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Research

Relationships of plasma total homocysteine (HCY), folates and vitamin B12 levels to vertebral fracture and bone mineral density in Moroccan healthy postmenopausal women

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Abstract

Introduction: a potential role of Homocysteine (HCY) in bone metabolism has been considered from the observation of high prevalence of osteoporosis in subjects with Homocystinuria about 50 years ago. But the mechanism linking the increased level of HCY to increased fracture risk is not clear. The objective of this study was to investigate this possible relationship between vertebral fractures and HCY level in Moroccan postmenopausal women. **Methods:** one hundred and twenty-two healthy postmenopausal women gave their informed consent to participate in this cross-sectional study. Women were recruited through advertisements and mouth to ear between January 2017 and May 2017. Bone mineral density was determined by a Lunar Prodigy Vision DXA system. Vertebral fracture assessment image was inspected visually by 2 clinicians. **Results:** we found that a high level of HCYor low levels of vitamin B12 and folates are not associated to the bone mineral density and are not risk factors for vertebral fractures in healthy postmenopausal women. Whereas, the presence of vertebral fracture was associated to the number of years since menopause and to the OC level. Probably this is due to the young age of the patients involved in this study. We also showed that high level of HCY is associated with the number of years since menopause and not age for women. **Conclusion:** we found that a high level of HCY or low levels of vitamin vitamin B12 and folates are not associated to the bone mineral density and are not risk factors for vertebral fracture in healthy Moroccan postmenopausal women.

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Introduction

Several factors are known to affect bone metabolism and to increase the risk of fracture, one of them is the high level of HCY [1]. Several studies in postmenopausal women has found an association between increased plasma concentrations of HCY and low bone mineral density (BMD) [2-4], while other studies showed no significant association [5-8]. For the fracture risk, some reports in older persons have shown an association between elevated plasma HCY and fracture risk [5, 8-10]. A potential role of HCY in bone metabolism has been considered from the observation of high prevalence of osteoporosis in subjects with homocystinuria about 50 years ago, Homocystinuria is a genetic disorder caused by mutation of the cystathionine beta-synthase gene [11]. But, the mechanism has not yet been elucidated. It has been shown in vitro that high concentration of HCY decreases the secretion of osteocalcin in preosteoblastic cells but enhances the secretion of osteopentin [12, 13]. Another mechanism is the increased osteoclast activity associated to the high level of HCY concentration.In fact, in-vitro study showed that the increased concentrations of HCY inhibit the activity of lysyloxidase, the enzyme involved in crosslinking of collagen [14-17], and interference in cross link formation would cause an altered bone matrix, resulting in more fragile bone [14-17]. The main objective of this study was to investigate the relationship between vertebral fracture and HCY level in Moroccan postmenopausal women.

Methods

Patients: one hundred and twenty-two healthy postmenopausal women gave their informed consent to participate in this cross-sectional study. Women were recruited through advertisements and mouth to ear between January 2017 and May 2017. The procedures of the study were in accordance with the Declaration of Helsinki, and the Ethics Committee of the Faculty of Medicine and Pharmacy of Rabat approval was obtained for the study. Original inclusion criteria were no previous osteoporotic fracture based on patient record, 24 months of amenorrhea and no previous use of hormone replacement therapy. Women with liver or renal disease, endocrine or metabolic abnormalities, and receiving medicine known to influence bone mineralization or levels of HCY were excluded. Each subject completed a standardized questionnaire designed to document putative risk factors of osteoporosis.

