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SNPs in the 3'UTR of the RANK gene determine site-dependent osteoporotic fracture

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Summary

Objectives: The RANK/RANKL/OPG system is involved in the determination of bone mineral density (BMD) and bone microarchitecture. Our study seeks to evaluate if there are SNPs in the 3'UTR region of the RANK gene associated with osteoporotic phenotypes.

Material and methods: Seven genetic variants in 1,098 women from the BARCOS cohort were genotyped, and their association with BMD and osteoporotic fractures evaluated. An interaction with SNP rs9594738 in the RANKL gene which was previously associated with BMD was tested.

Results: None of the SNPs were associated significantly with BMD. SNP rs78326403 was associated with wrist/forearm fractures (Log-additive model odds ratio (OR)=3.12 [IC 95%: 1.69 ; 5.75]; $p=7.16 \times 10^{-4}$), while SNP rs884205 was associated with fractures of the spinal column (OR=4.05 Recessive; [95% CI: 1.59 ; 10.35]; $p=8.24 \times 10^{-3}$). Lastly, an interaction was detected between SNP rs9594738 from RANKL and rs78326403 from RANK on the presence of fracture ($p=0.039$). The analysis of the effects of combined genotypes rs9594738 and rs78326403 pointed to an increase in the prevalence of fractures in subjects with a greater number of unfavourable alleles, the ORs being 2.76 [95% CI: 1.30 ; 5.81]; $p=0.007$) and 5.14 [95% CI: 1.37 ; 15.67]; $p=0.007$) for 2 and ≥ 3 unfavourable alleles respectively, in comparison with none/1.

Conclusions: Two SNPs in 3'UTR from the RANK gene predispose to site-dependent osteoporotic fracture. An interaction with SNP rs9594738 from RANKL suggests an additive effect of BMD and bone strength.

Key words: osteoporosis, fracture, SNPs, association studies.

Introduction

Osteoporosis is one of the most common problems in postmenopausal women, with a significant economic impact on Western society^{1,2}. According to the criteria of the World Health Organisation (WHO), osteoporosis is diagnosed non-invasively by measuring bone mineral density (BMD)³. Low impact traumas are the immediate consequence of osteoporosis and are a growing cause of hospitalisation, morbidity and mortality in old people⁴. However, the definition of these low trauma fractures as “osteoporotic fractures” may be misleading, since many of these patients have levels of BMD considered to be normal according to the WHO criteria^{5,6}. To improve the identification of subjects at high risk of fracture, a series of studies⁷⁻⁹ have proposed the clinical use of various predictors, including the WHO FRAX[®] algorithm¹⁰⁻¹², in place of only BMD. The identification of a number of predictors independent of BMD (such as family history of hip fracture) indicates that other factors, probably related to the microarchitecture or other elements of bone strength, play an important role in the definition of osteoporotic fractures.

The RANK/RANKL/OPG signalling system is fundamental to bone remodelling. The RANK ligand (RANKL) is a membrane protein of the pre-osteoblast cell or secreted by osteocytes which bond to the RANK receptor of the osteoclast precursor, thus promoting its differentiation and activation into a mature osteoclast. The osteoblast, in turn, also secretes the soluble protein OPG, which act as a decoy receptor and interacts with RANKL, impeding its union with RANK, thus inhibiting osteoclastogenesis. The equilibrium between OPG and RANKL and the union of the latter to its receptor RANK is key to determining the anabolic or catabolic state of bone. Thus the gene TNFRSF11A (locus 18q22.1), which codes for RANK, has a special significance in bone remodelling, since it determines the differentiation of the osteoclasts and their survival¹³. A change in this complex would provoke a deregulation of bone remodelling which may result in pathological states, such as osteoporosis.

Whole genome association studies¹⁴⁻¹⁷ and studies analysing interaction of the SNPs of RANK/RANKL¹⁸ provide evidence of the importance of the TNFRSF11A gene in the determination of BMD and the incidence of fractures. Changes in the 3'UTR region of a gene may affect its expression by modulating the bonding sites of microRNAs (miRNAs)¹⁹. Furthermore, it has been shown that miRNAs can specifically regulate osteogenesis²⁰. Therefore, the hypothesis of our study was that genetic variants in the 3'UTR regions of genes important to bone metabolism could be associated with osteoporotic phenotypes.

The objective was to identify SNPs in the 3'UTR of the RANK gene as possible functional genetic variants which may affect the risk of fracture. On the other hand, a possible interaction between the SNPs associated with fractures within the RANK gene and the SNP rs9594738 of the RANKL gene (previously associated with BMD)¹⁴ was also studied.

