



TNFRSF11B gene variants and bone mineral density in postmenopausal women in Malta

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Abstract

A number of polymorphisms in various genes have been identified and associated with bone mineral density (BMD) and with an increased risk of osteoporosis.

Objective: In this study, three single nucleotide polymorphisms (SNPs) within the TNFRSF11B gene were studied for association with an increased risk of osteoporosis in postmenopausal Maltese women ($n = 126$).

Methodology: Analysis was performed by PCR restriction fragment length polymorphism (RFLP) while BMD at the lumbar spine, femoral neck, Ward's triangle and trochanter was measured by DEXA.

Results: No significant association was observed between genotypes and BMD for all polymorphisms studied within this gene. Homozygotes CC (T⁹⁵⁰-C) were observed to have the highest BMD at all anatomical sites although statistical significance was not reached when comparing the three genotypes. A statistical significant difference was observed in the distribution of genotype frequencies for this polymorphism between normal individuals and those that were either osteopenic or osteoporotic at one or both anatomical sites, with the TT genotype associated more frequently with low BMD. The T⁹⁵⁰-C and G¹¹⁸¹-C polymorphisms were in strong linkage disequilibrium with each other but not with the A¹⁶³-G polymorphism further upstream in the OPG promoter. Statistical significance was reached when constructing haplotypes, where the A-T-G haplotype was found to be more frequent in individuals with low BMD.

Conclusions: These results indicate the possible role of TNFRSF11B gene variants in postmenopausal bone loss in women in Malta.

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1. Introduction

Complex diseases, such as osteoporosis, occur more frequently in populations than most monogenic disorders and therefore are a greater burden on society.

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Osteoporosis, which mostly affects the elderly population, is characterised by decreased bone mineral density (BMD), deterioration in the microarchitecture of bone and an increased risk of fractures [1]. Both environmental and genetic factors have been associated with peak bone mass reached early in life and with the pathogenesis of osteoporosis [2–4]. In a polygenic disease, like osteoporosis, multiple gene variants each having a small effect, contribute to an individual's increased susceptibility to the disease, although a major gene might also be involved [5]. A number of single nucleotide polymorphisms (SNPs) in candidate genes for osteoporosis have been studied and associated with BMD or fracture risk in different populations [4,6–10].

Osteoclast differentiation and activation is mediated by the interactions of receptor activator of nuclear factor- κ B (RANK) with RANK ligand (RANKL) and controlled by osteoprotegerin (OPG), a decoy receptor of the tumour necrosis factor (TNF) – receptor superfamily. OPG controls osteoclastogenesis by binding to RANKL on preosteoblast/stromal cells preventing cell–cell interactions with RANK present on osteoclast precursors [11–13]. Knockout mice for the TNFRSF11B gene were observed to have severe osteoporosis and an increased risk to fractures showing the importance of OPG in the regulation of bone mass [14].

The human TNFRSF11B gene is a single copy gene found on chromosome 8q23–24 which consists of five exons and spans a region of 29 kb [15]. It is known that the expression of OPG and RANKL is controlled by various cytokines, hormones and growth factors such as transforming growth factor (TGF)- β , insulin-like growth factor (IGF)-I, BMP, oestrogen and Cbfa1 [16–20], and it is the ratio of OPG/RANKL that determines the pool size of active osteoclasts [13]. A number of single nucleotide polymorphisms (SNPs) were analysed by a few investigators for any association with BMD and/or fracture risk. Conflicting results were however obtained [21–28]. Variations within the TNFRSF11B gene were also positively associated with other human diseases such as Paget's disease of bone, idiopathic hyperphosphatasia and vascular disease [29–32]. Genetic studies of OPG and related proteins are very important, since allelic variants within genes might also affect the patient's response to treatment. Administration of exogenous (recombinant) OPG in postmenopausal women was

found to be safe, well tolerated and had a very positive effect as an antiresorptive agent [33].

Two polymorphisms caused by transitions, an A¹⁶³–G and a T⁹⁵⁰–C, in the promoter region of the TNFRSF11B gene together with a G¹¹⁸¹–C transversion in the first exon resulting in a substitution of the third amino acid, lysine to asparagine in the signal peptide were analysed in a group of postmenopausal women in Malta for any correlation with BMD and an increased risk of osteoporosis. In a previous study on the same group of women no association was found between Vitamin D receptor gene polymorphisms and BMD [34].

