



Influence of homocysteine and its major genetic and nutritional determinants on bone mineral density

Influence de l'homocystéine et de ses principaux déterminants génétiques et nutritionnels sur la densité minérale osseuse

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ABSTRACT

Introduction: A potential role of hyperhomocysteinemia in bone metabolism has been considered from the observation of high prevalence of osteoporosis in subjects with homocystinuria about 50 years ago.

Aim: To examine the association of homocysteine level and its determinants Methylene tetrahydrofolate Reductase [MTHFR] C677T Polymorphism, folates and vitamin B12 levels with bone mineral density [BMD] and the prevalence of vertebral fractures [VF] on postmenopausal women.

Methods: Through a cross-sectional study, one hundred and twenty-two healthy postmenopausal women gave their informed consent to participate in this study. Women were recruited through advertisements and mouth to mouth between January 2017 and May 2017. One serum tube and one EDTA tube were collected from fasting patients. Bone mineral density was determined by a Lunar Prodigy® Vision DXA system®. Vertebral fracture [VF] assessment image was inspected visually by 2 clinicians.

Results: We found that a high level of homocysteine and low vitamin B12 and folate levels are not associated with bone mineral density and are not risk factors for VF in healthy postmenopausal women. Whereas, the presence of VF was associated with the number of years since menopause and with the osteocalcin levels.

Conclusion: The MTHFR C677T polymorphism, the high levels of HCY, or low levels of folate and vitamin B12 would not be risk factors for osteoporosis and VF in healthy postmenopausal women.

Keywords: Homocysteine, Methylene tetrahydrofolate Reductase C677T polymorphism, Bone mineral density, Vertebral Fracture.

RÉSUMÉ

Introduction : Le rôle de l'hyperhomocystéinémie dans la perturbation du métabolisme osseux a été suspecté à partir de l'observation d'une prévalence élevée d'ostéoporose chez les sujets atteints d'homocystinurie il y a environ 50 ans.

Objectif : Examiner l'association entre le taux d'homocystéine et de ses déterminants, le polymorphisme C677T de la méthylène-tétrahydrofolate-réductase (MTHFR), les taux de folates et de vitamine B12 avec la densité minérale osseuse (DMO) et la prévalence des fractures vertébrales (FV) chez les femmes ménopausées d'origine marocaine.

Méthodes : Il s'agit d'une étude transversale chez Cent vingt-deux femmes ménopausées en bonne santé. Toutes les femmes ont signé le consentement éclairé pour participer à cette étude. Les femmes ont été recrutées par le biais d'annonces ou de bouche à oreille entre janvier 2017 et mai 2017. Un tube sec et un tube EDTA ont été prélevés chez les patients à jeun. La densité minérale osseuse a été déterminée par un système Lunar Prodigy® Vision DXA system®. L'image d'évaluation de la FV a été inspectée visuellement par deux cliniciens.

Résultats : Nous avons constaté qu'un taux élevé d'homocystéine ou un faible taux de vitamine B12 et de folates n'étaient pas associés à la baisse de la densité minérale osseuse et ne constituaient pas des facteurs de risque de FV chez les femmes ménopausées en bonne santé. En revanche, la présence de FV a été associée à l'ancienneté de la ménopause et à l'ostéocalcinémie.

Conclusion : Le polymorphisme du MTHFR C677T, les taux élevés de l'homocystéine ou les faibles taux de folates et de vitamine B12 ne seraient pas des facteurs de risque d'ostéoporose et de FV chez les femmes ménopausées en bonne santé d'origine marocaine.

Mots clés : Homocystéine, polymorphisme C677T de la méthylène-tétrahydrofolate réductase, Densité minérale osseuse, fracture vertébrale.

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INTRODUCTION

Osteoporosis is defined as a skeletal disorder characterized by low bone mineral density and micro-architectural deterioration of bone tissue predisposing a patient to an increased risk of fracture [1]. A potential role of hyperhomocysteinemia in bone metabolism has been considered from the observation of the 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, results in the accumulation of homocysteine (HCY) in the blood.

Results from recent studies showed an association between increased plasma concentrations of HCY, bone mineral density (BMD), and increased risk of osteoporotic fractures [2–4], on the contrary, other studies did not show any significant association [5–7]. The inconsistencies in these results may be related to different BMD measurement sites, ethnicity, age [advanced age was significantly associated with elevated HCY levels] and food habits.

