

1 **Effect of two different sublingual dosages of vitamin B₁₂ on cobalamin nutritional status in**
2 **vegans and vegetarians with a marginal deficiency: a randomized controlled trial**

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27 **ABSTRACT**

28 *Background & Aims:* Vegetarians and vegans are more vulnerable to vitamin B₁₂ deficiency with
29 severe risks of megaloblastic anemia, cognitive decline, neuropathy, and depression. An easy and
30 simple method of supplementation consists of taking one weekly dosage of 2000 µg. **However, single**
31 **large oral doses of vitamin B₁₂ are poorly absorbed. The present research evaluates the ability of two**
32 **different sublingual dosages of vitamin B₁₂ (350 µg/week vs. 2000 µg/week) in improving**
33 **cyanocobalamin (vitamin B₁₂) nutritional status in vegans and vegetarians with a marginal deficiency.**

34 *Methods:* A 12-week randomized, double-blind, controlled, parallel intervention trial was performed.
35 Forty subjects with marginal vitamin B₁₂ deficiency were enrolled and randomly divided into two
36 groups: test group *Ld* (low dose, 350 µg/week) and control group *Hd* (high dose, 2000 µg/week)
37 vitamin B₁₂ supplementation. Blood samples were collected at baseline and after 15, 30, 60, and 90
38 days from the intervention for the determination of vitamin B₁₂, related metabolic markers, and blood
39 cell counts.

40 *Results:* **Two-way analysis of variance showed a significant effect of *time* ($P < 0.0001$) and of *time x***
41 ***treatment interaction* ($P = 0.012$) on serum concentration of vitamin B₁₂. In particular, 90 days of**
42 **supplementation increased the levels of cyanocobalamin (+81.8% in the *Ld* group and +144.0% in**
43 **the *Hd* group) compared to baseline. A significant increase was observed for the levels of**
44 **holotranscobalamin (+64.5% in the *Ld* group and +165.2% in the *Hd* group), while a decrease**
45 **occurred for the levels of methylmalonic acid (-72.3% in the *Ld* group and -69.4% in the *Hd* group),**
46 **homocysteine (-56.8% in the *Ld* group and -53.6% in the *Hd* group), and folate (-22.8% in the *Ld***
47 **group and -17.7% in the *Hd* group) compared to baseline (time effect, $P < 0.0001$). No difference**
48 **was observed between groups (*Ld* vs. *Hd*). No effect was detected for the other variables under study.**

49 *Conclusions:* **In our experimental conditions, both supplements were able to restore adequate serum**
50 **concentrations of vitamin B₁₂ and to improve the levels of related metabolic blood markers in subjects**
51 **with a marginal deficiency. The results support the use of a sublingual dosage of 50 µg/day (350**

52 $\mu\text{g}/\text{week}$) of cobalamin, instead of 2000 $\mu\text{g}/\text{week}$ (provided as a single dose), to reach a state of
53 nutritional adequacy of vitamin B₁₂ in this target population.

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

55

56 **Keywords:** vitamin B₁₂, metabolites, sublingual supplements, vegans, vegetarians

57

58 **1.Introduction**

59 Vitamin B₁₂ (cyanocobalamin) represents an important and essential water-soluble nutrient involved
60 in the formation of erythrocytes, in the maintenance of the central nervous system, and in cognitive
61 performance [1]. Cyanocobalamin is present in large amounts in animal products such as meat, organ
62 meats, shellfish, eggs, milk, and other dairy foods. Plant foods do not contain vitamin B₁₂ unless they
63 are fortified (e.g., some breakfast cereals); however, the body absorbs animal sources of vitamin B₁₂
64 much better than plant sources [1,2]. The physiological absorption of vitamin B₁₂ is mediated by the
65 glycoprotein intrinsic factor (IF). For its absorption, the formation of the IF-B₁₂ complex and the
66 transport of vitamin B₁₂ across the ileum is required [1,2]. Once absorbed, vitamin B₁₂ is mainly
67 accumulated in the liver and stored for years before using [1,2].

68 The recommendations for B₁₂ intakes vary significantly from country to country and individual to
69 individual [3]. Normally, in healthy individuals with an ordinary omnivorous diet, a daily
70 consumption of a few micrograms of vitamin B₁₂ is enough to preserve adequate levels of the vitamin
71 [3,4]. In Italy, the National Reference of Energy and Nutrient Intake Levels (LARN) identified an
72 average requirement of 2.4 µg a day for adults and up to 2.6 µg and 2.8 µg in pregnancy and lactation,
73 respectively [4]. A deficiency of vitamin B₁₂ could be the result of gastrointestinal disorders, celiac
74 disease, Crohn's disease, and genetic polymorphisms leading to malabsorption of the nutrient [1,2].
75 However, this condition is less frequent; elderly and vegetarians are more susceptible to the condition
76 of vitamin B₁₂ deficiency due to their limited intake of meat products [5,6]. On the contrary, vegans
77 that exclude animal products from their diet frequently become deficient in vitamin B₁₂. In this regard,
78 a recent systematic review evaluated the prevalence of vitamin B₁₂ deficiency in individuals adhering
79 to vegetarian and vegan diets [7]. The authors documented that adherence to a vegan diet was
80 associated with an increased risk of vitamin B₁₂ deficiency compared to a vegetarian diet [7]. These
81 findings were in line with the observations reported by other authors [8–11].

