RANK/RANKL/OPG Pathway: Genetic associations with Stress Fracture period prevalence in Elite Athletes

Ian Varley[a], David C. Hughes[a], Julie P. Greeves[b], Trent Stellingwerff[c], Craig Ranson[d], William D. Fraser[e] and Craig Sale[a].

[a]Biomedical Life and Health Sciences Research Centre, Nottingham Trent University, Clifton Lane, Nottingham, NG11 8NS, UK. ian.varley@ntu.ac.uk; david.hughes02@ntu.ac.uk; craig.sale@ntu.ac.uk

[b]Department of Occupational Medicine, Headquarters Army Recruiting and Training Division, UK. Julie.Greeves143@mod.uk

[c]Canadian Sport Institute - Pacific, Victoria, British Columbia, Canada. tstellingwerff@csipacific.ca

[d]Cardiff School of Sport, Cardiff Metropolitan University, Cardiff, Wales, UK. cranson@cardiffmet.ac.uk

[e]Norwich Medical School, University of East Anglia, UK. W.Fraser@uea.ac.uk

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Correspondence:

Dr Craig Sale,

Biomedical, Life and Health Sciences Research Centre,

School of Science and Technology,

Nottingham Trent University,

NG11 8NS,

UK.

E-mail: craig.sale@ntu.ac.uk

Telephone: +44 (0) 1158 483505

Abstract

Context: The *RANK/RANKL/OPG* signalling pathway is important in the regulation of bone turnover, with single nucleotide polymorphisms (SNPs) in genes within this pathway associated with bone phenotypic adaptations.

Objective: To determine whether four SNPs associated with genes in the *RANK/RANKL/OPG* signalling pathway were associated with stress fracture injury in elite athletes.

Design, Participants, and Methods: Radiologically confirmed stress fracture history was reported in 518 elite athletes, forming the Stress Fracture Elite Athlete (SFEA) cohort. Data were analysed for the whole group, and were sub-stratified into male and cases of multiple stress fracture group. Genotypes were determined using proprietary fluorescence-based competitive allele-specific PCR assays.

Results: SNPs rs3018362 (*RANK)* and rs1021188 (*RANKL*) were associated with stress fracture injury ($p<0.05$). 8.1% of stress fracture group and 2.8% of the non-stress fracture group were homozygote for the rare allele of rs1021188. Allele frequency, heterozygotes and homozygotes for the rare allele of rs3018362 were associated with stress fracture period prevalence ($p<0.05$). Analysis of the male only group showed 8.2% of rs1021188 rare allele homozygotes to have suffered a stress fracture while 2.5% of the non-stress fracture group were homozygous. In cases of multiple stress fractures, homozygotes for the rare allele of rs1021188, and individuals possessing at least one copy of the rare allele of rs4355801 (*OPG*) were shown to be associated with stress fracture injury ($p<0.05$).

Conclusions: The data support an association between SNPs in the *RANK/RANKL/OPG* signalling pathway and the development of stress fracture injury. The association of rs3018362 (*RANK)* and rs1021188 (*RANKL*) with stress fracture injury susceptibility supports their role in the maintenance of bone health, and offers potential targets for therapeutic interventions.

Keywords: stress fracture, bone, athletes, SNPs, genetics

1 Introduction

Stress fractures arise from the inability of bone to tolerate repeated mechanical loading, and are characterised by damage to the bone's micro-architecture (1). Repeated mechanical loading can cause an uncoupling of osteoblast bone formation and osteoclast bone resorption (1). This can lead to bone loss and subsequent micro-damage that can result in localised bone weakening, prompting stress fracture development (2). Stress fracture period prevalence in elite athletes and military recruits ranges from 14 to 21% (3,4), and most commonly manifests in the lower limbs (5).