Materials and methods

Characteristics of the BARCOS cohort

All the patients of this cohort are postmenopausal women who made an initial visit to outpatients at the Bone Metabolism Unit of the Hospital del Mar-Parque de Salud Mar in Barcelona, Spain, due to the menopause^{21,22}. The patients were registered consecutively, not selectively, and recruited prospectively, independently of their BMD values. Their age, weight, height, age at menarche, years since the menopause at the time of densitometry, months of breastfeeding and history of previous fractures (Table 1) were recorded. Women with metabolic or endocrine diseases, chronic renal insufficiency, chronic hepatic disease, cancer (except cancer of the surface of the skin), Paget's disease of bone, malabsorption syndrome or on treatment with hormone replacement, antiresorptive or anabolic agents, oral corticosteroids, antiepileptic drugs, lithium, heparin or warfarin, were excluded, as well as those who declined the invitation to participate and did not give their informed consent. Furthermore, those subjects with a history of early menopause (<40 years of age) were excluded from this analysis. The blood samples and written informed consent were obtained in accordance with the rules of the Hospital del Mar's Committee on Human Genetic Research.

Determination of BMD and fracture

Double energy X-ray densitometry, DXA (QDR 4500 SL, Hologic, Waltham, MA, EE.UU.), was used to measure BMD (g/cm²) in the lumbar spine (LS) L2-L4 and in the femoral neck (FN). The technique had a coefficient of variation (CV) of 1.0% for the measurement of the LS and 1.65% for the FN. The vertebral and non-vertebral clinical fractures were recorded. The non-vertebral fractures were validated through medical records, and those of the spine through X-rays if there was a history of diagnosis of vertebral fracture, loss of height, or back pain. Vertebral fractures were defined as those which occur after the age of 45 years and due to a low impact trauma. Fractures of the face, fingers, toes or cranium were excluded. Vertebral fractures were defined in accordance with the semiquantitative criteria of Genant et al.²³.

Extraction of DNA

The buffy coats were obtained from 3 ml of blood collected in tubes of EDTA and stored at -20°C. The genomic DNA were obtained from the leucocytes through a process of salting out²⁴ or by using Autopure LS (Qiagen), a robotised workstation for the automated purification of genomic DNA, in the laboratories of the Biomedical Laboratory Support Services IMIM, Barcelona (Spain), and stored at -20°C.

Selection of the SNP and genotyping

The Ensembl (www.ensembl.org) and Entrez SNP (<http://www.ncbi.nlm.nih.gov/sites/entrez>) databases were used to select the SNPs of 3'UTR of the RANK gene. Only those SNPs with a MAF >0.01 were included.

The genotyping for the polymorphisms were carried out at Kbioscience (Herts., England) using the Kaspar system v4.0 and the Kraken allele algorithm. For the quality control, 959 samples were genotyped for the SNP rs9594738 (around 11% of the total genotyping) using the SNPlex System (Applied Biosystem) of the CEGEN platform (Barcelona, Spain). There was 99.8% concordance between the results of the two techniques.

Statistical methods

The Hardy-Wienberg equilibrium (HWE) was calculated by means of the Chi-squared test and the p value calculated with the online calculator of Tufts University (<http://www.tufts.edu/~mcourt01/Documents/Court20lab%20-%20calculator.xls>).

To evaluate the association of the genotyped SNPs with BMD and fractures linear and logistic adjusted regression models, respectively, were used. The confusion factors considered for adjustment were the body mass index (BMI)²⁵ and the age for the association with fracture; and the age of menarche, years since the menopause at the time of densitometry months of breastfeeding and the BMI²⁵ for the BMD. With the hypothesis that the effect on the fracture phenotype of the different genetic variants of RANK may vary as a function of the bone architecture, the effect of the SNPs both in the predominantly trabecular sites of fracture (spine) and in sites with more cortical bone (wrist/forearm) were studied.

Separately, the interactions between rs9594738 of the RANKL gene and the SNPs associated with fracture of RANK on the introduction of multiplier terms in the regression equation, were tested.

All the analyses were of two tails and values of $p < 0.05$ were considered to be significant. The statistical analyses were carried out using SPSS for Windows version 13.0 and version R 2.13.2 with haplo.stats, epicalc, SNPassoc, foreign, rms, and genetics packs.

