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Polymorphisms in Genes Involved in the NF-κB Signalling Pathway Are Associated with Bone Mineral Density, Geometry and Turnover in Men

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Abstract

Introduction: In this study, we aimed to investigate the association between single nucleotide polymorphisms (SNPs) within two genes involved in the NF-κB cascade (GPR177 and MAP3K14) and bone mineral density (BMD) assessed at different skeletal sites, radial geometric parameters and bone turnover.

Methods: Ten GPR177 SNPs previously associated with BMD with genome-wide significance and twelve tag SNPs ($r^2 \geq 0.8$) within MAP3K14 (±10 kb) were genotyped in 2359 men aged 40–79 years recruited from 8 centres for participation in the European Male Aging Study (EMAS). Measurement of bone turnover markers (PINP and CTX-I) in the serum and quantitative ultrasound (QUS) at the calcaneus were performed in all centres. Dual energy X-ray absorptiometry (DXA), at the lumbar spine and hip, and peripheral quantitative computed tomography (pQCT), at the distal and midshaft radius, were performed in a subsample (2 centres). Linear regression was used to test for association between the SNPs and bone measures under an additive genetic model adjusting for study centre.

Results: We validated the associations between SNPs in GPR177 and BMD, previously reported and also observed evidence of pleiotrophic effects on density and geometry. Rs2772300 in GPR177 was associated with increased total hip and LS BMD, increased total and cortical vBMD at the radius and increased cortical area, thickness and stress strain index. We also found evidence of association with BMD, vBMD, geometric parameters and CTX-I for SNPs in MAP3K14. None of the GPR177 and MAP3K14 SNPs were associated with calcaneal estimated BMD measured by QUS.

Conclusion: Our findings suggest that SNPs in GPR177 and MAP3K14 involved in the NF-κB signalling pathway influence bone mineral density, geometry and turnover in a population-based cohort of middle aged and elderly men. This adds to the understanding of the role of genetic variation in this pathway in determining bone health.

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Introduction

Osteoporosis is characterized by reduced bone mass and deterioration in bone micro-architecture leading to bone fragility and increased risk of fracture [1]. It is a major health problem with the lifetime risk of fracture estimated to be about 50% and 20% at age 50 years in women and men, respectively [2]. Prospective studies suggest that there is a strong relationship between levels of bone mineral density (BMD) and subsequent risk of fracture in both men [3] and women [4,5].

Genetic factors are important determinants of bone mass. Family and twin studies suggest that up to 80% of areal BMD (BMDa) at the lumbar spine (LS) and hip is determined by genetic factors [6-8]. Genome-wide association studies (GWAS) have identified a number of loci associated with LS and hip BMDa, reaching genome-wide significance (GWS), including the genes involved in the RANKL/RANK/OPG signalling pathway [9-12], which have also been associated with markers of bone turnover [13]. The RANKL/RANK/OPG signalling pathway has an important role in bone turnover. Interaction of RANKL (receptor activator of NF-

b B ligand) with its receptor RANK (receptor activator of NF-

b) on the surface of osteoclast precursors [14] activates both canonical and non-canonical pathways of NF-
b. Subsequently, active NF-
b dimers move into the nucleus, bind to the DNA and cause gene transcription and osteoclast differentiation [15]. OPG acts as a decoy receptor for RANKL and can block its effects [14].

Rivadeneira et al. performed a meta-analysis of five GWAS of LS and femoral neck (FN) BMD, in approximately 20,000 subjects [10]. They strengthened the association of RANKL, RANK and OPG with BMDa, and also identified two new loci associated with the NF-
b pathway. GPR177 is located on chromosome 1p31.3 and single nucleotide polymorphisms (SNPs) within this gene were associated with LS and FN BMDa at the GWS level [10]. GPR177 which is also known as WNTLESS homolog is an activator of the NF-
b pathway [16]. It also induces osteoblast differentiation through the Wnt signalling pathway [17]. A single SNP on chromosome 17q12, rs9303521, which is located about 400 kb upstream of MAP3K14, was also associated with LS BMDa at the GWS level in the meta-analysis [10]. MAP3K14 encodes NIK (NF-
b inducing kinase) which induces production of active NF-
b dimers in the non-canonical pathway [15].