In athletes, stress fracture injury is likely to have a complex aetiology involving numerous factors, with, for example, prior training (6) and biomechanical variables (*e.g.*, running kinematics) (7) being implicated in stress fracture risk (for a wider review of this issue please see Bennell et al., 1999). Susceptibility may also have genetic origins, supported by reports of monozygotic twins developing similar stress fracture injuries (8), multiple stress fractures occurring in the same individual (9), stress fractures occurring in some individuals but not in others undertaking identical training protocols (3,4) and a family history of stress fracture injury acting as a risk factor (10).

Genetic associations with stress fracture period prevalence in military personnel have been investigated using a variety of single nucleotide polymorphisms (SNPs) previously associated with receptors known to influence bone mineralisation, remodelling (11) and endocrine abnormalities (12). Associations were shown for SNPs and haplotype blocks within the vitamin D receptor (13) and an androgen receptor repeat sequence (14). However, other studies have shown no association for the same SNPs in similar military populations (15). The reason for the disparity may be the range of SNPs analysed and small numbers of stress fracture cases in some studies (e.g. n=64, 16). There is a

need to examine this in a large cohort of elite athletes given that the pathogenesis of stress fracture might be different due to the phenotypic differences, training variables and fitness.

Given that disturbances in bone remodelling and the inability of bone to withstand repeated bouts of mechanical loading are implicated in the development of stress fracture injury (1), SNPs repeatedly shown to be associated with these bone phenotypes in large-scale studies are worthy of focused study. As all previous studies have used military personnel, studies involving alternative groups, such as athletes with a similarly high period prevalence of stress fracture injury, are required to provide further insights into both the aetiology and genetic disposition of stress fracture injury. Furthermore, no published literature exists in relation to genetic associations with stress fracture injury risk in elite athletes.

The receptor activator of nuclear factor-$_{K}$B (*RANK*), and its ligand (*RANKL*), a member of the tumour necrosis factor (TNF) superfamily, are integral to osteoclastogenesis as they stimulate osteoclast activation, formation and differentiation (17). Osteoprotegerin (*OPG*) acts as a decoy receptor for *RANKL* leading to the prevention of osteoclast precursor development into mature osteoclasts, resulting in the subsequent attenuation of bone resorption (17). These factors in combination make up the *RANK/RANKL/OPG* signalling pathway, an important system in the regulation of bone turnover, and in the potential mediation of stress fracture injury development in individuals with a high frequency and/or amplitude of mechanical loading.

The specific mechanisms of how SNPs within the *RANK/RANKL/OPG* signalling pathway influence bone health remain unknown. Several of these SNPs have been associated with bone phenotypic alterations, including changes in bone mineral density (BMD) (18, 19, 20, 21), bone cross sectional area (22), osteoporotic fracture risk (20) and bone resorption and formation (23), although none of these have been established in stress fracture injury.

This study examined whether SNPs within the *RANK/RANKL/OPG* signalling pathway were associated with stress fracture injury in elite athletes.

2 Methods

2.1 Participants

In total a convenience sample of, 518 elite athletes, 449 male and 69 female, were recruited by email and word of mouth from professional sports clubs and elite sporting associations based in North America and the United Kingdom from 2010-2013 to form the Stress Fracture in Elite Athletes (SFEA) group (see Table 1 for participant characteristics). Participating elite athletes competed in various sports including, football (n = 218), cricket (n = 156), track and field (n = 67, running events n = 62), rowing (n = 13), boxing (n = 2), tennis (n = 12), hockey (n = 26) and gymnastics (n = 7), with each sport having both stress fracture Cases and non-stress fracture Control participants. Elite athletes were mainly white Caucasian (83.2% in the stress fracture Cases and 79.9% in the non-stress fracture Controls). Professional athletes were classified as elite due to their full time participation in sport; non-professional athletes were classified as elite if they regularly competed at international or national level. Each participant completed a statement of informed consent and a health status questionnaire, which was followed by an athletic status questionnaire detailing age, playing position if applicable, the average hours trained per week, number of appearances for their country, the first time competed at an elite level and for how many years. A fracture history questionnaire was also completed containing questions on both fracture and stress fracture history, method of stress fracture confirmation, time, date, location and treatment of stress fracture, training prior to stress fracture, recurrence details and family history. To be classified as a stress fracture case, radiological scan (*e.g.*, X-ray, MRI, CT) confirmation was a prerequisite. Participants self-reported radiologically confirmed stress fracture injury occurrence, although individual scans were not directly scrutinised by experimenters. In 17 individuals there was a lack of stress fracture history clarity (e.g. reports of stress reactions) and thus these participants were removed from the statistical analysis. The control group was made up of athletes who had never had a stress fracture injury and had no reported history