Results

Seven genetic variants of 3'UTR of the RANK gene in the BARCOS cohort were genotyped (Table 2). All the SNPs, except rs72933640, were in HWE. However, the MAF of rs72933640 in BARCOS was similar to the MAF (0.108) published by the National Centre for Biotechnology Information

Table 1. Baseline characteristics of the cohort BARCOS

| Patient characteristics | Mean \pm SD | n |
|-----------------------------|------------------|------------|
| Age of menopause (years) | 48.46 \pm 4.06 | 1096 |
| BMI | 26.16 \pm 3.85 | 1088 |
| Lactation (months) | 7.73 \pm 12.79 | 1091 |
| LS densitometry age (years) | 56.04 \pm 8.49 | 1087 |
| Years since menopause LS | 7.59 \pm 8.26 | 1091 |
| BMD LS (g/cm ²) | 0.853 \pm 0.15 | 1092 |
| FN densitometry age (years) | 57.89 \pm 8.03 | 1003 |
| Years since menopause FN | 9.36 \pm 7.91 | 1007 |
| BMD FN (g/cm ²) | 0.683 \pm 0.11 | 1009 |
| Menarche, age (years) | 12.89 \pm 1.58 | 1081 |
| Fractures: | 152 (13.8%) | 1098 |
| Column | | 68 (44.7%) |
| Hip | | 8 (5.3%) |
| Wrist/Forearm | | 36 (23.7%) |
| Other | | 40 (26.3%) |

(NCBI) for the EU population. The MAF for all the SNPs genotyped was > 0.01 in our population.

The SNPs rs78326403 and rs78459945 were in total linkage disequilibrium (D) ($D' = 0.999$; $R^2 = 0.968$). The latter, in having a lower genotyping efficiency, was eliminated from the statistical analysis.

None of the SNPs studied were found to be associated with BMD (data not shown). On the other hand, the SNPs rs78326403 and rs884205 were significantly associated with the prevalence for fracture in our cohort (Figure 1 and Table 2). For the SNP rs78326403, the log-additive model gave a value of $p = 0.053$; OR=1.58 [95% CI: 1.00 ; 2.49]. For the SNP rs884205, the log-additive model gave a value of $p = 0.048$ with OR=1.40 [95% CI: 1.01 ; 1.95], while the recessive model produced $p = 4.9 \times 10^{-3}$; OR=3.28 [95% CI: 1.51 ; 7.13]. Only SNP rs884205 exceeded the Bonferroni correction (p value for a significant association: $p = 8.33 \times 10^{-3}$) (Figure 1). No significant interaction was detected between them ($p = 0.87$).

Then, both SNPs were analysed for their association with fractures of the spine or wrist/forearm separately (Table 3). SNP rs78326403 was associated with fractures of wrist/forearm (log-additive model, $p = 7.16 \times 10^{-4}$; OR=3.12 [95% CI: 1.69 ; 5.75]), but not with vertebral fractures ($p = 0.78$, log-additive model). On the other hand the SNP rs884205

Table 2. List of the genotypes SNPs, MAF and p values for the association with fractures under a log-additive model (or other model if indicated)

| SNP # | rs | Location ¹ | Efficiency | MAF BARCOS | HWE | p | OR (IC 95%) |
|-------|------------|-----------------------|------------|-------------------|--------------|---------------------------------------|------------------|
| 1 | rs78622775 | 18:60052935 | 0.95 | 0.01 ² | 0.73 | (0.99) ^c | |
| 2 | rs12455323 | 18:60053891 | 0.94 | 0.32 | 0.90 | 0.82 | |
| 3 | rs72933640 | 18:60054077 | 0.94 | 0.12 | 0.005 | 0.59 | |
| 4 | rs78326403 | 18:60054440 | 0.95 | 0.08 | 0.27 | 0.053 (0.022)^e | 1.83 (1.11-3.02) |
| 5 | rs78459945 | 18:60054757 | 0.94 | 0.08 | 0.39 | In DL with rs78326403 | |
| 6 | rs72933641 | 18:60054804 | 0.95 | 0.13 | 0.07 | 0.57 | |
| 7 | rs884205 | 18:60054857 | 0.94 | 0.19 | 0.32 | 0.048 (0.0049)^r | 3.28 (1.51-7.13) |

¹: ENSEMBL website, release 66, February 2012.