The mechanism linking hyperhomocysteinemia with the pathogenesis of osteoporosis has not yet been elucidated. Nevertheless, some mechanisms have been proposed: the increased osteoclast activity associated with the high level of HCY concentration, interference in cross-link formation would cause an altered bone matrix resulting in more fragile bone, and alteration of gene expression with reduced methylation. Also, some deficiencies in vitamins B6, B12, or folates cause increased serum levels of HCY because these vitamins act as co-factors for various enzymes involved in HCY metabolism. Finally, some polymorphism of genes can cause hyperhomocysteinemia, the most common and the most studied genetic cause is the MTHFR C677T polymorphism. The MTHFR gene is located on chromosome 1 (1p36.3) and has 11 exons spanning 22 kb within a region linked to the regulation of BMD [8]. The gene code for the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20), this enzyme acts as a carbon donor for the re-methylation of HCY to methionine. The S-adenosyl methionine (the derivative of methionine and adenosyltriphosphate) serves as the main methyl donor in humans. To our knowledge, this is the first study on the association of the MTHFR C677T polymorphism with the BMD, and the risk of fracture in the North African population.

The main objective of this study was to examine the association of homocysteine level and its determinants MTHFR C677T Polymorphism, folates, and vitamin B12 levels with BMD and the prevalence of vertebral fractures (VF) in Moroccan postmenopausal women.

METHODS

Study Subjects

122 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. Also, 182 healthy controls were genotyped from the DNA bank of the general population available in the Laboratory of Genetics and Molecular Pathology of our University.

The procedures of the study were in accordance with the Declaration

of Helsinki, and the Ethics Committee of the faculty of medicine. Inclusion criteria were: no previous osteoporotic fractures, a minimum of two years of amenorrhea, and no previous use of hormone replacement therapy. Exclusion criteria were as follows: women with liver or renal disease, endocrine or metabolic abnormalities, and women receiving medicine known to influence bone mineralization or levels of HCY. Each subject completed a standardized questionnaire designed to document putative risk factors of osteoporosis.

BMD measurements

BMD was determined by a Lunar Prodigy Vision DXA system (Lunar Corp., Madison, WI®). 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 the smallest detectable difference of 0.04 g/cm² (spine) and 0.02 (hips). Patients' BMD was measured at the lumbar spine (anteroposterior projection at L1–L4) and the femurs (i.e., femoral neck, trochanter, and total hip). The world health organization [WHO] classification system was applied, defining osteoporosis as T-score ≤ -2.5 and osteopenia as $-2.5 < \text{T-score} < -1$. Study participants were categorized by the lowest T-score of the L1–L4 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 in any 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 assigned a 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, vitamin B12, and serum parathyroid hormone were taken between 8 and 9 AM, in the fasting state, placed on ice, centrifuged within 1 h, 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 analyzed. 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.

DNA Extraction and PCR Analysis

Genomic DNA was extracted from peripheral blood leukocytes using the salting-out procedure. Spectrophotometry was used to quantify DNA. The C677T polymorphism of the MTHFR gene was performed using polymerase chain reaction (PCR) and restriction fragment

length polymorphism analysis (RFLP). Reactions were performed in a final volume of 25 µl. PCR products were cleaved overnight with HinfI at 37 °C and electrophoresed on a 3% agarose gel in the presence of a molecular weight marker ladder 100. After staining with ethidium bromide, ultraviolet was used on a transilluminator to visualize DNA bands. The mutant allele [TT] contains a HinfI restriction site allowing RFLP analysis of the digested products. Digestion of the amplified fragment at the HinfI site yields 175 and 23 bp fragments. The wild-type allele remains uncut.