of stress fracture symptoms or radiological investigations suggestive of a stress fracture. Ethical approval was granted by the Nottingham Trent University Ethical Review committee.

Table 1. Characteristics of elite athletes with and without radiologically confirmed stress fracture injuries.

Characteristics	Stress fracture (n=125)	Non-stress fracture (n=376)	*P*-value
Age (years)	27.7±7.5	24.4±5.4	<0.00001*
Height (m)	1.82±10	1.81±8.3	0.45
Body Mass (kg)	77.3±14.5	77.8±10.5	0.72
BMI	23.2±2.7	23.7±2.2	0.07
Age at elite (years)	18.2+4.2	17±2.2	0.01*
Training (h/week)	20±11.3	18.2±10.1	0.12
Alcohol consumption (units/week)	5.2±6.9	4.1±6.1	0.15

Male only

	Stress fracture (n=98)	Non-stress fracture (n=335)	*P*-value
Age (years)	27.2±6.9	24.2±5.5	<0.000001*
Height (m)	1.85±7.2	1.82±7.1	0.0005*
Body Mass (kg)	82.9±10.6	79.6±9.4	0.0063*
BMI	24.1±2.1	23.9±2.1	0.46
Age at elite (years)	18.2+4.3	17±2.2	0.009*
Training (h/week)	21.6±11.9	18.2±10.5	0.01*
Alcohol consumption (units/week)	5.6±7.3	4.2±6.2	0.12

Participants' characteristics. Values are expressed as mean ±SD.

2.2 Procedures

Genomic deoxyribonucleic acid (DNA) was derived from saliva deposited into a 5mL collection tube and subsequently mixed with 2 mL of preservative in accordance with manufacturer guidelines (Norgen Biotek Corp, Saliva DNA Collection kit Thorold, Canada). The collection tube was incubated in a water bath for 1h at 55ºC followed by inversion and gentle shaking. An aliquot of 500µL was then added to 10µL of proteinase K and mixed by vortex in a 1.5mL tube before incubating for a further 30 minutes at 55ºC. An equal sample volume of isopropanol was added and

mixed by inversion followed by 5 minutes centrifugation at 13,000 x g. The supernatant was then removed and replaced by an equal sample volume of 70% ethanol followed by further centrifugation for 1 minute at 13,000 x g. The ethanol was then removed and 100µL of Tris(hydroxymethyl)aminomethane ethylenediaminetetraacetic acid (TE) buffer was added to rehydrate the DNA. The final sample was mixed by vortex for 10 seconds and left overnight at room temperature to ensure complete rehydration. Samples were stored at -20^{o}C until subsequent genotyping.

Specific genes and SNPs were analysed based on a purported mechanism that might explain an association; in the present manuscript we present the findings relating to genes and SNPs analysed in relation to the *RANK/RANKL/OPG* signalling pathway. Four SNPs from the *RANK/RANKL/OPG* signalling pathway (rs3018362 located 27 kb downstream of *RANK* on chromosome 18q21, rs4355801 located on chromosome 8q24 near *OPG*, rs1021188 located ~20Kb upstream of RANKL on chromosome 13q14, rs9594738 located on chromosome 13q14 190kb upstream of *RANKL*) were selected based on their prominence and reproducibility among different populations in Genome wide association studies (GWAS) and meta-analyses. Genotyping was conducted using a proprietary fluorescence-based competitive allele-specific polymerase chain reaction assay (Local Government Chemists (LGC) genomics Herts, UK). LGC genomics staff were blind to the clinical status (Case or Control) of the genotyped individuals.