²: Due to low MAF for rs78622775, the only model possible is the codominant model. In the case in which a more significant p value is obtained in another model this is indicated between (). Bold type p<0.05; ^c: codominant; ^e: overdominant; ^r: recessive.

was associated with spine fractures (recessive model, $p=8.24 \times 10^{-3}$; OR=4.05 [95% CI: 1.59 ; 10.35]), but not with fractures of wrist/forearm ($p=0.66$, log-additive model). In this case both SNPs exceeded the Bonferroni correction. After an additional adjustment for BMD, both associations continued to be significant (Table 3) although the nominal association between rs884205 and spinal fractures after adjustment for LS BMD did not exceed the Bonferroni correction (recessive model $p=0.025$).

The analysis of the interaction between the SNP of RANKL rs9594738, previously associated with BMD, and rs78326403, considering as a variable wrist/forearm fractures, produced a significant result ($p=0.039$). Taking into account fractures of the spine, there was no interaction between rs9594738 and rs884205 ($p=0.39$). Nor was there any significant interaction found when the variable studied was BMD. The analysis of the effect of the genotypes composed of the SNPs rs9594738 and rs78326403 pointed towards an increase in the prevalence of fractures in subjects with a high number of unfavourable alleles (Table 4). Due to minor or zero differences between carriers with no, or only one, unfavourable allele on the prevalence of fractures, both categories are combined. Similarly, due to the small number of patients with 4 unfavourable alleles ($n=3$), this category was combined with the group of 3 unfavourable alleles. In general, the comparison was carried out in the following way: 0/1 vs 2 and 0/1 vs 3 or more unfavourable alleles. The analysis of the effect of these compound genotypes on the prevalence of fracture suggest and additive effect with the

corresponding adjusted ORs: 2.76 [95% CI: 1.30 ; 5.81], $p=7.4 \times 10^{-3}$; and 5.14 [95% CI: 1.37 ; 15.67]; $p=7.5 \times 10^{-3}$, for 2 and ≥ 3 unfavourable alleles, respectively (Table 4).