82 Vitamin B₁₂ deficiency has been associated with several metabolic disorders such as macrocytic
83 anemia, hyperhomocysteinemia, cardiovascular, cerebrovascular, and neurological disorders [12–

84 15]. However, despite the high risk of developing vitamin B₁₂ deficiency and related complications,
85 numerous vegans consider supplementation unnecessary. The deficiency appears after a long period
86 of depletion (can take years in some), due to the stocks of vitamin present in the liver [16]. Individuals
87 with serum levels of B₁₂ < 150 pmol/L are considered deficient [16,17], while subjects who have
88 values between 150 and 221 pmol/L are considered marginally deficient [18,19]. In this specific
89 situation, the integration of vitamin B₁₂ by the parenteral route is required. However, this approach is
90 poorly accepted because the results painful and expensive [20] as well as substituted by oral
91 formulations. However, this is not effective in subjects suffering from vomiting or diarrhea or are not
92 able to tolerate oral therapies [21]. Moreover, when high doses of vitamin B₁₂ are given orally, only
93 a small percentage seems to be absorbed. Recently, the administration of vitamin B₁₂ in sublingual
94 form has been developed [21]. Although sublingual vitamin B₁₂ is often promoted for better
95 absorption, inconsistent results have been obtained as to the effects of administration of low and high
96 doses of vitamin B₁₂.

97 The aim of the present study was to evaluate the ability of two different doses (350 µg/week vs. 2000
98 µg/week) of sublingual supplements in improving the nutritional status of cyanocobalamin in a group
99 of vegans and vegetarians with a marginal deficiency. The low dose (*Ld*) consisted of 7 sublingual
100 tablets each providing 50 µg/day (350 µg/week) of vitamin B₁₂, while the high dose (*Hd*) consisted
101 of 1 sublingual tablet (2000 µg) for the entire week. The latter represents the most common method
102 of supplementation, even if it is administered by the oral or parenteral route. In this regard, several
103 studies have shown low absorption following the intake of high doses [1,22]. In addition, this practice
104 could be less tolerated in some subjects; for example, some authors found adverse effects (e.g.,
105 hyperhidrosis and blurred vision) following supplementation with 1 mg/day of vitamin B₁₂ in
106 individuals with mild and moderate Alzheimer disease [23]. Our hypothesis is that the sublingual
107 administrations of low (350 µg/week) and high (2000 µg/week) doses of cyanocobalamin are both
108 able to restore the nutritional adequacy of vitamin B₁₂ within 90 days [24–26] in vegans and
109 vegetarians affected by a marginal deficiency.

110 **2. Materials and Methods**

111 ***2.1 Subject recruitment***

112 The screening of the participants was performed between March 2015 and July 2016 through
113 advertisements on bulletin boards, telephone, or e-mail. Subjects were visited for a routine medical
114 examination by a physician to assess their eligibility to participate in the trial. The eligibility was
115 assessed by a physician through an accurate examination and by means of a health/medical
116 questionnaire to exclude subjects with diseases such as diabetes, renal insufficiency, allergies, chronic
117 constipation, diarrhea, or any other gastrointestinal disorder. Moreover, a small aliquot of blood was
118 collected to ascertain vitamin B₁₂ nutritional status. Subjects were selected according to the following
119 inclusion criteria: vegan and vegetarian subjects in a condition of marginal vitamin B₁₂ deficiency (<
120 220 pmol/L) or full-blown (< 150 pmol/L), non-smokers or light smokers (maximum 5–6
121 cigarettes/day), and moderate alcohol consumption (up to 14 glasses of wine/beer per week). Subjects
122 with cardiovascular, coronary, diabetes, hepatic, renal, or gastrointestinal diseases were excluded.
123 Subjects were not included if using drugs, medications, and/or supplements at least one month before
124 the beginning of the experiment. Moreover, subjects were excluded if taking vitamin B₁₂ supplements
125 at least one year before the experiment. The study was performed in accordance with the ethical
126 standards established in the 2013 Declaration of Helsinki and approved by the Ethics Committee of
127 the University of Milan (March 4, 2015, ref. 11/15). The study was registered at www.isrctn.org as
128 ISRCTN75099618. All participants signed an informed consent form.

129 ***2.2 Experimental design***

130 A researcher who was not involved in the study and in sample analysis was appointed to allocate
131 patients to the different treatments according to a randomization list obtained through the center's
132 database. The number of participants who were randomly assigned to different study groups, the rate
133 of patients completing the study, and patients analyzed for the primary outcome are depicted in Figure
134 1. Forty subjects were enrolled and randomly divided into two groups of 20 subjects each for a 12-
135 week double-blind, randomized, controlled, parallel dietary intervention study. The study was

136 performed between May 2015 and October 2016. One group received the supplement at a low dose
137 (*Ld*; equivalent to 50 µg/day, 350 µg/week), while the other group (control) received the supplement
138 at a high dose (*Hd*; equivalent to 2000 µg/week in a single dose). Vitamin B₁₂ was provided to the
139 volunteers in one stock at the beginning of the study. Each subject received 13 boxes containing the
140 doses for a week in a blind condition. All tablets were packaged and numbered (from 1 to 7) in single-
141 dose blisters. Subjects were instructed to follow the sequence of numbers and to swallow one tablet
142 per day in the morning before breakfast. The *Ld* group ingested 7 sublingual tablets/week of
143 cyanocobalamin (50 µg each, equivalent to 350 µg), while the *Hd* group took only 1 sublingual tablet
144 of vitamin B₁₂ (2000 µg) and 6 sublingual tablets of placebo. For both groups (*Ld* and *Hd*), the tablets
145 of vitamin B₁₂ consisted of mannitol, maize starch, vegetable stearate magnesium, beet juice, and
146 sucralose. The placebo tablets matched the shape, size, color, flavor, and the composition of the
147 vitamin B₁₂ supplements. The sublingual vitamin B₁₂ tablets were obtained from bacteria with a
148 manufacturing process compatible with the strictly vegan dietary requirements. The crystalline form
149 of cyanocobalamin was used for the preparation of the tablets.

