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ORIGINAL ARTICLE

# Vitamin D receptor gene polymorphisms influence on clinical profile and bone mineral density at different skeletal sites in postmenopausal osteoporotic women

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## Abstract

Bone remodeling is marked by bone synthesis and absorption balance, and any altered dynamic in this process leads to osteoporosis (OP). The interaction of hormonal, environmental and genetic factors regulate bone metabolism. Since vitamin D displays a classic role in bone metabolism regulation, acting through vitamin D receptor (VDR), the genetic variants within *VDR* were the first ones associated with bone density and remodelling. Therefore, we investigated whether three single nucleotide polymorphisms (SNPs) within *VDR* were associated with OP differential susceptibility and clinical profile from postmenopausal versus healthy women from Northeast Brazil. Genetic association study enrolling 146 postmenopausal osteoporotic women as the patient group and 95 healthy age-matched women as the control group. We assessed three SNPs within *VDR* (rs11168268, rs1540339 and rs3890733), considering the clinical profile of all patients. Our results showed an association of rs11168268 G/G genotype with higher bone mineral density (BMD) mean for the total hip (A/A = 0.828 ± 0.09; A/G = 0.081 ± 0.13; G/G = 0.876 ± 0.12, $p = .039$), and the rs3890733 T/T genotype was associated with increased OP risk in patients below 60 years old (odds ratio [OR] = 5.12, 95% confidence interval [CI ]= 1.13–23.27, $p = .012$). The rs1540339 T/T genotype was associated with protection for individuals with low melanin deposition when compared to the high melanin deposition group (OR = 0.24, 95%CI = 0.06–0.94, $p = .029$). Additionally, 61% of patients presented deficient vitamin D serum levels. The SNP rs11168268 G/G was associated with a significantly increased mean total hip BMD in patients OP, highlighting this SNP and its relationship with BMD.

KEYWORDS
osteoporosis, SNP, VDR, vitamin D

## 1 | INTRODUCTION

Osteoporosis (OP) is an essential skeletal condition characterized by low bone mass, reduced bone strength and increased risk of fractures. The condition results from bone synthesis and reabsorption misbalance, regulated by endocrine, environmental and genetic factors (Brent Richards et al., 2012; Chandra & Rajawat, 2021). The multiple aetiologies of decreased bone mineral density (BMD) and metabolic bone

  

diseases development create major confounding factors, leaving the exact aetiology of OP poorly understood (He et al., 2015).

OP is a current concern in public health worldwide, and its prevalence increases as people live longer. Additionally, it affects both genders and displays bias towards postmenopausal women. The condition presents a genetic background characterized by the polygenic influence and many gene variations associated with low BMD and possibly facilitating fractures (Conti et al., 2015; Lovšin et al., 2018). Different endocrine pathways involved in skeletal ageing with BMD loss process have been described, leading to new possibilities for prognosis and effectiveness in disease therapies (Chandra & Rajawat, 2021; Larsson & Fazzalari, 2014; Y. Zhang et al., 2014).

Vitamin D is a secosteroid hormone with a major source in the skin, synthesized when 7-dehydrocolesterol reacts with ultraviolet B (UVB) light. The active form of vitamin D, known as $D_3$, acts through its receptor—vitamin D receptor (VDR)—and has a significant function in calcium (Ca) absorption and equilibrium, being the natural modulator of bone homeostasis (Goltzman, 2018; Holick, 2004; Y. Y. Zhang et al., 2003). *VDR*, located at chromosome 12 (12q12–q14) and displaying 14 exons, is a member of the nuclear receptor family of transcription factors, regulating essential bone metabolism genes such as osteocalcin, osteopontin and factor nuclear kappa B (NF-$\kappa$B) receptor (Haussler et al., 2010; He et al., 2015). *VDR* is a highly polymorphic gene with several polymorphisms described and the first gene known to be associated with bone density, remodelling and turnover (Conti et al., 2015; He et al., 2015).

*VDR* polymorphisms were first associated with OP because vitamin D and its metabolites play a significant role in the Ca absorption pathway and bone metabolism. However, most of the studied single nucleotide polymorphisms (SNPs; *TaqI*, *BmsI* and *ApaI*) within *VDR* are in non-coding regions or with no known function, except for *FokI* and *Cdx-2* (Mohammadi et al., 2014; Yang et al., 2020). Nevertheless, they have been associated with several pathologies from systemic autoimmune disorders, such as systemic lupus erythematosus to cancer, such as melanoma (Carvalho et al., 2015; Shahbazi et al., 2013; Zeljic et al., 2014).