2. Materials and methods

2.1. Subjects

One hundred and twenty-six (126) postmenopausal women were recruited from new subjects referred by medical practitioners to the Bone Density Unit at the Department of Obstetrics and Gynaecology, St. Luke's Hospital, Malta for an osteoporosis risk evaluation. All subjects studied were healthy Caucasian women between the ages of 40–75 years, with a mean (\pm S.D.) age of 55.6 \pm 7.1 years. Informed consent was obtained from all participants in the study that had been approved by the Research Ethics Committee of the Faculty of Medicine and Surgery, University of Malta. All participants answered a detailed questionnaire concerning medical conditions and the use of medications, family history of osteoporosis and dietary/lifestyle habits. Exclusion criteria were the same as those described in a previous study on the same but slightly extended group of postmenopausal women [34]. Menopause was defined as amenorrhoea of at least 6 months duration. Blood for DNA analysis was collected by venipuncture in a tube containing K₂-EDTA as an anticoagulant.

According to WHO criteria [35] for both lumbar and femoral BMD, 30 (24.4%) of the participants were osteoporotic at the lumbar spine (t -score < -2.5), 41 (33.3%) were osteopenic (t -score < -1.0 to -2.5) and 52 (42.2%) were normal. At the femoral neck, 4 (3.2%) individuals were osteoporotic, 45 (36.3%) osteopenic and 75 (60.5%) were normal. Our sample was divided into two main groups, one of normal individuals (41.1%) having both lumbar and femoral

t-score > -1.0 and a group of individuals that were osteopenic and/or osteoporotic at one or both anatomical sites (58.9%).

2.2. DNA analysis

Genomic DNA was extracted and purified from peripheral blood leucocytes by salting out [36]. Fragments within the TNFRSF11B gene were amplified by polymerase chain reaction (PCR) using 1.0 unit Taq DNA polymerase (Promega, Madison, WI, USA) in a DNA thermocycler (Cyclogene, Techne Ltd., Cambridge, UK), using appropriate primers as previously described by Langdahl et al. [21]. All reactions were performed in a total volume of 25 μ l, containing 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 200 μ M of each dNTP (Promega, Madison, WI, USA), 50–70 pmol of each primer and 100 ng genomic DNA. Amplification was performed using a 'hot-start' PCR protocol. The correct size of the amplified products was evaluated by electrophoresis of 5 μ l PCR product on 1.0% agarose gels. The PCR products (10 μ l) were digested using appropriate restriction enzymes *Asel* (A¹⁶³-G), *HpaI* (T⁹⁵⁰-C), *SmaI* (G¹¹⁸¹-C) according to manufacturer's instructions (New England Biolabs, Beverly, MA, USA). Following electrophoresis and staining with ethidium bromide, digested products were viewed and photographed under ultraviolet illumination (Polaroid DS-34). Nomenclature used refers to the actual nucleotide changes in the respective polymorphisms. Re-analysis of 20% of samples was performed to check for accuracy of genotyping. No discrepancies were found with the original genotyping.

2.3. Biochemical markers of bone turnover

Blood and urine samples were collected and analysed for biochemical markers of bone turnover. Urinary deoxypyridinoline (DpD) crosslinks and serum procollagen type I were used as markers of bone resorption and formation, respectively (Metra Biosystems Inc., CA, USA).

2.4. Bone densitometry

Bone mineral density was measured at the lumbar spine (L2–L4) and femoral neck using a Norland 486

dual-energy X-ray absorptiometer (Norland, Medical Systems Inc., New York, USA).

2.5. Statistical analysis

The normality of the population was tested using the non-parametric test Kolmogorov–Smirnov while the Levene's statistic was used to test homogeneity of variances. The chi-square test was used to compare observed genotype frequencies with those expected under Hardy–Weinberg and to test for differences in genotype frequencies between normal individuals and those with a low BMD.

The EH software by Terwillinger and Ott [37] (<ftp://linkage.rockefeller.edu/software/utilities>) was used to construct haplotypes of polymorphisms within the same gene, to test for association between alleles (linkage disequilibrium) and for association of haplotypes with low BMD by comparing the normal with the low BMD group. The linkage disequilibrium analyzer (LDA) software version 1.0 [38] was used to test for LD and calculate Lewontin's *D'* and likelihood ratio (LR) test.

