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ORIGINAL CONTRIBUTION

The association between high plasma homocysteine levels and lower bone mineral density in Slovak women: the impact of vegetarian diet

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Abstract
Background A long-term vegetarian diet is generally poor in vitamin B group. The lack of vitamin B_{12} together with vitamin B_6 and folate deficiency is closely related to homocysteine metabolism. Hyperhomocysteinemia was found to be associated with increased bone turnover markers and increased fracture risk. Thus, hyperhomocysteinemia, vitamin B_{12} and folate deficiency may be regarded as novel risk factors for micronutrient deficiency-related osteoporosis.
Aim of the study To assess the possible impact of a vegetarian diet on bone mineral density in cohort of Slovak vegetarian women.
Methods Fasting serum glucose, albumin, calcium, phosphorous and creatinine as well as bone markers, serum vitamin B_{12}, folate and plasma levels of total homocysteine were assessed in two nutritional groups (vegetarians vs. nonvegetarians) of apparently healthy women (age range 20–70 years). Bone mineral density of the femoral neck, trochanter, total femur and lumbar spine was measured in all subjects.
Results Vegetarians had a significantly lower weight ($p < 0.05$), higher PTH ($p < 0.01$) and homocysteine ($p < 0.001$). Vitamin B_{12} was significantly higher in nonvegetarians ($p < 0.001$). No differences were observed in folate levels. Univariate analysis showed significant association between homocysteine and B_{12} ($p < 0.01$), folate ($p < 0.001$), creatinine ($p < 0.001$), total proteins ($p < 0.049$), age ($p < 0.001$) and vegetarian food intake ($p < 0.001$). Vegetarians had a significantly lower TrFBMD ($p < 0.05$) and ToFBMD ($p < 0.05$). Age and CTx were significant predictors in all sites of measured BMD and PTH. A strong correlation between homocysteine and FNBMD ($r = -0.2009$, $p < 0.002$), TrFBMD ($r = -0.1810$, $p < 0.004$) and ToFBMD ($r = -0.2225$, $p < 0.001$) was found in all subjects.
Conclusion Homocysteine is one of the predictors of bone mineral density, and hyperhomocysteinemia is associated with lower bone mineral density. In healthy adults, homocysteine levels are dependent on age as well as on nutritional habits. Thus, elderly women on a vegetarian diet seem to be at higher risk of osteoporosis development than nonvegetarian women.

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Keywords Homocysteine · Folate · Vitamin B_{12} · Bone mineral density · Vegetarian diet

Background

Osteoporosis is a complex multi-factorial condition characterized by reduced bone mass and impaired microarchitectural structure, leading to an increased susceptibility to fractures. Several factors are thought to influence bone mass. These can be broadly grouped into factors that cannot be modified, such as gender, age, body (frame) size, genetics, and ethnicity; and those factors that can be modified, such as hormonal status (especially sex and calciotropic hormone status), lifestyle factors including physical activity levels, smoking and alcohol consumption patterns, and diet. Nutrition plays an important role in the acquisition and maintenance of skeletal integrity. The vegetarian lifestyle has become very popular in recent years. There is some evidence that a well-balanced vegetarian diet may be consistent with good health and can potentially reduce the risk of cardiovascular disease, hypertension, diabetes, cancer and some other chronic diseases [28, 30]. On the other hand, people following a long-term vegetarian diet very often suffer from nutrient shortage like calcium, phosphorous, iron, zinc, vitamin D or vitamin B_{12} [9, 31]. The lack of vitamin B_{12} together with vitamin B_6 and folate deficiency is closely related to homocysteine (Hcy) metabolism. Hyperhomocysteinemia was found to be associated with increased bone turnover markers [6] and increased fracture risk [17, 20, 25, 32]. Thus, homocysteine, vitamin B_{12} and folate may be regarded as novel risk factors for micronutrient deficiency-related osteoporosis [14]. Most of the individuals examined in these studies were postmenopausal women. High Hcy plasma levels in elderly people are due to age-related decline of renal function as well as low vitamin B status. In the elderly, high prevalence of atrophic gastritis results in less absorption of vitamin B_{12} with consequent vitamin B_{12} deficiency [33]. Unlike them, vegetarians suffer from deficiency of vitamin B and increasing plasma Hcy independent of age as a result of low intake of animal food.