In this study, we aimed to validate GPR177 SNPs associations with BMDa, and to determine if SNPs in MAP3K14 are associated with BMDa utilizing an independent population of middle-aged and elderly men recruited in the European Male Aging Study (EMAS) [18]. We also explored the influence of GPR177 and MAP3K14 SNPs on bone geometry, another important determinant of bone strength. As both genes investigated here are involved in bone turnover related pathways, one would expect that their effects on bone density and geometry would, at least partly, be through altering bone turnover. Therefore, we sought to test this hypothesis. We also investigated the combined effect of strongly associated SNPs in RANKL, RANK, OPG, GPR177 and MAP3K14 on BMDa.

Methods

Ethics statement

Ethical approval for the study was obtained in accordance with local institutional requirements in each centre: Florence (Ethical Committee of the Azienda Ospedaliera Careggi & University of Florence), Leuven (Commissie Medische Ethiek UZ Gasthuisberg & KU Leuven), Lodz (Bioethical Committee of Medical University of Lodz for Human Studies), Malmo (Ethical Committee of Lund University), Manchester (North West Multi Centre Ethical Research Committee, University of Manchester & CMMCUH), Santiago de Compostela (Comité Ético de Investigación Clínica de Galicia & Universidad de Santiago de Compostela), Szeged (Human Investigation Review Board, University of Szeged) and Tartu (Ethical Committee, Medical University of Tartu). All subjects provided written informed consent. Approval for the genetic analysis described here was obtained for seven of the eight centres. Therefore, analysis was restricted to subjects from these seven centres (all centres except for Malmo, Sweden).

Study Participants

Men aged 40–79 years were recruited from population registries in 8 European centres (Manchester, UK; Leuven, Belgium; Tartu, Estonia; Lodz, Poland; Szeged, Hungary; Florence, Italy; Santiago de Compostela, Spain; Malmo, Sweden) into the European Male Ageing Study, for further details see Lee at al. (2008) [19]. Blood samples were collected for genetic analysis. Quantitative ultrasound (QUS) at the calcaneus was performed in subjects at all centres. Dual energy X-ray absorptiometry (DXA) and peripheral quantitative computed tomography (pQCT) were performed in a subsample of subjects in two centres (Manchester, UK and Leuven, Belgium). Participants were excluded from this analysis if they reported that at least one of their parents or grandparents was born outside Europe or North America, or if they reported use of anti-osteoporotic medications or systemic glucocorticoids.

Bone Assessments

DXA. DXA scans were performed in Manchester and Leuven centres using a DXA QDR 4500A device (Hologic, Inc, Waltham, MA, USA). BMDa (g/cm²) was measured at LS (L1 to L4) and total hip (TH). All scans and measurements were performed by trained and experienced DXA technicians. The Hologic Spine Phantom was scanned daily to monitor the device performance and long-term stability. The precision of these measurements in the LS and TH were 0.57% and 0.56% in Leuven, and 0.97% and 0.97% in Manchester, respectively. Both devices were cross-calibrated with the European Spine Phantom [20].

pQCT. Peripheral QCT measurements were performed in the non-dominant radius using XCT-2000 scanners (Stratec, Pforzheim, Germany) in Manchester and Leuven centres following the manufacturer’s standard quality assurance procedures. Total and trabecular vBMD (mg/mm³), and bone cross-sectional area (mm²) were measured at the distal radius (%)(voxel size 0.4 mm). Cortical vBMD (mg/mm³); total, cortical and medullary area (mm²); cortical thickness (mm) and stress strain index (SSI) (mm²) were measured at the midshaft radius (50%) (voxel size 0.6 mm). The detailed methodology for these measurements has been described previously [21].