Comparisons of continuous measurements, such as age, body mass index (BMI) and BMD, showing a normal distribution, were performed using one-way analysis of variance (ANOVA), while the Bonferroni post-hoc test was used to correct for multiple comparisons. The independent sample *t*-test was also used for pair ways comparisons between genotypes. Alternatively, the Kruskal–Wallis and Mann–Whitney methods were used for those variables not showing a normal distribution when tested by the Kolmogorov–Smirnov. Rare genotypes that occurred less than 5% within the population were excluded from statistical analysis. Adjustments for age, BMI and years since menopause (YSM) were performed using a generalised linear model (GLM) univariate analysis of variance.

The independent sample *t*-test was used to analyse data in terms of genetic models: (A) dominant or (B) recessive allele, while a linear regression analysis was used to test for an allele-dose effect (C) of a risk or protective allele. All statistical tests were performed using the Statistical Package for Social Sciences (SPSS) for Windows student version 9.0 (SPSS, Chicago, IL). All tests were considered two tailed at a level of significance of 0.05 where the null hypothesis

(no difference between means) was rejected at $p < 0.05$.

3. Results

The distributions of genotypes for all polymorphisms studied are shown in Tables 1–3 and these were all in Hardy–Weinberg equilibrium ($p > 0.05$). Only one individual was identified as being homozygote GG (0.9%) for the A¹⁶³–G polymorphism, which was excluded from further analysis. The A¹⁶³–G polymorphism was not in linkage disequilibrium (LD) with either the T⁹⁵⁰–C ($\chi^2 = 1.91$; d.f. = 3; $p = 0.59$; $D' = 0.37$; LR: $p = 0.18$) or the G¹¹⁸¹–C ($\chi^2 = 0.41$; d.f. = 3; $p = 0.94$; $D' = 0.20$; LR: $p = 0.54$) polymorphisms. Strong LD was only observed between the T⁹⁵⁰–C and G¹¹⁸¹–C polymorphisms ($\chi^2 = 76.76$; d.f. = 3; $p < 0.001$; $D' = 0.80$; LR: $p < 0.001$).

3.1. TNFRSF11B polymorphisms and BMD

No influence of the A¹⁶³–G polymorphism within the TNFRSF11B gene on LS and FN BMD or bone turnover was observed in the group studied (Table 1).

For the T⁹⁵⁰–C polymorphism in the promoter region of the TNFRSF11B gene, the highest BMD at all anatomical sites was observed in CC homozygotes. Differences between the three genotypes in LS, FN, Ward's or trochanter BMD were not detected with statistical significance when tested by ANOVA even after adjusting for age, BMI and YSM. Homozygotes CC were also observed to have the lowest overall bone turnover when compared to the other groups (Table 2). Statistical significance was not reached when testing for genetic models: (A) dominant (B) recessive allele (Table 4), or when testing for an allele dose effect by linear regression ($p > 0.05$) (data not shown).

No statistical significance was observed when comparing BMD between the three genotypes for the G¹¹⁸¹–C polymorphism using ANOVA (Table 3). Statistical significance was almost reached for adjusted femoral and Ward's triangle BMD when comparing GC with CC homozygotes (Table 3). When comparing the same groups, it was observed that CC homozygotes had a statistically significant lower bone resorption. No statistical significance was reached for genetic models of dominant and recessive alleles (Table 4) or for allele dose effects at any anatomical site (data not shown).

Table 1
General characteristics, lumbar and femoral BMD in maltese postmenopausal women according to OPG A¹⁶³–G polymorphism

