Bone mineral density in independent elderly women with different physical and functional profiles and vitamin D receptor gene polymorphisms

Densidade mineral óssea de idosas independentes com diferentes perfis físico-funcionais e polimorfismos do gene do receptor da vitamina D

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Abstract – The aim of the study was to compare BMD among physically independent elderly women with different physical-functional profiles and vitamin D receptor gene (VDR) polymorphisms, as well as to analyze the effect of the interaction between these last two aspects on BMD. Overall, 165 elderly women had BMD assessed by bone densitometry. Handgrip and lower limb strength and functional exercise capacity (6MWT) were also assessed. VDR polymorphisms (TaqI, BsmI, ApaI and FokI) were analyzed by polymerase chain reaction. For analyses, elderly women were categorized according to their performance on physical-functional tests into low performance (LP; < 25th percentile), normal performance (NP; 25th percentile ≤ NP ≤ 75th percentile) and high performance (HP; > 75th percentile). Regarding functional exercise capacity, LP group showed lower BMD compared to HP and NP groups (p=0.003). With respect to handgrip strength, there was a trend for LP group to have lower bone mineral density compared to NP group (p=0.08). No differences were observed in femur and lumbar BMD in comparisons among the different VDR genotypes (0.07≤p≤0.94); among different groups regarding lower limb strength (p=0.49) and in the interaction analysis among variables (0.17≤p≤0.77). It was concluded that physically independent elderly women with low functional exercise capacity have lower bone mineral density than those classified as normal and high performance. However, apparently, there is no effect of the interaction between VDR gene polymorphisms and physical and functional factors on BMD.

Key words: Elderly; Bone density; Exercise tolerance; Genetics.

Resumo – O objetivo do estudo foi comparar a DMO entre idosas fisicamente independentes com diferentes perfis físico-funcionais e polimorfismos do gene do receptor da vitamina D (VDR), assim como analisar a interação entre estes dois últimos aspectos sobre a DMO. Participaram do estudo 165 idosas, as quais tiveram a DMO avaliada por meio da densitometria óssea. Foram avaliadas também a força de preensão manual (FPM), força de membros inferiores (FMI) e a capacidade funcional de exercício (TC6min). Os polimorfismos do gene VDR (TaqI, BsmI, ApaI e FokI) foram analisados pela reação em cadeia da polimerase. Para as análises, as idosas foram categorizadas de acordo com seu desempenho físico-funcional em baixo desempenho (BD; < percentil 25), desempenho normal (DN; percentil 25 ≤ DN ≤ percentil 75) e alto desempenho (AD; > percentil 75). Para a capacidade funcional de exercício, o grupo BD apresentou menor DMO em comparação ao grupo AD e DN (p=0.003). Em relação à FPM, o grupo BD apresentou uma tendência a menor DMO quando comparado ao grupo DN (p=0.08). Não foram observadas diferenças na DMO do fêmur e da lombar em comparações entre os genótipos do gene VDR (0.07≤p≤0.94); entre os grupos em relação à FMI (p=0.24) e na análise de interação entre as variáveis (0.17≤p≤0.77). Pode-se concluir que as idosas fisicamente independentes com baixa capacidade funcional de exercício apresentam menor DMO que aquelas com desempenho normal e alto desempenho. No entanto, aparentemente não existe interação entre os polimorfismos do gene VDR e o desempenho físico-funcional sobre a DMO.

Palavras-chave: Densidade óssea; Genética; Idosos; Tolerância ao exercício.
INTRODUCTION

Increasing age is associated with profound changes in the health status of individuals, such as sarcopenia and decreased bone mineral density (BMD)\textsuperscript{1}. With regard to BMD, literature has shown that genetic factors account for approximately 60 to 80\% of interindividual variability. In this sense, genetic research has grown, especially in relation to vitamin D receptor gene (\textit{VDR}). It is noteworthy that vitamin D along with its receptor is one of the most important components of bone metabolism; hence \textit{VDR} gene is one of the most important genes studied in this research field\textsuperscript{2}.