BMD measurements: bone mineral density was determined by a Lunar ProdigyVision DXA system (Lunar Corp., Madison, WI). TheDXA scans were obtained by standard procedures supplied by the manufacturer for scanning and analysis. All BMD measurements were carried out by experienced technicians. Daily quality control was carried out by measurement of a Lunar phantom. At the time of the study, phantom measurements showed stable results. The phantom precision expressed as the coefficient of variation percentage was 0.08. Moreover, reproducibility has been assessed in clinical practice and showed a smallest detectable difference of 0.04 g/cm^2(spine) and 0.02 (hips). Patient's BMD was measured at the lumbar spine (anteroposterior projectionat L1-L4) and at the femurs (i.e., femoral neck,trochanter, and total hip). The WHO classification system was applied, defining osteoporosis as T-score ≤ 2.5 and osteopenia as -2.5 < T-score < -1. Study participants were categorized by the lowest T-score of the L1eL4 lumbar spine, femur neck, or total femur. VFA (Vertebral fracture assessment) was classified using a combination of a semi quantitative (SQ) approach by Genant *et al*. and morphometry in the following manner: each VFA image was inspected visually by 2 clinicians to decide whether it contained a fracture inany of the vertebrae visualized. Each vertebra that was judged as fractured by visual inspection by any of the investigators was measured using built-in morphometry and assigneda grade based on Genant's SQ scale, grade 1 (mild) fracture is a reduction in vertebral height of 20 to 25%, grade 2 (moderate) a reduction of 26 to 40%, and grade 3 (severe) a reduction of more than 40%.

Biological measurements: all blood samples were collected under fasting conditions on the same day of acquisition of the VFA images. Blood samples for plasma HCY, folates, osteocalcin, vitaminB12, and serum parathyroid hormone (PTH) were taken between 8 and 9 am, in the fasting state, placed on ice, centrifuged within 1h, and the separated plasma was then immediately stored in 2 different tubes at -80°C until assayed. These were measured using commercially available kits on the Architect (Abbott Diagnostics®). Plasma HCY was analyzed by commercially available immune-nephelometry kits with BN Prospec (Siemens healthcare diagnostics®). The assay intra and inter-assay CVs of 4.2% and 6.1%, respectively.The Architect 25(OH)D assay showed excellent precision with a total Coefficient of variance (CV%) < 5% at the concentrations of quality control material analysed. In the present study, 25(OH) D values of ≤ 20 ng/ml were defined as vitamin D insufficiency and of ≤ 10 ng/ml as vitamin D deficiency.

Statistical analysis: results are presented as mean ± standard deviation (SD). To compare patients with and without VFs Student test was used. To compare patients according to the quartiles of HCY levels and according to the BMD, analysis of variance was used. Correlations between different variables were assessed using the non-parametric Spearman test. Stepwise multiple regression analysis was used to determine the predictors of BMD. Potential risk factors were entered in a stepwise conditional binary regression analysis, and the resulted odds ratios (ORs) with 95% confidence intervals (CIs) were reported. The level of significance was taken as $p \leq 0.05$. Excel 2007 (Microsoft Corp., Redmond, WA) and SPSS 15.0 (SPSS Inc., Chicago, IL) were used for statistical analysis.

Ethical Considerations: Ethics Committee of the Faculty of Medicine and Pharmacy of Rabat approval was obtained for the study

Results

In this cohort of 122 postmenopausal women, the mean ± SD age, years since menopause, Body mass index (BMI) and the number of pregnancies showed a significant difference between the groups of women with normal bone mineral density (BMD), osteopenia and osteoporosis. While no significant difference was shown between this groups for the HCY level, parathormone and Folates (Table 1). Among the 122 women, 23 (18.85%) had densitometric osteoporosis. Vertebral fracture (VF) was identified using vertebral fracture assessment (VFA) in 17 (13.93%) patients. Comparison of patients according to VF showed a significant difference only for the PTH ($p = 0.003$) and the osteocalcin ($p = 0.02$) (Table 2). Comparison of patients according to quartiles of HCY levels showed that women in the highest quartile had a lower level of B12 and higher level of PTH and a high number of years since menopause (Table 3). In addition to that, a high positive correlation was found between the number of years since menopause and HCY levels too (Table 4). The study showed aweak correlation between the T-score at the total hip and the HCY levels. The most important association is the high positive correlation found between the HCY levels and the PTH levels. Also, significant correlations were found between B12, age in years and HC (Table 4). Multiple regression analysis presented in Table 5 showed that age and B12 were the main predictors of BMD at the total hip, whereas the main predictor of BMD at the lumbar spine was the age. Stepwise regression analysis showed that presence of VF was independently related to osteocalcin and the number of years of menopause (Table 6).