For its role in bone metabolism and homeostasis, we evaluated whether the TagSNPs rs1168268, rs1540339 and rs3890733, covered by linkage disequilibrium in most *VDR* genes, were associated with postmenopausal OP in women. Additionally, we assessed all patients' vitamin D serum levels, BMD, clinical features and their relation to the *VDR* SNPs investigated.

## 2 | METHODS AND SUBJECTS

### 2.1 | Subjects

In this study, 146 osteoporotic women were enrolled based on clinical and laboratory diagnosis, all postmenopausal (mean age at diagnosis 59, SD ± 3.91, ranging from 50 to 65 years old). All patients were recruited from the Rheumatology Division at Clinical Hospital, Federal University of Pernambuco (UFPE), Recife, Pernambuco, Brazil. Menopause was defined according to the World Health Organization (WHO) criteria as amenorrhea for at least 1 year in women over 45 years old without any other pathological or physiological cause.

Since Brazilian populations are ancestrally genetically heterogeneous, it is not appropriate to divide them into different groups, such as Caucasian or African-derived (Coelho et al., 2015). However, skin melanin deposition is essential to evaluate vitamin D levels and their consequences. Therefore, we arbitrarily divided the OP patients into two main groups: group I—patients with moderate to high melanin deposition (dark to very dark skin) and group II—patients with very low and low melanin deposition (fair and fair light skin tone).

In the control group, 95 postmenopausal non-osteoporotic age-matched (mean age 57, SD ± 3.96, ranging from 49 to 64) women were enrolled. All individuals from the control group presented no medical history of secondary OP on physical examination and laboratory tests. Additionally, no subjects from the study were on hormone replacement therapy. In the absence of a fragility fracture, BMD by dual-energy x-ray absorptiometry was used to diagnose OP or osteopenia according to the WHO classification (Kanis & Kanis, 1994). In addition, plain x-rays of the dorsal-lumbar spine (LS) and hip were performed to diagnose osteoporotic fractures. All information was obtained directly from the patient's assessment and medical records.

All the participants provided written informed consent approved by the local Research Ethics Committee (CEP/CCS/UFPE No. 513/11), according to the 1964 Helsinki Declaration.

### 2.2 | Measurement of 25-hydroxyvitamin $D_3$ (25(OH)D) serum levels and BMD

25(OH)D were determined by LIAISON Chemiluminescent Immunoassay (CLIA) (DiaSorin, Stillwater, MN, USA). Normal 25(OH)D serum levels were defined as values ≥ 30 ng/mL, insufficiency as values 20–30 ng/mL and deficiency as values < 20 ng/mL, according to Yamada et al. (2001).

Measurement by the Dual Energy X-ray Absorptiometry (Lunar Corporation, Madison, WI, USA) was performed at the LS from L1 to L4 anteroposteriorly and the total hip, including the femoral neck, Ward's triangle and trochanter. The results are expressed in g/cm$^2$ and T-score. We used a local database (reference population aged 20 to 29 years) to calculate the T-score. The mean (± SD) of normal values for women was 1.085 g/cm$^2$ (± 0.1) at the LS, 0.913 g/cm$^2$ (± 0.12) at the femoral neck and 0.316 g/cm$^2$ (± 0.07) at the distal radius. The in vivo precision error of the equipment employed in the study expressed in percentage coefficient of variation (%CV = SD + mean BMD of repeated measurements) was 0.9% for the LS on the anteroposterior view and 1.2% for the femoral neck.

### 2.3 | SNPs selection and VDR genotyping

Genomic DNA was isolated from 5 mL of whole blood using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA), following the protocol according to the manufacturer's

guidelines. Polymorphisms were selected using the SNPBrowser software version 4.0 (Applied Biosystems, Foster City, CA, USA) and the HapMap database (http://hapmap.ncbi.nlm.nih.gov/). We selected three TagSNPs rs11168268, rs1540339 and rs3890733, tagging some of the most studied polymorphisms, namely, *Taq-I*, *Bsm-I*, *Apa-I*, and considering the 10% minimum allele frequency in Caucasian (Nothen European from Utah-CEU) and African-derived (Yoruba in Ibadan-YRI) subpopulation according to National Center for Biotechnology Information. A TagSNP is a representative SNP in a particular genome region, presenting high linkage disequilibrium with other polymorphisms within or not a gene (Stram, 2004). The list of all tagged SNPs by the ones assessed herein is shown in Supporting Information Data 1.