| General characteristics and genotypes | AA | AG | <i>p</i> -value |
|---|-------------|-------------|-----------------|
| <i>N</i> | 92 | 24 | – |
| % | 78.6 | 20.5 | – |
| Age (years) ^{a,b} | 56.4 (7.5) | 53.2 (5.3) | 0.06 |
| BMI (kg/m ²) ^a | 28.9 (4.0) | 27.7 (4.3) | 0.23 |
| BMD L2–L4 (g/cm ²) ^a | 0.95 (0.19) | 0.99 (0.21) | 0.45 |
| Adjusted ^c | 0.96 | 0.96 | 0.99 |
| BMD femoral (g/cm ²) ^a | 0.84 (0.13) | 0.86 (0.14) | 0.41 |
| Adjusted ^c | 0.84 | 0.84 | 0.77 |
| Ward's triangle (g/cm ²) ^a | 0.62 (0.14) | 0.66 (0.18) | 0.25 |
| Adjusted ^c | 0.63 | 0.62 | 0.92 |
| Trochanter (g/cm ²) ^a | 0.67 (0.12) | 0.71 (0.14) | 0.24 |
| Adjusted ^c | 0.68 | 0.68 | 0.92 |
| Procollagen (ng/ml) ^{a,b} | 96.4 (67.1) | 99.2 (42.0) | 0.52 |
| DpD (nmol/mmol creatinine) ^a | 9.0 (3.3) | 10.5 (3.2) | 0.14 |

^a Values are means \pm S.D. in parenthesis; *p*-values obtained by independent sample *t*-test.

^b Mann–Whitney *t*-test for age and procollagen.

^c Values are adjusted for age, BMI, YSM; 95% CI given by univariate analysis (not shown).

Table 2

General characteristics, lumbar and femoral BMD in maltese postmenopausal women according to OPG T⁹⁵⁰-C polymorphism

| General characteristics and genotypes | | | | p-value | | | |
|---|--------------|-------------|-------------|-----------|------|------|-----------|
| | TT | TC | CC | TT vs. TC | | | TC vs. CC |
| N | 24 | 71 | 29 | – | | | |
| % | 19.4 | 57.3 | 23.4 | – | | | |
| Age (years) ^{a,b} | 57.0 (8.7) | 55.9 (6.7) | 53.9 (6.8) | 0.24 | 0.48 | 0.15 | 0.17 |
| BMI (kg/m ²) ^a | 28.5 (3.6) | 28.7 (4.6) | 28.6 (3.6) | 0.99 | 0.88 | 0.93 | 0.94 |
| BMD L2–L4 (g/cm ²) ^a | 0.93 (0.14) | 0.96 (0.21) | 0.97 (0.19) | 0.74 | 0.46 | 0.39 | 0.79 |
| Adjusted ^c | 0.94 | 0.96 | 0.96 | 0.85 | 0.54 | 0.78 | 0.86 |
| BMD femoral (g/cm ²) ^a | 0.84 (0.13) | 0.83 (0.14) | 0.87 (0.15) | 0.42 | 0.71 | 0.46 | 0.19 |
| Adjusted ^c | 0.85 | 0.83 | 0.86 | 0.60 | 0.59 | 0.90 | 0.37 |
| Ward's triangle (g/cm ²) ^a | 0.61 (0.11) | 0.61 (0.15) | 0.68 (0.16) | 0.15 | 0.96 | 0.14 | 0.08 |
| Adjusted ^c | 0.63 | 0.61 | 0.66 | 0.24 | 0.63 | 0.50 | 0.12 |
| Trochanter (g/cm ²) ^a | 0.66 (0.09) | 0.67 (0.14) | 0.72 (0.13) | 0.20 | 0.79 | 0.09 | 0.12 |
| Adjusted ^c | 0.68 | 0.67 | 0.71 | 0.26 | 0.74 | 0.29 | 0.13 |
| Procollagen (ng/ml) ^{a,b} | 115.7 (65.9) | 95.7 (69.6) | 81.4 (23.4) | 0.16 | 0.12 | 0.06 | 0.66 |
| DpD (nmol/mmol creatinine) ^a | 10.1 (3.6) | 9.4 (3.3) | 7.8 (2.6) | 0.15 | 0.51 | 0.07 | 0.10 |

^a Values are means ± S.D. in parenthesis; p-values obtained by ANOVA or independent sample *t*-test.^b Kruskal–Wallis or Mann–Whitney *t*-test for age and procollagen.^c Values are adjusted for age, BMI, YSM; 95% CI given by univariate analysis (not shown).

