# Association of Genotype with Bone Metabolism, Skeletal Adaptation and Stress Fracture Injury Occurrence

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Abstract

Positive changes in bone metabolism, structural characteristics, size and mass are commonly associated with weight-bearing exercise. Despite this, negative effects of exercise on bone phenotypes, such as stress fracture injuries have been reported. Little is known about the extent of the genetic mediation of changes in bone characteristics, stress fracture injury and bone resorption in response to exercise. Accordingly, this thesis investigated: the genotype dependent changes in bone phenotypes in academy footballers before and after an increase in training volume; genetic associations with stress fracture injury in elite athletes and a preliminary investigation into genetic associations with bone resorption following 120 min of treadmill running.

The tibial bone characteristics of 80, full-time academy footballers was determined using pQCT before and after 12 weeks of increased volume football training. Genetic associations with baseline, post increased training and change in bone characteristics were then determined. Secondly, radiologically confirmed stress fracture history was reported in 518 elite athletes, forming the Stress Fracture Elite Athlete (SFEA) cohort. Genetic associations were analysed for the whole group, and were also sub-stratified. Finally, recreationally active healthy male participants (n=42) performed a 120 min run at 70% $\dot{V}O_{2max}$. Genetic associations with bone resorption at baseline, immediately, 24, 48 and 72 hours post run were investigated.

SNPs in the proximity of genes in *P2X7R* and the *RANK/RANKL/OPG* signalling and *Wnt* signalling pathways were associated with bone phenotypes before and following 12 weeks of increased volume football training ($P<0.05$). SNPs in close proximity to *SOST*, *P2X7R*, *RANK*, *RANKL*, *OPG*, Bradykinin and *VDR* genes were associated with stress fracture injury in the whole cohort and in various sub-classifications of elite athletes ($P<0.05$). No

associations were shown in bone resorption prior to, immediately following or in the 3 days following 120 min of treadmill running.

The data suggest a role for specific genes and SNPs in bone phenotypic changes as a result of exercise training and in the susceptibility to stress fracture injury. The association of SNPs in *P2X7R* and the *RANK/RANKL/OPG* signalling and *Wnt* signalling pathways with bone phenotypes and stress fracture injury susceptibility highlights their role in the maintenance of bone health, and offers potential targets for therapeutic interventions.

List of Abbreviations

| | |
|---|---|
| 1,25-$(OH)_2$D | 1,25-dihydroxyvitamin D |
| 25(OH)D | 25-hydroxyvitamin D |
| 3D | Three-dimensional |
| µCT | Micro-Computed Tomography |
| A | Athletes |
| ACE | Angiotensin I-Converting Enzyme |
| AF | Allele Frequency |
| ALSPAC | Avon Longitudinal Study of Parents and Children |
| ANOVA | Analysis of Variance |
| APC | Adenomatous polyposis coli |
| AT | Anaerobic Threshold |
| ATP | Adenosine Triphosphate |
| AUC | Area Under the Curve |
| BASE | Pre exercise |
| BK2R | Bradykinin type 2 receptor |
| BMC | Bone Mineral Content |
| BMI | Body Mass Index |
| BMU | Basic Multicellular Units |
| bone-ALP | Bone Alkaline Phosphatase |
| BMD | Bone Mineral Density |
| BMP | Bone Morphogenetic Protein |
| BSA | Bovine Serum Albumin |
| C | Cricketers |
| Ca+ | Calcium |
| CaMKII | Calmodulin-dependent protein kinase II |
| CHO | