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Low bone mineral density and bone mineral content are associated with low cobalamin status in adolescents

■ **Summary** *Background* Cobalamin deficiency is prevalent in vegetarians and has been associated with increased risk of osteoporosis. *Aim of the study* To examine the association between cobalamin status and bone mineral density in adolescents formerly fed a macrobiotic diet and in their counterparts.

Methods In this cross-sectional study bone mineral density (BMD) and bone mineral content (BMC) were determined by DEXA in 73 adolescents (9–15 y) who were fed a macrobiotic diet up to the age of 6 years followed by a lacto-(-ovo-) vegetarian or omnivorous diet. Data from 94 adolescents having consumed an omnivorous diet throughout their lives were used as controls. Serum concentrations of cobalamin, methylmalonic acid (MMA) and homocysteine were measured and calcium intake was assessed by questionnaire. Analysis of covariance (MANCOVA) was performed to calculate adjusted means for vitamin B₁₂ and MMA for low and normal BMC and BMD groups. *Results* Serum cobalamin concentrations were significantly lower (geometric mean (GM) 246 pmol/L vs. 469 pmol/L) and MMA concentrations were significantly higher (GM 0.27 μmol/L vs. 0.16 μmol/L) in the formerly macrobiotic-fed adolescents compared to their counterparts. In the total study population, after adjusting

for height, weight, bone area, percent lean body mass, age, puberty and calcium intake, serum MMA was significantly higher in subjects with a low BMD ($p = 0.0003$) than in subjects with a normal BMD. Vitamin B₁₂ was significantly lower in the group with low BMD ($p = 0.0035$) or BMC ($p = 0.0038$) than in the group with normal BMD or BMC. When analyses were restricted to the group of formerly macrobiotic-fed adolescents, MMA concentration remained higher in the low BMD group compared to the normal BMD group. *Conclusions* In adolescents, signs of an impaired cobalamin status, as judged by elevated concentrations of methylmalonic acid, were associated with low BMD. This was especially true in adolescents fed a macrobiotic diet during the first years of life, where cobalamin deficiency was more prominent.

■ **Key words** adolescents – bone mass – cobalamin deficiency – macrobiotic diet – vegetarian diet – analysis of covariance

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Introduction

Cobalamin deficiency has been suggested to affect bone metabolism [1]. Likewise, pernicious anemia, an autoimmune disorder resulting in cobalamin deficiency, has been identified as a risk factor for osteoporosis [2,

3]. Furthermore, a case report demonstrated that a patient with pernicious anemia and osteoporosis had a marked improvement of bone mineral density after combined cobalamin and etidronate therapy [4]. A high urinary methylmalonic acid (MMA) concentration, a metabolic marker of functional cobalamin deficiency, was observed in 55% of the children of a small macro-

biotic community [5]. Similarly, low cobalamin concentrations were observed in 26% of a vegetarian group and in 78% of a vegan group [6]. It is conceivable that cobalamin deficiency in these cases may contribute to the risk of reduced bone mass in subjects following these diets, although data about this relation are scarce [7].

Since 1985, we have been studying the nutritional status of Dutch children consuming a macrobiotic diet. A macrobiotic diet consists mainly of cereals, pulses and vegetables, and occasionally some fish. The consumption of meat, dairy products and vitamin D supplements is normally avoided. In a previous study, our group reported that infants who followed a macrobiotic diet had very low dietary intakes of cobalamin and calcium [8]. In addition, plasma cobalamin concentrations were low [9] and many infants had a marked vitamin D deficiency [10].

A follow-up study was carried out in 1995 and included most of the formerly investigated macrobiotic children. We here report on these children, at that time young adolescents (9–15 y), and investigated the potential influence of a macrobiotic diet in early childhood on bone mass in later life. After the age of six years, most of these subjects had switched to a lacto-(-ovo-)vegetarian or even omnivorous diet at the advice of the former investigators. Hereafter, we refer to these adolescents, who previously received a macrobiotic diet, as 'the macrobiotic group'. The macrobiotic group had a 3–8% lower bone mass as compared to age-matched controls who had consumed the omnivorous diet throughout their lives [11].

In an earlier report we found that a substantial portion of the macrobiotic adolescents still had signs of an impaired cobalamin status [12] as indicated by their low serum concentrations of cobalamin and/or serum elevated concentrations of methylmalonic acid (MMA), a sensitive and specific marker of functional cobalamin deficiency [13]. Our findings of a higher prevalence of osteoporosis in elderly women with a low cobalamin status compared to those with a normal cobalamin status [14] and the high prevalence of an inadequate cobalamin status among the macrobiotic group motivated us to test the hypothesis whether the low BMD frequently found in adolescents previously fed a macrobiotic diet was associated with signs of impaired cobalamin status.

Materials and methods

Subjects and design

The subjects included in this study have been characterized in detail previously [11]. In summary, bone mass was measured in 195 adolescents aged 9–15 y between May and July 1995. The macrobiotic adolescents were recruited from an existing group of macrobiotic families

affiliated with the Division of Human Nutrition, Wageningen University. Ninety-three adolescents (50 boys and 43 girls), born from macrobiotic mothers and receiving a macrobiotic diet from birth until about the age of 6 years, were included in the study and are referred to as the "macrobiotic group or macrobiotic subjects". The control group consisted of 102 adolescents (42 boys and 60 girls) aged between 9 and 15 years, having consumed an omnivorous diet throughout their lives. We excluded twenty-five subjects from the present study as they either did not give their consent for blood sampling or the amount of serum obtained from them was insufficient for the biochemical analysis of cobalamin status parameters. Thus, complete data of 170 adolescents (76 macrobiotic subjects and 94 controls) were available for further statistical analyses. All subjects were Caucasian, in good health and without any medication known to affect bone or calcium metabolism. Socio-economic status was determined by the Attwood scores, a five-point scale based on occupation and highest level of education attained by both parents. The external Medical Ethical Committee of the Division of Human Nutrition and Epidemiology of Wageningen University approved the study and written informed consent was obtained from the subjects and their parents.

Anthropometry

Subjects were weighed (in underwear) to the nearest 0.1 kg using a digital scale (ED-60T, Berkel, Rotterdam, The Netherlands). Standing height was measured (without shoes) to the nearest 0.1 cm using a microtoise. Pubertal stage was determined by one investigator, according to the Tanner method, using the development of breast in girls and that of pubic hair in boys [15]. One subject refused observers' assessment and therefore a self-assessment value was used.

Lean body mass and fat mass were determined using a dual energy X-ray absorptiometer (DEXA, model DPX-L, Lunar Radiation Corp., Madison, WI) with software version 1.31. All the measurements were obtained from the total body DEXA scan. Percentage lean body mass ($[\text{non-bone lean body mass/body weight}] \times 100$) was used as a measure of body composition in data analysis.

Bone measurements

Bone mineral content (BMC, g), bone area (BA, cm²) and resulting bone mineral density (BMD, g/cm²) were determined using DEXA. A spine phantom, provided by the manufacturer, was scanned weekly throughout the study period and gave coefficients of variation of 0.65% for L1-L4 BMC and 0.73% for L1-L4 area bone density

(BMD, g/cm²). In vivo precision was assessed using repeated scans of six adults, which gave coefficients of reproducibility for BMD ranging from 0.6 % (for the total body) to 3.2 % (for the trochanter).

■ Biochemical measurements

Non-fasting blood specimens were taken (90 % taken from mid May–mid June 1995, followed by other 10 % in the subsequent month). Samples were allowed to clot and were then centrifuged (1190 x g for 10 min at 4 °C). Serum was separated from blood cells within 60 min and separated serum samples were stored at –80 °C until further analysis. Serum concentrations of cobalamin and folate were measured by using a microparticle-based enzyme immunoassay (IMx system; Abbott Laboratories, North Chicago, IL). The coefficient of variation (CV) for intra- and interassay of cobalamin were below 5 %. The intra-assay CVs of the folate assay remained between 3 % and 6 % and the interassay CVs ranged between 6 % and 10 % depending on the folate concentration. MMA was measured by capillary electrophoresis, with intra- and interassay coefficients of variation below 12 % at low physiological concentrations [16]. Concentrations of total homocysteine (tHcy) were assayed by a method based on HPLC and fluorescence detection, with a between-day CV < 4 % [17]. 1,25-dihydroxyvitamin D (1,25(OH)₂D) was measured by immunoassay (IDS, Bolden Business Park, Bolden, UK). Intra- and interassay coefficients of variation were 5–8 % and 9 % respectively.