150 Subjects were instructed to maintain their dietary and lifestyle habits as declared before enrollment.
151 Moreover, they were encouraged to abstain from consuming sources of vitamin B₁₂ (e.g., spirulin,
152 yeast, fortified foods). A 24-hour record of food consumption was kept by each volunteer the day
153 before blood collection to check compliance with the dietary instructions. Every 2 weeks, subjects
154 returned the empty blisters (as evidence of the consumption of the tablets) and received the new
155 supplements. A 3-day food record and a weekly direct interview were also scheduled randomly during
156 the experimental period to check compliance with the dietary instructions and to assure the
157 consumption of the tablets. The day of the experiment, after an overnight fast, subjects reported to
158 the laboratories of the University of Milan. Blood samples were collected at baseline (time 0) and
159 after 15, 30, 60, and 90 days of intervention.

160 2.3 Study variables

161 The improvement of serum levels of vitamin B₁₂ was considered the primary endpoint. The other
162 variables under study were as follows: holotranscobalamin, methylmalonic acid, succinic acid,
163 methionine, homocysteine, vitamin B₆, folic acid, and complete blood count. Since the amount of
164 cobalt provided through the supplement was negligible with respect to the circulating blood levels,
165 this variable was not evaluated.

166 *2.4 Sampling and analysis of biochemical parameters*

167 Blood was collected **in the morning by a phlebotomist**. Samples were drawn into evacuated tubes
168 with or without K₂EDTA. **Serum was separated within 1 hour, while plasma was separated** within 30
169 minutes (min) after collection by centrifugation (15 min at 2300 X g at 4 °C). Plasma and serum were
170 aliquoted and stored at -80 °C until analysis. All the samples were analyzed blind. Blood cell count
171 was evaluated by routine laboratories assessment.

172 Vitamins B₁₂ levels were measured by a competitive test principle using IF specific for this
173 vitamin. **Vitamin B₁₂ was analyzed by** electrochemiluminescence immunoassay (ECLIA) using
174 Cobas immunoassay analyzers (Roche Diagnostics, North America). **Also, the assessment of serum**
175 **folate was performed with electrochemiluminescence immunoassay (ECLIA) using Cobas**
176 **immunoassay analyzers (Roche Diagnostics, North America).**

177 Holotranscobalamin concentration were determined in serum by immunoenzymatic assay kit
178 (BIOHIT HealthCare, Helsinki, Finland). Briefly, the microtiter plate wells were coated with a highly
179 specific monoclonal antibody for BIOHIT Active B₁₂ (holoTC). During the first incubation, holoTC
180 specifically bound to the surface coated with the antibody. Successively, the conjugate was added for
181 the binding of holoTC; the wells were then washed to remove unbound components and holoTC was
182 detected following the incubation with the substrate. Before the analysis, a stop solution was added
183 and the absorbance was read at 405 nm (mod. F200 Infinite, TECAN Milan, Italy).

184 Serum vitamin B₆ concentrations were evaluated by **high performance liquid chromatography**
185 method using the relevant commercial kit (Chromsystems Instruments & Chemicals, Munich,
186 Germany) [27]. Homocysteine (HCy), methionine (Met), methylmalonic acid (MMA), succinic acid

187 (SA), tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl), methanol, and formic acid were
188 obtained from Sigma-Aldrich (St. Louis, MO, USA). Water was obtained from the Milli-Q apparatus
189 (Millipore, Milford, MA). The determination of HCy, Met, MMA, and SA was performed according
190 to Fu et al. [28], with slight modifications. Briefly, 200 μ L of heparinized plasma was added to 100
191 μ L of water and 100 μ L of TCEP-HCl (0.1 M). The mixture was vortexed for 10 seconds (s),
192 incubated for 15 min at room temperature, and transferred to an Amicon 10K Da filter. The filter was
193 centrifuged at 9000 g for 30 min, the filtrate was transferred to a microvial, and 5 μ L injected into the
194 **Ultra Performance Liquid Chromatography (UPLC)-high resolution (HR)-mass spectrometers (MS).**
195 The analysis was carried out on an UHPLC model Acquity (Waters) coupled with a High-Resolution
196 Fourier Transform mass spectrometer (Orbitrap) model Exactive (Thermo Scientific) equipped with
197 an HESI-II probe for electrospray ionization and a collision cell (HCD). The column was a 1.8 μ m
198 HSS T3 C₁₈ (150 x 2.1 mm, Waters), flow rate was 0.45 mL/min, and the eluents were 0.1% formic
199 acid in water (A) and acetonitrile (B). The column and sample were kept at 60 °C and 15 °C,
200 respectively. The UHPLC separation was performed by the following linear elution gradient: 100 %
201 of A for 5 min, 0 to 100 % B in 1 s, 100 % B for 2 min, from 100 % to 0% B in 1 min, and then
202 isocratic for 2 min.

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

207 For MMA and SA (3.2–5 min) the operative conditions were spray voltage -3.0 kV, sheath
208 gas flow rate 55, auxiliary gas flow rate 20, capillary temperature 320 °C, capillary -35 V, tube lens
209 -70 V, skimmer -16 V, and heater temperature 120 °C. The acquisition was performed in full-scan
210 mode in the range $(m/z)^-$ 60–130 u and the ions with m/z 91.0038, corresponding to the formic acid
211 dimer [2M-H]⁻ that was used as the lock mass. The isolation window, **automatic gain control** target,
212 injection time, mass resolution, energy, and gas in the collision cell were ± 2 ppm, 1×10^6 , 100 ms,

213 50 K, 20 V, and N₂, respectively. The MS data were processed using Xcalibur software (Thermo
214 Scientific). The peak identity was ascertained, evaluating the accurate mass and the fragments
215 obtained in the collision cell. Calibration curves were in the range 0.15–14.8, 0.13–33.5, 0.17–42.5,
216 and 0.25–44 μMolar for HCy, Met, MMA, and SA, respectively. Finally, the wellness parameter was
217 calculated according to the Fedosov formula [29]: “wellness parameter”: $w = \log_{10}(\text{holoTC}_n) +$
218 $\log_{10}(\text{B}_{12n}) - \log_{10}(\text{MMA}_n) - \log_{10}(\text{HCy}_n)$, where concentrations are normalized (e.g., $\text{MMA}_n =$
219 MMA/MMA_n normal).