Table 3

General characteristics, lumbar and femoral BMD in maltese postmenopausal women according to OPG G¹¹⁸¹-C polymorphism

| General characteristics and genotypes | | | | p-value | | | |
|---|--------------|-------------|-------------|-----------|------|------|-----------|
| | GG | GC | CC | GG vs. GC | | | GC vs. CC |
| N | 30 | 61 | 22 | – | | | |
| % | 26.5 | 54.0 | 19.5 | – | | | |
| Age (years) ^{c, b} | 56.8 (8.4) | 56.0 (6.4) | 53.1 (7.9) | 0.15 | 0.67 | 0.12 | 0.06 |
| BMI (kg/m ²) ^a | 28.8 (4.3) | 28.9 (4.4) | 28.1 (3.3) | 0.76 | 0.92 | 0.55 | 0.47 |
| BMD L2–L4 (g/cm ²) ^a | 0.94 (0.20) | 0.97 (0.20) | 0.92 (0.17) | 0.60 | 0.57 | 0.69 | 0.33 |
| Adjusted ^c | 0.96 | 0.97 | 0.91 | 0.35 | 0.69 | 0.21 | 0.16 |
| BMD femoral (g/cm ²) ^a | 0.83 (0.15) | 0.86 (0.13) | 0.82 (0.12) | 0.43 | 0.34 | 0.86 | 0.25 |
| Adjusted ^c | 0.84 | 0.86 | 0.81 | 0.22 | 0.41 | 0.39 | 0.07 |
| Ward's triangle (g/cm ²) ^a | 0.60 (0.15) | 0.65 (0.15) | 0.59 (0.13) | 0.17 | 0.14 | 0.90 | 0.13 |
| Adjusted ^c | 0.62 | 0.65 | 0.58 | 0.15 | 0.39 | 0.42 | 0.06 |
| Trochanter (g/cm ²) ^a | 0.66 (0.15) | 0.69 (0.11) | 0.66 (0.12) | 0.63 | 0.45 | 0.96 | 0.41 |
| Adjusted ^c | 0.68 | 0.68 | 0.66 | 0.74 | 0.92 | 0.70 | 0.38 |
| Procollagen (ng/ml) ^{a,b} | 104.4 (58.4) | 90.7 (33.9) | 83.9 (19.7) | 0.52 | 0.35 | 0.24 | 0.88 |
| DpD (nmol/mmol creatinine) ^a | 9.1 (3.2) | 9.8 (3.5) | 7.7 (1.9) | 0.15 | 0.50 | 0.19 | 0.01 |

^a Values are means ± S.D. in parenthesis; p-values obtained by ANOVA or independent sample *t*-test.^b Kruskal–Wallis or Mann–Whitney *t*-test for age and procollagen.^c Values are adjusted for age, BMI, YSM; 95% CI given by univariate analysis (not shown).

Table 4
Effects of OPG alleles on BMD in postmenopausal women assuming genetic models of a dominant or recessive allele