Discussion

This study showed that HCY, vitamin B12 and folates levels are not associated to the BMD and are not risk factors for VF in healthy postmenopausal women.Whereas, the presence of VF was associated to the number of years since menopause and to the OC level. We also showed in this study that high level of HCY is associated with the number of years since menopause and age for women. Patients with VFs compared with those without VF had significantly higher levels of PTH and OC. Also, we showed that presence of VF was independently related to OC level and the number of years of menopause. Several studies confirmed our results, thereis no or only a weak relation between HCY and BMD. In fact, BMD does not reflect the current status of bone metabolism and presents only poor information about themicroarchitecture of the bone matrix. The link between high level of HCY and fracture risk cannot be attributed to a reduced bone mineralisation [13, 18-20]. We found that high level of HCY was associated to high level of OC, and Avbersek-Luznik *et al* reported that increased levels of bone formation markers are associated with a significantly greater rate of bone loss in postmenopausal women [21]. In this study, The PTH level is highly and significantly elevated in women with VF and HCY level was positively correlated to the Parathyroid Hormone (PTH) level. This high PTH levels may indicate increased bone turnover in postmenopausal women with high level of HCY. In fact, the PTH level is increased to maintain calcium homeostasis over greater bone turnover [14]. We did not show a significant association between the plasma HCY level and fracture risk. In fact, the presence of vertebral fractures was associated to the OC level and the number of years since menopause in women in our study. Previous studies investigating the fracture risk have yielded contradictory results. In fact, some studies did not show a significantassociation between the plasma HCY level and fracture risk [22, 23]. But other studies found a significant positiverelation between HCY plasma levels and fracturerisk especially at the hip [5, 12, 24, 25]. These discrepancies in the results are due to difference in mean ages of women involved in these studies (from 57 to 70 year-old). To date, the mechanism linking HCY to increased fracture risk have not yet been clarified. B12 and folates are the main determinants in the metabolism of HCY. Some studies trials have found that supplementation of folic acid (0.5 - 5 mg day-1) has

resulted in reducing the levels of HCY in blood up to 25% and the co-supplementation of folic acid and Vitamin B12 (0.5-5 mg day-1 and 500 mcg day-1, respectively) provided a further reduction of 7% with a decrease in serum total HCY by 32% [26]. Also, high concentrations of HCY and low levels of B12 and folates, have been associated with low BMD and a higher risk of fractures in the elderly [26]. Some in vitro studies have found that high concentrations of HCY increased bone resorption by increasing osteoclast activity [14, 27]. Others in vitro studies have shown that elevated concentrations of HCY inhibit the activity of the lysyloxydase (involved in cross-linking of the collagen) and consequently would lead to an altered bone matrix and enhance bone fragility [7-9]. In another meta-analysis of observational studies, structural deterioration of bone tissue have been associated with elevated HCY levels and low vitamin B12 and folates levels, and people with hip fractures have severe deficiency in folates [26]. In this study, we showed that in postmenopausal women the number of years since menopause and not age reported in previousstudies is the major determinant of the fracture risk. Our study has strengths and limitations. All of DXA measurements were conducted with a single bone densitometerand all of biochemical exams were done in a single biochemistry laboratory, with very careful quality controls in place. The assessment of fracture was carefully conducted using standard procedures of acquisition and standard reading of all VFAs. All the morphometric assessments were made by 2 experienced investigators after training sessions and a previous global visualization. The main limitations lie in the cross-sectional nature of the study and the procedures used to select subjects, who were all volunteers and ambulatory.

Conclusion

Finally, it is not clear the effect of high HCY level on bone, some authors report a relationship between HCY level and bone, but others find no association. In addition to that, it is difficult to demonstrate whether this is related to HCY level or to the vitamins which are required of its metabolism such as B12, B6, folates.