Statistical Analysis

Results are presented as mean ± standard deviation (SD). To compare normal patients, patients with osteopenia and osteoporosis, and also to compare according to MTHFR genotype, ANOVA-test was used. Correlations between continuous variables were calculated using Pearson correlation coefficients. Potential risk factors were entered into a stepwise conditional binary regression analysis and the resulting odds ratios with 95% confidence intervals were reported. The level of significance was taken as p ≤ 0.05. Excel 2007 (Microsoft Office 2007) and SPSS 15.0 (Statistical Package for the Social Sciences) was used for statistical analysis.

found between the levels of HCY, vitamin D, vitamin B12, folates, PTH, osteocalcin in the three groups. The osteoporotic group is older, with a low body mass index and a high number of years since menopause. For the C677T MTHFR polymorphism, the genotypic and allelic frequencies were 64.3, 28.6, and 7.1%, respectively, for the CC, CT, and TT genotypes, and 78.6 and 21.4%, respectively, for the 677C and 677T alleles among the cases. In the control group, the frequencies were 52.2, 41.8, and 6%, respectively, for the CC, CT, and TT genotypes, and 73.1 and 26.9%, respectively, for the 677C and 677T alleles (Table 2). The MTHFR C677T polymorphism was determined in 43 women with osteopenia, 22 women with osteoporosis, and 55 normal women. The gene frequencies of group N were 0.78 for the C allele and 0.22 for the T allele. Statistical analysis showed that the distribution frequency of the CC, TT and CT genotypes [p² + 2pq + q² = 1] and C, T alleles [p + q = 1] was in Hardy–Weinberg equilibrium [x² = 1.93, P=0.16].

Table 2. C677T MTHFR polymorphism frequencies and genetic distribution among the cases and controls.

MTHFR	Patients n [%]	Controls n [%]	p-value	OR	95% IC
Genotype distribution					
CC	54 [64.3]	94 [51.65]		1	
CT	24 [28.6]	77 [42.3]	0.03	0.54	0.30 to 0.95
TT	6 [7.1]	11 [6.05]	0.92	0.94	0.33 to 2.71
Allele frequency					
C	132 [78.6]	265 [73]		1	
T	36 [21.4]	99 [27]	0.15	0.73	0.47 to 1.12

OR: Odds ratio
IC: interval of confidence

RESULTS

Table 1 summarizes the variables distributed between the groups ranked according to BMD in three groups (Normal, osteopenia, and osteoporosis). There was no significant difference in the percentage of VF between the three groups. Also, no significant difference was

Table 1. Demographic and measured analytes according to BMD status [Mean ±SD] [n=120].

	Normal [n=55] Means ±SD	Osteopenia [n=43] Means ±SD	Osteoporosis [n=22] Means ±SD	p
Homocysteine [mg/L]	9.11±8.79	7.8881±3.87	9.7195±5.61	0.530
Folates [mg/L]	6.679±2.62	9.007±8.07	6.000±2.22	0.045
Vitamin B12 [pg/ml]	384.34±188.03	476.63±278.72	377.05±97.79	0.081
Vitamin D in ng/mL	14.00±6.41	16.73±16.35	13.24±7.19	0.053
Osteocalcin [µg/l][OC]	23.2760±20.83	20.9526±5.49	27.1563±17.64	0.392
Parathormone [pg/L][PTH]	17.78±6.08	15.43±6.02	14.00±6.02	0.399
Age in years	61.164±5.37	63.142±6.66	65.465±7.18	0.02
Years since menopause[YSM]	9.84±6.88	14.71±7.08	14.65±5.34	0.001
Body mass index in Kg/m ² : m [SD][BMI]	31.06±5.02	29.71±3.88	27.46±3.70	0.005
Spine BMD in g/cm ²	1.11369±0.13	0.99065±0.13	.81243±0.07	<0.001
Lumbar spine T-score	-0.222±0.92	-1.300±1.09	-2.878±0.56	<0.001
Total hip BMD in g/cm ²	0.99413±0.10	0.91944±0.14	0.80091±0.11	<0.001
Total hip T-score	-0.160±0.80	-0.840±1.23	-1.787±0.92	<0.001
Vertebral fracture [%]	10.75	2.15	5.38	0.57
Frequency of MTHFR CC genotype [%]	52	72	75	
Frequency of MTHFR CT genotype [%]	39	23	17	0.38
Frequency of MTHFR TT genotype [%]	9	5	8	

Mean ± standard deviation

Except for the HCY level, we didn't find any significant differences in demographic and measured analytes according to MTHFR C677T genotype (**Table 3**), even for the body mass index, the lumbar spine, the total hip, and the percentage of VF.

A positive correlation was found between HCY level and age. However,

we recorded a negative correlation between HCY and vitamin B12 levels. No correlations were found between the HCY level on one hand and BMD, BMI, vitamin D level on the other hand (**Table 4**). Stepwise regression analysis showed that BMD was independently related to vitamin B12 level and age in years (**Table 5**).