The pioneering study by Morrison et al.\textsuperscript{3} demonstrated that recessive genotype of the \textit{VDR} gene was associated with higher BMD. Houston et al.\textsuperscript{4} have found association between recessive genotype and lower BMD. In contrast, other studies have shown that genetics was not related to BMD\textsuperscript{5,6}.

It is known that in addition to \textit{VDR} gene polymorphisms, BMD is influenced by environmental factors and the interaction of both \textsuperscript{7}. In this context, physical and functional factors can be highlighted as important contributors of BMD. Some studies\textsuperscript{8-10} have investigated the relationship between physical performance and BMD and found modest associations between variables. Zimmernan et al.\textsuperscript{9} and Foley et al.\textsuperscript{10} showed no correlation between handgrip strength and BMD. Thus, how physical performance in physical and functional tests is related to BMD is still unclear in literature.

Regarding the interaction between \textit{VDR} gene polymorphism and physical and functional performance, studies have shown that the dominant effect is associated with increased BMD in physically active women\textsuperscript{7,11}. Moreover, Tsuritani et al.\textsuperscript{11} found that the recessive allele is more responsive to physical exercise. Jarvinen et al.\textsuperscript{12} found no interaction between genetics and physical activity, demonstrating that the bone tissue benefited from physical activity regardless of VDR gene.

Importantly, to date, the six-minute walk test, which assesses the functional exercise capacity, widely covered by the world literature, has not been used in studies analyzing the physical and functional factors related to BMD. Thus, the 6MWT deserves to be better investigated as it is a simple and inexpensive test\textsuperscript{13}. In addition, its functional feature may reflect the potential to perform activities of the daily living\textsuperscript{13}, which makes the results of the 6MWT and possible links with BMD highly relevant.

Thus, considering that there are still gaps with respect to the effect of the interaction between \textit{VDR} gene polymorphisms and physical and functional tests on BMD, the aim of this study was to compare the BMD among physically independent elderly women with different physical and functional profiles and \textit{VDR} gene polymorphisms and to analyze the effect of the interaction between these last two aspects on BMD.

METHODS

Study design and subjects
A cross sectional study was carried out from September 2009 to January 2013.
The convenience sample consisted of elderly women of an interdisciplinary project (EELO Project; Study on Aging and Longevity) developed at the University of Northern Parana (UNOPAR) that aimed to evaluate socio-demographic factors and indicators of the health conditions of this population in Londrina, Paraná, Brazil. Further information is available at http://www2.unopar.br/sites/eelo page. The EELO project had a total sample of 508 older adults, which is representative of the total population of 43,610 older adults in Londrina. This population of Londrina represents 12% of the total population, which is similar to what has been described in other countries.

Later, from the database of the EELO project, the sample was selected for the performance of bone density examination. The selection criteria for performing bone densitometry were being physically independent and / or active according to functional status classification of Spirduso (levels 3 and 4, respectively). According to Spirduso, elderly classified as Level 3 are able to develop light physical work, but are sedentary and have low functional reserve. Those classified as Level 4 perform regular physical activity and functional capacity are above average; have performed three physical and functional tests (handgrip strength, sitting and standing test and six-minute walk test) and do not make use of drugs that interfere in bone metabolism. Of the 323 individuals who met the inclusion criteria, 42 were not found, 28 refused to participate and nine died. Thus, 244 elderly individuals performed bone densitometry examination. For the analysis of this study, however, only women were considered (n = 165). This study was approved by the Ethics Research Committee of UNOPAR (PP070 / 09) and all participants signed the free informed consent form.

**Anthropometric data**

Initially, individuals were evaluated for body mass and height with an anthropometric scale (Filizola®, São Paulo, Brazil), and then the body mass index (BMI) was calculated using the formula (body mass / height^2). The protocol used was that of Guedes & Guedes.

**Ethnic evaluation**

Ethnic evaluation was performed by the same team of students and teachers, in this case the appraiser, to define the racial group that the individual belongs according to skin color.

