Relationship of homocysteine levels with lumbar spine and femur neck BMD in postmenopausal women

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ABSTRACT

Objective: The focus of several studies in recent years has been the association between increased plasma concentrations of homocysteine (Hcy), reduced bone mineral density and increased risk of bone fractures. Nevertheless, inconsistencies persist in the literature. Thus, the objective of this study was to investigate the possible relationship between serum Hcy and vitamin B12 status, and bone mineral density, on a group of post-menopausal women.

Materials and methods: One hundred thirty-nine postmenopausal women were recruited to enter this cross-sectional study. Bone mineral density (BMD) of total hip, femoral neck and lumbar spine was measured by dual-energy X-ray absorptiometry (DXA) and serum Hcy, vitamin B12, parathyroid hormone (PTH), total calcium and magnesium levels were determined. In addition, we investigated the relationship of Hcy and vitamin B12 and BMD using a meta-analysis approach.

Results: Serum Hcy levels were significantly higher in osteoporotic women when compared to other BMD groups, and were inversely related to lumbar spine BMD and femur neck BMD. Body mass index and serum Hcy levels were shown to be significant predictors of BMD at lumbar spine, femur neck and total hip. The performed meta-analysis showed that serum Hcy levels were significantly higher in osteoporotic subjects compared to normal BMD subjects.

Conclusion: This study shows that Hcy status, but not vitamin B12 status, is associated with BMD in this cohort of postmenopausal women. We therefore confirm that high Hcy levels are an independent risk factor for osteoporosis. BMD evaluation in women at post menopause with high Hcy levels may be helpful in advising precautionary measures.

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INTRODUCTION

Osteoporosis, a major health problem in ageing population, is a chronic skeletal disorder characterized by reduction of bone mineral density (BMD) and compromised bone strength that leads to increased susceptibility to bone fractures[1]. Results from recent investigations show an association between increased plasma concentrations of homocysteine (Hcy) and reduced BMD and increased risk of bone fractures[2-10]. Actually, hyperhomocysteinemia (HHcy) has been considered as a new independent risk factor for osteoporosis and fractures, especially in the elderly[11,13]. Several mechanisms have been proposed to link HHcy with the pathogenesis of osteoporosis: reduction of bone formation by inducing apoptosis in human bone marrow stromal cells[12], osteoclastogenesis stimulation[13,14], bone blood flow reduction and consequently changes on bone biomechanical properties[15], decline in physical functioning[19], alteration of gene expression with reduced methylation capacity[20], oxidative damage on trabecular structures[21], and reduction of bone strength by interfering with collagen cross-links[5,22]. Despite this, there is continuing inconsistency concerning the relationship between Hcystatus and BMD, with some studies showing an association of high plasma concentrations of Hcywith low BMD[4,6,16,17,28] while other studies showed no significant association[3,5,8,30]. In general, plasma concentrations of Hcy are age-dependent with higher levels in elderly people,

especially in postmenopausal women[18].

Vitamin B12 is an important determinant of total Hcy status and may have an indirect impact on BMD via tHcy[4,9,28]. In addition, vitamin B12 may have direct impact on BMD by affecting osteoblasts activity[31]. Several studies have analyzed the relationship between vitamin B12 levels and BMD[3-5,9,28,33]. However, the results of these studies are at odds with each other.

The study main purpose of the study is to investigate a possible relationship between serum Hcy and vitamin B12 status and BMD on a group of post-menopausal women. In addition, we aimed to estimate the relationship of Hcy and vitamin B12 with osteoporosis risk using a meta-analysis approach.