2.3 Statistical Analysis

Student's *t*-test was used for the analysis of descriptive variables. Pearson Chi-square test ($\chi 2$) was used to assess the observed frequency of each genotype with what would be expected in accordance with Hardy-Weinberg equilibrium. Odds ratios and corresponding 95% confidence intervals were calculated for stress fracture injury risk. *Post hoc* logistic regression models were created for each individual SNP genotyped, with sex, age at sample collection, age at elite status acting as other

covariates. *P* <0.05 was considered statistically significant in the principal analysis. Multiple comparisons testing was not applied due to the conservative nature of the Bonferroni correction increasing the likelihood of a type I error and the absence of an appropriate statistical test to consider previous and future analysis. Due to the relatively low numbers of females in the study, it was decided that the entire group and a male only group would be analysed in order to allow for differences in stress fracture aetiology that may be gender specific. Data were also sub-stratified into cases of multiple stress fractures for the purposes of statistical analysis. All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 13.0 (SPSS, Inc., Chicago, IL, USA).

3 Results

Call rates for *RANKL* rs1021188, *RANKL* rs9594738, *RANK* rs3018362 and *OPG* rs4355801 were 96.2%, 96%, 95.8% and 95.6%. All SNPs were within Hardy-Weinberg equilibrium apart from *OPG* rs4355801 (*P*=0.04). The method of genotyping is robust and a high level of internal validation and reliability make errors in genotyping an unlikely reason for the deviance.

3.1 Participant characteristics

Analysis was carried out on 125 (98 men and 27 women) athletes with a radiologically confirmed stress fracture injury and 376 (335 men and 41 women) athletes who reported no stress related bone injury. The stress fracture group were significantly older than the non-stress fracture group at the time of collecting the saliva sample and at the age at which the elite level was reached across the whole group (p<0.01) (Table 3). These differences remained significant along with height, body mass and hours training when only males were analysed (*P* <0.01).

The average age at stress fracture was 19.9±3.9y and occurrence was recorded at various anatomical sites; lower limb (56.8%), lumbar spine (33.5%), rib (6.5%) pelvic area (1.6%) and upper limb (1.6%).

3.2 Genetic analysis

3.2.1 *RANKL*

A significant association between SNP rs1021188 and stress fracture period prevalence was shown in the whole, male only and multiple stress fracture groups (P <0.05). 8.1% (whole group), 8.2% (male only) and 10% (multiple stress fracture) of athletes were homozygote for the rare allele in the stress fracture injury groups, and in only 2.5% (whole group) and 2.8% (male only) of the non-stress fracture groups. No significant associations were shown between *RANKL* SNP rs9594738 and stress fracture injury in the whole, male only or multiple stress fracture groups (P >0.05) (Table 2, 3, 4).

3.2.2 *RANK*

A significant association between stress fracture period prevalence and SNP rs3018362 was shown in the whole group, and significant associations persisted in the male only group (P <0.05). Heterozygotes combined with those homozygous for the rare allele were positively associated with stress fracture period prevalence in the whole group (65.2%) and men only sub-stratification (66.0%) in comparison to the Control group (55.0% whole group; 54.3% men only) (P <0.05). A copy of the variant *A* allele was also associated with stress fracture period prevalence in the whole group (41.5%) and men only sub-stratification (42.5%) in comparison to the Control group (33.4% and 33.2%) (P <0.05) (Table 2 and 3). No significant associations were shown when comparing the multiple stress fracture group to non-stress fractures (Table 4).