The European Forearm Phantom (EFP) was measured for cross-calibration between the two centres; 10 repeat measurements were taken in slices 1–4. The differences were less than precision error for total, trabecular and cortical vBMD, and cortical area. Therefore, no cross-calibration was performed between the two centres. The short term precision of 2 repeat measurements with repositioning were: 2.1% and 1.3% for total vBMD; 1.27% and 1.42% for trabecular vBMD; 0.77% and 0.71% for cortical vBMD; and 2.4% and 1.3% for cortical area; in Manchester (n = 22) and Leuven (n = 40), respectively.

QUS. BMD at the left calcaneus was estimated by QUS using the Sahara Clinical Sonometer (Hologic, Bedford, MA, USA) in...
all centres following a standardized protocol. Outputs included broadband ultrasound attenuation (BUA) (dB/MHz) and speed of sound (SOS) (m/s). Calcaneal eBMD was calculated (g/cm²) using the following formula: $BMD = 0.002592 \times (BUA + SOS) - 3.607$. The CVs were 2.4%, 0.3% and 3.4% for BUA, SOS and BMD, respectively as described previously [13].

**Bone Turnover Markers.** Serum N-terminal propeptide of type I procollagen (PINP) (ng/ml) and C-terminal cross-linked telopeptide of type I collagen (CTX-I) (ng/ml) levels were measured in Leuven, using electrochemiluminescence immunoassay (ECLIA) (Roche Diagnostics) on samples from all centres. The detection limit of PINP assay was <5 ng/ml, and the intra-assay and inter-assay coefficients of variations (CVs) were 0.8–2.9 and 1.8–2.9%, respectively. The detection limit of CTX-I assay was 0.01 ng/ml, and the intra-assay and inter-assay CVs were 2.0–5.5% and 1.0–4.6%, respectively.

**SNPs Selection**

**GPR177.** The SNPs within GPR177 associated with LS or FN BMDₐ at the genome-wide significant level (p<5×10⁻⁸) in a previous GWAS meta-analysis [10] were selected for genotyping. Where SNPs were in high linkage disequilibrium (LD) ($r^2$≥0.8) based on HapMap Phase II CEPH SNP data (http://www.hapmap.org), only one of them was genotyped.

**MAP3K14.** Pair-wise tag SNPs ($r^2$≥0.8) were selected for SNPs in HapMap Phase II CEPH SNP data (http://www.hapmap.org) with a minor allele frequency (MAF) of greater than 5% within MAP3K14 and its 10 kb flanking regions using Tagger implemented in Haplovip 4.0 [22].

**Genotyping and Quality Control**

SNPs were genotyped using SEQUENOM MassARRAY technology following the manufacturer’s instructions (http://www.sequenom.com). Sample and assay quality control thresholds were set to 90%. The SNPs deviating from Hardy-Weinberg equilibrium (HWE), p≤0.05, were excluded from the analysis.

**Statistical Analysis**

The outcome variables were standardised, $z = (x - \mu) / \sigma$ where $x$ = raw value of bone measure, $\mu$ = mean of bone measure and $\sigma$ = standard deviation of bone measure.

The association between the SNPs and the standardised outcome variables (DXA: LS and TH BMDₐ, pQCT: radius vBMD and geometric parameters, QUS: calcaneal BMD, and bone turnover markers: PINP and CTX-I) was tested using linear regression under an additive genetic model using PLINK (1.05) [23]. The results were adjusted for study centre and also for study centre, age, height and weight. The results are presented as a percentage change ($\beta$) in a standard deviation (SD) with 95% confidence intervals for each copy of the minor allele. For significantly associated SNPs, the interaction between the SNP and centre was tested to see if the relationship between the SNP and the outcome differed across centres.

Pairs of SNPs associated with BMDₐ and located in different genes (RANKL, RANK, OPG, GPR177 and MAP3K14) at the same skeletal site with a p-value<0.01 were tested to determine if carrying the risk allele (allele associated with lower BMD) for both SNPs was associated with a greater effect on BMDₐ. For RANKL, RANK and OPG, data from previous analysis in the EMAS cohort was used [13]. In order to determine the combined effect of the two SNPs, a new combined genotype variable was defined based on the number of risk alleles each individual carried for either SNP. This new variable could have a value between 0 to 4: 1: having no copy of risk alleles for both SNPs, 2: having two copies of risk alleles for either SNPs, 3: having two copies of risk allele for one SNP and one copy of risk allele for the other one, and 4: having 2 copies of risk alleles for both SNPs. Subsequently, linear regression was used for testing the association between BMDₐ and this new variable adjusting for centre in STATA (9.2).