In contrast to fracture risk, the association between Hcy and BMD is still in doubt [5–7, 20, 21, 32]. In addition, we did not find any work in the literature, where the relationship between Hcy and BMD is evaluated directly in vegetarians. The majority of authors dealing with the questions of bone metabolism in vegetarians focus mostly on possible insufficiency of several nutrients such as vitamin D, Ca or animal proteins.

The aim of our study was to assess the possible impact of a vegetarian diet on bone mass density in a cohort of Slovak vegetarian women. We hypothesized that increased concentrations of homocysteine in vegetarian women can significantly increase the risk of decreased BMD, especially in older women, compared with nonvegetarian women.

Methods

The study was performed on a group of 141 women on long-term ovo-lacto-vegetarian diet and a control group of 131 women on standard western diet. All women were apparently healthy non-smokers with normal weight, similar physical load and living standard. Both groups were age matched. The study was carried out during spring. No supplementation of vitamins, mineral and trace elements was allowed. The Regional Ethics Committee approved the study, and all participants gave their written informed consent.

Fasting blood samples were taken for the measurements of serum biochemical parameters. Serum glucose (Glu), albumin (Alb), calcium (Ca), phosphorous (P) and creatinine (Cr) concentrations were assessed by a standard laboratory method on Vitros 250 autoanalyzer (Johnson & Johnson, NY, USA). Parathormone (PTH; Immunotech, France) was determined by RIA method with intra-assay variability 7.5% and interassay variability 11%. The detection limit was 2 pg/ml. Measurement of osteocalcin (OC) was performed by sandwich immunoradioassay (OSTEO-RIACT, Cis bio international, France) with intra-assay variability 1.2–2.8%, inter-assay variability 3.6–5.2% and detection limit 0.4 ng/ml. C-terminal telopeptides of type-I collagen (CTx) were determined by using commercial competitive enzyme immunoassay (Serum CrossLaps ELISA, Nordic Bioscience Diagnostic A/S, Denmark). The intra-assay and inter-assay variability of CTx were 6.5–8.1 and 5.0–5.4%, respectively. The detection limit was 0.02 ng/ml. Serum vitamin B_{12} and folate were determined using Elecsys 2010 System (Roche Diagnostics, Switzerland). The intra-assay and inter-assay variability of vitamin B_{12} were 3.0–8.7 and 3.7–9.4%, respectively. The detection limit was 22 pmol/l. The intra-assay variability of folate was 3.0–7.0% and inter-assay variability was 5.0–13.3%. The detection limit was 1.45 nmol/l. Plasma levels of total homocysteine (Hcy) was measured by HPLC method [15]. The intra-assay and inter-assay variability were <3 and <4%, respectively with detection limit 1.0 μmol/l.

Bone mineral density (BMD) of the femoral neck (NFBMD), trochanter (TrFBMD), total femur (ToFBMD) and lumbar spine (L_{1-4}BMD) was measured in all probands by dual-energy X-ray absorptiometry (DEXA) using a

Lunar DPX-L bone densitometer (Lunar Co., Madison, WI, USA). BMD was expressed in absolute values (g/cm^2).

Data analysis

The statistical evaluation was performed using Statistical Package for the Social Sciences software (SPSS 12.0, Chicago, IL, USA). The distribution of investigated variables were estimated using Kolgomorov–Smirnov test. For descriptive purposes, the quantitative data is presented as mean ± SD. The significance of differences was determined by unpaired Student's t test, two tailed. The univariate linear model was used to calculate the predictors of Hcy concentrations. The stepwise multiple regression model was applied to evaluate the relationship between BMD in all measured sites and various variables. The relationship between serum values of Hcy concentrations and BMD were investigated by linear regression analysis and Pearson's correlation coefficient (p). p values < 0.05 were considered to indicate statistical significance.

Results

Demographics and clinical characteristics of studied women are summarized in Table 1. Probands were divided into two groups according to nutritional habits. Vegetarians did not differ from nonvegetarians in age, height and BMI, but their weight was found to be significantly lower ($p < 0.05$). Basic biochemical parameters were in normal range in all study probands with no differences between separate groups. CTx and OC levels were similar in both groups. Vegetarian women had a significantly higher PTH ($p < 0.01$).