**Diagnosis of Osteoporosis**

Bone mineral density was assessed by the dual-energy densitometer unit (QDR 4500, Hologic Inc., Bedford, USA) in the regions of the lumbar spine (L1-L4) and femoral neck. The interpretation for diagnosis was performed according to WHO criteria: normal BMD with T-score of up to -1.0 SD, osteopenia T-score ranging from -1.0 to -2.4 SD and osteoporosis T-score ≤ -2.5 SD at any bone site. The bone mineral density values in the lumbar spine (L1-L4) were also analyzed in g.cm^2. Examinations were performed in a private clinic in the city of Londrina, Paraná.
For the analysis of BMD in g/cm², 46 elderly women were excluded from the sample, since according to the T-score, they showed worse diagnosis in the femur region (region where the values in g/cm² are not obtained). Thus, 119 participants were considered in these analyses.

**Collection of material for DNA analysis**

DNA was obtained from peripheral blood leukocytes collected with EDTA using PureLink Genomic DNA extraction kit (Invitrogen, Carlsbad, USA). The extracted DNA was stored in a freezer at -80°C until time of analysis of polymorphisms.

Evaluation of the DNA quality and quantity was performed by analysis of absorbance in a spectrophotometer (NanoDrop 2000 – Thermo Scientific) at 260nm and 280nm. Subsequently, DNA dilution was performed in ultrapure Milli-Q® water to final concentration of 30ng / uL.

**Polymerase chain reaction (PCR) and analysis of vitamin D receptor gene (VDR) polymorphisms**

For the analysis of single nucleotide polymorphisms (SNPs) of the VDR gene, amplification technique was performed of DNA fragments by polymerase chain reaction (PCR) in real time by TaqMan® system (Applied Biosystems, Foster City, USA). Four polymorphisms were observed: TaqI (rs731236), BsmI (rs1544410), Apal (rs7975235) and FokI (rs2228570). The standard reaction used contained 20μL final volume as follows: 10μl of Taqman® Genotyping Master Mix (1x), 0.5μL probe (1x) (Applied Biosystems, Foster City, USA) 7.5μL ultrapure Milli-Q® water and 2μL DNA (30ng / uL). Thermocycler StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, USA) was used with the following cycling: 60°C for 30 seconds (pre denaturation), 95°C for 10 minutes for initial denaturation, 50 cycles of 95°C for 15 seconds (denaturation) and 60°C for 1 minute and 30 seconds (pairing of primers) and final extension cycle of 30 seconds at 60°C. The evaluation of results was performed by the StepOne Software v2.3.

**Handgrip strength**

Handgrip strength (HGS) was evaluated by manual dynamometer (Takei, Kiki, Kogyo, Japan), according to protocol proposed by Vianna et al.17.

Individuals remained standing with arm outstretched and dynamometer next to the body17. Two maximum handgrip strength measurements in both upper limbs were alternately performed, with an interval of 30 seconds, and the best result was chosen for analysis 17.

**Lower limb strength**

Lower limb strength (LLS) was evaluated using the sitting and standing test. This test consists of sitting and standing from a chair without support, with arms folded across the chest the fastest as possible for 30 seconds18. The test was performed only once after familiarization and the number of complete repetitions was recorded.
**Functional exercise capacity**

Functional exercise capacity was assessed using the six-minute walk test (6MWT), according to protocol of the American Thoracic Society (ATS)\textsuperscript{13}. Two tests were performed in a hallway of thirty meters with minimum interval of thirty minutes, and the highest value was used for analysis. The reference values of Britto et al.\textsuperscript{19} were used.

**Categorization of physical and functional tests**

Older women were categorized according to their performance in physical and functional tests as low performance (LP) (below the 25\textsuperscript{th} percentile), normal performance (NP) (within 25-75\textsuperscript{th} percentile) and high performance (HP) (above 75\textsuperscript{th} percentile), based on the study by Rikli & Jones \textsuperscript{20}.

**Statistical analysis**

Data distribution was analyzed using the Shapiro-Wilk test. Considering the normality of the data, parametric or non-parametric tests were applied. For comparison of bone mineral density (T-score and / or g.cm\textsuperscript{2}) among groups with different performance in physical and functional tests, one-way ANOVA and Kruskal-Wallis tests were used with Tukey and Dunn post-tests, respectively. The interaction analysis was performed using the two-way ANOVA test and Bonferroni post-test. The chi-square test was used to compare categorical variables. Correlations were evaluated using the Pearson and Spearman correlation coefficient.