| OPG T ⁹⁵⁰ -C polymorphism | Dominant model* | | | Recessive model* | | |
|---|-----------------|-------------|------|------------------|-------------|------|
| | TT | TC and CC | P* | TT and TC | CC | P* |
| N | 24 | 98 | – | 93 | 29 | – |
| BMD L2–L4 (g/cm ²) ^a | 0.93 (0.14) | 0.96 (0.20) | 0.47 | 0.95 (0.19) | 0.97 (0.19) | 0.64 |
| Adjusted ^b | 0.94 | 0.96 | 0.59 | 0.96 | 0.95 | 0.98 |
| BMD femoral (g/cm ²) ^a | 0.84 (0.13) | 0.84 (0.14) | 1.00 | 0.83 (0.13) | 0.87 (0.15) | 0.20 |
| Adjusted ^b | 0.85 | 0.84 | 0.72 | 0.84 | 0.86 | 0.41 |
| Ward's triangle (g/cm ²) ^a | 0.61 (0.11) | 0.63 (0.16) | 0.63 | 0.61 (0.14) | 0.68 (0.16) | 0.05 |
| Adjusted ^b | 0.63 | 0.63 | 0.93 | 0.62 | 0.66 | 0.12 |
| Trochanter (g/cm ²) ^a | 0.66 (0.09) | 0.68 (0.14) | 0.46 | 0.67 (0.13) | 0.72 (0.13) | 0.08 |
| Adjusted ^b | 0.68 | 0.68 | 0.89 | 0.67 | 0.71 | 0.11 |
| OPG G ¹¹⁸¹ -C polymorphism | GG | GC and CC | P* | GG and GC | CC | P* |
| N | 29 | 82 | – | 89 | 22 | – |
| BMD L2–L4 (g/cm ²) ^a | 0.94 (0.20) | 0.96 (0.19) | 0.76 | 0.96 (0.20) | 0.92 (0.17) | 0.40 |
| Adjusted ^b | 0.96 | 0.95 | 0.98 | 0.97 | 0.91 | 0.17 |
| BMD femoral (g/cm ²) ^a | 0.83 (0.15) | 0.85 (0.13) | 0.49 | 0.85 (0.14) | 0.82 (0.12) | 0.40 |
| Adjusted ^b | 0.83 | 0.84 | 0.73 | 0.85 | 0.81 | 0.13 |
| Ward's triangle (g/cm ²) ^a | 0.60 (0.15) | 0.64 (0.15) | 0.27 | 0.64 (0.15) | 0.59 (0.13) | 0.26 |
| Adjusted ^b | 0.62 | 0.63 | 0.67 | 0.64 | 0.58 | 0.09 |
| Trochanter (g/cm ²) ^a | 0.66 (0.15) | 0.68 (0.12) | 0.56 | 0.68 (0.13) | 0.66 (0.12) | 0.57 |
| Adjusted ^b | 0.68 | 0.67 | 0.96 | 0.68 | 0.66 | 0.45 |

* Mean BMD compared between genotype groups assuming hypothesis: (A) dominant T or G alleles and (B) recessive T or G alleles; *p*-values obtained by unpaired independent sample *t*-test (two-tailed).

^a Values are means ± S.D. in parenthesis.

^b Values are adjusted for age, BMI, YSM; 95% CI given by univariate analysis (not shown).

No significance was reached when the Bonferroni correction for multiple comparisons was performed at a level of significance of 0.05.

3.2. Genotype and haplotype frequencies in normal individuals and those with a low BMD

The distribution of genotype frequencies between a group of normal individuals (*t*-score >–1.0) and those having low BMD at the lumbar and/or femoral sites (*t*-score <–1.0) using a chi-square test was analysed. A statistical significant difference was observed for the T⁹⁵⁰-C polymorphism in the promoter region of the TNFRSF11B gene ($\chi^2 = 9.68$; *p* = 0.01, d.f. = 2), where the TT genotype occurred more frequently in women with low BMD, whereas the CC genotype was more prevalent in the normal group (Fig. 1). A significant odds ratio of 2.11 (95% CI: 1.26–3.55)

was only obtained when assuming the T allele for the T⁹⁵⁰-C polymorphism in the TNFRSF11B gene as a risk for low BMD. Genotype distribution for the G¹¹⁸¹-C polymorphism between normal individuals and those with low BMD did not reach significance ($\chi^2 = 5.47$; *p* = 0.07, d.f. = 2), although GG homozygotes were 52% more frequent in the group with low BMD. The odds ratio obtained when assuming the G as the risk allele was 1.56 (95% CI: 0.91–2.66). There was no difference in the distribution of genotypes for the A¹⁶³-G polymorphism between normal individuals and those having a low BMD ($\chi^2 = 1.47$; *p* = 0.48, d.f. = 2).

When combining genotypes, the most common genotypes were AA/TC/GC (41.9%) and AA/TT/GG (17.1%) with the most frequent haplotypes being A–T–G (44.6%) and A–C–C (40.4%). On comparing haplotype frequencies between normal individuals and