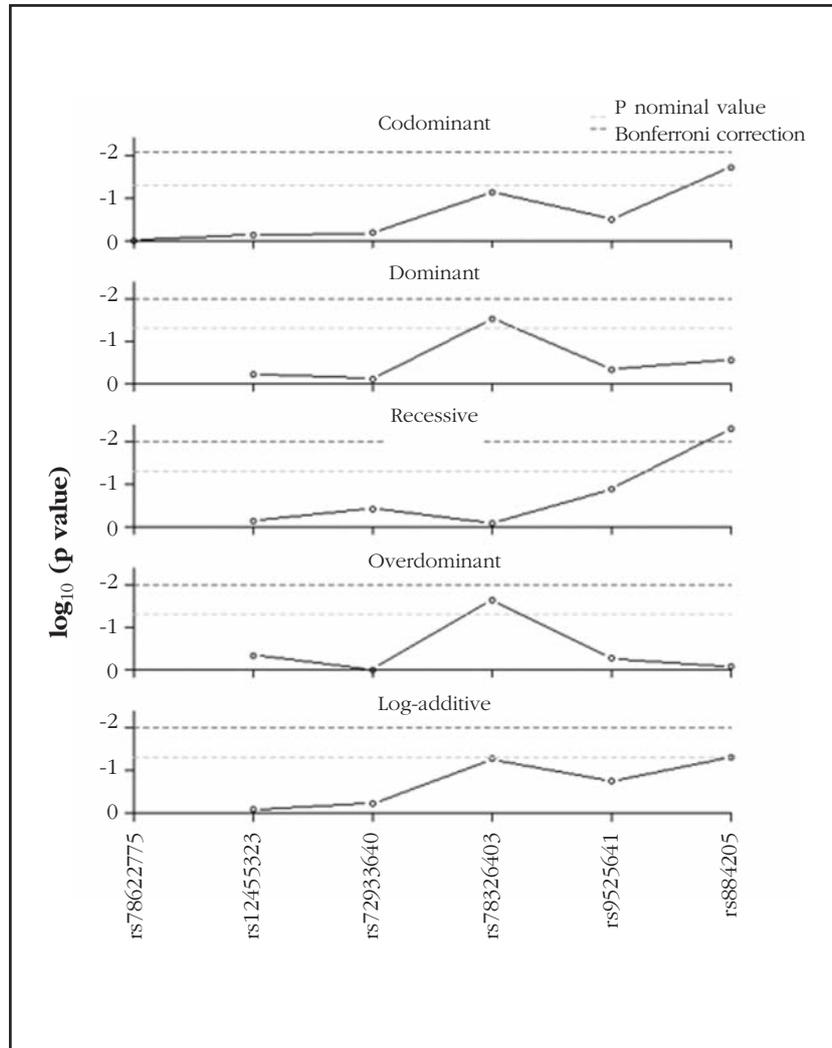
Discussion

The equilibrium of bone remodelling is, in part, regulated by the RANK/RANKL/OPG system. It is for this reason that this system has been well studied in the field of osteoporosis. In the light of this growing interest in the miRNAs as an epigenetic regulatory element, this study was centred on SNPs, situated in the 3'UTR of the RANK gene, which may affect the bonding of miRNAs. 7 SNPs in this region have been genotyped, resulting in two of them having been associated with osteoporotic fracture. The databases available to date offer little information, most of them in silico and based on algorithms, in terms of the binding sites of miRNAs in RANK's 3'UTR region. These databases, furthermore, do not hold any information about the genomic sequences which the SNPs associated with fractures contain. Therefore we do not have data regarding miRNAs which can bind to the region which contains the significant SNPs, and therefore functional studies would be necessary to clarify this question.

In recent years there has been a deepening in the study of genomics and the events which occur from gene expression to the final protein, which is to say, all those steps and molecules involved from the point at which gene expression takes place in the primary miRNA up to the operative functional protein, passing through the whole process of regulation, both of transcription and of

translation. The microRNAs (miRNAs or miRs) form a part of this dimension in gene regulation. The miRNAs are short single-stranded RNAs, ssRNAs) of 19-25 nucleotides generated from endogenous transcriptions with a hairpin shape, and have been evolutionarily preserved. Their function is the post-transcriptional silencing of genes through pairing with target mRNA in the 3'UTR region, resulting in the removal of this mRNA and thus the repression of its translation. A particular miRNA may act on a number of genes, and various different miRNAs may act on the same gene. If the pairing between the miRNA and the mRNA is only partial, a translational repression and later degradation results. If the pairing between the miRNA and mRNA is perfect (or nearly so) removal and degradation occurs. The majority of the targets of the miRNAs identified are in the 3'UTR of the messenger RNAs, which include transcription factors, receptors and kinases. The miRNAs regulate the protein translation and the stability of the mRNA, and thereby can modify numerous pathways related to development and diseases. Different studies have shown that genetic variants in the sequences of the miRNAs, as well as in their target sequences in the 3'UTR of the genes, may alter the mechanism regulating gene expression, and as a consequence, lead to a range of pathologies¹⁹. Lei et al. identified 3 polymorphisms located in potential binding sites for 9 miRNAs in the 3'UTR of the FGF2 gene which were associated with BMD in the hip²⁶. We have identified 2 SNPs in the 3'UTR region of the RANK gene, rs78326403 and rs884205, which are associated with osteoporotic fractures in the BARCOS cohort. In a later analysis, each SNP showed a stronger association as a function of the site of the fracture studied: rs78326403 was found to be associated only with fractures of the wrist/forearm, while rs884205 was only associated with fractures of the spinal column. Furthermore, adjusting the results for the corresponding BMD (FN-BMD for rs78326403 and LS-BMD for rs884205) did not change the associa-

Figure 1. Results of the study of the association of the genotyped SNPs with the prevalence of osteoporotic fracture in the BARCOS cohort for all the statistical models, presented as $-\log_{10}$ (p value). In each of the graphics, the lowest threshold line represents $p=0.05$, and the highest line represents the p value necessary after the Bonferroni correction ($p=8.33 \times 10^{-3}$)



tion of rs78326403, while the association of rs884205 with fracture was attenuated. These findings suggest that different SNPs in the RANK gene, even situated in the same region, may have a differential influence in a particular fracture site (cortical vs, trabecular bone). These associations with different types of fracture suggest different mechanisms of regulation of bone metabolism in predominantly cortical bone and in bone which is predominantly trabecular. Other factors have also been shown to be capable of differentially regulating cortical and trabecular bone, such as the GH-IGF-1²⁷⁻³⁰ axis or the gonadal hormones³¹⁻³⁴. It should be mentioned that these hormones regulate bone remodelling by means of RANK/RANKL/OPG³⁵⁻³⁹. Therefore, it seems plausible that the different hormonal signals may effect their action through different elements of the RANK/RANKL system.