MATERIALS AND METHODS

STUDY DESIGN AND SUBJECTS

The study was conducted at the University of Prishtina and Kosovo Occupational Health Institute between April 2013 and July 2013. One hundred thirty-nine postmenopausal women, aged between 41 and 65 years, were recruited prospectively to enter this cross-sectional study (Figure 1). The research protocol was in accordance with the Declaration of Helsinki and was approved by the local ethical committee. All the subjects provided their written informed consent before inclusion in the study. Postmenopausal women were defined as those whose menses had stopped more than

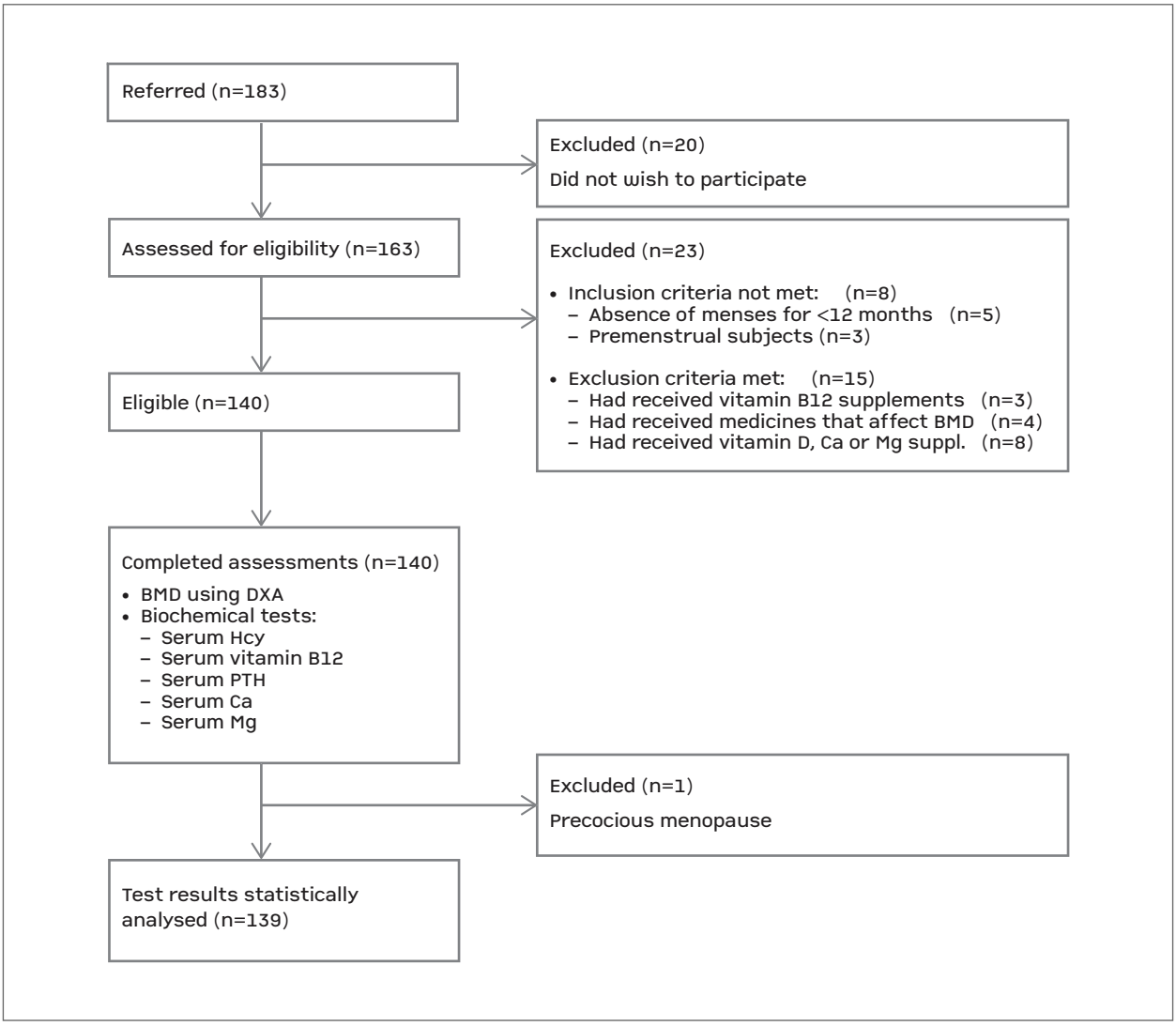

FIGURE 1. Flow chart of the study participants

1 year before inclusion in the study.