■ Lifestyle parameters

Current calcium intake (mg/d) was estimated using a validated food frequency questionnaire [18], to which several questions were included regarding non-dairy sources of calcium that are present in a macrobiotic diet. The reference period of the questionnaire was one month before, and food intake was estimated in terms of standardized household portion sizes. Daily calcium intake was computed using the values from the Dutch Food Composition Table released in 1993 [19].

Physical activity was assessed by asking every subject about the time spent on physical activity (sports) during and after the school time. The total number of minutes spent per week on sporting activities was calculated.

■ Statistical analyses

Means and standard deviations (SD) were calculated for demographic data and lifestyle factors. For serum cobalamin, MMA, tHcy and folate concentrations, geometric

means (GM) and 5th and 95th percentiles were calculated from lognormal transformed data because of skewed distributions. Differences in demographic data, lifestyle factors, biochemical and bone composition variables between the macrobiotic and control group (separately for boys and girls) were investigated using the two-sample Student's t test.

The 25th percentile of the control subjects was used as cut-off for definition of 'low' or 'normal' total body BMC and 'low' or 'normal' total body BMD. Analysis of covariance (MANCOVA) was performed to calculate adjusted means of vitamin B₁₂, MMA and Hcy for the low and normal BMC and BMD groups. Sex, weight, height, percent lean body mass, age and (stage of) puberty were added as covariates. Calcium intake was added as a covariate to the regression models as well, since it was an independent predictor of total BMD. Bone area was also added to the regression model to calculate the adjusted means for the different BMC groups. Boys (n = 76) and girls (n = 91) were analyzed together because there was no interaction between the variables of interest and sex. Also no different results between boys and girls were observed when the data were analyzed separately instead of together with sex as a covariate.

To exclude a possible group effect, separate MANCOVA analyses were also performed for the macrobiotic cohort alone.

The investigated associations were corroborated with multiple regression analyses (SAS GLM procedure). Regression models were constructed with (continuous) BMD as the dependent variable; weight, height, percent lean body mass, calcium intake, age and (stage of) puberty (treated as a discrete variable) were added simultaneously as covariates; and serum concentrations of cobalamin and MMA as the (independent) variables of interest. These multiple regression analyses were also used in order to know which variables were needed to adjust for in the MANCOVA analysis.

Data were analyzed using SAS system release 8.0 (SAS Institute Inc., Cary, NC, USA). In all analyses, a probability of 0.05 was considered significant.

Results

Descriptive characteristics, lifestyle parameters, and blood and bone parameters for macrobiotic and control subjects are shown in Table 1. Macrobiotic boys were on average one year older than the control boys. Yet, weight was similar for macrobiotic and control adolescents, whereas %lean body mass was significantly higher in macrobiotic adolescents than in the control adolescents. Socio-economic status and physical activity level were similar for all groups. In both sexes, calcium intake was significantly lower in macrobiotic than in control subjects. Serum cobalamin concentrations were signifi-

Table 1 Characteristics of Dutch adolescents participating in a study on cobalamin status and bone mass^{1,2}