Homocysteine concentrations were beyond the normal range in both groups (<12.0 μmol/l) but significantly higher in vegetarians ($p < 0.001$). Hyperhomocysteinemia was found in 78% of vegetarians and in 48% of nonvegetarians. The mean vitamin B_{12} levels were in normal range in both groups (>220 pmol/l) with significantly higher concentrations in nonvegetarians ($p < 0.001$). Up to 48% of vegetarians and 28% of nonvegetarians did not reach a normal value of plasma vitamin B_{12}. Folate concentrations were in normal range with no significant differences between groups. Results from univariate analysis show the variables associated with plasma homocysteine (Table 2). Homocysteine was significantly associated with vitamin B_{12} ($p < 0.01$), folate ($p < 0.001$), creatinine ($p < 0.001$), total proteins ($p < 0.049$), age ($p < 0.001$) and a vegetarian diet ($p < 0.001$).

The mean BMD values of the femoral neck (NFBMD), femoral trochanter (TrFBMD), total femur (ToFBMD) and lumbar spine ($L_{1–4}$BMD) are given in Table 3. Vegetarians had a significantly lower TrFBMD ($p < 0.05$) and ToFBMD ($p < 0.05$) and did not differ in NFBMD and $L_{1–4}$BMD. A stepwise multiple regression analysis was performed to determine the main predictors of the femur and lumbar spine BMD. (Table 4). Age and CTx were significant predictors in all sites of measured BMD, PTH in

Table 1 Clinical and biochemical characteristics of the subjects (means ± SD)

	Vegetarians	Nonvegetarians	$p<$
N	141	131	
Age (years)	41.9 ± 19.7	40.8 ± 19.8	NS
Age range (years)	20-70	20-70	
Height (m)	165.3 ± 7.0	165.7 ± 6.6	NS
Weight (kg)	62.8 ± 10.6	67.1 ± 14.2	0.05
BMI (kg/m^2)	23.9 ± 4.7	25.0 ± 5.8	NS
Glu (mmol/l)	5.4 ± 0.8	5.5 ± 0.7	NS
Alb (mmol/l)	44.5 ± 2.7	44.8 ± 3.3	NS
Total protein (g/l)	77.7 ± 4.8	78.7 ± 4.3	NS
Ca (mmol/l)	2.34 ± 0.12	2.36 ± 0.16	NS
P (mmol/l)	1.17 ± 0.15	1.14 ± 0.16	NS
Creatinine (μmol/l)	77.4 ± 10.4	81.7 ± 14.7	NS
CTx (ng/ml)	0.66 ± 0.34	0.61 ± 0.25	NS
OC (ng/ml)	26.2 ± 10.6	26.1 ± 8.8	NS
PTH (pg/ml)	52.1 ± 34.0	42.9 ± 23.5	0.01
Homocysteine (μmol/l)	16.5 ± 5.6	12.5 ± 4.5	0.001
Hyperhomocysteinemia (%)	78	45	
Vitamin B_{12} (pmol/l)	246.9 ± 161.3	301.6 ± 137.4	0.001
Vitamin B_{12} deficiency (%)	47	28	
Folate (μg/l)	23.8 ± 10.4	23.7 ± 8.6	NS
Folate deficiency (%)	2	2	

Table 2 Univariate model of factors associated with plasma homocysteine

Dependent variable: Hcy					
Source	Type III sum of squares	df	Mean square	F	Sig.
Model	58,995.37	7	8,427.91	402.20	0.000
Vitamin B_{12}	140.45	1	140.45	6.70	0.010
Folate	354.93	1	354.93	16.94	0.000
Creatinine	279.55	1	279.55	13.34	0.000
Total protein	81.61	1	81.61	3.89	0.049
Age	321.32	1	321.32	15.33	0.000
Veg/nonveg	1,116.54	2	558.27	26.64	0.000
Error	5,511.09	263	20.95		
Total	64,506.4596	270			

$R^2 = 0.915$ (adjusted $R^2 = 0.912$)

Table 3 Comparison of BMD between vegetarians and nonvegetarians (means ± SD)

	Vegetarians	Nonvegetarians	$p<$
NFBMD (g/cm^2)	0.9182 ± 0.1419	0.9409 ± 0.1358	NS
TrFBMD (g/cm^2)	0.7456 ± 0.1229	0.7744 ± 0.1161	0.05
ToFBMD (g/cm^2)	0.9574 ± 0.1370	0.9916 ± 1272	0.05
$L_{1\text{-}4}$BMD (g/cm^2)	1.0845 ± 0.1916	1.1026 ± 0.1589	NS

TrFBMD and ToFBMD, Hcy in TrFBMD and ToFBMD and serum creatinine in $L_{1\text{-}4}$BMD. Homocysteine significantly correlated with FNBMD ($r = -0.2009$, $p < 0.002$), TrFBMD ($r = -0.1810$, $p < 0.004$) and ToFBMD ($r = -0.2225$, $p < 0.001$) in all groups, after the selection of vegetarians and nonvegetarians, significant correlations were confirmed only in nonvegetarian women (Table 5). No significant correlations were found between vitamin B_{12} and BMD as well as between folate and BMD.