Table 3. Significant results regarding the association between the SNPs rs884205 and rs78326403 and the site of fracture

| SNP | Site fracture | n | n fractures | p | OR | IC 95% | p ¹ | OR ¹ | 95% CI ¹ |
|------------|---------------|-------|-------------|------------------------------------|------|------------|-----------------------------------|-----------------|---------------------|
| rs78326403 | Wrist/forearm | 1,033 | 34 | 7.16x10 ⁻⁴ ^a | 3.12 | 1.69-5.75 | 5.8x10 ⁻⁴ ^a | 3.21 | 1.74-5.94 |
| rs884205 | Column | 1,029 | 62 | 8.24x10 ⁻³ ^r | 4.05 | 1.59-10.35 | 0.025 ^r | 3.31 | 1.24-8.82 |

¹: Result after correction for BMD: BMD in the femoral neck for rs78326403 and in the spinal column rs884205; ^r: recessive; ^a: log-additive.

Table 4. Analysis of the effect of the genotypes composed of the SNPs rs9594738 and rs78326403: 0/1 unfavourable alleles as a reference group vs 2 and ≥3 unfavourable alleles

| Interaction | n unfavourable alleles | n | n individuals with fractures (%) | Grups | p | OR | IC 95% |
|-------------------|------------------------|-----|----------------------------------|-----------------|----------------------|------|------------|
| | 0 | 266 | 7 (2.6%) | Reference group | | | |
| rs9594738x | 1 | 476 | 9 (1.9%) | | | | |
| rs78326403 | 2 | 244 | 14 (5.7%) | 0/1 vs 2 | 7.4x10 ⁻³ | 2.76 | 1.30-5.81 |
| | 3 | 33 | 4 (12.1%) | 0/1 vs 3/4 | 7.5x10 ⁻³ | 5.14 | 1.37-15.67 |
| | 4 | 3 | 0 (0%) | | | | |

This is the first time that the SNP rs78326403 has been associated with fractures; SNP rs8844205 had previously been associated with osteoporotic phenotypes^{16,40}. In a recent meta-analysis carried out by the GEFOS-GONOMOS consortium⁴¹, which included the BARCOS cohort, rs884205 was associated with BMD, but not with fractures. This difference may be due to the heterogeneity between the different cohorts in the evaluation of the fracture on the part of the different groups of the consortium. A future replication should clarify the association of this SNP with the risk of fracture.

Finally, a significant interaction between the SNP rs9594738 of RANKL, which is associated with BMD, and rs78326403, suggests an epistatic effect between RANK and RANKL. In agreement with these results, in a study of the compound genotypes it was confirmed that there was an additive effect of the alleles of the two SNPs. So, the carriers of more unfavourable alleles have a higher risk (OR=5.14) of suffering fractures with respect to the carriers of the more favourable genotypes.

In agreement with our data, numerous genetic association studies have shown a different inheritability for BMD and fracture (or bone quality)¹⁴⁻¹⁶. However, it is widely known that these osteoporotic phenotypes are closely related, and our results reinforce this premise. Consequently, it is necessary to consider both low BMD and other

additional measurements of bone quality or microarchitecture to evaluate with precision the risk of fracture in a clinical setting. These findings may be clinically relevant in the future for a more specific approach for different types of fracture, both to better understand their underlying mechanisms as well as to seek more specific therapeutic strategies. Our study has various limitations. The sample available is relatively small (1,098 women) having a limited number of fractures, and this reduces the statistical power of the study and, therefore, our ability to identify and analyse rare genotypes. Furthermore, our results could be specific to the population studied, which was limited to postmenopausal Caucasian Mediterranean women. Other studies in larger cohorts with different characteristics could determine if the associations which we have reported are replicable.

In conclusion, we have identified 2 SNPs in the 3'UTR of the RANK gene (rs78326403 and rs884205), which are associated with osteoporotic fractures. In our cohort, each of the SNPs is associated with a specific site of fracture. It was also found that there was a significant interaction between rs78326403 and an SNP (rs9594738) in the RANKL gene associated with BMD, highlighting the importance of BMD and microarchitecture as genetically determined predictors of the risk of fracture.

Declaration of interest: All authors declare no conflicts of interest.