	Boys		Girls	
	Macrobiotic (n = 39)	Control (n = 39)	Macrobiotic (n = 37)	Control (n = 55)
Age (y)	12.7±2.2 ^a	11.7±1.5	11.9±1.6	11.7±1.7
Height (m)	1.57±0.14	1.53±0.12	1.51±0.12	1.52±0.11
Weight (kg)	42.7±11.4	41.2±9.2	38.9±9.4	41.3±8.6
% lean body mass	84.4±3.0 ^c	79.4±7.5	78.2±5.3 ^b	74.2±6.9
Pubertal stage ⁴	2.6±1.4	2.3±1.1	2.2±1.3	2.4±1.2
Physical activity (min/wk)	280±148	279±94	234±165	260±102
Socio-economic status	1.9±0.5 ^a	2.3±0.8	2.2±0.7	2.3±0.8
Calcium intake (mg/d)	665±421 ^{c,5}	1056±380	518±321 ^{c,5}	1030±348 ⁵
Cobalamin (pmol/l) ³	212 (103–370) ^c	484 (259–813)	286 (115–580) ^c	458 (219–850)
MMA (µmol/l) ³	0.30 (0.11–0.94) ^c	0.15 (0.05–0.34)	0.25 (0.09–0.76) ^c	0.17 (0.07–0.28)
Folate (nmol/l) ³	18.0 (9.5–26.0) ^b	14.7 (8.4–22.0)	18.7 (10.0–27.0) ^c	14.5 (9.1–26.0)
Total homocysteine (µmol/l) ³	8.3 (5.3–15.5) ^b	7.0 (4.2–10.8)	7.6 (4.7–16.7)	7.2 (4.6–14.5)
Vitamin D (pmol/l)	138±40	133±40	152±31	149±44
Total body BMD (g/cm ²)	0.97±0.09	0.98±0.06	0.93 ^a ±0.09	0.97±0.08
Total body BMC (g)	1770±554	1681±418	1510±432	1633±395

¹ Unless otherwise indicated values are mean ± SD of raw data

² Two-sample Student's t test: Significance of difference between macrobiotic and control subjects (separately for boys and girls): ^a p < 0.05, ^b p < 0.01, ^c p < 0.001

³ Values are geometric mean (P5–P95); percentiles estimated from lognormal distribution

⁴ Range pubertal stage (1 to 5)

⁵ n = 34, 34 and 54, respectively for macrobiotic boys and girls, and control girls

cantly lower, and concentrations of MMA and folate were significantly higher in macrobiotic subjects compared to controls. Total homocysteine concentrations were only significantly higher in macrobiotic boys as compared to controls. Serum 1,25-dihydroxyvitamin D showed no significant differences between the groups.

Table 2 shows that the adjusted mean (SD) of serum vitamin B₁₂ concentration for the subjects in the group with low total body BMD was significantly lower (p = 0.0035) than the adjusted mean for the subjects in the normal BMD group, 344 (24) pmol/L vs. 442 (18) pmol/L, respectively. After dividing the subjects into two groups of total body BMC, the adjusted means of vitamin B₁₂ concentration were again significantly different between the low and normal BMC (p = 0.0038). More-

over, the adjusted mean MMA concentration was higher for the subjects in the low BMD group than in the normal BMD group (p = 0.0003). Adjusted mean concentration of MMA was not significantly different between the low and normal BMC groups (p = 0.32). Also, no significant differences in Hcy values were found between the low and normal BMD (p = 0.25) or BMC (p = 0.59) groups (data not shown).

Table 2 also shows the adjusted means of vitamin B₁₂ and MMA for the macrobiotic group alone. The mean (P5, P95) MMA concentrations were significantly different (p = 0.0148) between the low and normal total body BMD, 0.40 (0.32, 0.49) and 0.22 (0.13, 0.31) µmol/L, respectively.

Results of multiple regression analyses demonstrated

Table 2 Adjusted mean (SD or P5, P95) concentrations of vitamin B₁₂ and MMA for different groups of BMD and BMC in omnivorous- and previously macrobiotic-fed Dutch adolescents (n = 161) and separately for previously macrobiotic-fed Dutch adolescents (n = 68)¹

	Low BMD	Normal BMD	Low BMC	Normal BMC
All subjects, n	60	101	50	111
Vitamin B ₁₂ (pmol/L)	344 (24)*	442 (18)	323 (31)*	442 (18)
MMA (µmol/L)	0.31 (0.26, 0.35)*	0.20 (0.16, 0.23)	0.26 (0.21, 0.32)	0.22 (0.19, 0.26)
Only macrobiotic-fed adolescents, n	36	32	28	40
Vitamin B ₁₂ (pmol/L)	269 (23)	279 (25)	286 (32)	266 (24)
MMA (µmol/L)	0.40 (0.32, 0.49)*	0.22 (0.13, 0.32)	0.26 (0.14, 0.39)	0.35 (0.26, 0.45)