3.2.3 *OPG*

No association was shown between SNP rs4355801 and stress fracture period prevalence in both the whole group, and the male only group (P >0.05) (Table 2 and 3). However, individuals possessing at least one copy of the rare allele of rs4355801 (*OPG*) had a greater risk of multiple stress fracture injury (76.6%) compared to the non-stress fracture Control group (63.5%) (P <0.05) (Table 4).

3.2.4 Sport sub-groups

No association was shown when cricketers were analysed independently, for any of the SNPs. When footballers were combined with hockey players (invasion sports), allele frequency of *RANKL* SNPs rs1021188 and rs9594738 were associated with stress fracture injury (P <0.05). *RANKL* SNP rs9594738 was also associated with stress fracture injury when heterozygotes were combined with those homozygous for the rare allele (P <0.05). When only runners were analysed individuals possessing at least one copy of the rare allele of rs4355801 (*OPG*) had a greater risk of stress fracture (P <0.05). In the same population, heterozygotes combined with those homozygous for the rare allele of *RANK* SNP rs3018362 were positively associated with stress fracture injury (P <0.05). No significant associations were shown for any of the other sports analysed.

3.2.5 Logistic regression

After adjusting for sex, age at elite status and age at sample collection only *RANK* rs3018362 remained significantly associated with stress fracture injury (P <0.05; OR 1.42; 95% CI 1.04-1.95).

Table 2. Distribution and percentage of stress fracture Cases and Controls in the whole group. Odds ratio (OR) and 95% confidence intervals comparing the most frequent genotype to heterozygotes and homozygotes for the variant allele. *indicates significance P<0.05.

SNP	Genotype	Stress fracture N (%)	Non-stress fracture N (%)	OR	(95% CI)	$\chi 2$ p- value
RANKL rs9594738	CC	45(37.2)	114(31.6)			
	TC	54(44.6)	192(53.2)	0.71	(0.45-1.13)	

	TT	22(18.2)	55(15.2)	1.01	(0.55-1.85)	0.265
	TC or TT	76(62.8)	247(68.4)	0.78	(0.51-1.20)	0.256
	Allele					
	C	144(59.5)	420(58.2)			
	T	98(40.5)	302(41.8)	0.95	(0.70-1.27)	0.674
rs1021188	GG	85(69.1)	249(69)			
	GA	28(22.8)	102(28.3)	0.80	(0.49-1.31)	
	AA	10 (8.1)	10(2.8)	2.93	(1.18-7.28)	0.024*
	GA or AA	38 (30.9)	112(31)	0.99	(0.64-1.55)	0.978
	Allele					
	A	48(19.5)	122(16.9)			
	G	198(80.5)	600(83.1)	1.19	(0.82-1.73)	0.273
RANK						
rs3018362	GG	41(34.7)	163(45.0)			
	GA	56 (47.5)	156(43.1)	1.43	(0.90-2.26)	
	AA	21 (17.8)	43(11.9)	1.9	(1.04-3.62)	0.084
	GA or AA	77 (65.2)	199(55)	1.54	(0.99-2.37)	0.049*
	Allele					
	A	98(41.5)	242(33.4)			
	G	138(58.5)	482(66.6)	1.41	(1.05-1.91)	0.008*
OPG						
rs4355801	AA	43 (35.2)	138(38.5)			
	GA	53 (44.4)	156(43.6)	1.09	(0.69-1.73)	
	GG	26(21.3)	64(17.9)	1.30	(0.74-2.31)	0.658
	GA or GG	79(64.8)	220(61.5)	1.15	(0.75-1.77)	0.518
	Allele					
	A	139 (57)	432 (60.3)			
	G	105 (43)	284(39.7)	1.15	(0.86-1.54)	0.282

Table 3. Distribution and percentage of male stress fracture Cases and Controls. Odds ratio (OR) and 95% confidence intervals comparing the most frequent genotype to heterozygotes and homozygotes for the variant allele. *indicates significance $P<0.05$.

