Physical Activity, Cdx-2 Genotype, and BMD

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Abstract
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The present study investigated the interaction between Cdx-2 polymorphism and physical activity level over bone mineral density (BMD) variation in Brazilian postmenopausal women. One hundred and ninety women volunteered to participate in the study (66.6 ± 5.3 years, 64.58 ± 11.74 kg and 151.94 ± 6.36 cm). Physical activity level (PAL) was assessed using the international physical activity questionnaire (IPAQ). Lumbar spine (L2 – L4), femoral neck, great trochanter and Wards' triangle bone mineral density (BMD) were measured by dual-energy X-ray absorptiometry (DXA). The Cdx-2 polymorphism was genotyped by minisequencing, using the SNaPshot™ Multiplex System (Applied Biosystems, Foster City, CA, USA). Overall, no significant association was found between Cdx-2 polymorphism and adjusted BMD at any site. However, the results revealed a significant interaction between PAL and Cdx-2 genotype on adjusted femoral neck and Wards' triangle BMD. Active women carrying the Cdx-G/G genotype showed higher adjusted femoral neck and Wards' triangle BMD than inactive women carrying the same genotype, thus suggesting a larger chronic response to physical activity. These results suggest that, in postmenopausal women, the Cdx-2 polymorphism does not influence BMD by itself; however, it seems to affect the BMD response to physical activity since only the Cdx-G/G genotype carriers presented significant differences between active and inactive.

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Introduction
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Fractures are increasingly recognized as an important public health problem in the modern societies, imposing a heavy social and economic burden and leading to serious impairment in the elderly [15,25]. Recent studies revealed that this scenario tends to get worst, some authors estimated that the number of patients suffering the consequences of hip fractures might reach more than 20 million in the world by the year 2050 [15]. Bone mineral density (BMD) has been suggested as an excellent proxy to identify individuals at high risk of fracture in a preventive context [21]. Although age is an important predictor of BMD, the mechanisms leading to BMD loss are multifactorial, encompassing nutritional, medical, hormonal, environmental and genetic factors, as well as their interaction [25].

The results from family and twin studies have shown that genetic factors accounted for up to 85% of interindividual variations observed in BMD at the lumbar spine and hip [10,29]. Despite the large number of genes related to bone phenotypes [19], studies performing segregation analyses reinforced the hypothesis of a major gene responsible for most of the interindividual variation in BMD [9,19]. In this context, the vitamin D receptor (VDR) gene has been one of the most studied because of the biological effect of vitamin D in bone metabolism [19,28].

In 1999, Yamamoto et al. [34] described a binding site to the specific transcription factor Cdx-2 in the promoter region of the VDR gene. Later, Arai et al. [4] identified a polymorphism at this site, defined by the substitution of guanine by adenine and the alleles were named Cdx-G or Cdx-A, according to the base present. This mutation is supposed to be functional, because it seems to influence VDR expression at the small intestine. In the studies of Arai et al. [4] and Fang et al. [11], the Cdx-A allele showed a higher affinity with the Cdx-2 protein, which led to a higher transcriptional activity in this allele in comparison to Cdx-G. There are few studies associating this polymorphism to BMD and they are limited to

Table 1   Characteristics of the subjects

Variable	Mean ± standard deviation
N	190
Age (years)	66.6 ± 5.3
Body weight (kg)	64.58 ± 11.74
Height (cm)	151.94 ± 6.36
BMI (g/m^2)	27.95 ± 4.48
% body fat	38.4 ± 6.3
BMD L2 – L4 (g/cm^2)*	0.978 ± 0.178
BMD FN (g/cm^2)	0.869 ± 0.136
BMD WT (g/cm^2)	0.684 ± 0.158

BMI: body mass index; BMD: bone mineral density; FN: femoral neck; WT: Wards' triangle; * n = 189 (one volunteer failed to complete the measurement)

Europeans [11] and Japanese [4, 24]. No study was found with Latin American women. Research regarding the Cdx-2 polymorphism is controversial; some studies suggest a higher BMD for subjects with the Cdx-A/A genotype [4]; however, this hypothesis was not confirmed by other studies [11, 24].