The exclusion criteria were: having received vitamin B12 and/or folic acid supplements or other medicines known to influence levels of Hcy or vitamin B12 or bone mineralization during the preceding one year (glucocorticoids, thiazide diuretics, anticoagulants, anticonvulsants, estrogen therapy); having received bisphosphonates, contraceptives, vitamin D or calcium, magnesium or iron supplements during the preceding six months. Patients were also excluded if they had metabolic bone diseases or endocrine abnormalities (other than diabetes mellitus), malabsorptive disorders, having neoplastic diseases during previous five years, chronic liver or renal diseases, severe cognitive impairment or severe psychological problems and if they were drug or alcohol abusers, or current smokers.

Weight and height were measured with a balanced scale and attached stadiometer (Seca model 700-220, Vogel & Halke Gmbh & Co., Hamburg, Germany) in light indoor clothing without shoes before performing DXA, and body mass index (BMI) was calculated as kilograms per square meters (kg/m^2).

BMD MEASUREMENT

Total hip, femoral neck and lumbar spine (L1-L4) BMD was measured in all subjects included in the study by dual-energy X-ray absorptiometry (DXA) using the same Hologic® DXA scanner (model QDR 4500) and was expressed in absolute values as grams of mineral content per square centimeters of bone area (g/cm^2). Quality control of the scanner was performed daily by measuring a phantom's density that showed stable results at the time of the study. Coefficients of variation of DXA measurements were 1%. Consistent with World Health Organization (WHO) classification system, osteoporosis is defined as T-score at least -2.5 standard deviations below the mean (≤ -2.5 SD) and osteopenia or low bone mass is defined as a T-score between -2.5 and -1.0 standard deviations below the mean.

BLOOD SAMPLING AND SERUM ANALYSES

Fasting blood samples for measurement of serum Hcy, vitamin B12, total calcium, magnesium, and parathyroid hormone (PTH) were drawn from the antecubital vein between 8 and 9 AM on the same day of the BMD measurements. Samples were centrifuged within one hour, and the separated serum was immediately stored at -20 ˚C until assayed.

Serum Hcy, total calcium and magnesium concentrations were determined using automated analyzer, COBAS Integra 400 Plus (Roche Diagnostics, Switzerland), while serum vitamin B12 and PTH concentrations were determined simultaneously using Electrochemiluminescence Immunoassay kits on the Elecsys 2010 system (Roche Diagnostics, Switzerland).

STATISTICAL ANALYSIS

Results of continuous variables are reported as means ± SD. Chi-square test was used to compare proportions (percentages). One-way ANOVA was used to analyze differences between groups for continuous variables followed with post hoc test. Pearson's correlation analysis was carried out to measure the association of BMD with the study variables.

Multiple regression analysis was performed to determine the relationship between BMD at different regions, age, BMI as well as serum levels of Hcy and vitamin B12.

Studies cited in this article were included in the meta-analysis if they met all of the following criteria: (1) used a cross-sectional, cohort or case-control design, (2) study participants were postmenopausal women, (3) evaluated the association between serum Hcy and/or vitamin B12 levels and BMD, (4) provided sufficient information on Hcy and/or vitamin B12 levels in osteoporotic patients and normal BMD participants, (5) BMD was measured by DXA, (6) the mean and standard deviation of Hcy and/or vitamin B12 levels were used to calculate the mean difference and 95% CI. Other cited studies in this article that observed or did not observe an association between serum Hcy and/or vitamin B12 and BMD were not included in the meta-analysis because necessary information for analysis was unavailable. Potential between-study heterogeneity of the studies included in the meta-analysis was assessed using Chi-square based Q test and the degree of heterogeneity was assessed using I^2 test, which was considered significant for I^2>50%. When the effects were assumed to be homogeneous (I^2<50%), the fixed-effects model was used to estimate the pooled mean differences and their corresponding 95% confidence interval (95% CI). Otherwise, the random-effects model was used. Begg's funnel plots were used to assess potential publication bias. Pooled weighted mean difference of Hcy and vitamin B12 in osteoporotic patients and normal BMD participants was assessed using the Z-test.

Statistical analysis was performed using SPSS software version 16 (SPSS Inc., Chicago, USA) and Comprehensive Meta-Analysis (CMA) software version 2 (Biostat, Inc., Englewood, USA). A p value of less than 0.05 was considered as statistically significant.