For the study of genetic variables, the chi-square test was used to analyze the Hardy-Weinberg equilibrium. The level of statistical significance adopted for all analyses was p <0.05.

The sample power was calculated using the GPower \textsuperscript{3.1} software. Correlation between BMD and functional exercise capacity (6MWT) was used, alpha 0.05, correlation for null hypothesis of 0, sample size of 119, resulting in sample power of 0.80.

The statistical programs used for analyses were GraphPadPrism 5.0 (GraphPad Software Inc., San Diego, USA), Statistical Package of Social Science (SPSS) 20.0 (SPSS Inc., Chicago, USA), Statistica (StatSoft Inc., Tulsa, USA).

**RESULTS**

The sample consisted of 165 physically independent elderly women. In relation to bone mineral density, most had osteopenia (n = 92; 56%), 52 participants (31\%) had osteoporosis and 21 (13\%) had normal BMD. The general sample characteristics are presented in Table 1.

**BMD and functional exercise capacity, handgrip strength and lower limb strength**

With regard to functional exercise capacity, LP group had lower BMD of the lumbar spine (g.cm\textsuperscript{2}) compared to HP and NP groups (p = 0.003)
Correlation was observed between lumbar spine BMD (g.cm$^2$) and percentage of the predicted value in the 6MWT ($r = 0.22; p = 0.02$). Comparing the lumbar spine BMD (g.cm$^2$) among groups with different performance in the HGS test, LP group showed tendency to lower BMD when compared to NP group ($p = 0.08$) (Figure 2). In addition, no correlation was found between lumbar spine BMD (g.cm$^2$) and HGS ($r = 0.09; p = 0.32$). Regarding LLS, there was no significant difference in BMD in the comparison among groups ($p = 0.24$) (Figure 3) and there was no correlation between BMD and LLS ($r = 0.12; p = 0.22$). Correlations between BMD of the lumbar spine (g.cm$^2$) and age ($r = -0.24; p = 0.01$), body mass ($r = 0.47; p = 0.0001$) and BMI ($r = 0.47; p = 0.0001$) were found.

**Comparison of BMD among VDR gene polymorphisms**

In relation to bone mineral density (BMD) in the different genotypes (dominant, recessive and heterozygous) of VDR gene polymorphisms (TaqI, BsmI, ApaI and FokI), there was no significant difference in the specific site comparisons to the bone site of the lumbar spine and femoral neck, as shown in Table 2.
There was no effect of the interaction between genetic polymorphisms (TaqI, BsmI, ApaI and FokI) of the \textit{VDR} gene and functional exercise capacity (6MWT) on BMD ($p = 0.58$), as well as with other physical and functional factors. 

### Table 2. Comparison of bone mineral density (BMD) in different vitamin D receptor gene (VDR) polymorphisms

<table>
<thead>
<tr>
<th>VDR Gene Polymorphisms</th>
<th>TaqI</th>
<th>BsmI</th>
<th>ApaI</th>
<th>FokI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample (n)</td>
<td>63</td>
<td>23</td>
<td>79</td>
<td>60</td>
</tr>
<tr>
<td>BMD Site L (g.cm$^{-2}$)</td>
<td>0.883</td>
<td>0.899</td>
<td>0.843</td>
<td>0.871</td>
</tr>
</tbody>
</table>

Data are expressed as mean and standard deviation; absolute frequency; n: number of individuals; Site L: lumbar spine bone mineral density; Site F (lap): femoral neck BMD; Normal BMD (T-score $>-1.0$ SD); Osteopenia: T-score between -1.01 and -2.49 SD; Osteoporosis: T-score $\leq -2.5$ SD; $p$: Kruskal Wallis test value. $n = 165$ for classification according to the T-score; $n = 119$ for lumbar spine BMD in g.cm$^2$. 