**Results**

**Subject Characteristics**

Of the 2658 men who consented to participate in the genetic analysis, 299 were excluded: 180 failed sample quality control, 21 reported at least one of their parents or grandparents was born outside Europe or North America, and 98 reported use of anti-osteoporotic medications or systemic glucocorticoids. In total, 2359 men, mean (±SD) age 60±11 years, were included in the analysis. Bone turnover markers and ultrasound BMD were measured in almost all men (N = 2244 and 2314, respectively). DXA and pQCT were performed in a subsample of men from 2 centres (N = 586 and 560, respectively). Mean values of the bone turnover markers, QUS, DXA and pQCT parameters are presented in Table 1.

| Table 1. Mean (SD) of bone turnover markers, DXA, pQCT and QUS parameters. |
|----------------|----------------|----------------|
| **Bone turnover markers** | **DXA BMDₐ** | **pQCT** |
| PINP (ng/ml) | 2244 | 42.91 | 21.72 |
| CTX-I (ng/ml) | 2242 | 357.72 | 181.37 |
| Lumbar spine (g/cm²) | 588 | 1.06 | 0.18 |
| Total hip (g/cm³) | 587 | 1.02 | 0.14 |
| Femoral neck (g/cm²) | 587 | 0.81 | 0.13 |
| **DXA BMDₐ** | **Total vBMD (mg/mm³)** | **Total area (mm²)** |
| | 560 | 400.66 | 71.21 |
| | 560 | 203.82 | 42.72 |
| Cortex (mm) | 560 | 374.62 | 66.31 |
| **pQCT** | **Trabecular vBMD (mg/mm³)** | **Trabecular thickness (mm)** |
| | 560 | 1214.47 | 29.68 |
| | 560 | 3.24 | 0.42 |
| **QUS** | **Total area (mm²)** | **Medullary area (mm²)** |
| | 560 | 149.31 | 20.72 |
| | 560 | 106.44 | 13.63 |
| | 560 | 42.87 | 15.71 |
| | 560 | 337.56 | 63.87 |
| | **BMD at calcaneus (g/cm²)** | 2314 | 0.54 | 0.14 |

PINP: N-terminal propeptide of type I procollagen, CTX-I: C-terminal cross-linked telopeptide of type I collagen, DXA: dual energy X-ray absorptiometry, BMDₐ: areal bone mineral density, pQCT: peripheral quantitative computed tomography, vBMD: volumetric bone mineral density, SSI: stress strain index, QUS: quantitative ultrasound.
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MAP3K14. Forty-three SNPs with a MAF>0.05 were identified in MAP3K14 and its 10 kb flanking regions. These SNPs were tagged by 13 SNPs. Of these, 12 SNPs were successfully genotyped and passed quality control covering 98% of the selected SNPs with a MAF of more than 5% in the region. Rs2067316 failed genotyping.

Genetic Association Analysis

For the bone turnover markers and ultrasound BMD, with \( \alpha = 0.05 \), there was greater than 80% power to detect differences of 0.2 SD for SNPs with a MAF >0.05 and 0.1 SD for SNPs with a MAF =0.45 under an additive genetic model. For DXA and pQCT outcomes, with \( \alpha = 0.05 \), there was greater than 80% power to detect differences of 0.4 SD for SNPs with a MAF =0.05 and 0.2 SD for SNPs with a MAF =0.45 under an additive genetic model. Statistical power was calculated using Quanto v1.2.3 software [24].