Discussion

Hyperhomocysteinemia (HHcy) is a condition of elevated plasma Hcy concentration. It is a sensitive functional marker of inadequate cellular folate and vitamin B_{12} concentrations. In the general population, mild HHcy is a common condition. Higher levels of plasma homocysteine are caused either by genetic defects in the enzymes involved in homocysteine metabolism (cystathionine β-synthase, MTHFR) or by nutritional deficiencies in vitamin cofactors. It has been speculated that deficiencies of B-group vitamins contribute to approximately two-thirds of all cases of HHcy [27]. In our study, 45% of controls and up to 78% of vegetarian women were found to attain homocysteine concentrations above the 12 µmol/l cutoff [29]. Twenty-eight percent of controls and 47% of vegetarian women suffered from vitamin B_{12} deficiency. This data is similar to those of Majchrzak et al. [18] and Koebnick et al. [16]. HHcy is age dependent and in elderly people is also related to age-dependent decline in renal function [24]. Our results confirmed a strong association between Hcy and variables including vitamin B_{12}, folate, age, creatinine and vegetarian diet.

HHcy is recognised as an important marker for cardiovascular risk [19] as well as for cognitive impairment [30]. Recently, homocysteine has been linked to be a potential risk factor for osteoporosis. This hypothesis was suggested from the observation of a high prevalence of osteoporosis in patients with homocystinuria [8, 22]. Most of published data is concerned with the relationship between elevated Hcy values and higher prevalence of fracture risk, even though some of them are contradictory [17, 20, 23, 32]. In an Iranian study with 271 postmenopausal women, Golbahar et al. [7] found a significant negative association of plasma Hcy with BMD at both femoral neck and lumbar spine and a positive association of plasma folate with BMD at the femoral neck. These associations remained significant when adjusted for age, BMI and creatinine. Gerdhem et al. [5] reported significant associations between high Hcy, high bone marker levels and low BMD at the femoral neck and trochanter in 996 women 75 years old. The negative association of Hcy with BMD was also confirmed in the study of Elshorbagy et al. [4] on 5,238 men and women in two groups aged 40–42 and 65–67 years as well as in the study of Bozkurt et al.[2] in Turkish postmenopausal women. These results are inconsistent with those of van Meurs et al. [32] and Herrmann et al. [10]. They reported that neither hip nor lumbar spine DXA results were associated with Hcy levels. An association between low vitamin B_{12} and low BMD was also reported [3, 7, 21]. Our study showed that Hcy, together with age, CTx and PTH, is the main predictor of BMD in both trochanter and total femur. Comparing all measured sites, vegetarians had a significantly lower TrFBMD and ToFBMD. Linear regression analysis confirmed the negative correlation between Hcy and FNBMD, TrFBMD and ToFBMD in vegetarians, but not in nonvegetarians. To our knowledge, there is a lack of data confined to the direct relation of Hcy and BMD in vegetarians. There is only one study on the relationship of vitamin B_{12} and BMD in adolescents formerly fed a macrobiotic diet [3]. In those probands, signs of an impaired cobalamin status, as judged by elevated concentrations of methylmalonic acid, were associated with low BMD.