SNP	Genotype	Stress fracture N (%)	Non-stress fracture N (%)	OR	(95% CI)	$\chi 2$ p- value
RANKL						
rs9594738	CC	37(38.9)	101(31.7)			
	TC	42(44.2)	168(52.7)	0.68	(0.41-1.13)	
	TT	16(16.8)	50(15.7)	0.87	(0.44-1.72)	0.265
	TC or TT	58(61.1)	218(68.3)	0.73	(0.45-1.17)	0.256
	Allele					
	C	116(61.1)	370(58)			

	T	74(38.9)	268(42)	0.75	(0.47-1.19)	0.674
rs1021188	AA	66(68.0)	223(69.0)			
	GA	23(23.7)	92(28.5)	0.84	(0.50-1.44)	
	GG	8(8.2)	8(2.5)	3.38	(1.22-9.35)	0.028*
	GA or GG	31(32.0)	100(31.0)	1.05	(0.64-1.71)	0.852
	Allele					
	A	155(41.7)	538(39.0)			
	G	39(58.3)	108(61.0)	1.25	(0.83-1.88)	0.206
RANK						
rs3018362	GG	32(34.0)	147(45.7)			
	GA	44(46.8)	136(42.2)	1.49	(0.89-2.48)	
	AA	18(19.1)	39(12.1)	2.12	(1.08-4.17)	0.072
	GA or AA	62(66.0)	175(54.3)	1.63	(1.01-2.63)	0.045*
	Allele					
	A	80(42.5)	214(33.2)			
	G	108(57.4)	430(66.8)	1.49	(1.07-2.08)	0.006*
OPG						
rs4355801	AA	35(36.5)	125(39.7)			
	GA	42(43.8)	134(42.5)	1.12	(0.67-1.87)	
	GG	19(19.8)	56(17.8)	1.21	(0.64-2.30)	0.825
	GA or GG	61(63.5)	190(60.3)	1.15	(0.71-1.84)	0.518
	Allele					
	A	112(58.3)	384(61.0)			
	G	80(41.1)	246(39.0)	1.11	(0.80-1.55)	0.457

Table 4. Distribution and percentage of multiple stress fracture cases. Odds ratio (OR) and 95% confidence intervals comparing the most frequent genotype to heterozygotes and homozygotes for the variant allele.*indicates significance $P<0.05$. n/a indicates insufficient sample size to carry out odds ratio analysis.

SNP	Genotype	Stress fracture N (%)	OR	(95% CI)	χ^2 p- value
RANKL					
rs9594738	CC	16(33.3)			
	TC	23(47.9)	0.85	(0.43-1.68)	
	TT	9(18.7)	1.17	(0.48-2.80)	0.741
	TC or TT	32(66.7)	0.92	(0.49-1.75)	0.806
	Allele				
	C	55(57.3)			
	T	41(42.7)	1.04	(0.67-1.59)	0.861
rs1021188	GG	38(76.0)			
	GA	7(14.0)	0.45	(0.19-1.04)	
	AA	5(10.0)	3.28	(1.06-10.11)	0.006*
	GA or	12(24.0)	0.7	(0.35-1.39)	0.310

	AA				
	Allele				
	A	12(24.0)			
	G	38(76.0)	1.55	(0.79-3.06)	0.978
RANK					
rs3018362	GG	15(33.3)			
	GA	26(57.8)	1.81	(0.92-3.55)	
	AA	4(8.9)	n/a	n/a	0.175
	GA or AA	30(66.7)	1.64	(0.85-3.15)	0.136
	Allele				
	A	34(37.8)			
	G	56(62.2)	1.21	(0.77-1.9)	0.381
OPG					
rs4355801	AA	11(23.4)			
	GA	26(55.3)	2.09	(0.99-4.39)	
	GG	10(21.3)	1.96	(0.79-4.85)	0.127
	GA or GG	36(76.6)	2.05	(1.01-4.17)	0.042*
	Allele				
	A	48(51.1)			
	G	46(48.9)	1.46	(0.95-2.24)	0.076

Exploratory analysis of allele combinations was conducted to examine how potential gene-gene interactions may affect stress fracture injury risk. However, the combining of alleles reduced the number of participants in each group and meaningful data from this sub-analysis could not be derived.