GPR177. Of the 10 SNPs successfully genotyped, 6 were associated with total hip BMD\(_a\), Rs1430742, which was the most significantly associated SNP in this locus in a GWAS meta-analysis of LS and FN BMD [10], showed the largest effect with a significantly associated SNP in this locus in a GWAS meta-analytic(ld) association of LS and FN BMD\(_a\), with \( \alpha = 0.05 \) per allele resulting in a 0.18 SD (95%CI 0.04, 0.33) \( p = 0.015 \). Rs891257 was also significantly associated with increased LS BMD\(_a\) (0.05, 0.32) \( p = 0.007 \) per allele). Other SNPs showed a change in total hip BMD\(_a\) but was not associated with LS or TH BMD\(_a\) (Table 4). Two SNPs (rs2566759 and rs2195682 were associated with cross-sectional area at the distal radius. Rs277230 and rs1430742 were associated with increased cortical area at the mid-shaft radius and rs2772300 was also associated with increased cortical thickness and SSI. Rs7554551 was associated with cortical thickness at the mid-shaft radius. There was no association between SNPs in GPR177 and bone turnover markers or ultrasound vBMD.

MAP3K14. Two SNPs showed evidence of association with BMD. Rs8065345 was significantly associated with a 0.25 SD (95%CI 0.10, 0.41) \( p = 0.002 \) per allele increase in LS BMD\(_a\) and was also associated with increased cortical area and cortical thickness. Rs7215764 was significantly associated with increased total hip BMD\(_a\) and cortical vBMD at the mid-shaft radius. Three further SNPs were associated with geometric parameters, rs11651968 was associated with decreased cortical area at the mid-shaft radius and rs1785379 was associated with decreased medullary area and increased cortical thickness at the mid-shaft radius. Rs16939940 was associated with total area at the mid-shaft radius; however, this association became non-significant after further adjustment for age, height and weight (Table 5). Further adjusting the associations between MAP3K14 SNPs and bone health parameters for age, height and weight gave broadly similar results with effect estimates of similar magnitude. LD between the associated SNPs was weak, \( r^2 \leq 0.13 \). The results for all MAP3K14 SNPs with LS and total hip BMD\(_a\) and total and cortical vBMD are given in Tables S1 & S2 respectively.

In contrast to GPR177, there were three MAP3K14 SNPs associated with CTX-I serum levels; rs7929479 (0.07 SD (95%CI 0.01, 0.12) \( p = 0.013 \), rs4792594 (0.06 SD (95%CI 0.00, 0.12) \( p = 0.042 \) and rs4329483 (0.08 SD (95%CI 0.03, 0.14) \( p = 0.003 \). None of the SNPs were associated with PINP serum levels. There was a moderate LD between these three SNPs (0.69 < \( r^2 \) > 0.39). There was no association between SNPs in MAP3K14 and ultrasound BMD.

The combined effect of BMD associated SNPs in different pathway genes

A single pair of SNPs, rs9594738 in RANKL and rs8065345 in MAP3K14, which were both associated with LS BMD\(_a\) \( p = 0.001 \) met the inclusion criteria for examining their combined effect. Five

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Table 2. Genetic association between GPR177 SNPs and BMD\(_a\).