220

221 *2.5 Statistical analysis*

222 Sample size was estimated, based on previous studies, in order to detect significant differences in the
223 serum vitamin B₁₂ levels [24–26]. Sixteen subjects per group were considered sufficient to
224 demonstrate at least a 70% improvement in the levels of vitamin B₁₂ after supplementation with a p
225 value of 0.05 and a power of 80%. The calculation was based on the assumptions that the mean ±
226 standard deviation (SD) baseline vitamin B₁₂ concentration was 140 ± 40 μmol/L and that the
227 treatment would increase the levels of cyanocobalamin up to 240 μmol/L. This value represents the
228 mean found in an Italian blood donor population [4].

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

238

239 **3. Results**

240 **3.1 Baseline characteristic of the study population**

241 Baseline characteristics of the subjects enrolled in each group are reported in Table 1. Four subjects
242 (2 for each group) were lost during the follow-up period due to personal reasons. All subjects ($n =$
243 36) showed a marginal deficiency of vitamin B₁₂ (< 220 pmol/L) [3]. Regarding the other biomarkers
244 of cobalamin status: 27 out of 36 subjects had serum levels of MMA above 750 nmol/L (cut-off above
245 which cobalamin deficiency is diagnosed), while 14 out of 36 subjects documented moderate
246 hyperhomocysteinemia (range 17.6–33.8 μ mol/L) with plasma total homocysteine (HCy-pt) value \geq
247 15 μ mol/L [3]. Moreover, six subjects had folate levels (range 7–9 nmol/L) below 10 nmol/L,
248 suggesting a folate deficiency [30]. Two subjects showed low vitamin B₆ levels (< 21.3 nmol/L) and
249 one also had low holotranscobalamin levels (< 21 pmol/L) [3]. No abnormalities in blood cell count
250 were observed. The age, sex, hemoglobin level, platelet and white blood cell counts, mean
251 corpuscular volume, and serum cobalamin levels were not significantly different between groups
252 (Table 1).

253 **3.2 Compliance**

254 Subjects were highly motivated to participate in the intervention and confirmed the consumption of
255 the tablets. The compliance was verified during a weekly direct interview, as previously reported, and
256 confirmed by returning the empty blisters (100% compliance). Not one participant declared adverse
257 effects following the supplementation.

258 **3.3 Effect of supplementation on serum levels of total, active, and inactive form of vitamin B₁₂**

259 The serum levels of total vitamin B₁₂, measured at baseline (time 0 day) and after 15, 30, 60, and 90
260 days from the start of supplementation, are reported in Figure 1. Subjects increased the serum
261 concentrations of total vitamin B₁₂ to above 240 pmol/L according to our hypothesis. On the whole,
262 repeated measures of ANOVA did not show a significant effect of *treatment*, but revealed a
263 significant effect of *time* ($P = 0.008$) and of *time* \times *treatment* interaction ($P = 0.012$) for circulating
264 levels of total vitamin B₁₂ that increased following the treatments. In particular, post-hoc analysis

265 showed a significant enhancement after 15 days from the start of the intake of the supplements (+
266 51.7% in *Ld* group vs. +74.2% in *Hd* group; $P < 0.0001$). The values increased over time and appeared
267 significantly different between groups after 30 days until the end of the experimental period ($P <$
268 0.01). Figures 2A and 2B show the levels of active (holotranscobalamin, HoloTC) (2A) and inactive
269 forms (2B) of vitamin B₁₂ measured at baseline and after 15 and 90 days from the start of
270 supplementation. The analysis at 15 and 90 days was performed based on the prominent absorption
271 observed in vitamin B₁₂. On the whole, ANOVA did not show a significant effect of *treatment* and
272 of *time x treatment interaction*, but revealed an effect of *time* ($P < 0.0001$) for serum circulating levels
273 of active and inactive vitamin B₁₂ that increased during the treatments.

274 **3.4 Effect of supplementation on serum levels of methylmalonic acid and homocysteine**

275 The serum levels of MMA and HCy were measured at baseline (time 0 day) and after 15, 30, 60, and
276 90 days from the start of supplementation, are reported in Figures 3A and 3B. ANOVA revealed only
277 a significant effect of *time* ($P < 0.0001$) for serum circulating levels of MMA and HCy that decreased
278 over time following both treatments.

279 **3.5 Effect of supplementation on serum concentrations of methionine, succinic acid, vitamin B₆** 280 **and folate, blood cell count, and wellness parameter**

281 The serum levels of Met, SA, vitamin B₆, and folate, measured at baseline (time 0 day) and after 15,
282 30, 60, and 90 days from the start of supplementation, are reported in Table2. ANOVA revealed only
283 a significant effect of *time* for serum circulating levels of folate ($P < 0.0001$), Met ($P < 0.0001$) and
284 SA ($P < 0.0001$). In particular, folate showed a significant decrease over time, while Met and SA has
285 significant increases.

286 In Table 2 are reported the values of the wellness parameter measured at baseline (time 0 day) and
287 after 15 and 90 days from the start of supplementation are reported in Table 2. Since the index derives
288 from a formula that also takes into consideration the levels of holoTC, this parameter was measured
289 only at times for which the levels of holoTC were detected. On the whole, repeated measures ANOVA
290 did not show a significant effect of *treatment*, but revealed a significant effect of *time* ($P < 0.0001$)

291 and *time* × *treatment* interaction ($P = 0.046$). In particular, post-hoc analysis documented a significant
292 improvement over time following the intake of both the supplements, with a difference between
293 groups only at specific and independent time points.