**Effect of the interaction among genetic, physical and functional factors on BMD**

There was no effect of the interaction between genetic polymorphisms (TaqI, BsmI, ApaI and FokI) of the \textit{VDR} gene and functional exercise capacity (6MWT) on BMD ($p = 0.58$), as well as with other physical and functional factors.
DISCUSSION

This study showed that physically independent elderly women with low functional exercise capacity had lower lumbar spine BMD compared to elderly women with normal performance and high performance. In addition, BMD correlated with functional exercise capacity and anthropometric factors in this population. However, there was no difference in BMD in relation to VDR gene polymorphisms (TaqI, BsmI, ApaI and FokI) and there was no effect of the interaction between VDR gene polymorphisms and physical and functional tests on BMD.

With regard to the association between BMD and exercise capacity, Taaffe et al. showed that white men with poor aerobic capacity assessed by 400-meter walking test were 2.78 times more likely to have osteoporosis. Although the methodology used by Taaffe et al. is different from that of the present study, results were similar, that is, poor physical performance is associated with lower BMD. It is noteworthy that the present study showed for the first time association between 6MWT (test widely used in the evaluation of functional exercise capacity in healthy subjects and patients with chronic diseases) and BMD.

In relation to lower BMD in elderly women with low functional physical performance, studies have shown an association between handgrip strength and BMD. It is known that the adjustment observed in bone mass to typical mechanical use demonstrates the mechanisms that control longitudinal growth, and bone modeling and remodeling directly determines bone mass, a mechanism known as Frost Theory. This theory has been used to explain the association between physical activity and increased bone mineral content due to mechanical stress on the muscle contraction bone tissue. Indeed, elderly women with better physical and functional performance regarding handgrip strength were those more likely to have higher BMD values. It could be hypothesized that these individuals have more preserved muscle strength because they have higher levels of habitual physical activity, which could contribute to the preservation of BMD. However, the level of habitual physical activity unfortunately has not been evaluated in this study. Accordingly, other studies have shown relationship between peripheral muscle strength assessed by another test (sitting and standing test) and BMD. In the present study, there was no difference in BMD among groups with different performance on the sitting and standing test. It could be inferred that this test evaluates the lower limb physical function, involving different aspects of performance, such as, lower limb muscle strength and balance. Thus, there is no way to isolate the muscle component, because as the evaluation is performed dynamically, balance interferes considerably in the outcome. Thus, the specific analysis between lower limb muscle strength and BMD is compromised using the...
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sitting and standing test. However, it is noteworthy that studies related to BMD and aging involve mostly large samples; thus, simpler methodologies end up by being used. Since this study involves a subsample of another population study, a test for the evaluation of muscle strength with simple methodology was used so that it could be applied to a large number of individuals without the need for expensive equipment of difficult mobility.

Other studies have shown no association between BMD and peripheral muscle strength \(^9,10\), as in the present study. In the study of Zimmermann et al.\(^9\), this may have occurred due to sample size (56 subjects), which may have been insufficient to identify the association, a factor that should also be considered in this research. Foley et al.\(^10\) have reported that the lack of association between peripheral muscle strength and BMD is due to that fact that the muscles evaluated were not adjacent to the site where BMD was measured. However, literature shows that the simple handgrip strength measure may represent the peripheral muscle strength in a generalized way\(^24\). In addition, the great advantage of assessing handgrip strength is the evaluation method because it is simple, easy, noninvasive, and is important predictor of functionality, nutritional status and mortality in the elderly, which use is highly relevant in clinical practice\(^24\). Other studies used peripheral muscle strength tests such as the sitting and standing test also found no correlation with BMD. This is the case of the results of Lindsey et al.\(^22\) and those of the present study. The lack of association between these variables may be related to the nature of this functional test\(^18\), with dynamic characteristics that involve other skills such as balance, coordination, agility, and may not reflect specifically the peripheral muscle strength, as previously mentioned.

Modest and positive correlation between anthropometric variables (body mass and body mass index) and BMD was observed. Kang et al.\(^25\) and Salamat et al.\(^26\) also found positive correlation between body mass and BMD in their studies. Regarding BMI, Salamat et al.\(^26\) found an association between BMD and body mass index, showing that lower BMI is associated with lower BMD. Higher body mass, whether in relation to lean or fat body mass, leads to greater mechanical stress due to the muscle work to support body or locomotion, which consequentially results in stimulation for bone formation \(^27\), explaining the relationships between body mass, BMI and BMD.