| SNP          | Alleles | MAF       | Total Hip BMD\(_a\) | LS BMD\(_a\) |
|--------------|---------|-----------|---------------------|-------------|
|              |         |           | \( \beta (SD) (95\% CI)\) | \( \beta (SD) (95\% CI)\) |
|              |         |           | \( p^a \)            | \( p^a \)    |
| rs2566784    | G>T     | 0.25      | 0.13 (0.01, 0.26)    | 0.063       |
|              |         |           | 0.18 (0.06, 0.29)    | 0.003       |
| rs3762371    | G>A     | 0.4       | 0.07 (0.19, 0.05)    | 0.261       |
|              |         |           | 0.03 (0.14, 0.07)    | 0.565       |
| rs2566759    | A>G     | 0.5       | 0.12 (0.04, 0.00)    | 0.049       |
|              |         |           | 0.09 (0.20, 0.01)    | 0.088       |
| rs2566758    | T>C     | 0.46      | 0.13 (0.24, 0.01)    | 0.039       |
|              |         |           | 0.10 (0.21, 0.00)    | 0.053       |
| rs1367447    | C>T     | 0.19      | 0.15 (0.01, 0.30)    | 0.062       |
|              |         |           | 0.09 (0.05, 0.23)    | 0.208       |
| rs1430742    | T>C     | 0.23      | 0.18 (0.04, 0.33)    | 0.015       |
|              |         |           | 0.17 (0.04, 0.30)    | 0.011       |
| rs2195682    | T>C     | 0.39      | 0.16 (0.28, 0.04)    | 0.007       |
|              |         |           | 0.14 (0.24, 0.03)    | 0.011       |
| rs891527     | A>T     | 0.42      | 0.05 (0.07, 0.17)    | 0.411       |
|              |         |           | 0.06 (0.04, 0.17)    | 0.242       |
| rs2772300    | G>A     | 0.28      | 0.15 (0.02, 0.29)    | 0.026       |
|              |         |           | 0.18 (0.06, 0.30)    | 0.003       |
| rs7554551    | T>C     | 0.33      | 0.17 (0.05, 0.30)    | 0.005       |
|              |         |           | 0.19 (0.08, 0.29)    | 0.001       |
|              |         |           | 0.14 (0.03, 0.26)    | 0.016       |

*adjusted for study centre, age, height and weight.

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The combined effect of BMD associated SNPs in different pathway genes

A single pair of SNPs, rs9594738 in RANKL and rs8065345 in MAP3K14, which were both associated with LS BMD\(_a\) \( p = 0.001 \) met the inclusion criteria for examining their combined effect. Five
of which showed significant association with total hip BMD\textsubscript{a} in the same direction in EMAS. Two of these SNPs were also significantly associated with LS BMD\textsubscript{a} and another 2 showed suggestive association (p<0.1) with LS BMD\textsubscript{a}. This weaker evidence of association with LS BMD\textsubscript{a} may have arisen due to the presence of concomitant disease such as osteoarthritis which can artificially raise BMD of the spine in an elderly cohort, making it harder to detect an association, particularly in a cohort of modest sample size. The results confirm the GWAS meta-analysis findings and show that SNPs in \textit{GPR177} influence BMD\textsubscript{a} at osteoporotic sites in a population of middle aged and elderly men at that their effects can be detected in a relatively modest sample size.

In addition, SNPs in \textit{GPR177} were also associated with total and cortical BMD at the radius, which is a novel finding, however despite testing in a larger sample size, there was no evidence of association with calcaneal eBMD as measured by ultrasound, a pattern which has been observed for other BMD susceptibility genes [13]. The findings reported here also suggest that these SNPs may have pleiotrophic effects on bone as SNPs associating with cortical vBMD were also associated with geometric parameters at the radius. Rs2772300, which was associated with increased BMD\textsubscript{a} at the hip and LS and total and cortical vBMD at the radius, was also associated with increased cortical area, thickness and stress strain index. It seems unlikely that these effects are mediated by bone turnover as no association was observed between these SNPs and the bone turnover markers CTX-I and PINP, in a larger sample size. Variation in the level of markers may be one explanation for this. We attempted to reduce variability in the bone turnover markers by taking fasting blood samples and measuring them in a single laboratory (to reduce technical variability). Further, the bone turnover markers selected here (PINP and CTX-I) both had low intra-assay and inter-assay CVs minimizing their analytical variability. Turnover marker levels, however, reflect current skeletal turnover and may not necessarily reflect previous levels, such misclassification may in part explain the absence of any observed significant associations.