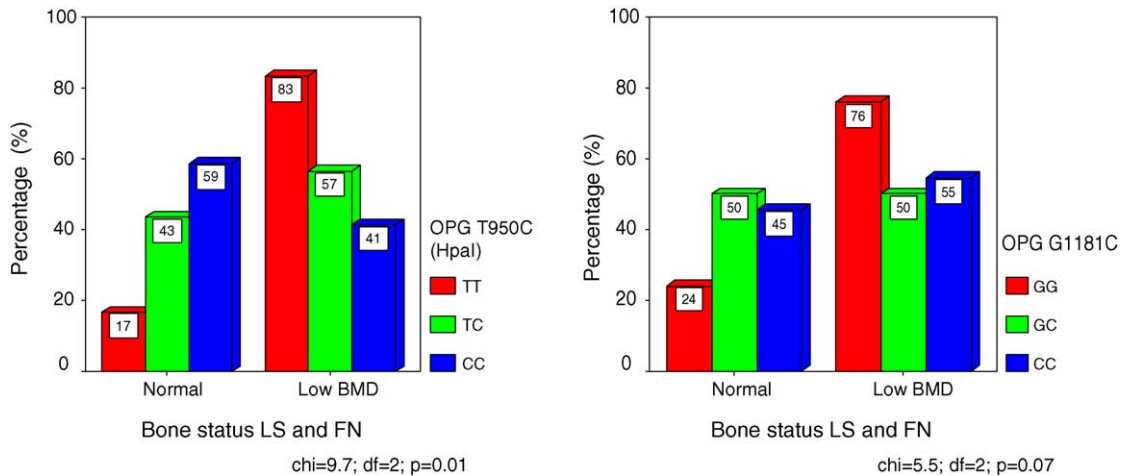


Fig. 1. Genotype distributions between normal postmenopausal women and women with low LS and/or FN BMD.

Table 5
Haplotype analysis in cases and controls

| Haplotypes | Low BMD (n=59) (%) | Normal (n=44) (%) |
|------------|--------------------|-------------------|
| A–T–G | 51.7 | 34.1 |
| A–T–C | 0.0 | 1.5 |
| A–C–G | 5.2 | 1.2 |
| A–C–C | 34.7 | 49.6 |
| G–T–G | 0.0 | 0.8 |
| G–T–C | 5.1 | 2.3 |
| G–C–G | 2.4 | 10.5 |
| G–C–C | 0.9 | 0.0 |

Significance: $\chi^2 = 24.66$; $p < 0.001$, d.f. = 7.

those with low LS and/or FN BMD, statistical significance was reached ($\chi^2 = 24.66$; $p < 0.001$, d.f. = 7) with the A–T–G haplotype being more frequent in those individuals with low BMD (51.7% versus 34.1%). Conversely, the A–C–C and G–C–G haplotypes were more prevalent in normal individuals than in those with low BMD (Table 5), perhaps indicative of a protective role.

4. Discussion

Bone metabolism is influenced by both genetic and environmental factors and is tightly controlled by various cytokines and hormones. The onset of menopause results in an increase in bone resorption, partly due to an increase in proresorptive cytokines along with an impaired production of antiresorptive cytokines brought about by the lack of oestrogen. The

TNFRSF11B gene is of particular interest since osteoprotegerin together with RANKL are directly involved both in the proliferation/differentiation of osteoclast precursors as well as in osteoclast apoptosis, which are both affected by changes brought about by the onset of menopause [13].

In this study, no significant association was found between any of the three TNFRSF11B polymorphisms and BMD in this group of Maltese postmenopausal women. For the T⁹⁵⁰–C polymorphism the highest BMD at all anatomical sites was observed in CC homozygotes while TT homozygotes had the lowest BMD. These observations agree with results of previous studies where the CC genotype was significantly associated with higher LS BMD in a cohort of postmenopausal women from Denmark [21]. A similar trend was also observed by other investigators although this did not reach significance [22,24,25], while contrasting results were obtained in other studies [26]. Genotype frequencies for the T⁹⁵⁰–C polymorphism in our cohort of postmenopausal women compared well with those observed in Caucasian women [21–23], but differed from those observed in Swedish men [24] and in pre/postmenopausal Japanese women [26,28]. Observations for the G¹¹⁸¹–C polymorphism agree with results obtained for the Irish and Japanese populations where individuals having the C allele had the lowest BMD [23,28]. This is in contrast to what has been reported for the Danish population where homozygotes CC had the highest BMD [21].

The A¹⁶³-G polymorphism further upstream in the TNFRSF11B promoter does not seem to have any significant effect on BMD in our group of postmenopausal women. This contrasts with results obtained by Langdahl et al. [21] who reported that this polymorphism was the most significant variant responsible for an increased risk for osteoporosis. As has been observed in the Danish study, this polymorphism is rare in the Maltese population, with only one individual identified as GG homozygote [21,27].