294 No effect was documented for serum circulating levels of vitamin B₆ and blood cell count (data not
295 shown).

296

297 **4. Discussion**

298 In the present study, we documented that as a little as 350 µg per week of vitamin B₁₂ supplementation
299 was enough to correct a marginal deficiency of cobalamin and to improve holoTC, MMA, and Hcy
300 (biomarkers of cobalamin status) in a group of vegans and vegetarians. The results obtained support
301 the use of a sublingual supplement at low doses as an effective and non-invasive method to improve
302 the cobalamin status in this target population.

303 It has been reported that the absorption of vitamin B₁₂ from supplements **does not depend only on the**
304 **dose and frequency of the intake but also on the health status of the subjects. In particular, it is widely**
305 **recognized that subjects suffering from gastric or small intestine resections, inflammatory bowel**
306 **disease, and other complications related to intestinal absorption may become deficient [31].**
307 **Moreover,** the capacity of absorption is strictly dependent on saturable active transport and on the
308 efficiency of the aspecific route. In this regard, different studies have shown that the absorptive
309 capacity of vitamin B₁₂ is high when the amount introduced is low. For example, **the oral**
310 **administration of different doses (1 µg, 10 µg, 50 µg, 500 µg, and 1000 µg) of vitamin B₁₂ are**
311 **absorbed with an efficiency of 56%, 16%, 3%, 2%, and 1.3%, respectively [32].** A plethora of studies
312 investigated the effect of a supplementation on the levels of vitamin B₁₂ and related cardiovascular
313 markers; however, most of them were performed in the elderly [6,15], **those with**
314 **hyperhomocysteinemia [33,34], and undernourished children [35,36], while very few are involving**
315 **vegetarians and/or vegans. A recent 12-week randomized, placebo-controlled trial performed in**

316 vegans documented that the use of a vitamin B₁₂-fortified toothpaste (about 100 µg/g depending on
317 the number of brush sessions) improved serum and plasma concentrations of cobalamin and related
318 associated markers [37]. Yajnik et al. [26] found that supplementation of vitamin B₁₂ (500 µg/day),
319 over a 6-week period, significantly increased plasma vitamin B₁₂ concentration (from 125 to 215
320 pmol/L) in a group of healthy, lacto-vegetarian women. The improvement was observed within the
321 first 2 weeks of intervention, and the levels maintained stability up to 4 weeks. Sharabi and coworkers
322 documented similar findings following sublingual and oral administration of 500 µg of cobalamin in
323 subjects with a deficiency [38].

324 In our experimental conditions, supplementation with low and high doses (350 µg/week vs. 2000
325 µg/week) of cobalamin significantly improved circulating serum levels of vitamin B₁₂, suggesting the
326 efficiency and efficacy of both supplements in restoring the levels of the vitamin (> 240 pmol/L) [3].
327 However, serum levels of vitamin B₁₂ above the cut-off point does not necessarily indicate an
328 adequate nutritional status. In fact, there is inconsistency among the scientific community regarding
329 the identification of reference values for cyanocobalamin. Future studies should be performed in order
330 to identify the cut-offs according to individual variability (i.e., age, sex, etc.) and lifestyle habits (i.e.,
331 vegans, vegetarians). Holotranscobalamin represents the metabolically active form of vitamin B₁₂
332 that delivers cobalamin to the target cells. Recently, it has been recognized as an early and reliable
333 marker to discriminate an impaired cobalamin status [39]. However, discrepancies remain about
334 mode of application and assignment of these cut-off values to diagnose a deficiency. Based on
335 different populations and criteria, cut-off values from 21 to 45 pmol/L have been proposed as
336 “suboptimal” [3]. In our study, subjects have shown levels of holoTC within the range of normality.
337 This is in line with the characteristics of our population that included only individuals with a marginal
338 cobalamin deficiency. The supplementation with both dosages significantly increased the levels of
339 holoTC. The improvement was comparable between groups, since only an effect of time, but not of
340 treatments, was observed. The impact of vitamin B₁₂ supplementation on levels of holoTC has been
341 evaluated in different studies [40,41]. In a double-blind, placebo-controlled trial, 12 and 24 weeks of

342 supplementation with 1000 µg vitamin B₁₂ or 1000 µg vitamin B₁₂ + 400 µg folic acid significantly
343 increased the levels of cobalamin as well as those of holoTC in elderly subjects [40]. Brito et al., [41]
344 reported that a single intramuscular injection of 10 mg vitamin B₁₂ (providing 100 mg pyridoxine and
345 100 mg thiamine) significantly increased, after 4 months, serum vitamin B₁₂ and holotranscobalamin
346 levels in a group of 27 community-dwelling elderly Chileans.

347 Other biomarkers of cobalamin status include hematological changes and the metabolites MMA and
348 Hcy. These variables can add valuable information in conjunction with serum holoTC and/or
349 cobalamin for assessment of B₁₂status. MMA is considered a biomarker of cobalamin function with
350 regard to its role in the functioning of methylmalonyl-CoA mutase. Serum MMA concentration
351 increases following an insufficient supply of cobalamin. As previously reported values above 750
352 nmol/L are used to discriminate a cobalamin deficiency [3].