Besides the relevance of the genetic component, there are other factors known to influence bone phenotypes. Among lifestyle factors, physical activity is considered to play a central role in BMD determination [27]. Indeed, studies have reported that physically active lifestyles are related to increased BMD [7, 28] and lower fracture incidence [8, 12]. In addition, a study employing a meta-analytic approach suggested that physical exercise might slow the rate of bone loss in postmenopausal women [16]. However, the evidence of physical activity influence on BMD is controversial [5], which could be related to the interaction between genes and environment [19]. Recent studies have indicated that health-related response to exercise, including BMD response, may be mediated in large part by variation in genes, leading to an increased interest in knowledge of the relation between physical activity and genes [6, 33].

With regard to the Cdx-2 polymorphism at the VDR gene, it was found that only one study analyzed the interaction between physical activity and its genotypes. Morita et al. [24] studied a large sample of Japanese women and reported a significant interaction between Cdx-2 genotype and previous participation in sports (high school) on BMD in postmenopausal women. The results suggested that women carrying the Cdx-G/G had a better response to physical activity, which is in contrast with the previous hypothesis of a favorable effect for the Cdx-A allele [4]. It is important to note that physical activity history was not assessed by a validated questionnaire because it was not the main purpose of the study. Additionally, the analysis did not involve the recent level of physical activity. Therefore, the purpose of the present study was to analyze the interaction between physical activity level and Cdx-2 polymorphism on BMD of Brazilian postmenopausal women. Aware of potential complications when performing association studies in admixed populations, we genotyped 14 ancestry informative SNPs and took the estimated genetic ancestry values as covariates during the analysis.

Methods
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Subjects
The present investigation design is classified as a cross-sectional observational association study. The postmenopausal women were recruited from a social project sponsored by the University,

which offers physical exercise, psychological, medical and nutritional assistance to the local elderly population, independent of socioeconomic status. Volunteers were residents in the Brasilia, DC area and were invited to participate in this investigation by phone call, when the first visit was scheduled for those who were interested. Initially, four hundred phone calls were made, with two hundred and twenty-four acceptances (56%). The main reasons for nonacceptance included phone number alteration, acute illness, lack of interest and fear to provide a blood sample. In addition, from the two hundred and twenty-four individuals that went to the first visit, only one hundred and ninety were considered for subsequent analysis because of the exclusion criteria and difficulties in blood collection.

In the fist visit, volunteers arrived at the Physical Education and Health Study Laboratory (LEEFS) at approximately 8:00 a.m. and procedures were conducted in the following order: 1) Women answered a face-to-face questionnaire addressing medical history, comorbities, hormonal replacement therapy (HRT) status, skin color and lifestyle habits such as smoking status, alcohol consumption and others; 2) Another questionnaire was applied at a face-to-face interview to asses physical activity level (see below); 3) Blood samples were obtained from each participant for subsequent genotype analysis; and 4) At the end of the visit, a second visit was scheduled for anthropometric and body composition measurements. The period between the two visits was no longer than ten days. The biological material collected was stored at the LEEFS until all volunteers had completed the first visit, then blood samples were moved to the Genomic Science and Biotechnology Laboratory of the University, where DNA extraction and amplification as well as Genotyping were carried out.

The characteristics of the subjects are listed in ◉ **Table 1**. Women impaired to perform the DXA scanning, in pre- or perimenopausal stage, foreign born, implanted with metallic prosthesis or affected by metabolic/endocrine dysfunctions that alter bone metabolism were excluded. Before the beginning of the study, all volunteers were invited to read and sign a consent form. The Catholic University of Brasilia Ethics Committee approved the study procedures.

Physical activity level (PAL) was assessed by the international physical activity questionnaire (IPAQ). The official Portuguese short version of IPAQ previously validated in the Brazilian population [22] was used in the present study. The subjects were divided into two groups based on IPAQ scores: physically inactive (IA) and physically active (AT). Classification of IA or AT was based on the definitions proposed by the American College of Sports Medicine, Centers for Disease Control and Prevention [26] and the U. S. Surgeon General's Report [32], as previously done by Hallal et al. [14]. The questionnaires were applied face-to-face by a trained interviewer.