It was identified that age is negatively correlated with BMD. This finding corroborates the findings of Kang et al.\(^25\) that advancing age is associated with lower BMD. It is known that age is a risk factor for low BMD, because after the age of thirty years, BMD has a declining rate of one percent per year\(^1\). However, the correlation between age and BMD found in this study was weak \((r = -0.24; \ p = 0.01)\). This finding can be explained by the sample size, the small age variability and the profile of individuals included, i.e., all were physically independent, showing no aspect of fragility that could directly influence BMD.

There were no differences in BMD in relation to \(VDR\) genotypes.
However, literature has demonstrated relationships between polymorphic variations of the VDR gene and BMD. Other studies found no association between VDR gene polymorphisms (TaqI, ApaI, BsmI) and BMD, which corroborates our findings. It is important to highlight how TaqI, BsmI and ApaI polymorphisms affect the vitamin D receptor action, as they have no effect on the end product of the protein encoded by the VDR gene, but modulate gene expression. In the present study, it could be inferred that the effects of VDR polymorphisms were discrete in relation to all factors affecting BMD, in addition to the sample size.

Regarding the association of VDR gene polymorphisms and physical and functional tests and bone mineral density, this study found no interaction among such variables. Similarly, Jarvinen et al. also found no interaction between VDR gene polymorphism and physical activity in premenopausal women. The authors of that study showed that bone benefited from physical activity regardless of genotype. Moreover, Blanchet et al. and Gentil et al. showed that the dominant genotype is associated with increased BMD in physically active women. Tsuritani et al. found that the recessive genotype is more responsive to exercise. These contradictions show that more studies are needed to confirm the interaction between VDR gene polymorphisms, physical and functional tests and BMD. In addition, in relation to polymorphisms, they should be evaluated not only in isolation, but using their combination through the haplotype study.

It should be also mentioned that in addition to physical and functional factors, other environmental components can also influence BMD. Among them, smoking, alcohol, low calcium intake, vitamin D insufficiency, estrogen deficiency, late menarche and low weight. These aspects, associated with genetics, make the study of the interaction between physical and functional performance and VDR gene polymorphisms on BMD quite complex, given the numerous existing environmental factors.

According to the authors, this is the first study investigating the interaction between VDR gene polymorphisms, muscle strength and functional exercise capacity measured by objective methods. Until then, interaction studies described in literature were limited to the investigation of physical aspects through questionnaires. Furthermore, the main VDR gene polymorphism (TaqI, ApaI, BsmI and FokI) were investigated, highlighting the methodological relevance of this study.

Regarding the study limitations, it could be said that the groups showed strong tendency of age differences, which may have contributed to accentuate the findings in the study variables. However, the age of all participants was not discrepant (66 [63-71] years), which can reduce the effect of aging on variables. In addition, the sample may not have been sufficient to detect interactions among variables. It is possible that, for more conclusive statements about the genetic profile, it was necessary to consider a larger number of individuals and the variants of the VDR gene should be assessed using their combination by means of the haplotype study. Furthermore, the quantification of the protein levels of VDR or even the...
transcript of this gene (mRNA) should be performed in order to confirm the bioactivity hypothesis of this protein: how VDR gene polymorphisms could modulate the gene expression and the production of this receptor, which could explain why carriers of certain alleles would show lower BMD. The lack of assessment of physical activity in the daily life can be considered as another limitation, since it could add important information about the physical condition of these individuals.

With regard to the clinical application of the results of this study, the presence of association between functional exercise capacity testing and BMD shows that based on the physical performance assessment of older women, it is possible to identify individuals more likely to have osteopenia and osteoporosis. Thus, for subjects with lower BMD and hence increased risk of fracture, a more specific and early therapeutic approach should be recommended.

CONCLUSIONS

In short, physically independent elderly women with low functional exercise capacity have lower bone mineral density than those with normal and high performance. However, there is no apparent interaction between VDR gene polymorphism and physical and functional performance on bone mineral density.

REFERENCES