We also looked at the association of common SNPs (MAF=0.05) in \textit{MAP3K14} with bone health parameters as this gene was previously highlighted as a good candidate, however there is no LD between the SNPs tested here in the \textit{MAP3K14} gene and the 17q12 SNP associated with BMD\textsubscript{a} [10]. There was

| SNP       | Alleles | MAF   | Mean Total vBMD (4%) | p* | Mean Total vBMD (4%) | p* | Mean Cortical vBMD (50%) | p* |
|-----------|---------|-------|----------------------|----|----------------------|----|--------------------------|----|
| rs2566784 | G>T     | 0.25  | 0.08 (0.04, 0.19)    | 0.178 | 0.09 (0.02, 0.20) | 0.099 | 0.04 (0.09, 0.18) | 0.52 |
| rs2195682 | C>T     | 0.39  | -0.10 (-0.21, 0.00) | 0.053 | -0.06 (-0.16, 0.04) | 0.222 | -0.09 (-0.21, 0.03) | 0.133 |
| rs891527  | A>T     | 0.42  | -0.04 (-0.07, 0.14) | 0.504 | 0.02 (-0.08, 0.12) | 0.72 | 0.16 (0.04, 0.28) | 0.011 |
| rs2772300 | G>A     | 0.28  | 0.13 (0.01, 0.23)   | 0.036 | 0.10 (-0.01, 0.22) | 0.074 | 0.18 (0.05, 0.32) | 0.007 |
| rs7554551 | T>C     | 0.33  | 0.12 (0.01, 0.23)   | 0.028 | 0.10 (0.00, 0.21) | 0.047 | 0.03 (-0.09, 0.15) | 0.641 |

*Adjusted for study centre; age, height and weight.

\textbf{Table 3. Genetic association between \textit{GPR177} SNPs and BMD\textsubscript{a}.}
Evidence of association between MAP3K14 SNPs and BMD, vBMD and radius geometric parameters, however unlike GPR177, there were no SNPs showing association with BMD and geometric parameters, therefore the findings should be interpreted with caution. This is the first time that genetic variation in the MAP3K14 gene has been investigated with bone health parameters and in a modest sample size, therefore replication of these findings is required in larger cohorts.

Notably, we also found that individuals carrying risk alleles for SNPs in the MAP3K14 and RANKL genes had a significantly lower LS BMD, suggesting that, despite the modest effects of single SNPs, in individuals carrying multiple risk alleles for SNPs in the RANKL/RANK/OPG and NF-kB signalling pathways they could have a profound effect on bone health. This finding also requires replication in independent cohorts.

Important potential limitations of this study are that false positive associations might have been produced due to population stratification and multiple testing. We tried to minimize the probability of population stratification by excluding subjects of non-European ancestry and did not observe any between centre heterogeneity. However, we were unable to explore population substructure using methods such as genomic control or principal component analysis as these require data on a large number of SNPs. Based on the method described by Li and Ji [25], 19 independent SNPs (GPR177: 8 and MAP3K14: 11 SNPs) were investigated in this study. The majority of the positive findings would not remain significant if Bonferroni correction (p<0.0026, 0.05/19) was applied to correct for multiple testing. On the other hand, we may have missed some genuine associations due to low statistical power. Lack of replication for association of some SNPs with BMD may also be due to the relatively small number of subjects in our DXA subsample compared with the GWAS meta-analysis. In addition, EMAS is a male population whereas the GWAS meta-analysis data was based on studies of both men and women.

Assessment of cortical vBMD using pQCT is subject to the partial volume effect; the thinner the cortices the lower vBMD. In our analysis, we used a higher threshold (900 mg/cm^3) for analysis of cortical vBMD rather than the traditional 710 mg/cm^3 threshold [26] to reduce the likelihood of this effect. In addition, most of the SNPs associated with cortical vBMD were also associated with areal and/or volumetric BMD at other skeletal sites but they were not associated with cortical thickness suggesting that they are real findings rather than false positive results arising due to technical artefact.

In summary, our findings suggest that SNPs within GPR177 and MAP3K14 involved in the NF-κB pathway influence BMD at both axial (LS and Hip) and peripheral sites (radius) and they also influence radius geometry. We also found some evidence of association between SNPs within MAP3K14 and bone turnover.