It is not clear whether homocysteine influences the bone directly or if it only reflects the action of its cofounders like B vitamins. Herrmann et al. [11] found a strong accumulation of Hcy in bone tissue of hyperhomocysteinemic rats that was accompanied by significant bone loss and reduction of bone strength. This confirms the hypothesis that HHcy has direct detrimental effects on the bone. The majority of Hcy in bone tissue was bound to extracellular collagen. Hcy is known to disturb enzymatic collagen crosslinking by the inhibition of lysyl oxidase [26]. Blouin et al. [1] reported a significant correlation between plasma Hcy levels and collagen crosslinks ratio in bone forming areas in elderly women. This was independent of the bone mineral content and/or bone mineral distribution pattern. Recently, Herrmann et al. [13] has found that increased Hcy concentrations stimulate human osteoclast activity in vitro, suggesting a mechanistic role of Hcy for bone resorption. Herrmann et al. also found moderate stimulation of primary human osteoblast activity by increasing concentrations of Hcy. However, the magnitude of this effect seems to be less

Table 4 Predictors of BMD: stepwise multiple regression analysis

Dependent variable	Standardized coefficient		Standardized coefficient Beta	*t*	Sig.
	B	SE			
NFBMD					
Constant	1.143	0.150		7.634	0.000
Age	−0.004	0.000	−0.588	−9.526	0.000
Vitamin B_{12}	0.000	0.000	−0.040	−0.733	0.464
Folate	0.000	0.001	−0.028	−0.516	0.606
Hcy	−0.002	0.001	−0.093	−1.652	0.100
PTH	0.000	0.000	0.104	1.756	0.080
CTx	−0.086	0.027	−0.172	−3.223	0.001
Ca	0.004	0.064	0.003	0.057	0.955
Cr	0.000	0.001	0.039	0.688	0.492
TrFBMD					
Constant	0.901	0.152		5.945	0.000
Age	−0.001	0.000	−0.209	−2.908	0.004
Vitamin B_{12}	0.000	0.000	−0.052	−0.813	0.417
Folate	−0.001	0.001	−0.047	−0.745	0.457
Hcy	−0.004	0.001	−0.178	−2.705	0.007
PTH	0.001	0.000	0.140	2.037	0.043
CTx	−0.073	0.027	−0.168	−2.709	0.007
Ca	−0.035	0.065	−0.036	−0.540	0.590
Cr	0.001	0.001	0.123	1.892	0.060
ToFBMD					
Constant	1.210	0.159		7.598	0.000
Age	−0.003	0.000	−0.404	−5.899	0.000
Vitamin B_{12}	0.000	0.000	−0.010	−0.165	0.869
Folate	0.000	0.001	−0.032	−0.533	0.595
Hcy	−0.003	0.002	−0.134	−2.140	0.033
PTH	0.001	0.000	0.152	2.313	0.022
CTx	−0.099	0.028	−0.207	−3.500	0.001
Ca	−0.039	0.068	−0.036	−0.566	0.572
Cr	0.001	0.001	0.069	1.112	0.267
L_{1-4}BMD					
Constant	1.518	0.202		7.496	0.000
Age	−0.004	0.001	−0.482	−7.312	0.000
Vitamin B_{12}	0.000	0.000	−0.023	−0.400	0.689
Folate	0.000	0.001	−0.001	−0.015	0.988
Hcy	0.000	0.002	0.003	0.044	0.965
PTH	0.000	0.000	0.010	0.160	0.873
CTx	−0.150	0.036	−0.237	−4.155	0.000
Ca	−0.129	0.087	−0.090	−1.485	0.139
Cr	0.002	0.001	0.144	2.409	0.017

pronounced than his observations on osteoclasts, suggesting an imbalance between osteoblasts and osteoclasts in favour of osteoclasts [12].

In summary, this study has shown that in our population of healthy Slovak women, Hcy is one of the predictors of BMD and HHcy is associated with bone loss. Additionally, HHcy is much higher in women on a vegetarian diet than in nonvegetarians. These results are consistent with increasing experimental evidence for an effect of Hcy on bone metabolism and confirm the hypothesis that Hcy plays a role not only in the pathogenesis of osteoporotic fractures but also in lower bone mineral density. Thus, because of

Table 5 Correlation between Hcy and BMD at the neck femur, trochanter, total femur and lumbar spine

Dependent variable	Independent variable Hcy					
	All		Vegetarians		Nonvegetarians	
	r	*p*	*r*	*p*	*r*	*p*
NFBMD	−0.2009	0.002	−0.2167	0.02	−0.1492	NS
TrFBMD	−0.1810	0.004	−0.2195	0.02	−0.0721	NS
ToFBMD	−0.2225	0.001	−0.2406	0.01	−0.1292	NS
$L_{1\text{-}4}$BMD	−0.1018	NS	−0.0701	NS	−0.0584	NS

HHcy, elderly women on a vegetarian diet seem to be at higher risk of low bone mineral density than nonvegetarian women.

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