Abstract

Fractures are the feared consequences of osteoporosis and fractures of the proximal femur (FPF) are those that involve the highest morbidity and mortality. Thus far, evaluation of bone mineral density (BMD) is the best way to determine the risk of fracture. Genetic inheritance, in turn, is one of the major determinants of BMD. A correlation between different genotypes of the vitamin D receptor (VDR) and BMD has been recently reported. On this basis, we decided to determine the importance of the determination of VDR genotype in the presence of an osteoporotic FPF in a Brazilian population. We studied three groups: group I consisted of 73 elderly subjects older than 65 years (78.5 ± 7.2 years) hospitalized for nonpathological FPF; group II consisted of 50 individuals older than 65 years (72.9 ± 5.2 years) without FPF and group III consisted of 98 young normal Brazilian individuals aged 32.6 ± 6.6 years (mean ± SD). Analysis of VDR gene polymorphism by restriction fragment length polymorphism (RFLP) was performed by PCR amplification followed by BsmI digestion of DNA isolated from peripheral leukocytes. The genotype distribution in group I was 20.5% BB, 42.5% Bb and 37% bb and did not differ significantly from the values obtained for group II (16% BB, 36% Bb and 48% bb) or for group III (10.2% BB, 47.6% Bb and 41.8% bb). No differences in genotype distribution were observed between sexes or between the young and elderly groups. We conclude that determination of VDR polymorphism is of no practical use for the prediction of FPF. Other nongenetic factors probably start to affect bone mass, the risk to fall and consequently the occurrence of osteoporotic fractures with advancing age.

Key words
- Osteoporosis
- Fracture of proximal femur (FPF)
- Vitamin D receptor polymorphism
Introduction

About 25% of American menopausal women are expected to suffer a bone fracture due to osteoporosis (1). Fractures of the proximal femur (FPF) are associated with a larger number of deaths, with disability and greater medical costs than all other osteoporotic fractures taken together (2,3) and therefore represent a considerable socioeconomic problem. The risk for osteoporotic fractures is inversely correlated with the amount of bone mass in an individual and genetic inheritance is considered to be a major determinant responsible for 60 to 80% of bone mass (4). The search for a genetic marker for osteoporosis interests many researchers worldwide in view of the socioeconomic impact of this disease. Morrison et al. (5) suggested that the alleles of the vitamin D receptor (VDR) may be of high predictive value in the quantification of bone mass in an individual. Although later studies conducted on different racial groups in different countries have produced conflicting results (6-8), we observed the same correlation as that reported by Morrison et al. in a population of 126 premenopausal Brazilian women from the city of São Paulo (9). We reported that a group of women with a BB genotype had, on average, a lower bone mineral density (BMD) than women with the bb genotype and this difference was more significant in a region of the neck of the femur.

Few studies, however, are available about the determination of VDR polymorphism in the presence of osteoporotic fractures and all of them concern vertebral fractures. No study has correlated VDR polymorphism with osteoporotic FPF. Thus, the objective of the present study was to determine the practical usefulness of the determination of VDR genotype for the prediction of FPF risk in a Brazilian population.

Patients and Methods

Three groups were evaluated in the present study. Group I consisted of 73 patients aged 65 years or older consecutively admitted to the Orthopedics Department of two teaching hospitals in the city of São Paulo for FPF. Patients whose fracture was due to the presence of local neoplasia or bone metastases were excluded from the study. The patients reported their history of disease and of use of medications and the data were confirmed in the medical records during hospitalization. The associated diseases of the patients studied were: diabetes mellitus (DM) in 17 (23.2%), systemic arterial hypertension (SAH) in 27 (37%), heart disease in 9 (12.3%), senile dementia in 6 (8.2%), osteoarthritis in 5 (6.8%), and treated primary hypothyroidism in 1 (1.4%). Only 8% of these patients were aware of the fact that they had osteoporosis and were taking calcium, vitamin D and/or calcitonin. Eleven patients (15%) took thiazides, 9 (12.3%) took oral hypoglycemic agents, 13 (17.8%) took anti-hypertensive agents, and 3 (4.1%) took anxiolytic and/or psychotropic agents.