4 Discussion

To our knowledge, this is the first study to examine the genetic associations with stress fracture injury in elite athletes, with all other studies to date being from cohorts of military personnel. This study is the first, from any population, to show that SNPs within the *RANK/RANKL/OPG* signalling pathway are associated with stress fracture injuries; SNPs rs3018362 and rs1021188, which are not in linkage disequilibrium (19), located near *RANK* and *RANKL* were associated with stress fracture injury in our whole cohort, as well as in the men only group. A copy of the minor allele of SNP rs4355801 was also associated with increased risk of multiple stress fractures.

Although, the specific function of the SNPs genotyped is not known, variant alleles of SNPs within the *RANK/RANKL/OPG* signalling pathway are known to be associated with osteoclast differentiation and activation (17) subsequently causing a decrease in bone resorption (23). It has also been suggested that allelic differences in the *RANK/RANKL/OPG* SNP may have a mediatory role in the process by which vitamin D up regulates *RANKL* expression in osteoblast and osteoblast precursor cells (24). *RANKL* is vital for the differentiation of osteoclasts into mature multi-nucleated cells, as well as their activation and longevity (17). The importance of *RANKL* is highlighted by Denosumab, a monoclonal antibody to *RANKL*, which has been shown to prevent bone loss in osteoporotic patients (25). The association of rs1021188 with stress fracture injury may be explained by the minor allele of rs1021188 being associated with increased concentrations of circulating *RANKL* (19), possibly increasing osteoclastogenesis in carriers of the minor allele and, consequently, increasing bone resorption. Although speculative, this could suggest an uncoupling of bone turnover resulting from increased bone resorption, providing a mechanistic explanation for our findings (26).

As well as being associated with stress fracture injury in the present study, the SNPs analysed have previously been associated with other bone phenotypes. The minor allele of SNP rs3018362 has been associated with Paget's disease (27) and lower BMD at the tibia measured by peripheral quantitative computed tomography (pQCT) (18), although other studies have reported no associations with rs3018362 (20,28). The rare allele of SNP rs1021188 has been shown to be associated with increased circulating free *RANKL* (18), decreased cortical porosity (28) and BMD (18, 28) at the tibia, (all measured by pQCT) in meta-analyses of GWAS. Homozygotes for the rare allele of SNP rs4355801 have also previously been shown to be associated with low BMD and osteoporotic fracture period prevalence (21). High recombination rates have been shown between rs9594738 and rs1021188 (18). GWAS have previously shown associations between the variant allele of rs9594738 and bone

phenotypes such as low BMD (20) and osteoporotic fracture (29), although no significant association with rs9594738 and stress fracture injury period prevalence was observed in the present study.

Pathophysiological differences between osteoporotic fracture and stress fracture injury may explain why the findings of these studies are not in agreement. Although not associated with stress fracture injury in the present study, bone phenotypic associations with rs9594738 have been shown. However, these have been in areas mainly consisting of trabecular bone, whereas stress fractures in athletes occur predominantly in the tibial diaphysis and metatarsals (30), sites mainly comprised of cortical bone. The influence of exercise on the *RANK/RANKL/OPG* signalling pathway, circulating *RANKL* and *OPG* concentrations and *RANK* density may also introduce confounding effects. Circulating free *RANKL* is notoriously difficult to measure (31), whereas the influence of exercise on *OPG* is more widely reported (32, 33). The outcome of exercise on *OPG* concentrations is variable; concentrations of serum *OPG* have been shown to increase following running of long and short distances in recreationally active males (32,33) and elevated in habitually active females (34). Conversely, *OPG* concentrations were unaltered in obese males following a 6-month training programme (35). As the SFEA group was comprised of elite athletes from different sports, the different training regimes undertaken may have influenced *OPG* concentrations and therefore confounded any genetic associations. There is always the possibility that *OPG* fluctuations do not solely relate to effects in bone, since *OPG* is not bone specific and can be produced by muscle and endothelial cells (36), as well as osteoblasts, which secrete *OPG* in response to exercise through inflammation or muscle damage (37).