RESULTS

Clinical and biochemical features from the study population expressed in means ± SD with regard to the lowest T-score are summarized in Table I. According to the lowest T-score in any of the skeletal sites where BMD was measured, 49 subjects had osteoporosis, 57 had low bone mass and 33 had normal BMD values. While there were no differences between BMD groups regarding age (p=0.324), the mean BMI in normal BMD group was statistically higher than in osteoporosis group (p<0.001) and the mean duration of menopause was significantly longer in the osteoporosis group compared to the mean duration of menopause in low bone mass and normal BMD groups (p=0.001). The prevalence of HHcy (Hcy>15 µmol/l) in the study population was 33.09%. The subjects in osteoporosis group had significantly higher serum Hcy levels (16.84±11.71 vs. 13.95±2.98 and 13.09±2.53, respectively, p=0.040) and increased prevalence of HHcy when compared to the subjects in low bone mass and normal BMD groups (51% vs. 28.1% and 15.2%, respectively, p=0.002). There was no statistically significant difference between the groups in serum vitamin B12, PTH, total calcium and magnesium levels (p>0.05).

The results of Pearson's correlation analysis between BMD and the study variables are shown in Table II. Lumbar spine BMD, femur neck BMD and total hip BMD were positively correlated with BMI, while no significant correlations were found between BMD (at all sites where it was measured) and age. Serum levels of Hcy were inversely related to lumbar spine BMD (r= -0.163, p<0.05) and femur neck BMD (r= -0.164, p<0.05) whereas at total hip the association didn't reach statistical significance (r = -0.154, p>0.05). No significant associations were found between serum vitamin B12, PTH, total calcium and magnesium levels and BMD (Table II). Highly significant negative correlation between Hcy and vitamin B12 (r=-0.244, p<0.001) was found (data not shown in the table).

Multiple linear regression analysis in Table III shows the possible influence of age, BMI, serum Hcy and vitamin B12 levels in predicting BMD. According to β coefficients of age-adjusted linear regression analysis, main significant predictors of BMD at lumbar spine, femur neck and total hip were BMI and serum Hcy levels; age itself was a significant predictor at the femoral neck only.

Because there was a significant between-study heterogeneity in the meta-analysis of the association between Hcy and BMD (χ^2=42.92; df=5; p<0.001; I^2=88.35) the weighted mean difference (WMD) estimates of BMD (in g/cm^2) were calculated as DerSimonian and Laird estimators using random-effects model; the WMD for normal BMD versus osteoporosis was 0.826 (95% CI, 0.375-1.276).The forest plot of six

TABLE I. CLINICAL AND BIOCHEMICAL CHARACTERISTICS OF 139 POSTMENOPAUSAL WOMEN, DIVIDED ACCORDING TO THE LOWEST T-SCORE

	Total subjects (n=139)	Osteoporosis (n=49)	Osteopenia (n=57)	Normal (n=33)	p value
Age (years)	56.53±5.84	57.53±4.97	56.11±6.46	55.79±5.89	0.324
Weight (kg)	75.04±12.40	70.65±12.23	74.42±11.29	82.60±11.23	p<0.001
BMI (kg/m^2)	30.01±4.88	28.65±4.80	30.01±4.69	32.03±4.76	0.08
Duration of menopause (years)	8.73±6.24	11.22±6.83	7.82±5.65	6.58±5.11	0.001
Lumbar spine BMD (g/cm^2)	0.86±0.17	0.69±0.89	0.87±0.08	1.06±0.12	p<0.001
Femur neck BMD (g/cm^2)	0.74±0.12	0.66±0.91	0.73±0.08	0.88±0.10	p<0.001
Total hip BMD (g/cm^2)	0.86±0.14	0.78±0.12	0.85±0.09	0.99±0.13	p<0.001
Homocysteine (5.5-15 µmol/l)	14.77±7.43	16.84±11.71	13.95±2.98	13.09±2.53	0.040
HHcy (Hcy>15 µmol/l)	n=46 (33.09%)	n=25 (51%)	n=16 (28.1%)	n=5 (15.2%)	0.002
Vitamin B12 (191-663 pg/ml)	426.13±135.66	399.62±145.79	438.26±129.36	444.54±128.51	0.232
PTH (10-65 pg/ml)	53.52±56.12	67.10±90.09	46.14±18.25	46.08±19.71	0.108
Total Ca (2.25-2.75 mmol/l)	2.41±0.25	2.38±0.25	2.41±0.26	2.46±0.23	0.333
Mg (0.66-1.07 mmol/l)	0.82±0.09	0.81±0.10	0.82±0.09	0.83±0.09	0.655