Group II consisted of 50 individuals without current or previous FPF of a similar age range (≥ 65 years) consecutively seen at the Geriatrics Outpatient Clinic of one of the teaching hospitals. Patients known to have neoplasias were excluded from this group of aged subjects without FPF. In this group, 7 patients (14%) had DM, 27 (54%) had SAH, 2 (4%) had heart disease, 5 (10%) had dyslipidemias, 3 (6%) had chronic bronchitis, 3 (6%) had osteoarthritis, and 1 (2%) had treated primary hypothyroidism. Among the medications taken by these individuals were thiazides (9 individuals, 18%), oral hypoglycemic drugs (4 individuals, 8%), antihypertensive drugs (14 individuals, 28%), and anxiolytics (1 individual, 2%).
Finally, group III consisted of 98 young healthy volunteers ranging in age from 18 to 45 years, all of them employees of the Federal University of São Paulo.

Because of the many races and rich miscegenation occurring in Brazil, we divided the individuals into white, black, crossbred (white with black and/or Indian) and oriental.

All individuals in groups I and II were submitted to a questionnaire that evaluated racial characteristics and daily ingestion of dairy calcium. Ingestion of dairy calcium has been characterized as the number of glasses of milk drunk in a day as well as the ingestion of milk by-products (yoghurt, cheese, clabber). According to these data, the patients were classified into three groups: 1) those ingesting less than 500 mg calcium/day; 2) those ingesting 500 to 1000 mg/day, and 3) those ingesting more than 1000 mg/day. The questionnaires were filled out by the same interviewer.

The protocol was approved by the Medical Ethics Committee of the Federal University of São Paulo, and all subjects gave informed consent to participate in the study.

A blood sample was collected from each subject for extraction of genomic DNA. All laboratory tests were carried out in the Endocrinology Laboratory of the Federal University of São Paulo.

Restriction fragment length polymorphism method (RFLP): genomic DNA was extracted from peripheral blood leucocytes using a commercial kit (Puregene, Gentra Systems, Inc., Minneapolis, MN). The desired fragments of the receptor gene were amplified by the polymerase chain reaction (PCR) using the same primers as described by Morrison et al. (10). The PCR product was submitted to digestion with the BsmI restriction enzyme for 3 h and the fragments were separated by agarose gel electrophoresis and photographed. The genotype denoted BB does not have the restriction site recognized by the enzyme, and therefore presents a band of 800 bp. The bb genotype appears as two bands of 650 and 150 bp, and the Bb heterozygote is recognized by presenting 3 bands of 800, 650 and 150 bp.

Statistical analysis

The data are reported as means ± SD and medians. Statistical analysis for comparison of two groups of data was performed by the nonparametric Mann-Whitney test since most of the data did not present normal distribution. The variation in the distribution of the three VDR genotypes (BB, Bb and bb) in the groups studied was compared by the chi-square test. The level of significance was set at P≤0.05. The crude odds ratio and the ratio adjusted for sex, age and race were obtained by 95% point and confidence intervals using unconditional logistic regression. Dairy calcium ingestion data were compared by the chi-square test.

Results

Of the 73 patients in group I, 56 (76.7%) were women and 17 (23.3%) were men, with a female/male ratio of 3.3/1. Age ranged from 65 to 94 years, with a mean ± SD of 78.52 ± 7.2 years and a median of 78 years. There was a geometric increase in fractures with age range, with 42.4% of them occurring in patients 80 years or older. When the patients were divided into subgroups according to different age levels within sex, the largest number of cases of FPF in men was found to occur from the age of 75 years, when the number of FPF doubled (58.7%), while in women the frequency almost doubled later, starting at the age of 80 years (48.2%) (Figure 1). With respect to racial distribution, there was a lower predominance of fractures among blacks and a higher predominance among orientals than in the group II. The racial composition of the group was 56 whites (76.7%), 12 crossbreds (16.4%), 4 orientals (5.3%) and only one black (1.4%).
A.C. Ramalho et al.

Of the 50 individuals in group II, 36 (72%) were women and 14 (28%) were men. Mean ± SD age was 72.9 ± 5.2 years and the median was 72 years (range: 64-85 years). The racial composition of the group was 38 whites (76%), 5 blacks (10%), 7 crossbreds (14%), and no orientals.