Bibliography

- Knapp KM, Blake GM, Spector TD, Fogelman I. Can the WHO definition of osteoporosis be applied to multi-site axial transmission quantitative ultrasound? *Osteoporos Int* 2004;15:367-74.
- Riggs BL, Melton LJ, 3rd. The worldwide problem of osteoporosis: insights afforded by epidemiology. *Bone* 1995;17:505S-11S.
- Kanis JA, Melton LJ, 3rd, Christiansen C, Johnston CC, Khaltaev N. The diagnosis of osteoporosis. *J Bone Miner Res* 1994;9:1137-41.
- Shepstone L, Fordham R, Lenaghan E, Harvey I, Cooper C, Gittos N, et al. A pragmatic randomised controlled trial of the effectiveness and cost-effectiveness of screening older women for the prevention of fractures: rationale, design and methods for the SCOOP study. *Osteoporos Int* 2012;23:2507-15.
- Schuit SC, van der Klift M, Weel AE, de Laet CE, Burger H, Seeman E, et al. Fracture incidence and association with bone mineral density in elderly men and women: the Rotterdam Study. *Bone* 2004;34:195-202.
- Hillier TA, Cauley JA, Rizzo JH, Pedula KL, Ensrud KE, Bauer DC, et al. WHO absolute fracture risk models (FRAX): do clinical risk factors improve fracture prediction in older women without osteoporosis? *J Bone Miner Res* 2011;26:1774-82.
- Sornay-Rendu E, Munoz F, Garnero P, Duboeuf F, Delmas PD. Identification of osteopenic women at high risk of fracture: the OFELY study. *J Bone Miner Res* 2005;20:1813-9.
- Baim S, Leslie WD. Assessment of Fracture Risk. *Curr Osteoporos Rep* 2012;10:28-41.
- Leslie WD, Morin S, Lix LM, Johansson H, Oden A, McCloskey E, et al. Fracture risk assessment without bone density measurement in routine clinical practice. *Osteoporos Int* 2012;23:75-85.
- Sornay-Rendu E, Munoz F, Delmas PD, Chapurlat RD. The FRAX tool in French women: How well does it describe the real incidence of fracture in the OFELY cohort? *J Bone Miner Res* 2010;25:2101-7.
- González-Macías J, Marín F, Vila J, Díez-Pérez A. Probability of fractures predicted by FRAX(R) and observed incidence in the Spanish ECOSAP Study cohort. *Bone* 2012;50:373-7.
- Kanis JA, Oden A, Johansson H, Borgstrom F, Strom O, McCloskey E. FRAX and its applications to clinical practice. *Bone* 2009;44:734-43.
- Liu C, Walter TS, Huang P, Zhang S, Zhu X, Wu Y, et al. Structural and functional insights of RANKL-RANK interaction and signaling. *J Immunol* 2010;184:6910-9.
- Styrkarsdottir U, Halldorsson BV, Gretarsdottir S, et al. Multiple genetic loci for bone mineral density and fractures. *N Engl J Med* 2008;358:2355-65.
- Styrkarsdottir U, Halldorsson BV, Gretarsdottir S, Gudbjartsson DF, Walters GB, Ingvarsson T, et al. New sequence variants associated with bone mineral density. *Nat Genet* 2009;41:15-7.
- Rivadeneira F, Styrkarsdottir U, Estrada K, Halldorsson BV, Hsu YH, Richards JB, et al. Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. *Nat Genet* 2009;41:1199-206.
- Duncan EL, Danoy P, Kemp JP, Leo PJ, McCloskey E, Nicholson GC, et al. Genome-wide association study using extreme truncate selection identifies novel genes affecting bone mineral density and fracture risk. *PLoS Genet* 2011;7:e1001372.
- Zupan J, Mencej-Bedrac S, Jurkovic-Mlakar S, Prezelj J, Marc J. Gene-gene interactions in RANK/RANKL/OPG system influence bone mineral density in postmenopausal women. *J Steroid Biochem Mol Biol* 2010;118:102-6.
- Landi D, Gemignani F, Landi S. Role of variations within microRNA-binding sites in cancer. *Mutagenesis* 2012;27:205-10.
- Dong S, Yang B, Guo H, Kang F. MicroRNAs regulate osteogenesis and chondrogenesis. *Biochem Biophys Res Commun* 2012;418:587-91.
- Bustamante M, Nogués X, Agueda L, Jurado S, Wesseliuss A, Cáceres E, et al. Promoter 2 -1025 T/C polymorphism in the RUNX2 gene is associated with femoral neck BMD in Spanish postmenopausal women. *Calcif Tissue Int* 2007;81:327-32.
- Bustamante M, Nogués X, Mellibovsky L, Agueda L, Jurado S, Cáceres E, et al. Polymorphisms in the interleukin-6 receptor gene are associated with bone mineral density and body mass index in Spanish postmenopausal women. *Eur J Endocrinol* 2007;157:677-84.