Table 3. Demographic and measured analytes according to MTHFR genotype [Mean \pm SD] [n=88].

	CC genotype [n=54] Means \pm SD	CT genotype [n=24] Means \pm SD	TT genotype [n=6] Means \pm SD	p
Years since menopause [YSM]	13.98 \pm 6.76	13.38 \pm 7.16	12.50 \pm 7.80	0.85
Age in years	63.94 \pm 7.01	62.95 \pm 6.11	61.15 \pm 6.08	0.57
Homocysteine [mg/L]	9.60 \pm 4.40	7.40 \pm 4.05	21.70 \pm 21.10	<0.001
Parathormone [pg/L][PTH]	18.67 \pm 6.74	15.75 \pm 4.57	26.00 \pm 0.001	0.35
Vitamin D in ng/mL	14.16 \pm 6.93	20.52 \pm 21.14	16.50 \pm 12.22	0.16
Folates [mg/L]	7.33 \pm 2.67	9.10 \pm 10.67	8.25 \pm 4.38	0.52
Vitamin B12 [pg/ml]	447.10 \pm 270.21	429.13 \pm 206.13	335.33 \pm 218.48	0.58
Osteocalcin [μ g/l][OC]	21.28 \pm 5.68	30.91 \pm 31.86	32.96 \pm 30.71	0.09
Body mass index in Kg/m ² : m [SD][BMI]	30.16 \pm 4.33	30.46 \pm 4.35	28.35 \pm 4.85	0.57
Spine BMD in g/cm ²	0.99 \pm 0.16	1.05 \pm 0.15	1.01 \pm 0.17	0.35
Lumbar spine T-score	-1.31 \pm 1.27	-0.88 \pm 1.25	-1.00 \pm 1.64	0.37
Total hip BMD in g/cm ²	0.91 \pm 0.14	0.94 \pm 0.12	0.94 \pm 0.18	0.46
Total hip T-score	-0.92 \pm 1.11	-0.62 \pm 0.98	-0.70 \pm 1.52	0.53

Mean \pm standard deviation

Table 4. Correlations between homocysteine, bone mineral density, demographics and measured analytes

	Homocysteine	Vitamine D	Folates	B12	OC	Age	BMI	YSM
Vitamin D in ng/mL [VitD]	-0.027							
Folates [mg/L] [Fol]	0.034	0.002						
Vitamin B12 [pg/ml] [B12]	-0.198*	-0.053	0.061					
Osteocalcin [μ g/l][OC]	0.056	0.047	-0.147	0.093				
Age in years	0.206*	0.1	-0.062	0.08	0.069			
Body mass index in Kg/m ² : m [SD][BMI]	-0.133	0.044	0.108	-0.018	-0.175	-0.068		
Years since menopause[YSM]	0.118	-0.086	-0.086	0.054	0.07	0.522**	-0.092	
Lumbar Spine BMD in g/cm ²	0.014	-0.056	0.021	0.066	-0.116	-0.169	0.363**	-0.240**
Lumbar spine BMD [LS] T-score	0.003	-0.05	0.001	-0.004	-0.03	-0.190*	0.379**	-0.287**
Total hip [TH]BMD in g/cm ²	-0.094	-0.101	0.044	0.024	-0.159	-0.353**	0.352**	-0.327**
Total hip BMD [TH] T-score	-0.119	-0.111	0.042	0.019	-0.136	-0.365**	0.330**	-0.349**

** Correlation is significant at the <0.01 level [bilateral].

* Correlation is significant at the <0.05 level [bilateral].

B12: vitamin B12; BM: Body mass index; YSM: Years since menopause

Table 5. Multiple linear regression analysis with BMD as the dependent variable

	Unstandardized coefficients		Standardized coefficients	p-value	95 % Confidence interval	
	B	standard error	Beta		LOWER	HIGHER
Vitamin B12	-0.008	0.003	-0.235	0.012	-0.014	-0.002
Age in years	0.252	0.106	0.219	0.019	0.042	0.463

DISCUSSION

We found that the MTHFR C677T polymorphism, the high level of HCY, and low levels of folates and vitamin B12 are not risk factors for osteoporosis and VF in young healthy Moroccan postmenopausal women. Likewise, our study demonstrated that increasing age and low vitamin B12 levels were the most important independent factors associated with low BMD. We concluded that further studies are needed to find at which HCY concentration we will have a high fracture risk.