Of the 98 individuals in group III, 67 (68.4%) were women and 31 (31.6%) were men. Mean ± SD age was 32.6 ± 6.6 years and the median was 33 years (range: 18-45 years). The racial composition of the group was 64 whites (65.3%), 11 blacks (11.2%), 21 crossbreds (21.4%), and 2 orientals (2.0%).

The distribution of the various VDR genotypes (BB, Bb and bb) was similar for the three groups (Figure 2). A slight predominance of genotype BB (20.5%) was observed in group I compared to group II (16%) and group III (10.2%), but the difference was not statistically significant (P = 0.23). Allele B was detected in 41.7% of the individuals in group I and in 34% of the individuals in group II, the difference again being nonsignificant (P = 0.3). To better compare the frequency of BB and bb in group I and group II, we obtained the odds ratio of 1.67 with a confidence interval of 0.6 to 4.62, a nonsignificant value (P>0.32). The presence in the model of sex, race and age did not affect this result. Using the odds ratio to compare the frequency of BB in group I with the frequency in groups II and III (patients without FPF), we did not find a statistically significant difference (odds ratio = 2.0; confidence interval = 0.88 to 4.55; P = 0.1), even when considering variables such as sex, race and age.

The frequency of BB was higher in the elderly groups (18.6% for groups I and II as a whole) compared to group III (10.2%), but the difference was not statistically significant (P = 0.17). Using the odds ratio to compare the frequency of BB in the elderly groups (group I and group II) with the young group (group III), we did not find significant differences (odds ratio = 1.85; confidence interval = 0.79 to 4.32; P = 0.16).

Genotype distribution was similar for the two sexes: men presented 40.3% bb, 43.4% Bb and 11.3% BB and women presented 42.1% bb, 41.5% Bb and 16.3% BB (P = 0.52). The analysis of the distribution of VDR genotype only in group I and II women did not show a significant difference (P = 0.39), even when considering only white women (P = 0.62) (Table 2).

Dairy calcium ingestion was similar for groups I and II, in which 34.5% of patients with fractures and 40% of subjects with no fractures ingested <500 mg calcium/day (P = 0.36). Also, there was no significant difference in calcium ingestion between the various genotypes (P = 0.2).
Discussion

The present study was the first in which the correlation between VDR polymorphism and the occurrence of FPF was evaluated. It was centered on the role of the VDR genotypes described by Morrison et al. (5) in the occurrence of osteoporotic FPF. Although our data showed a larger number of BB genotypes in the group of patients with fractures, the chi-square test showed that this difference was not sufficient to rule out random sampling variability. The other two genotypes were also equally frequent in the group of patients with fractures compared to the other groups. Since most studies in the literature refer to VDR genotype in women, we analyzed our data only in women and we did not find any predominant VDR genotype in women with FPF compared to women without FPF. Thus, the determination of VDR genotype would be of no predictive value for the risk of fracture of the proximal femur of an individual. However, it is important to consider that there was a higher frequency of BB in group I that we believe would lead to statistical significance if the number of patients were higher. We excluded the influence of variables such as sex, age and race from the model, and these variables did not influence the results.

In agreement with our results, other authors did not find a correlation between VDR genotypes and fractures. However, all the previous studies concern only vertebral fractures. Hayes et al. (11) evaluated the correlation between the genotypes determined with another restriction enzyme, TaqI, and the length of the femoral head, a characteristic linked to the risk of local fracture, but did not demonstrate the importance of genotype among individuals who had already suffered a fracture. Houston et al. (12), in the UK, evaluated the correlation of VDR polymorphism with the occurrence of vertebral fractures and did not identify any predominant genotype in this type of fracture. In another study, Francis et al. (13) did not identify any predominant genotype in a group of men with vertebral crush fractures. In contrast, Yanagi et al. (14), in a study on Japanese women, demonstrated that the frequency of BB genotypes increases considerably from 3% in the control group to 24% in a group of women with osteoporosis with vertebral fractures.

Although we did not measure BMD, we believe that a low BMD is of little significance if it does not imply a larger number of fractures. Most of the studies on VDR polymorphisms are based on the correlation between BMD and the genotypes defined. In the present study, our concern was to objectively analyze the significance of genotype in terms of the prediction of the risk of fracture in an individual, and our results showed that this factor cannot be used to define a higher risk of FPF.