A significant difference was observed in the distribution of genotypes between normal and women with low BMD, for the T⁹⁵⁰-C polymorphism. The TT genotype was found more frequently in the group of postmenopausal women with low BMD. Also, for the polymorphism in the first exon, the GG genotype was more frequently found in the group of women with low BMD, although this was not statistically significant. Similar observations were made in a study by Langdahl et al., where the G allele was found to be significantly more common in osteoporotic patients than in normal controls [21]. In our study, the lack of significant association of any of these polymorphisms with BMD might be partly due to the age of our postmenopausal population (mean age 55.6 years) which is relatively young when compared to that of other studied populations with a mean age ranging from 60 to 73 years [21–23,25,26,28]. At the relatively younger mean age of our population, it might be too early to observe any significant bone loss as a result of increased osteoclast activity influenced by TNFRSF11B gene variants. Deoxypyridinoline crosslinks, were observed to be higher in individuals having either one or two copies of the risk alleles T (T⁹⁵⁰-C) and G (G¹¹⁸¹-C) indicating an increased resorption influenced by these variants.

A common problem with association studies is the lack of consensus between studies in different populations. Possible reasons for inconsistency include different sample sizes, bias and population stratification. Inadequate sample power might be the reason why no association with BMD was observed in this study. After calculating statistical power for this study, it was found that it would take a sample size larger than 1500 to show statistical differences in LS and FN BMD with 80% statistical power as influenced by TNFRSF11B polymorphisms. The chances to replicate results and sample size depend on a number of

factors including the frequency of the susceptibility allele within the population and the relative risk [39]. A larger sample will be needed to detect susceptibility alleles that are less frequent and with a very low relative risk.

Besides technical reasons, there are also biological phenomena that might result in inconsistency such as genetic and clinical heterogeneity. Another reason might be due to the very small effects of these polymorphisms on BMD that might be masked by the effects of other polymorphisms and/or genes together with environmental factors.

Theoretically, allelic variants within the TNFRSF11B gene promoter might affect gene expression, although the T⁹⁵⁰-C polymorphism is not located in a known responsive area but a few base pairs away from that responsive to TGF- β and only 129bp upstream of the TATA-box [16,24]. Allelic variations within coding regions of the gene might result in non-synonymous amino acid changes that affect the overall function of the protein or its interaction with RANKL. In this study, the nearby G¹¹⁸¹C polymorphism in exon 1 was found to be in strong linkage disequilibrium with the T⁹⁵⁰-C polymorphism agreeing with what was observed in the Danish and Irish populations [21,23]. The G¹¹⁸¹C results in an amino acid change, lysine to asparagine, in the signal peptide which might affect the behaviour of the protein by some mechanism that is as yet unknown.

The overall effect at the phenotypic level can also be influenced by other variants in related genes such as RANKL and RANK. Further studies are required to identify novel polymorphisms within these two genes and in genes encoding for proteins in the signal transduction cascade including members of the TNF receptor-associated factors (TRAF) family such as TRAF6 [40], all of which play a part in osteoclast differentiation and activation.

The TNFRSF11B gene is of particular interest since not only is it expressed in bone and found to be involved in bone diseases such as Paget's [29,30], but it is expressed in other tissues such as kidneys, lungs, liver and brain [41], and has also been linked to other diseases such as vascular disease [42]. Results obtained from in vitro studies and mouse genetics showed that osteoprotegerin-deficient mice developed early onset osteoporosis as well as arterial calcification of the aorta and renal arteries [14]. Also, polymorphisms within the

TNFRSF11B gene including the T⁹⁵⁰-C and G¹¹⁸¹-C polymorphisms have been associated with vascular morphology and function [32,43] showing that the OPG/RANKL system might be a common mechanism that links osteoporosis with an increased risk of vascular disease.

In conclusion, no significant associations were found between polymorphisms in the TNFRSF11B gene and BMD. However, a significant difference was observed between the distribution of genotype frequencies for the T⁹⁵⁰-C polymorphism and between TNFRSF11B haplotypes in normal individuals and those with low BMD. Results obtained from this study might be useful for meta-analysis of polymorphisms studied. Further studies are suggested to confirm these findings in other populations and to identify the biological mechanisms by which these polymorphisms affect BMD.

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