### Table 4. GPR177 SNP associations with radius geometric parameters.

| SNP       | Alleles | MAF | Phenotype            | Skeletal Site | β(SD) (95% CI) | p*    | β(SD) (95% CI) | p*    |
|-----------|---------|-----|----------------------|---------------|----------------|-------|----------------|-------|
| rs2566759 | A>G     | 0.5 | Cross-sectional area | 4% radius     | 0.11 (0.01, 0.21) | 0.037 | 0.10 (0.00, 0.20) | 0.045 |
| rs2566758 | T>C     | 0.46| Cross-sectional area | 4% radius     | 0.11 (0.01, 0.22) | 0.030 | 0.09 (0.00, 0.19) | 0.060 |
| rs1430742 | T>C     | 0.23| Cortical area        | 50% radius    | 0.17 (0.02, 0.32) | 0.031 | 0.14 (0.00, 0.27) | 0.053 |
| rs2772300 | G>A     | 0.28| Cortical area        | 50% radius    | 0.17 (0.03, 0.30) | 0.015 | 0.18 (0.05, 0.30) | 0.005 |
| rs7554551 | T>C     | 0.33| Cortical Thickness   | 50% radius    | 0.17 (0.04, 0.31) | 0.012 | 0.17 (0.04, 0.30) | 0.011 |

### Table 5. MAP3K14 SNP associations with bone mineral density and radius geometric parameters.

| SNP       | Alleles | MAF | Location                | Phenotype | Skeletal Site | β(SD) (95% CI) | p*    | β(SD) (95% CI) | p*    |
|-----------|---------|-----|-------------------------|-----------|---------------|----------------|-------|----------------|-------|
| rs8065345 | A>G     | 0.15| 3’ downstream           | BMDa       | Lumbar spine  | 0.25 (0.10, 0.41) | 0.002 | 0.21 (0.06, 0.35) | 0.006 |
|           |         |     | Cortical area           | 50% radius | 0.21 (0.05, 0.38) | 0.011 | 0.16 (0.01, 0.31) | 0.041 |
|           |         |     | Cortical thickness      | 50% radius | 0.24 (0.07, 0.40) | 0.004 | 0.22 (0.06, 0.38) | 0.006 |
| rs1165196 | C>T     | 0.48| 3’ downstream           | Cortical area | 50% radius | −0.13 (−0.25, −0.01) | 0.031 | −0.11, −0.22, 0.01 | 0.062 |
| rs7215764 | C>G     | 0.25| 3’ downstream           | BMDa       | Total hip     | 0.17 (0.03, 0.31) | 0.021 | 0.10 (−0.03, 0.22) | 0.136 |
|           |         |     | Cortical vBMD           | 50% radius | 0.17 (0.03, 0.32) | 0.020 | 0.17 (0.03, 0.31) | 0.016 |
| rs17685379| C>G     | 0.14| Intronic                | Medullary area | 50% radius | −0.17 (−0.35, 0.00) | 0.048 | −0.19 (−0.36, 0.02) | 0.031 |
|           |         |     | Cortical thickness      | 50% radius | 0.18 (0.01, 0.35) | 0.043 | 0.22 (0.05, 0.38) | 0.011 |
| rs16993948| T>C     | 0.05| Intronic                | Total area  | 50% radius    | 0.29 (0.01, 0.57) | 0.044 | 0.23 (−0.03, 0.50) | 0.089 |

BMDa: areal bone mineral density, vBMD: volumetric bone mineral density;

*aadjusted for study centre;

*badjusted for study centre, age, height and weight.

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Table S2 Genetic association between MAP3K14 SNPs and BMD. (DOC)

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Author Contributions

Conceived and designed the experiments: DR KLH TWO WT. Performed the experiments: DR. Analyzed the data: DR. Wrote the paper: DR. Interpretation of results: KLH TWO WT SRP TSH MEL NP FCW. Conceived and designed population-based study: SB HB DV IT/H/JEA KAW GB FFC JDF GF AG KK MP. Revision/final approval of the manuscript: KLH TWO WT SB HB DV IT/H/JEA KAW GB FFC JDF GF AG KK MP.

Table S1 Genetic association between MAP3K14 SNPs and BMD. (DOC)

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