Bone mineral density assessment
Measurements of BMD were carried out at the Image Laboratory placed at the Laboratory of Physical Education Studies from the University. The BMD was measured at the lumbar spine (L2 – L4), great trochanter, femoral neck and Wards' triangle by dual energy X-ray absorptiometry (DXA) in a Lunar DPX-IQ scanner (Lunar Corp. Madison, WI, USA). The following instructions were given to the subjects: to avoid calcium supplement ingestion on the test day; to not perform radiological contrast exams 14 days before the test; to not perform nuclear medicine exams three

Table 2 Characteristics of the subjects (crude values) according to the Cdx-2 genotype and physical activity level (mean ± standard deviations)

	IA			AT			$p^†$	$p^‡$
	Cdx-G/G	Cdx-G/A	Cdx-A/A	Cdx-G/G	Cdx-G/A	Cdx-A/A		
N	28	37	10	51	50	14		
Age (years)	66.7 ± 5.2	66.7 ± 5.1	65.8 ± 3.3	66.0 ± 4.5	66.6 ± 6.0	68.9 ± 4.1	0.244	
Body weight (kg)	63.21 ± 9.62	65.88 ± 13.38	68.80 ± 15.80	63.11 ± 11.43	66.48 ± 9.72	57.62 ± 9.87	0.11	
Height (cm)	151.21 ± 6.28	152.38 ± 5.43	150.60 ± 5.48	151.98 ± 7.35	152.71 ± 5.47	150.77 ± 8.20	0.947	
BMI (g/m^2)	27.61 ± 3.60	28.32 ± 5.18	30.14 ± 5.81	27.24 ± 4.01	28.48 ± 3.68	25.28 ± 3.27	0.09	
% body fat	40.1 ± 6.3	37.8 ± 7.0	41.3 ± 8.3	37.4 ± 5.4	39.2 ± 5.4	34.8 ± 7.3	0.051	
BMD L2 – L4 (g/cm^2)	0.912 ± 0.173	0.980 ± 0.165	1.008 ± 0.214	0.977 ± 0.152	0.998 ± 0.206	0.997 ± 0.212	0.534	0.702
BMD FN (g/cm^2)	0.831 ± 0.097	0.892 ± 0.124	0.863 ± 0.181	0.891 ± 0.137	0.868 ± 0.125	0.831 ± 0.160	0.038*	0.035*
BMD TR (g/cm^2)	0.756 ± 0.097	0.792 ± 0.138	0.801 ± 0.162	0.779 ± 0.108	0.776 ± 0.130	0.751 ± 0.140	0.346	0.455
BMD WT (g/cm^2)	0.636 ± 0.123	0.704 ± 0.134	0.701 ± 0.199	0.708 ± 0.162	0.679 ± 0.148	0.660 ± 0.170	0.036*	0.049*

IA: physically inactive; AT: physically active; BMI: body mass index; BMD: bone mineral density; FN: femoral neck; WT: Wards' triangle; TR: trochanter; [†] non-adjusted p for the interaction between physical activity and Cdx-2 genotypes; [‡] adjusted p for the interaction between physical activity and Cdx-2 genotypes; [*] Cdx-G/G-AT > Cdx-G/G-IA

days before the test; and to be in a 4-hour fast and wear clothes without metal accessories.

Anthropometric assessment

Body weight was measured in a weight beam scale with 0.1 kg precision (Filizola, São Paulo, Brazil). Height was measured on a stadiometer with 0.1 cm precision (Filizola, São Paulo, Brazil). Body mass index (BMI) was calculated as the weight divided by the height squared (kg/m^2). Fat mass and fat-free mass were assessed by DXA.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes following the method proposed by Miller et al. [23]. The polymorphic sites were amplified by polymerase chain reaction (PCR). The 10 µl reaction mixture contained: buffer 1 ×, $MgCl_2$ 1.5 µM, dNTPs 250 µM, primer 1 µM, Taq DNA polimerase 1 U and 10 – 20 ng de DNA. PCR was performed in a termocycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA, USA) under the following conditions: 05 minutes at 95 °C; 14 cycles (01 minute 95 °C – 01 minute at 63 °C – 01 minute at 72 °C); 25 cycles (01 minute at 95 °C – 01 minute at 56 °C – 01 minute at 72 °C); 10 minutes at 72 °C; and maintained at 10° until the reaction removal from the termocycler.