BMD: Bone mineral density; BMI: Body mass index; HHcy: Hyperhomocysteinemia; PTH: Parathormone

TABLE II. PEARSON CORRELATION COEFFICIENTS BETWEEN BMD AND THE STUDY VARIABLES (R)

	Lumbar spine BMD (g/cm²)	Femur neck BMD (g/cm²)	Total hip BMD (g/cm²)
Age (years)	-0.040	-0.180*	0.012
Body mass index (kg/m²)	0.311**	0.429**	0.530**
Homocysteine	-0.163*	-0.164*	-0.152
Vitamin B12	0.028	0.109	0.050
PTH	-0.116	-0.079	-0.049
Total Ca	0.128	0.059	0.071
Mg	0.049	0.023	0.033

*p <0.05; **p<0.01

studies (Figure 2) with 821 participants shows that serum Hcy levels were significantly higher in osteoporotic subjects compared to normal BMD subjects (Z=3.590; p=0.0003; 95% CI, 0.375-1.276).

For the vitamin B12 analysis the largest source of heterogeneity stemmed from the study of Bucciarelli *et al*.[6] After removing this study from the meta-analysis the heterogeneity decreased from 98.65% to 11.99% (χ^2=5.681; df=5; p=0.338; I^2=11.99). The forest plot of six studies with 643 participants shows that serum vitamin B12 levels were lower in osteoporotic subjects (WMD=-0.007) but the difference was not statistically significant under fixed-effect model (Z=-0.082; p=0.935; 95% CI, -0.165-0.152).

DISCUSSION

A high serum level of homocysteine has been recently recognized as a risk factor for osteoporosis and osteoporotic fractures[2,7,11,13,19]. The role of Hcy is yet not clearly understood, despite the fact that there are several suggested mechanisms for the effect of HHcy in bones[5,12-15,19-21]. Earlier studies that observed high prevalence of osteoporosis in patients with homocysteinuria[23,24], followed by studies investigating the influence of methylenetetrahydrofolate reductase (MTHFR) polymorphism in BMD[25,26,27] were forerunners of more