Although BMD is known to play an im-

### Table 1 - VDR genotype distribution among different races in the groups of young individuals, elderly subjects without fractures and elderly subjects with fractures.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>White</th>
<th>Black</th>
<th>Crossbred</th>
<th>Oriental</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB</td>
<td>25 (15.8%)</td>
<td>4 (23.5%)</td>
<td>4 (10%)</td>
<td>0 (0%)</td>
<td>33 (14.9%)</td>
</tr>
<tr>
<td>Bb</td>
<td>71 (44.9%)</td>
<td>3 (17.6%)</td>
<td>21 (52.2%)</td>
<td>2 (33.3%)</td>
<td>97 (43.9%)</td>
</tr>
<tr>
<td>bb</td>
<td>62 (39.2%)</td>
<td>10 (58.8%)</td>
<td>15 (37.5%)</td>
<td>4 (66.6%)</td>
<td>91 (41.2%)</td>
</tr>
<tr>
<td>Total</td>
<td>158 (100%)</td>
<td>17 (100%)</td>
<td>40 (100%)</td>
<td>6 (100%)</td>
<td>221 (100%)</td>
</tr>
</tbody>
</table>

**χ² = 8.52. P = 0.2027.**

### Table 2 - VDR genotype distribution in women with and without fractures of the proximal femur (FPF).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>With FPF</th>
<th>without FPF</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB</td>
<td>13 (23.2%)</td>
<td>7 (19.4%)</td>
</tr>
<tr>
<td>Bb</td>
<td>23 (41.1%)</td>
<td>11 (30.5%)</td>
</tr>
<tr>
<td>bb</td>
<td>20 (35.7%)</td>
<td>18 (50%)</td>
</tr>
<tr>
<td>Total</td>
<td>56 (100%)</td>
<td>36 (100%)</td>
</tr>
</tbody>
</table>

**χ² = 1.8. P = 0.39.**
A.C. Ramalho et al.

important role in the risk of fracture, the occurrence of FPF is not justified simply by a reduction in BMD. Other factors that do not depend on BMD are also involved, such as those that increase the risk of falls for an elderly individual (15).

There is a variation in the distribution of VDR alleles among different racial groups. In oriental countries such as Japan the frequency of BB is low in the general population (16). In agreement with this fact, we did not detect any BB genotype in the 6 individuals of oriental extraction who participated in the present study. Although the number of subjects involved is small, these data agree with those observed in another study conducted on a population of premenopausal women in the city of São Paulo (9), where the B allele was found only in 16.6% of orientals, in contrast to 60.8% of whites and 74.9% of blacks and crossbreds.

Although there are clear population differences in the distribution of VDR genotypes, the environmental or life style factors that might explain them are unknown. Calcium ingestion and assimilation may be one of these factors, as previously demonstrated (17,18). In the present study there was no difference in the ingestion of dairy calcium between groups I and II. Also, there was no significant difference in calcium ingestion between the various genotypes.

The study also permitted us to determine some of the epidemiologic characteristics of FPF in the city of São Paulo, Brazil, which had not been studied previously. The hospitals where the patients were studied are large public teaching hospitals which are representative of the entire population.

The female/male ratio of osteoporotic FPF was 3.3/1, a value similar to the 3.8/1 ratio reported for the La Plata region of Argentina (19). It should be pointed out that in Brazil, a country of continental dimensions and with a wide variety of ethnic groups, costumes and dietary habits, if the populations of the North or Center of the country were studied, the data obtained may differ from those reported here. Almost half the FPF of our patients occurred after 80 years of age and the exponential increase in FPF with age observed in our group has been previously reported by several investigators (2,19,20). Only one black patient was observed in the group of subjects with FPF (1.4%), whereas groups II and III included approximately 10% black subjects. This is in agreement with the literature, which emphasizes the greater protection against osteoporosis and fractures among blacks (21,22).

The discovery of VDR polymorphism as a factor associated with reduced BMD in many of the populations studied has led to an advance in the study of osteoporosis by finally providing a genetic marker, even though a weak one, although its physiopathological significance has not been determined. However, the use of genotype determination as a predictive factor for the risk of proximal femur fracture did not prove to be valid in the present study.

References