Genotyping was done by minisequencing primer extension reaction as previously described [18], using the SNaPshot™ Multiplex System (Applied Biosystems, Foster City, CA, USA). Briefly, the reaction consists in extend a primer that anneal immediately adjacent to the polymorphic site with a single fluorescently labeled nucleoside triphosphate complementary to the polymorphic site. Fragments were then electrophoresed at the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and results analyzed with Genescan 3.7 and Genotyper 3.7 softwares (Applied Biosystems, Foster City, CA, USA). The following amplification and extension primers were specifically designed to the reactions:

▶ PCR Primer F (5′-3′) – CATTGTAGAACATCTTTTGTATCAGGA;
▶ PCR Primer R (5′-3′) – GAGAAAAAGGATCAGGGATGA;
▶ Primer Extension (5′-3′) – CCTGAGTAAACTAGGTCACA.

Genotyping of 14 ancestry informative markers (AIM) was also performed using single base extension methodology. Based on parental population (European, African and Amerindian) allele frequencies for 14 AIMs, individual maximum likelihood estimation of admixture proportion was carried out using the program IAE3CI. This approach has been previously demonstrated to provide similar information on the accuracy of ancestral estimates when compared to other methods [31]. The percentage of genomic contribution from African parental population to one's genome, here termed Africanicity, was used in our analysis.

Statistical analysis

Standard statistical procedures were used to calculate means and standard deviation (SD). The χ2 test was used to test if the studied sample was in Hardy-Weinberg equilibrium. The interaction between Cdx-2 genotypes and PAL on BMD was assessed by factorial ANCOVA. To adjust for differences related to confounding variables, stepwise multivariate regression analyses were performed using percent body fat, BMI, age, HRT, self-reported skin-color, smoking, calcium supplementation and Africanicity to identify predictors for BMD at each site, and the factors included in the models were used as covariates. Multiple comparisons with confidence interval adjustment by the Bonferroni procedure were used as post hoc tests when necessary. Statistical significance was established at p < 0.05. Statistical analyses were performed using SPSS 8.0 for Windows (SPSS, Inc., Chicago, IL, USA).

Results
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The utilization of a BMI higher than 30 Kg/m² [13] to define obesity revealed that 28.6 % of the volunteers were obese. Only 4 % of the subjects were smokers and 16.8 % were in hormone replacement therapy. The colors "white" and "brunette" were self-reported by 41.1 % of the subjects, 16.3 % reported themselves as "black" and 1.6 % as "red colored". IPAQ results revealed that 60.9 % of the subjects were classified as AT and 39.1 % as IA. The R^2 values of the multivariate stepwise regressions were 0.267 and 0.255 for femoral neck and Wards' triangle BMD, respectively. The variables included in the models for these two sites were: BMI, HRT status and smoking. Lumber spine BMD, it included BMI, age and Africanicity (R^2 = 0.172). The model for trochanter BMD included BMI, HRT and age (R^2 = 0.279). The variables included in the models were used as covariates in further analyses.

The results of the $χ^2$ test revealed that the genotype distribution was in Hardy-Weinberg equilibrium. Subjects BMD according to Cdx-2 polymorphism and PAL are reported in ◦ **Table 2**. Allelic