353 Plasma Hcy is not a specific marker of cobalamin status since it is affected also by dietary factors,
354 such as folate, choline and betaine, as well as renal insufficiency, lifestyle factors (e.g. alcohol
355 consumption) and age [42-43]. However, elevated plasma Hcy concentration is commonly observed
356 in subjects with a cobalamin deficiency. In our experimental conditions, most of the subjects showed
357 baseline levels of MMA and Hcy above the cut-off values, while only few subjects showed low levels
358 of folate. For these reasons, those biomarkers, together with the levels of folate, vitamin B₆, Met and
359 SA, can be considered a valid support for the assessment of the nutritional status of cobalamin in
360 vegans and vegetarians. In fact, we were able to document a statistically significant decrease in the
361 levels of MMA and Hcy, and a significant increase in the levels of Met and SA. These results were
362 in line with those obtained by other authors showing a general improvement after cobalamin
363 supplementation [26,36,40,42]. An improvement in cobalamin nutritional status and a reduction of
364 Hcy and MMA may be also effective in the prevention of cardiovascular risk and neurological
365 disorders. However, some studies failed to observe a significant modulation in Hcy levels. For
366 example, Sharabi and colleagues [38] did not document a decrease in Hcy and MMA following 8

367 weeks of intervention with 500 µg/day of sublingual and oral B₁₂ administration in subjects with a
368 cobalamin deficiency.

369 As previously reported, there is an interrelationship between vitamin B₁₂ and folate; in particular,
370 vitamin B₁₂ deficiency can lead to lowered levels of methionine synthetase, which results in folate
371 deficiency and an increased proportion of the 5-methyl derivative. In our experimental conditions,
372 we did not quantify the levels of the 5-methyl derivative, but only folate that significantly reduced
373 following cobalamin supplementation. These results are complex to explain; we may hypothesize that
374 the improvement in B₁₂ status, also in terms of MMA and HCy, did not require high amounts of folate
375 to compensate for a cobalamin deficiency. However, we cannot exclude that these fluctuations were
376 attributed mainly to physiological changes, since the overall vitamin status was maintained within the
377 range of normality.

378 **A recent and robust biochemical indicator of cyanocobalamin status is the wellness parameter**
379 **conceived by** Fedosov that takes into consideration the levels of total and active B₁₂ forms and those
380 of MMA and HCy [29]. The cut-off to discriminate the wellness parameter are as follows: deficiency
381 $w = -1.49$; transition $w = -0.516$; normal $w = -0.0$, and excellent $w = +0.445$. In our experimental
382 conditions, subjects showed a low **wellness parameter** at baseline (-1.0 for *Ld* group and -1.3 for *Hd*
383 group), documenting a state of marginal deficiency. The supplementation of vitamin B₁₂ significantly
384 improved the **wellness parameter** in both the intervention groups.

385 **Finally, we observed no significant effect on blood cell count both at the beginning of the study (see**
386 **Table 1) and after the intervention (data not shown). These results are not surprising, since our**
387 **subjects were in stage 2–3 of vitamin B₁₂ deficiency and this condition does not affect the levels of**
388 **mean corpuscular volume and hemoglobin [43].**

389 **Study limitations**

390 A possible limitation of the study is the lack of a real control group (**vegans/vegetarians** with a
391 marginal deficiency who did not take supplements). However, by considering that our subjects were
392 **affected by a marginal** vitamin B₁₂ deficiency, the inclusion of a real placebo group

393 (vegans/vegetarians without supplements) would not have been possible for ethical reasons. A second
394 limitation of the study is the lack of a follow-up period post-supplementation in order to verify the
395 changes in the levels of vitamin B₁₂ and related metabolic markers along the time.

396 **Conclusions**

397 In conclusion, the results obtained have shown that both supplements were able to bring the levels of
398 vitamin B₁₂ from a marginal deficiency to an adequate nutritional status. In particular, we have
399 documented an increase of serum concentrations of vitamin B₁₂ and holoTC, and a reduction of MMA
400 and Hcy as markers of vitamin B₁₂ metabolism. These results are in line with the elevation of the
401 wellness parameter that provides further support for the improvement of the nutritional vitamin B₁₂
402 status.

403 Our observations emphasize the importance of supplementation in vegetarians and vegans with a
404 marginal deficiency, but it should be emphasized that the use of pharmacological doses is unnecessary
405 in this target group. Moreover, the absence of a consensus on vitamin B₁₂ cut-off values and the high
406 individual variability make it difficult to identify the real needs for vegans and vegetarians. Further
407 studies are necessary in order to confirm our findings and verify the effects of sublingual
408 supplementation in vegans and vegetarians with a severe deficiency and in those affected by
409 malabsorption and/or impaired metabolism of vitamin B₁₂.

410

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413

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417 design, conduct, or interpretation and reporting.

418

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427 All authors provided input into and read and approved the final version of the manuscript.