¹ Adjusted for: bone area (only in BMD groups), weight, height, percent lean body mass, age, puberty, sex, total physical activity and calcium intake

* The concentration of vitamin B₁₂ or MMA is statistically different between the low and normal BMC or BMD (p < 0.05)

that serum MMA was inversely associated with BMD at total body ($\beta = -0.018$, $p = 0.02$), after adjusting for weight, height, %LBM, age, puberty, calcium intake and sex. No significant associations of serum cobalamin or tHcy with bone mineral density were observed, but there was a tendency of a cobalamin association with total body BMD ($\beta = 0.018$, $p = 0.06$) as was the case for MMA.

In the separate regression analysis of the macrobiotic adolescents a significant inverse relation was observed between serum MMA and total body BMD ($\beta = -0.025$, $p = 0.04$), after adjusting for weight, height, %LBM, age, puberty, calcium intake and sex. No associations between cobalamin or tHcy with BMD were observed.

Similar results were found for the association between MMA and total body BMC ($\beta = -0.017$, $p = 0.02$) and also between vitamin B12 and total body BMC ($\beta = 0.016$, $p = 0.06$).

Discussion

The present study shows that serum cobalamin concentrations were significantly lower and MMA concentrations significantly higher in previously macrobiotic-fed adolescents as compared to the controls. Moreover, irrespective of the dietary group, adjusted vitamin B₁₂ concentrations were significantly lower in subjects of the low BMD or BMC group compared to subjects of the normal BMD or BMC group. Adjusted MMA concentrations were significantly higher in the low BMD group than in the normal BMD group.

Most studies only take the bone mineral density into account when investigating bone health or osteoporosis. Analyzing BMC as a dependent variable is preferred above the use of bone mineral density (BMD, g/cm²) or bone mineral area density (BMAD, g/cm³) by some groups because no assumptions are made about the relationships between BMC and BA, and potential size-related artefacts are avoided [20]. The similarity in associations found between vitamin B₁₂ or MMA with BMC, strengthen our findings of the associations with BMD.

The association between MMA and total body BMD remained present in the separate analysis of macrobiotic subjects (boys and girls), whereas this association was not found in control subjects. This leads to the suggestion that the observed association was confined to the vitamin B12 status and not concomitant to other characteristics of the macrobiotic group. We assume that other dietary and lifestyle factors were similar for all macrobiotic-fed adolescents. This implies that these and other (unknown) factors that may be different between macrobiotic-fed adolescents and control subjects cannot disturb the association between MMA and BMD. The association between MMA and BMD in the total study group is mostly driven by the macrobiotic subjects

in whom impaired cobalamin status was prevalent. Our results with respect to cobalamin status and bone mass are in line with earlier published data [2, 3, 7].

Remarkably, the concentrations of vitamin B₁₂ and MMA in the separate analysis of macrobiotic subjects were somewhat more favorable in the low BMC group than in the normal BMC group. The nonexpected reversed concentrations of vitamin B₁₂ and MMA in the low and normal macrobiotic groups were probably only found coincidentally due to the low number of subjects, and furthermore, these concentrations were not significantly different from each other.

We have previously reported [12] that a substantial number of the formerly strict macrobiotic-fed adolescents still showed signs of an inadequate cobalamin status, even after they had changed their diet. About 41 % of the macrobiotic subjects had cobalamin values below the 5th percentile of the control group (< 229 pmol/l). Similarly, 41 % of the macrobiotic subjects had serum MMA concentrations above the 95th percentile of the control group (> 0.29 μ mol/l). The inadequate cobalamin status is most likely the result of a low present dietary cobalamin intake in combination with low body stores of cobalamin in general as a result of long-term insufficient cobalamin supply during pregnancy and childhood. The macrobiotic diet is known to contain only very small amounts of cobalamin. Although our formerly strict macrobiotic-fed subjects had changed their diet at an average age of 6 years, the amounts of cobalamin supplied by the modified diet were obviously not sufficient to restore a normal cobalamin status in later life.