frequency was 0.647 for Cdx-G and 0.353 for Cdx-A alleles. Genotype frequency was 41.9% for Cdx-A/A; 45.5% for Cdx-G/A and 12.6% for Cdx-A/A. Cdx-2 polymorphism did not influence BMD at the femoral neck [$f_{(2,188)} = 0.630$, $p = 0.533$)], Wards' triangle [$f_{(2,188)} = 0.055$, $p = 0.946$], lumbar spine [$f_{(2,187)} = 0.328$, $p = 0.721$] and great trochanter [$f_{(2,188)} = 0.068$, $p = 0.934$)]. There were no interactions between Cdx-2 polymorphism and PAL for BMD at the lumbar spine [$f_{(2,187)} = 0.163$, $p = 0.850$] nor at the great trochanter [$f_{(2,188)} = 0.792$, $p = 0.455$]. However, the interaction between Cdx-2 polymorphism and PAL was significant for BMD at the Ward's triangle [$f_{(2,188)} = 3.068$, $p = 0.049$] and femoral neck [$f_{(2,188)} = 3.411$, $p = 0.035$]. Comparisons within genotypes revealed that carriers of the Cdx-G/G genotype classified as AT had higher BMD than those bearing the same genotype and classified as IA. These differences did not occur in the Cdx-G/A or Cdx-A/A genotypes.

Discussion
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The present study brings the first known report of the Cdx-2 genotype distribution in Brazilian subjects. The genotype distribution found in the studied subjects (Cdx-G/G 41.6%; Cdx-G/A 45.8% and Cdx-A/A 12.6%) was close to that found in Japanese women [24], but different from that reported by Fang et al. [11] in a large sample of white Northern Europeans.

According to the present results, the Cdx-2 polymorphism did not influence BMD at any site when analyzed independently of PAL. This is in agreement with the findings of Fang et al. [11] and Morita et al. [24], but contrary to the results reported by Arai et al. [4]. The study of Fang et al. [11] involved 2848 subjects and did not find differences in BMD among the Cdx-2 genotypes. Similar results were reported by Morita et al. [24] after analyzing 1340 Japanese women. In a previous study, however, Arai et al. [4] reported higher BMD at the lumbar spine for postmenopausal women with the Cdx-A/A genotype. This led the authors to suggest that the Cdx-2 polymorphism affects BMD only in cases of estrogen deficiency. The present study was not able to test this hypothesis because it did not evaluate estrogen levels; however, the results were adjusted by the status of hormone replacement and all women were postmenopausal.

With regard to physical activity, Morita et al. [24] reported a significant interaction between Cdx-2 genotypes and participation in a sports club at high school age. The authors reported that postmenopausal women bearing the Cdx-G/G genotype who had participated in sports clubs showed significantly greater BMD at the lumbar spine than subjects who had not participated in any sport club. This difference was not evident within the other genotypes. Similarly, the present study found significant interaction between BMD and PAL, analyzed as the recent history of physical activity. According to our results, only women with the Cdx-G/G genotype classified as AT had higher BMD at the femoral neck and Wards' triangle than carriers of the same genotype classified as IA. These results suggested a better response for the Cdx-G/G genotype.

Our results are contrary to the initial hypothesis of Arai et al. [4], who had suggested a favorable effect for the Cdx-A allele, due to a higher transcriptional activity. However, even if this functional difference was confirmed, it is possible that it is not relevant to the BMD status. The Cdx-2 polymorphism would affect the expression of VDR in the small intestine, and previous studies have suggested that higher calcium absorption does not necessarily

reflect a higher bone mineralization [1,2]. In agreement with this hypothesis, studies with VDR null animals showed that animals provided with an enriched diet had normal bone development even in the absence of VDR [3,17]. Additionally, a recent study suggested that VDR may be an inhibitor of osteoblast activity [30], therefore, the higher transcriptional activity of the Cdx-A allele may have a negative impact on bone response to physical activity.

Therefore, we concluded that the Cdx-2 polymorphism is not associated with BMD in postmenopausal women by itself. The combined analysis of the Cdx-2 genotypes and PAL revealed a significant interaction between both factors on femoral neck and Wards' triangle BMD, bringing the hypothesis that the Cdx-G/G genotype may be related to a better bone response to mechanical stress. However, future researches addressing the role of VDR in bone mechanotransduction are necessary in order to provide insight on the physiological mechanisms associated with the interaction observed in the present study. Studies comparing the response of bone tissue to an exercise training intervention among Cdx-2 genotypes are also necessary.

Conflict of Interest
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The authors report no conflict of interest.

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