- Genant HK, Wu CY, van Kuijk C, Nevitt MC. Vertebral fracture assessment using a semi-quantitative technique. *J Bone Miner Res* 1993;8:1137-48.
- Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988;16:1215.
- Prieto-Alhambra D, Premaor MO, Fina Avilés F, Hermosilla E, Martínez-Lagunamd D, Carbonell-Abella C, et al. The association between fracture and obesity is site-dependent: a population-based study in postmenopausal women. *J Bone Miner Res* 2012;27:294-300.
- Lei SF, Papasian CJ, Deng HW. Polymorphisms in predicted miRNA binding sites and osteoporosis. *J Bone Miner Res* 2011;26:72-8.
- Yakar S, Rosen CJ, Beamer WG, Ackert-Bicknell CL, Wu Y, Liu JL, et al. Circulating levels of IGF-1 directly regulate bone growth and density. *J Clin Invest* 2002;110:771-81.
- Hamrick MW, Ding KH, Ponnala S, Ferrari SL, Isaacs CM. Caloric restriction decreases cortical bone mass but spares trabecular bone in the mouse skeleton: implications for the regulation of bone mass by body weight. *J Bone Miner Res* 2008;23:870-8.
- Moyer-Mileur LJ, Slater H, Jordan KC, Murray MA. IGF-1 and IGF-binding proteins and bone mass, geometry, and strength: relation to metabolic control in adolescent girls with type 1 diabetes. *J Bone Miner Res* 2008;23:1884-91.
- Yakar S, Canalis E, Sun H, Mejia W, Kawashima Y, Nasser P, et al. Serum IGF-1 determines skeletal strength by regulating subperiosteal expansion and trait interactions. *J Bone Miner Res* 2009;24:1481-92.
- Windahl SH, Vidal O, Andersson G, Gustafsson JA, Ohlsson C. Increased cortical bone mineral content but unchanged trabecular bone mineral density in female ERbeta(-/-) mice. *J Clin Invest* 1999;104:895-901.
- Lorentzon M, Swanson C, Andersson N, Mellstrom D, Ohlsson C. Free testosterone is a positive, whereas free estradiol is a negative, predictor of cortical bone size in young Swedish men: the GOOD study. *J Bone Miner Res* 2005;20:1334-41.
- Taes Y, Lapauw B, Griet V, De Bacquer D, Goemaere S, Zmierzak H, et al. Prevalent fractures are related to cortical bone geometry in young healthy men at age of peak bone mass. *J Bone Miner Res* 2010;25:1433-40.
- Vanbillemont G, Lapauw B, Bogaert V, Goemaere S, Zmierzak HG, Taes Y, et al. Sex hormone-binding globulin as an independent determinant of cortical bone status in men at the age of peak bone mass. *J Clin Endocrinol Metab* 2010;95:1579-86.
- Rubin J, Ackert-Bicknell CL, Zhu L, Fan X, Murphy TC, Nanes MS, et al. IGF-I regulates osteoprotegerin (OPG) and receptor activator of nuclear factor-kappaB ligand in vitro and OPG in vivo. *J Clin Endocrinol Metab* 2002;87:4273-9.
- Zhao HY, Liu JM, Ning G, Zhao YJ, Chen Y, Sun LH, et al. Relationships between insulin-like growth factor-I (IGF-I) and OPG, RANKL, bone mineral density in healthy Chinese women. *Osteoporos Int* 2008;19:221-6.
- Taxel P, Kaneko H, Lee SK, Aguila HL, Raisz LG, Lorenzo JA. Estradiol rapidly inhibits osteoclastogene-

- sis and RANKL expression in bone marrow cultures in postmenopausal women: a pilot study. *Osteoporos Int* 2008;19:193-9.
38. Michael H, Harkonen PL, Vaananen HK, Hentunen TA. Estrogen and testosterone use different cellular pathways to inhibit osteoclastogenesis and bone resorption. *J Bone Miner Res* 2005;20:2224-32.
 39. Hofbauer LC, Kuhne CA, Viereck V. The OPG/RANKL/RANK system in metabolic bone diseases. *J Musculoskelet Neuronal Interact* 2004;4:268-75.
 40. Guo Y, Wang JT, Liu H, Li M, Yang TL, Zhang XW, et al. Are bone mineral density loci associated with hip osteoporotic fractures? A validation study on previously reported genome-wide association loci in a Chinese population. *Genet Mol Res* 2012;11:202-10.
 41. Estrada K, Styrkarsdottir U, Evangelou E, Hsu YH, Duncan EL, Ntzani EE, et al. Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. *Nat Genet* 2012;44:491-501.