Genotyping was performed with commercially available fluorogenic allele-specific Taqman Probes (Applied Biosystems, Foster City, CA, USA) using the ABI7500 Real-Time PCR system (Thermo Fisher, Madison, WI, USA). Allelic discrimination was followed as recommended by the manufacturer and analysed using the SDS software version 2.3 (Applied Biosystems, Foster City, CA, USA).

### 2.4 | Statistical analysis

Allelic and genotypic frequencies and Hardy–Weinberg equilibrium were performed using the SNPStats tool (available online: http://bioinfo.iconcologia.net/SNPstats). The exact Fisher test was applied to determine the statistical significance of all comparisons. Haploview Software version 4.2 was used for haplotype associations. 'SNPassoc' R software package version 2.12.2, developed for genetic studies, was used for evaluating the association between SNPs and postmenopausal OP susceptibility and all clinical features (González et al., 2007). The multivariate analysis logistic regression was performed to investigate the association between the variables and dependent variables binary: SNPs and Ca, vitamin D and BMD of total hip and femoral neck in IBM SPSS statistic software version 18.0 (IBM Corp, Armonk, NY, USA). *p*-values < .05 were considered statistically significant.

## 3 | RESULTS

### 3.1 | Clinical characterization of the OP patients

In total, 146 patients with postmenopausal OP were included in our analysis. Additionally, all the clinical and laboratory findings and anti-OP therapy from the patients' group are depicted in Table 1, with a mean level of 25(OH)D of 27.99 ng/mL. Low serum levels of 25(OH)D were observed in 89/146 (60.95%) and vitamin D deficiency in 68/146 (45.58%). Furthermore, 9/146 (6.2%) presented historical fractures, whereas 137/146 (93.8%) patients had no fractures (Table 1).

### 3.2 | *VDR* allelic and genotyping frequencies

*VDR* allelic and genotypic frequencies from the selected SNPs were in Hardy–Weinberg equilibrium in OP patients and healthy controls, and all assessed frequencies are shown in Table 2. No association was identified when assessing the patients and control groups overall. However, we identified associations by subgroups when stratifying the patient's group by clinical features and SNPs presence.

When comparing patients below and above 60 years old, the SNP rs3890733 T/T genotype was associated with the group of individuals below 60 years old (odds ratio [OR] = 5.16, 95% confidence interval [CI] = 1.1–24.1, $p = .043$ and OR = 5.12, 95%CI = 1.13–23.27, $p = .012$) in the codominant and recessive model, respectively (Table 3).

**TABLE 1** Clinical features from all assessed osteoporotic postmenopausal women.

| Characteristic | *N* (%) |
|---|---|
| Moderate to high skin melanin deposition | 84 (57.5) |
| Low to very low skin melanin deposition | 62 (42.5) |
| Age (range) | 59 (50–65) |
| Mean of years since menopause (range) | 11 (2–27) |
| Mean of body mass index mean (range) | 26.24 (18.37–41.65) |
| **Obesity (%)** | |
| Present | 87 (59.6) |
| Absent | 59 (40.4) |
| **Smoking (%)** | 55 (37.7) |
| Smoking duration, years, mean (range) | 20 (5–45) |
| **Mean bone mineral density in g/cm$^2$ (range)** | |
| Femoral neck | 0.739 (0.482–0.987) |
| Total hip | 0.828 (0.518–1.182) |
| Lumbar spine (LS) | 0.768 (0.484–0.923) |
| **Site of osteoporosis (OP)[a] (%)** | |
| Femoral neck | 26 (17.8) |
| Total hip | 16 (10.96) |
| LS | 142 (97.26) |
| **Osteoporotic fractures (%)** | |
| Present | 9 (6.2) |
| Absent | 137 (93.8) |
| **Vitamin D serum levels (%)** | |
| Low serum level of 25-hydroxyvitamin $D_3$ | 89 (60.95) |
| Vitamin D insufficiency | 21 (14.38) |
| Vitamin D deficiency | 68 (45.58) |

[a]Some patients presented OP in more than one site.

### 3.3 | *VDR* SNPs and clinical features from postmenopausal osteoporotic patients

Moreover, no association was found between *VDR* polymorphisms and vitamin D levels. Regarding BMD, we observed a statistically significant higher BMD mean of total hip among patients for the SNP rs11168268 G/G genotype when compared with A/G genotype (A/A = 0.828 ± 0.09; A/G = 0.081 ± 0.13; G/G = 0.876 ± 0.12, $p = .039$; Supporting Information Data 2).