In this study, hyperhomocysteinemia was not associated with VF in young postmenopausal Moroccan women. We did not find an association because of the youngest age of our patients in comparison to other studies. But in Japan, and 1475 postmenopausal Japanese women [mean age 66.6 years], HCY level contributed to the risk of severe [grade 3] vertebral fracture [OR = 1.27, 95% CI 1.04–1.58, $p = 0.021$]. In another study, the serum level of HCY was an independent risk factor for moderate to severe VF [9]. Dhonukshe-Rutten et al found in women with high HCY and/or low vitamin B12 concentrations had an adjusted RR for fractures of 2.8 [1.3–5.7] [10]. A High level of HCY impairs the bone structure and increases the risk of fractures. Also, a high level of HCY may inhibit the cross-linking through its binds to the aldehydic groups of allylsine and hydroxyallylsine [11,12]. It is well known that HCY acts as a negative regulator of lysyl oxidase activity [13]. McLean and al studied 825 men [69.5±6.9 range: 59–90] and 1174 women [69.5±6.9 range: 59–90] found that the homocysteine concentration, is an important risk factor for hip fracture in older persons [14].

Our findings that the polymorphism of the C677T in the MTHFR gene was not associated with the BMD of this cohort of Moroccan postmenopausal women are in agreement with some studies [15], and in contrast with others. The Danish Osteoporosis Prevention Study shows that healthy postmenopausal women with the TT genotype have 0.1-0.3 standard deviation [SD] reduced BMD at most measurement sites and that one in nine fractures in this age group can be attributed to this genetic polymorphism alone [3]. In two meta-analyses [16,17], the authors suggested that postmenopausal women with the TT genotype had lower femoral neck BMD than that of women with the CC/CT genotype, but the lumbar spine BMD with the TT genotype had no significant difference compared with the CC/CT genotype.

The polymorphism of the C677T in the MTHFR gene was not associated with to high risk of fracture in this cohort, we believe that this is due to the young age of our population and the small number of patients with severe VF, also, mutation of the gene of the MTHFR didn't reach the statistical power needed to show such association. Previous studies have found that this association in postmenopausal women is controversial [1,2,18]. In fact, in 502 postmenopausal ambulatory Japanese women, the effect of MTHFR C677T polymorphism on fracture susceptibility was studied, subjects with the TT genotype had a higher incidence rate of fractures and higher plasma level of HCY than subjects with the non-TT genotype [19]. In a meta-analysis[20], a significant association was found between

C677T polymorphism and fracture risk among East Asians but not Europeans, this may be due to the frequency of C677T polymorphism TT genotype being much lower in Europeans than East Asians [HapMap data]. The frequencies of CC, CT, and TT were about 59.3%, 33.9%, and 6.8%, respectively, in the European population, while the frequencies of CC, CT, and TT were about 26.7%, 44.4%, and 28.9%, respectively in the Chinese population [20].

Moreover, at least 15 other genes can be assigned as confirmed osteoporosis susceptibility genes [VDR, ESR1, ESR2, LRP5, LRP4, SOST, GRP177, OPG, RANK, RANKL, COLIA1, SPP1, ITGA1, SP7, and SOX6] [21]. Also, environmental factors such as physical activity, diet, and smoking have been shown to influence BMD, osteoporosis, and the risk of fractures.

Our study has strengths and limitations. This is the first study in the North African population to the best of our knowledge. All DXA measurements were conducted with a single densitometer and all the biochemical exams were done in a single laboratory. The assessment of fracture was carefully conducted using standard procedures of acquisition and standard reading of all the vertebral fracture assessments. All morphometric assessments were made by 2 experienced investigators after training sessions and a previous global visualization. The limitations of this study are the small sample size and the small proportion of the TT genotype in our study.

CONCLUSION

This study didn't confirm that the MTHFR C677T polymorphism, the high level of HCY, or low levels of vitamin B12 and folates are risk factors for VF in young healthy postmenopausal women. Therefore, further large studies are needed to determine how MTHFR C677T polymorphism, HCY, and vitamins B may impact the fracture risk in postmenopausal women. Also, to better understand the inconsistency in the results from different ethnicities and to understand at which HCY levels we will have high fracture risk.

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