To keep sufficient power we did not perform gender-specific analyses of our data. The serum cobalamin concentrations were higher in girls than in boys, which could explain why elevated MMA concentrations were less common in girls than in boys. Serum MMA is assumed to be a better functional indicator of cobalamin status than serum cobalamin concentration itself [21], which may explain the lack of associations between BMD and serum cobalamin in our data. However, a larger sample size would have most likely given significant associations.

As opposed to MMA, not only vitamin B12, but several B-vitamin deficiency states and a great number of confounders may influence homocysteine concentrations [22]. The vegetarian diet is rich in folate and low in methionine. We have also observed higher serum folate concentrations in the macrobiotic-fed adolescents than in the controls. These factors may obscure potentially existing interactions between homocysteine and low cobalamin intake. Although there is a strong correlation between homocysteine and cobalamin concentrations [23], homocysteine status may be a less strong functional indicator of cobalamin status in vegetarians, as the homocysteine status is influenced by the intake of

folic acid and methionine. Thus, an association of homocysteine status with bone mass may be less likely found in people following a vegetarian diet.

We did not observe an association between vitamin D status and BMD. This may be due to similar vitamin D concentrations for macrobiotics and controls and because the production of 1,25-dihydroxyvitamin D is under tight feedback control [24, 25]. Therefore, 25-hydroxyvitamin D would have been a more reliable indicator of vitamin D status. Also, in contrast to cobalamin status, it is possibly easier to restore vitamin D status. In our first study of these macrobiotic children we frequently observed vitamin D deficiency at the age of 6–18 mo [26]. This vitamin D deficiency could have had a negative influence on BMC and BMD in earlier childhood and it is possible that the catch-up growth of BMC and BMD is slower than that of the vitamin D status.

The mechanism of cobalamin-dependent changes in bone mass was investigated in 1964 by Van Dommelen and Klaassen [27] and by Carmel et al. [1]. Van Dommelen and Klaassen reported that serum concentrations of total alkaline phosphatase were lower in patients with cobalamin deficiency than in matched controls; these values rose after cobalamin replenishment. Carmel et al. [1] demonstrated that serum concentrations of skeletal alkaline phosphatase and osteocalcin were decreased in cobalamin-deficient patients and returned to normal after therapy with vitamin B₁₂. Osteocalcin is a marker of osteoblast activity and alkaline phosphatase is a marker for bone formation. Furthermore, *in vitro* studies of calvarial cells from chicken embryos showed that the alkaline phosphatase content was cobalamin-dependent [1]. These findings indicate that osteoblast function depends on an adequate supply of cobalamin.

The strengths of our study are the use of a combina-

tion of two biochemical markers (cobalamin and MMA) to measure cobalamin status, and the adjustment for important covariates like height, weight, percent lean body mass, age, puberty and calcium intake. In addition, a separate analysis was carried out for the macrobiotic group alone to exclude influence of other group effects. This implies that other dietary and lifestyle factors that can differ between macrobiotic and control subjects, such as vitamin D status, fiber intake, and physical activity, are unlikely to have confounded the association between cobalamin status and bone mass.

In conclusion, our data suggest that signs of low functional cobalamin status are associated with low bone mineral density especially in adolescents who were fed a strict macrobiotic diet during the first years of life. These findings do not give a clarification of cause and consequence of the low vitamin B₁₂ status and low BMD and BMC. A causal relation may nevertheless exist between vitamin B₁₂ status and bone health in these adolescents, as the low vitamin B₁₂ status is by and large a prolonged condition and might consequently be a causal factor for low bone health. Further research is strongly advocated to study whether these results can be extrapolated to the elderly population. We have recently shown an association of vitamin B₁₂ status with bone mineral content and bone mineral density in frail elderly women [14] and an association between homocysteine status with fractures in a Dutch population [28] which was corroborated in an American study [29]. It is important to study the causality of these associations.

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