HCy, did not require high amounts of folate to compensate for a cobalamin deficiency. However, we cannot exclude that these fluctuations were attributed mainly to physiological changes, since the overall vitamin status was maintained within the range of normality.

A recent and robust biochemical indicator of cyanocobalamin status is the wellness parameter conceived by Fedosov that takes into consideration the levels of total and active B_{12} forms and those of MMA and HCy [28]. The cut-off to discriminate the wellness parameter are as follows: deficiency $w = -1.49$; transition $w = -0.516$; normal $w = -0.0$, and excellent $w = +0.445$. In our experimental conditions, subjects showed a low wellness parameter at baseline (−1.0 for *Ld* group and −1.3 for *Hd* group), documenting a state of marginal deficiency. The supplementation of vitamin B_{12} significantly improved the wellness parameter in both the intervention groups.

Finally, we observed no significant effect on blood cell count both at the beginning of the study (see Table 1) and after the intervention (data not shown). These results are not surprising, since our subjects were in stage 2–3 of vitamin B_{12} deficiency and this condition does not affect the levels of mean corpuscular volume and hemoglobin [42].

5. Study limitations

A possible limitation of the study is the lack of a real control group (vegans/vegetarians with a marginal deficiency who did not take supplements). However, by considering that our subjects were affected by a marginal vitamin B_{12} deficiency, the inclusion of a real placebo group (vegans/vegetarians without supplements) would not have been possible for ethical reasons. A second limitation of the study is the lack of a follow-up period post-supplementation in order to verify the changes in the levels of vitamin B_{12} and related metabolic markers along the time.

6. Conclusions

In conclusion, the results obtained have shown that both supplements were able to bring the levels of vitamin B_{12} from a marginal deficiency to an adequate nutritional status. In particular, we have documented an increase of serum concentrations of vitamin B_{12} and holoTC, and a reduction of MMA and HCy as markers of vitamin B_{12} metabolism. These results are in line with the elevation of the wellness parameter that provides further support for the improvement of the nutritional vitamin B_{12} status.

Our observations emphasize the importance of supplementation in vegetarians and vegans with a marginal deficiency, but it should be emphasized that the use of pharmacological doses is unnecessary in this target group. Moreover, the absence of a consensus on vitamin B_{12} cut-off values and the high individual variability make it difficult to identify the real needs for vegans and vegetarians. Further studies are necessary in order to confirm our findings and verify the effects of sublingual supplementation in vegans and vegetarians with a severe deficiency and in those affected by malabsorption and/or impaired metabolism of vitamin B_{12}.

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Author disclosure

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Conflict of interest

Q3 None.

Uncited reference

Q7 [43].

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