**TABLE 2** Allelic and genotypic distribution from all TagSNPs assessed within vitamin D receptor OP patients and controls.

| Polymorphism | Patients *N* (%) | Controls *N* (%) | OR (95% CI) | *p*-value |
|---|---|---|---|---|
| **rs11168268** | | | | |
| **Allele** | **140** | **92** | | |
| A | 159 (57%) | 113 (61%) | | |
| G | 121 (43%) | 71 (39%) | 1.21 (0.81–1.80) | .33 |
| **Genotype** | | | | |
| A/A | 48 (34.28%) | 35 (38.04%) | 1.00 | |
| A/G | 63 (45%) | 43 (46.74%) | 1.07 (0.57–1.99) | .88 |
| G/G | 29 (20.72%) | 14 (15.22%) | 1.52 (0.65–3.56) | .33 |
| **rs1540339** | | | | |
| **Allele** | **142** | **84** | | |
| C | 204 (72%) | 119 (71%) | | |
| T | 80 (28%) | 49 (29%) | 0.95 (0.61–1.48) | .83 |
| **Genotype** | | | | |
| C/C | 73 (51.41%) | 41 (48.81%) | 1.00 | |
| C/T | 58 (40.85%) | 37 (44.05%) | 0.88 (0.48–1.60) | .66 |
| T/T | 11 (7.74%) | 6 (7.14%) | 1.03 (0.32–3.65) | 1.00 |
| **rs3890733** | | | | |
| **Allele** | **141** | **88** | | |
| C | 197 (70%) | 126 (72) | | |
| T | 85 (30%) | 50 (28%) | 1.08 (0.70–1.68) | .75 |
| **Genotype** | | | | |
| C/C | 74 (52.48%) | 47 (53.41%) | 1.00 | |
| C/T | 49 (34.75%) | 32 (36.36%) | 0.97 (0.52–1.80) | 1.00 |
| T/T | 18 (12.77%) | 9 (10.23%) | 1.27 (0.49–3.48) | .66 |

Abbreviations: CI, 95% confidence interval; OR, odds ratio; *p*-value.

**TABLE 3** Patient stratification analysis according to age: Above and below 60 years old and genotype distribution.

| rs3890733 | >60 years N = 50 (%) | <60 years N = 91(%) | OR, CI and *p*-value |
|---|---|---|---|
| **Codominant** | | | |
| C/C | 29 (58%) | 45 (49.5%) | OR = 1 |
| C/T | 19 (38%) | 30 (33%) | OR = 1.02,CI = 0.49–2.13 |
| T/T | 02 (4%) | 16 (17.5%) | OR = 5.16,CI = 1.1–24.10, ***p* = .043**[a] |
| **Recessive** | | | |
| C/C + C/T | 48(96%) | 75(82.4%) | OR = 1 |
| T/T | 02(4%) | 16(17.6%) | OR = 5.12,CI = 1.13–23.27, ***p* = .012**[a] |

Abbreviations: CI, 95% confidence interval; OR, odds ratio; *p*-value.
[a]Statistically significant *p*-value.

We also evaluated haplotype combinations in our data, but no association either to OP susceptibility or gene–gene interactions (epistasis) was detected in this study (Supporting Information Data 3). However, when considering the *VDR* SNPs and skin deposition melanin in the patients' group, SNP rs1540339 T/T genotype was associated with low susceptibility for individuals from group II (fair and fair light skin tone) in both codominant and recessive models (OR = 0.2, 95%CI = 0.05–0.8, $p = .043$ and OR = 0.24, 95%CI = 0.06–0.94, $p = .029$; Supporting Information Data 4).

Multivariate analysis of genotypes with clinical features such as Ca, vitamin D and BMD of total hip and femoral neck has not presented statistically significant results (data not shown).

## 4 | DISCUSSION

This study assessed the TagSNPs rs3890733, rs11168268 and rs1540339 within *VDR* and their role in OP susceptibility and its traits. *VDR* polymorphisms have been associated with both OP susceptibility and BMD in several populations (Horst-Sikorska et al., 2013; Mohammadi et al., 2014; Pouresmaeili et al., 2013; Singh et al., 2013; Wang et al., 2021).

Age-related bone loss is usually asymptomatic, with different genetic and environmental influences on BMD in several sites throughout the skeleton (Pouresmaeili et al., 2013). However, we identified TagSNP rs3890733 T/T genotype association with an increased risk of presenting OP at a younger age (< 60 years old) in both codominant and dominant analysis, which could relate to a more intense loss in bone turnover over the years due to the fact that in the first 10 years after menopause, women show a marked loss of bone mass (Finkelstein et al., 2008). Estrogen is one of the main elements in physiological bone remodelling. Its deficiency after menopause causes increased production of cytokines such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor (TNF), which increase the half-life of the osteoclast and the differentiation of pre-osteoclastic cells in mature osteoclasts. The lack of the hormone also decreases the number of osteoblasts and osteocytes, which impairs the detection of microdamage and immediate repair of bone mass (Pacifici, 1996; Rahnama et al., 2013).