What is known about this topic

- Several studies in postmenopausal women had found an association between increased plasma concentrations of HCY and low bone mineral density (BMD);
- Other studies showed no significant association;
- For the fracture risk, some reports in older persons have shown an association between elevated plasma HCY and fracture risk.

What this study adds

- First study in the north African population on the association between HCY, vitamin B12, folates levels and vertebral fractures;
- Hcy, vitamin B12 and folates levels are not associated to the BMD and are not risk factors for VF in healthy postmenopausal women;
- We showed that presence of VF was independently related to OC level and the number of years of menopause; patients with VFs compared with those without VF had significantly higher level of PTH and OC were observed among.

Competing interests

The authors declare no competing interests.

Authors' contributions

Aissam El Maataoui and Zahra Ouzzif contributed to study design, data analysis/interpretation and in drafting or critically revising the manuscript. Lamiae Ennefah, Aziza Mounach, Abdellah El Maghraoui were involved in study design, results interpretation, and in drafting or critically revising the manuscript. All authors read and approved the final manuscript.

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Table 1: demographic and measured analytes according to BMD status (Mean ±SD) (n = 122)

	Normal (n=55)	**Osteopenia (n=43)**	**Osteoporosis (n=23)**	**p**
Vitamin B12 (pg/ml)	384,34±188,026	470,95±277,78	377,05±97,79	0,105
Osteocalcin (µg/l)	23,27±20,83	20,95±5,48	27,15±17,64	0,399
Vitamin D in ng/mL	14±6,04	16,51±16,2	13,24±7,19	0,437
Age in years	61,1±5,36	63,14±6,65	65,46±7,18	0,02
BMI in Kg/m²	31,06±5,016	29,7±3,88	27,46±3,69	0,005
Number of pregnancies	4±1,743	4,63±2,912	5,74±3,09	0,021
Years since menopausein years	9,84±6,88	14,72±7,082	14,65±5,348	0,001
Spine BMD in g/cm²	1,11±0,125	0,99±0,129	0,812±0,065	<0.001
Spine T-score	-0,222±0,913	-1,11±1,35	-1,3±1,08	<0.001
Total hip BMD in g/cm²	0,99±0,1	0,919±0,14	0,8±0,1	<0.001
Total hip T-score	-0,16±0,787	-0,84±1,227	-1,78±0,9167	<0.001
HCY (mg/L)	9,11±8,79	7,88±3,86	9,7±5,61	0,53
Parathormone (pg/L)	15,93±5,622	16,21±6,908	22,88±16,957	0,224
Folates (mg/L)	6,679±2,62	8,893±8,008	6±2,217	0,055

Table 2: comparison between patients according to vertebral fractures (Mean ±SD) (n = 122)			
	WITHOUT VF (n=105)	**WITH VF (n=17)**	**p**
Osteocalcin (µg/l)	21,3428±6,22824	25,6624±18,52965	0,02
Parathormone (pg/L)	16,76±6,220	25,40±25,40	0,003
HCY (mg/L)	9,0220±7,78670	10,4741±5,94436	0,86
Vitamin D in ng/mL	15,20±12,145	12,59±5,853	0,57
Folates (mg/L)	7,674±6,4513	6,076±2,1539	0,45
Vitamin B12 (pg/ml)	408,17±251,579	397,29±144,342	0,53
Spine BMD in g/cm^2	1,03789±0,154932	1,01024±0,175752	0,59
Lumbar spine T-score	-1,011±1,2745	-1,276±1,4078	0,67
Total hip BMD in g/cm^2	0,94853±0,126947	0,93076±0,127610	0,94
Total hip T-score	-0,620±1,0828	-0,782±1,1069	0,99