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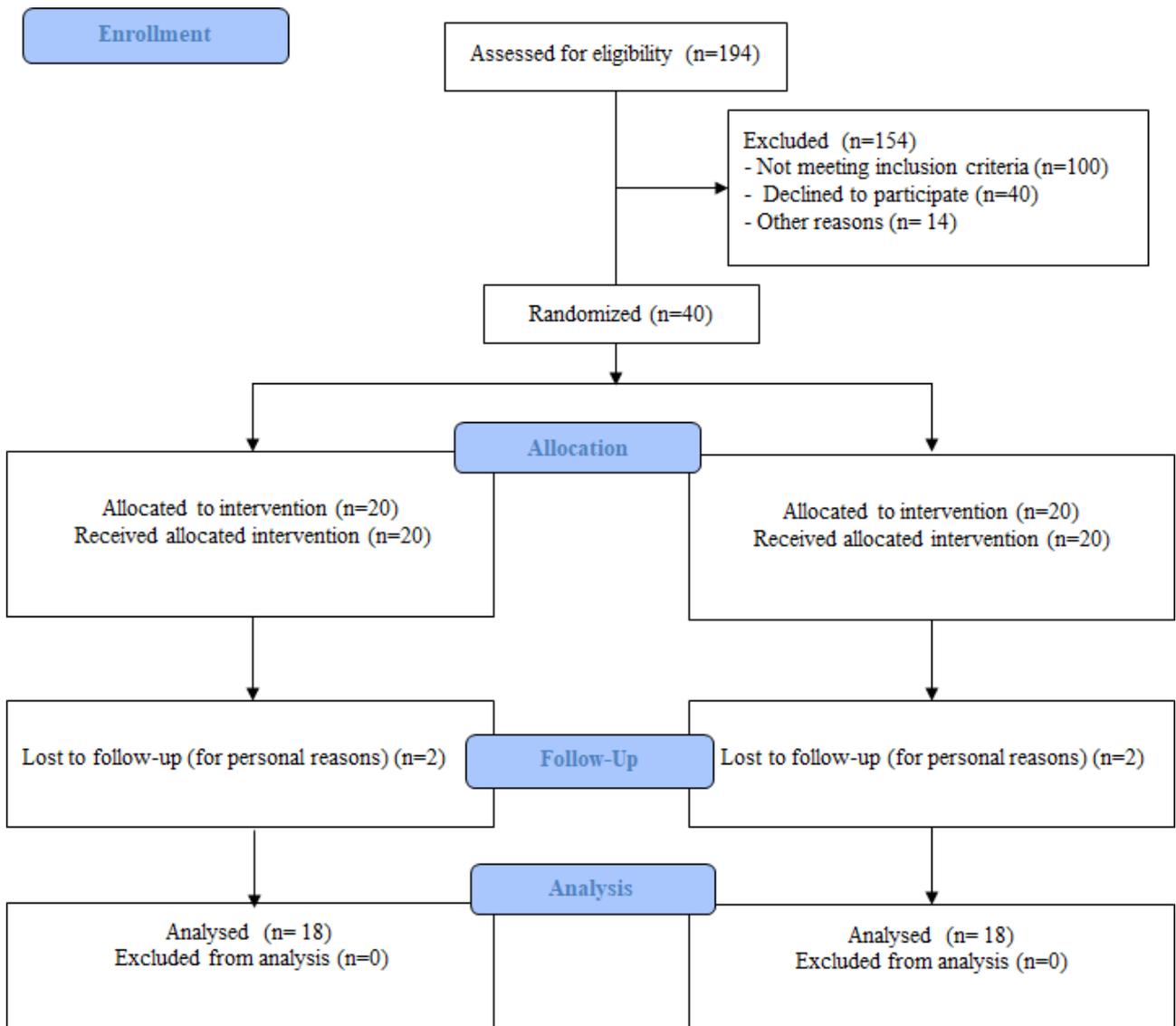
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554 **Figure 1-** Study flow-chart showing the process of patient selection and enrolment, allocation to the
 555 two study groups, and rate of patients completing the study. Ld: group treated with low dosage of
 556 vitamin B₁₂ (350 µg/week); Hd: group treated with high dosage of vitamin B₁₂ (2000 µg/week).

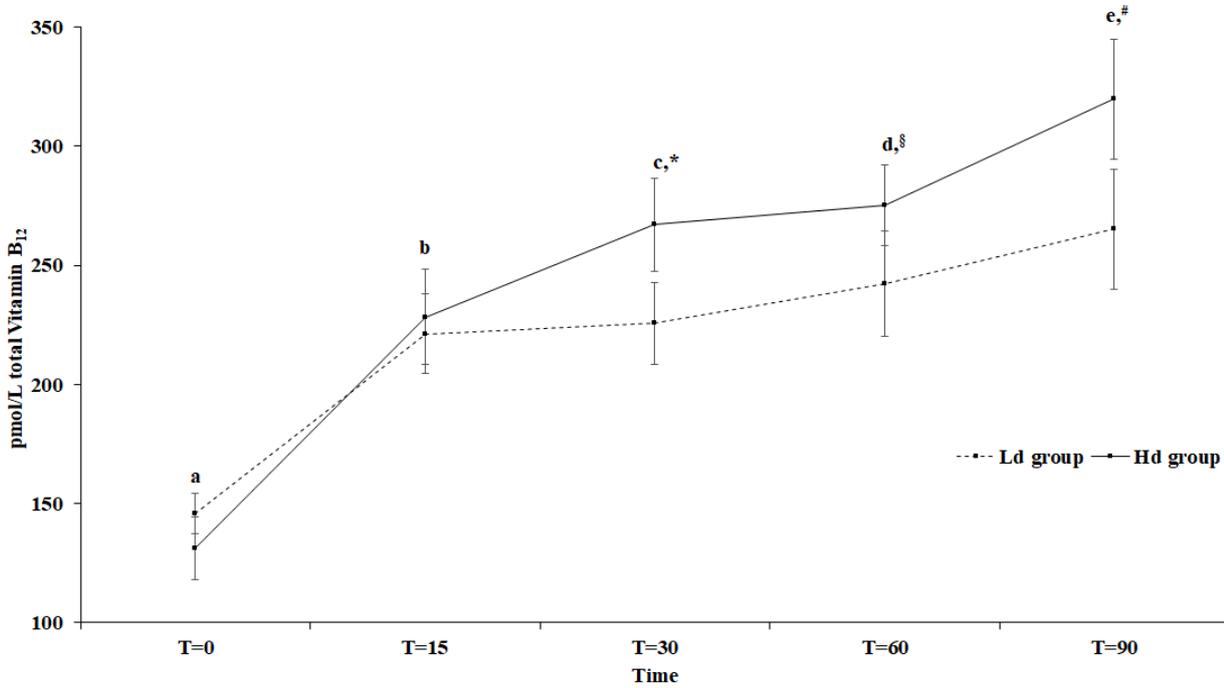
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CONSORT 2010 Flow Diagram



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563 **Figure 2-** Effect of supplementation on serum circulating levels of total vitamin B₁₂ in the two
564 intervention groups (Ld vs Hd). The concentrations were measured at baseline (T0) and after 15, 30,
565 60 and 90 days.