The SNP rs1168268 G/G was associated with increased BMD in this study. Noteworthy, this particular SNP tags the extensively studied SNP *BsmI* (rs1544410). The *BsmI* is an intronic variant with unknown protein consequence; however, it is in strong linkage disequilibrium with the polyA variable number of tandem repeats in the 3′ untranslated region (3'UTR) (Ingles et al., 1997). Therefore, it may influence *VDR* transcript (Uitterlinden et al., 2004), which in turn enhances vitamin D proper function and leads to increased BMD. So, the G/G genotype could be an attenuating factor for patients with hip OP. Therefore, this TagSNP could improve the BMD in these patients. Furthermore, it covers three well-recognized SNPs (Apa-I, Bsm-I and Taq-I) that may potentially influence the stability of RNAm at the VDR gene (Chen et al., 2020). Some studies observed an association between the presence of these restriction sites (Apa-I, Bsm-I and Taq-I) with increased BMD, higher peak bone mass and even a decrease of bone loss in Iranian, Turkish, Chinese and Dutch populations (Creatsa et al., 2011; Jakubowska-Pietkiewicz et al., 2012; Li et al., 2012; Özaydin et al., 2010; Pouresmaeili et al., 2013; Qin et al., 2004). However, the finding of this study is unusual in the Brazilian population.

The TagSNP rs1540339 T/T genotype was associated with lower susceptibility to OP in group II (very low to low melanin skin deposition), compared to group I (moderate to high melanin skin deposition). Our data disagree with those of Horst-Sikorska, which show that decreased susceptibility to OP and its fractures are substantially lower in African-derived subjects—which usually present moderately high melanin when compared to Caucasian and Asian-derived origin, with very low to low melanin skin deposition (Horst-Sikorska et al., 2013). This particular TagSNP rs1540339, located in the intron 4 within *VDR* and has no precise function, has previously been associated by our research group with Type I Diabetes (De Azevêdo Silva et al., 2013) and tags three other SNPs rs2239181, rs2239179 and rs886441. At the same time, the rs2239179 has been associated with lower susceptibility to melanoma in a Caucasian-derived population (Ogbah et al., 2013). Melanoma is a type of cancer strongly influenced by sun exposure, and melanin is known to act as a biological protector against UV light damage to DNA (Böhm et al., 2005). Therefore, Caucasian-derived individuals with less melanin than African-derived with moderate to high melanin may display advantages in vitamin D production but with increased UV light exposure consequences (Lupsa & Insogna, 2015).

In this study, we observed that 61% of all patients presented low vitamin D levels, which agrees with most studies showing that low vitamin D status is a risk factor for BMD loss in postmenopausal women (Chang & Lee, 2019; van der Wielen et al., 1995). Estrogen has an essential role in increasing the activity of the enzyme responsible for activating vitamin D; therefore, declining estrogen levels during menopause could lead to vitamin D deficiency (LeBlanc et al., 2014). Furthermore, even though 25-hydroxyvitamin D serum level of 20 ng/mL seems to be enough for homeostasis of bone metabolism, it is considered low for the non-classical roles of vitamin D, leading to other pathologies (Lupsa & Insogna, 2015).

Low vitamin D levels are known to contribute to reducing BMD, increasing the risk of fractures and falls in the elderly. However, in this study, the absence of fractures was more prevalent (93.8%) than its presence. In addition, the patient recruitment was carefully carried out to age-matched healthy controls. Therefore, it is probable that the younger the OP patient, the lower the risk of falling with subsequent fractures. Nevertheless, low vitamin D levels were still inversely associated with increased parathyroid hormone, alkaline phosphatase and osteocalcin levels in postmenopausal women leading to accelerated bone mass loss and low BMD (Capatina et al., 2014; Lips et al., 2001).

In summary, our data show that *VDR* variants are associated with higher BMD and increased susceptibility to OP in patients under 60 years old. Although this study has a limited number of samples and a '*p*-value' close to borderline statistical significance, it is the first study that evaluated SNPs in linkage disequilibrium in a Brazilian population, which emphasizes the role of VDR in the clinical features of the disease in this population.

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### CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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