Table 3: comparison between patients according to the quartiles of HCY (Mean ±SD) (n = 122)					
	Quartile 1	**Quartile 2**	**Quartile 3**	**Quartile 4**	**p**
Vitamin B12 (pg/ml)	399,2±105,3	500,31±314,7	430,97±201,4	327,75±163,2	0,023
Osteocalcin (µg/l)	19,53±6,4	20,78±5,02	27,03±27,12	24,35±15,33	0,34
Vitamin D in ng/mL	15,47±7,4	14,52±7,17	15,67±19,29	13,68±6,207	0,9
Age in years	61,1±5,7	61,33±6,45	63,36±6,19	64,43±6,3	0,11
BMI in Kg/m^2	29,95±3,79	30,9±4,84	29,31±5,09	29,45±4,53	0,53
Number of pregnancies	4,59±2,88	4,1±2,36	4,47±2,78	5,03±2,198	0,56
Years since menopause	10,79±6,7	10,13±7,4	13,03±5,56	15,45±7,159	0,012
Spine BMD in g/cm^2	1,02±0,16	1,027±0,15	1,022±0,189	0,98±0,139	0,6
Total hip BMD in g/cm^2	0,96±0,16	0,95±0,15	0,91±0,125	0,89±0,149	0,2
Lumbar spine T-score	-1,01±1,34	-0,97±1,25	-0,927±1,59	-1,452±1,14	0,39
Total hip T-score	-0,403±1,2	-0,47±1,02	-0,857± 1,268	-1,052±1,036	0,087
Parathormone (pg/L)	13,75±4,351	15,25±6,02	19,2±7,85	27±16,32	0,019
Folates (mg/L)	8,277±9,4	7,497±2,8	6,85±2,47	6,8±2,93	0,69

Table 4: correlations between BMD and biological parameters (n=122)										
	HCY	**VITD**	**FOLATES**	**B12**	**OC**	**PTH**	**Age**	**BMI**	**NP**	**YSM**
Vitamin D in ng/mL(VITD)	-0,128									
Folates (mg/L)	-0,05	-0,004								
Vitamin B12 (pg/ml)(B12)	-0,240**	0,025	0,156							
Osteocalcin (µg/l)(OC)	0,166	-0,022	-0,208*	-0,049						
Parathormone (pg/L)(PTH)	0,622**	0,088	-0,156	-,392*	0,311					
Age in years	0,297**	0,131	-0,028	-0,052	0,003	0,107				
Body mass index in Kg/m^2: m (SD)(BMI)	-0,073	0,07	0,127	0,025	-0,17	0,102	-0,08			
Number of pregnancies(NP)	0,111	-0,101	0,073	-0,096	0,056	-0,06	0,331**	-0,177		
Years since menopause(YSM)	0,285**	-0,16	-0,029	0,015	0,139	-0,047	0,530**	-0,099	0,262**	
Spine BMD in g/cm^2	-0,084	0,042	0,08	-0,129	-0,115	-0,157	-0,17	0,333**	-0,181*	-0,266**
Lumbar spine T-score	-0,083	0,037	0,033	-0,083	-0,146	-0,182	-0,182*	0,334**	-0,215*	-0,311**
Total hip BMD in g/cm^2	-0,156	-0,107	0,029	0,035	-0,286**	-0,136	-0,325**	0,305**	-0,182*	-0,350**
Total hip T-score	-0,184*	-0,11	0,035	0,027	-0,300**	-0,127	-0,329**	0,283**	-0,205*	-0,361**
*P < 0.05; **P < 0.01										

Table 5: multiple regression analysis of the predictors of the bone mineral density at the lumbar spine and the total hip (n=122)									
Total hip BMD					**Lumbar spine and total hip BMD**				
	Unstandardized coefficients		**Standardized coefficients**	**p**		**Unstandardized coefficients**		**Standardized coefficients**	**p**
	B	Standard error	Beta			B	Standard error	Beta	
Age in years	-0,015	0,004	-0,537	0,001	**Age in years**	-0,012	0,004	-0,456	0,008
Vitamin B12	0,000	0,000	0,345	0,021					

Table 6: regression logistic analysis with the presence of vertebral fracture as the dependent variable (n = 122)		
	OR total population	**p**
Osteocalcin (µg/l)	1,066(0.974–1.167)	0,163
Years since menopause	1,424(1.006–2.015)	0,046