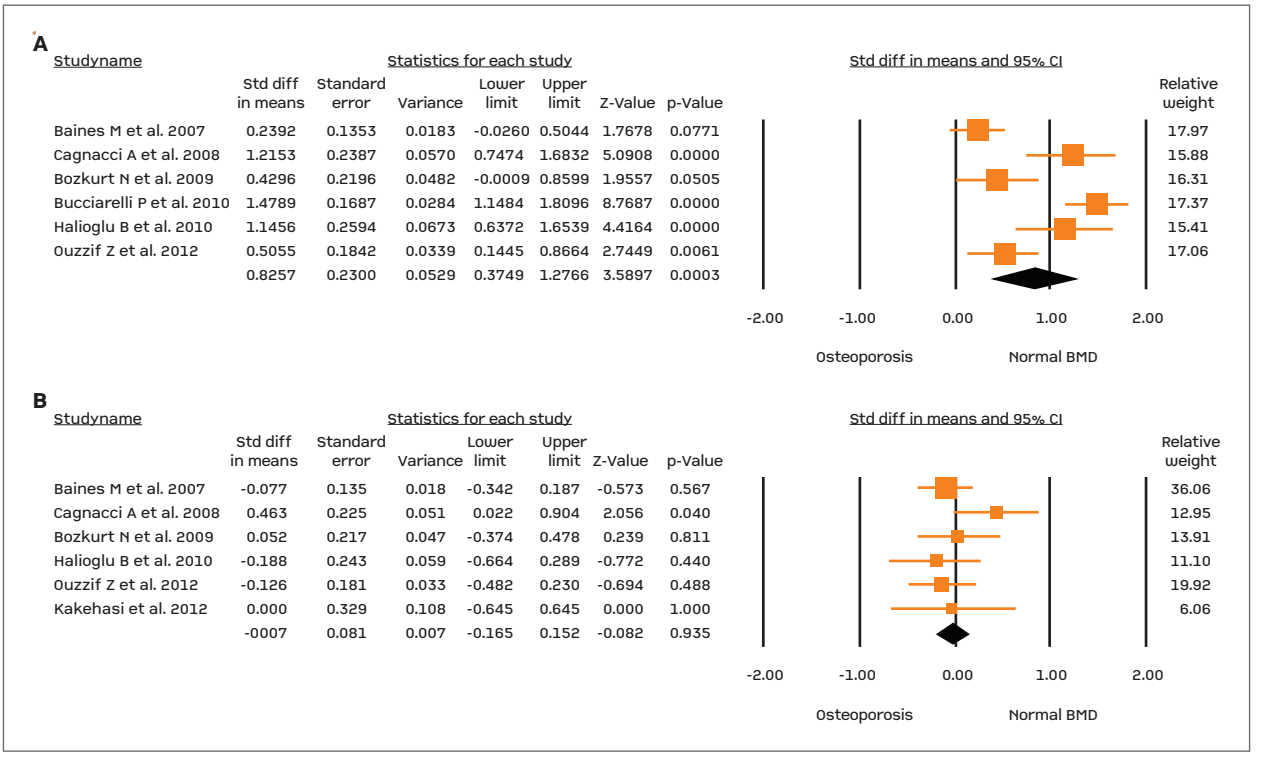

FIGURE 2. Forest plots of the meta-analysis of the association between homocysteine (A) and vitamin B12 (B) and bone mineral density

TABLE III. MULTIPLE LINEAR REGRESSION ANALYSIS OF THE PREDICTORS OF LUMBAR SPINE, FEMUR NECK AND TOTAL HIP BMD

	Lumbar spine BMD (R2=0.131, p=0.001)			Femur neck BMD (R2=0.268, p<0.001)			Total hip BMD (R2=0.308, p<0.001)		
	β	SE	p value	β	SE	p value	β	SE	p value
Age	-0.002	0.002	0.333	-0.005	0.002	0.003	-0.001	0.002	0.475
BMI	0.011	0.003	<0.001	0.012	0.002	<0.001	0.015	0.002	<0.001
Homocysteine	-0.004	0.002	0.040	-0.003	0.001	0.045	-0.003	0.001	0.035
Vitamin B12	-0.00003	0.0001	0.758	0.0004	0.00006	0.535	-0.000003	0.00007	0.968

BMD: Bone mineral density; BMI: Body mass index

recent studies that investigated the relationship between serum levels of Hcy and BMD[4,6,7,17,27-30]. Nevertheless, the inconsistencies persist as to whether HHcy is associated with an increased risk of developing osteoporosis.

In the present cross-sectional study of postmenopausal women, it was demonstrated that serum Hcy levels were significantly higher in osteoporotic women when compared to those with low bone mass or normal BMD. Results of this study have also demonstrated that high serum Hcy levels were inversely associated to lumbar spine BMD and femur neck BMD and that high Hcy levels were a significant factor to predict BMD at lumbar spine, femur neck and total hip. These findings are in agreement with those of Baines *et al.*[7] that showed a significant association of tHcy with BMD as well as significantly higher tHcy levels in the osteoporosis group. In the same way, Bozkurt *et al.*[4] found that plasma Hcy levels were associated with osteoporosis in a sample of Turkish postmenopausal women. Bucciarelli *et al.*[6] results also showed a negative association between Hcy levels and BMD of total femur in a large cohort of postmenopausal women. Similarly, Ouzzif *et al.*[28] concluded that Hcy as well as vitamin B12 levels are independent risk factors for osteoporosis in Moroccan postmenopausal women. Our results are in accordance with these studies in reporting significantly higher Hcy levels in osteoporotic subjects as well as in demonstrating a negative association between Hcy levels and BMD. Further supporting our findings are two longitudinal studies; Zhu *et al.*[17] found that a high Hcy was associated with greater hip bone loss in elderly women aged 70 to 85. Similarly, Kim *et al.*[29] reported detrimental effect of Hcy in femur in men and premenopausal women. Regarding results in these studies and current data, scientific evidence suggests an association between HHcy and low BMD. The results of the meta-analysis provided additional evidence to suggest that high Hcy levels contribute to low BMD and increase the risk of developing osteoporosis.