There are a lack of studies investigating the association of genotype with stress fracture injury in elite athletes, which may relate to the low period prevalence of stress fracture injury in some sports (*e.g.*, 0.5; 38) and the difficulties associated with obtaining samples from elite athletes due to them comprising only a minute proportion of the global population. Stress fracture period prevalence in the present study (24.9% in the entire group) was higher than previously reported (0.5% in elite football players; 38), although this might be due to the fact that participation may have been more likely if

there was a history of stress fracture injury within their own organisation/club. The predominant site of stress fracture was in the lower limb (56.8%), which is consistent with previously published data concerning athletes (30).

The stress fracture group were on average 3.3 years older at data collection (Table 1), although we suggest that the additional time this allowed to have suffered a stress fracture injury is unlikely to have affect the findings of the study given that the average age at stress fracture injury was 20±4 years. The stress fracture group were also significantly older at the age of achieving elite status. Potential explanations for this could be that absence from training might have delayed athletic development, given that stress fracture injuries have been shown result in an average of 169 days of lost training time (39) a finding supported by the current study, with an average of 110 days of training absence reported by participants.

This study is not without its limitations, whilst heterogeneity in sport type and training load are acknowledged as variable factors in the present study, it is currently unavoidable given to low number of elite athletes available to participate in such studies and the difficulty in recruiting participants due to perceived disruption of training schedules. Investigation of single sport groups in future will be required to confirm or refute our findings. As with all retrospective studies there is a possibility that recall bias may have occurred, although in the present study we believe this unlikely since all stress fractures were confirmed radiologically and caused a prolonged absence from training and competition, meaning that such events were likely to be well recalled by the athlete. As part of the athletic status questionnaire given to participants to provide information on the responder population, we recorded sex, age at elite status and age at sample collection. Given the potential for these factors to acts as covariates, they were included in a *post hoc* logistical regression model. After adjusting for these covariates, significant associations between stress fracture injury and genotype persisted in rs3018362 only. Other factors recorded (e.g., height, weight and hours training) in the questionnaire

were not included in the model, as these were not recorded at the time of stress fracture injury. Although challenging to undertake, future prospective studies investigating environmental and genetic factors associated with stress fracture injury would be useful to further elucidate to factors that might mediate genetic associations with stress fracture injury.

After correcting for multiple comparisons none of the data remained significant. However, the Bonferroni correction is known to be very conservative and is recognised to be more likely to lead to dismissal of a true positive rather than a false one. Given the number of comparisons made it is possible that a type II error might have occurred, although we suggest that this is unlikely given that the direction of the effects shown are consistent with previously published data related to bone phenotypes. If the findings were to have occurred by chance, effects in different directions would be expected.

In conclusion, SNPs located near genes in the *RANK/RANKL/OPG* signalling pathway are significantly associated with stress fracture injury. These data together with previously reported associations with other bone phenotypes suggest an important role for SNPs within the *RANK/RANKL/OPG* signalling pathway in the regulation of bone strength and the adaptation to mechanical loading. Further studies are needed to establish the specific mechanisms of how these SNPs are associated with stress fracture injury and how these allelic mutations influence bone adaptations and subsequently escalate stress fracture risk.

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