566

567 *Figure Legend:*

568 N=18 for each group.

569 Data are expressed as mean \pm SEM. ^{a,b,c,d,e}Data with different letters are significantly different within

570 the same treatment (time effect; P<0.05). ^{*,§,#}Data with different symbols are significantly different

571 between treatment (treatment effect; P<0.05)

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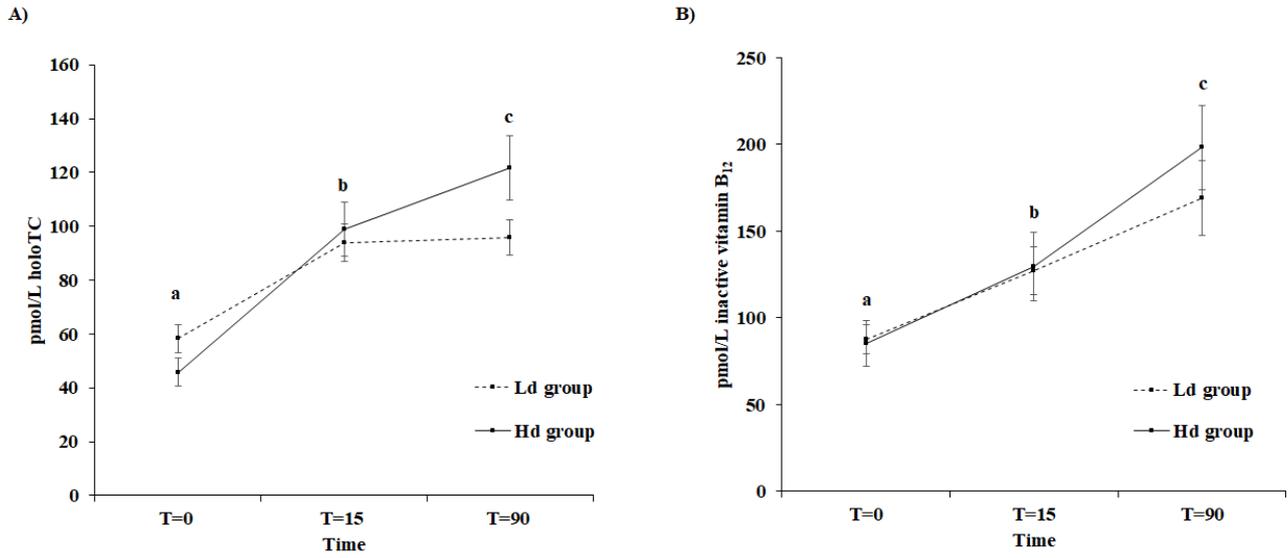
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578 **Figure 3**-Effect of supplementation on serum circulating levels of active (A) and inactive (B) form
579 of vitamin B₁₂ in the two intervention groups (LdvsHd). The concentrations were measured at baseline
580 (T0) and after 15 and 90 days from the supplementation. Data are expressed as mean ± SEM.



581

582 *Figure Legend:*

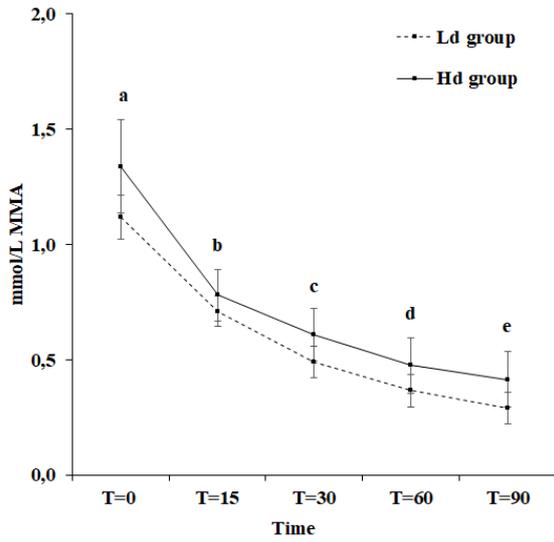
583 N=18 for each group.

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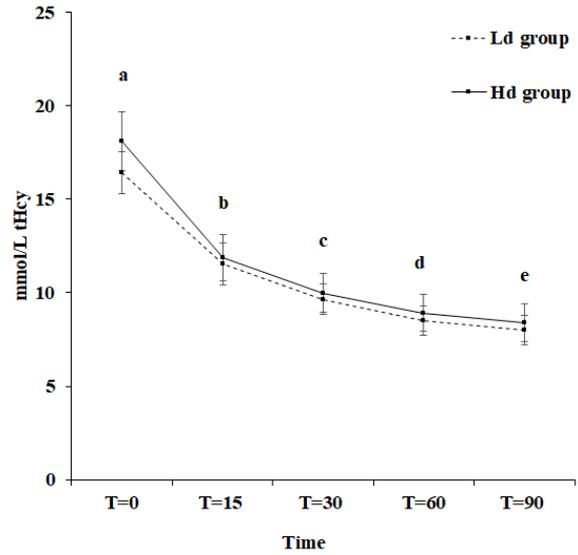
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586 **Figure 4**-Effect of supplementation on serum circulating levels of MMA (A) and tHcy (B) in the two
587 intervention groups (Ld vs Hd). The concentrations were measured at baseline (T0) and after 15 and
588 90 days from the supplementation.

A)



B)



589

590 *Figure Legend:*

591 N=18 for each group.

592 Data are expressed as mean \pm SEM. MMA, methylmalonic acid; tHcy, total homocysteine

593