On the contrary, there are results from studies that did not find a significant association between Hcy levels and BMD. Among earlier studies, Van Meurs *et al.*[30] failed to find evidence of association of Hcy and BMD of femoral neck and lumbar spine, and results from Cagnacci *et al.*[3] observed no direct relation between levels of Hcy and vertebral BMD in a sample of Italian postmenopausal women. In addition, results from a more recent study of Haliloglu *et al.*[5] showed that serum Hcy and vitamin B12 levels were not related to lumbar spine BMD in postmenopausal women. Finally, Rumbak *et al.*[8] results showed that in a population of Croatian postmenopausal women Hcy and vitamin B12 levels were not related to BMD regardless of the measurement site. The proposed mechanistic link between HHcy and osteoporosis and osteoporotic fractures is much more complicated and cannot be explained only through BMD[5,8]. The inconsistencies in these results may be related to different socio-demographic factors, dietary habits, age of participants and BMD measurement sites.

The focus of several studies has been also on vitamin B12 levels as far as it is related to the metabolism of Hcy. In a study of Naharci *et al.*[32] the protective effect of normal levels of vitamin B12 in femur neck BMD was demonstrated. A non-statistically significant difference in serum vitamin B12 concentrations was found in the current study in the osteoporosis group compared to low bone mass and normal BMD groups (399.62±145.79 vs. 438.26±129.36 and 444.54±128.51, respectively; p=0.232), likewise no significant association with BMD at any of the measurement sites.

These findings are consistent with the results of Kakehasi *et al*.[33] that suggested a lack of relationship between low levels of vitamin B12 and BMD in a sample of seventy Brazilian postmenopausal women. Similarly, data from the studies of Cagnacci *et al*.[3], Haliloglu *et al*.[5], and Rumbak *et al*.[7], reported no association between vitamin B12 status and BMD. In contrast, our findings are inconsistent with the results of Bozkurt *et al*.[4] and of Ouzzif *et al*.[28] In addition, the results of El Maghraoui *et al*.[9] reported increased bone loss at total femur among elderly women with low serum vitamin B12 levels. Findings of current study and the results of the meta-analysis, which pooled the data on the association between vitamin B12 levels and BMD, suggest that the relation is probably weak; hence vitamin B12 can be considered a less significant contributor to BMD.

The strength of this study is that all the measurements are performed with a single DXA scanner and by a single biochemistry lab. In addition, the pooled results of the previous studies in the meta-analysis provided additional evidence that HHcy is associated with an increased risk of osteoporosis. Our study has limitations that should be considered. Sample size makes it difficult to generalize the results to the overall population. The lack of data on vitamin D and folate status in these subjects made it impossible to analyze their potential influence in predicting BMD. Similarly, lack of data on biochemical markers of bone turnover made it impossible to assess potential influence of Hcy on them. Studies with a large sample size are needed to provide definitive evidence for a cause-effect relation of Hcy and vitamin B12 status and BMD.

In conclusion, results from this study show that Hcy status, but not vitamin B12 status, is associated with BMD in this cohort of postmenopausal women. Therefore, our results indicate that high Hcy levels are an independent risk factor for osteoporosis. At the present time there is limited evidence to fully support the hypothesis of osteoporosis development as a consequence of low vitamin B12 levels. BMD evaluation in women at post menopause with high Hcy levels may be helpful in advising precautionary measures and thus give a substantial contribution to low bone mass or osteoporosis prognosis.

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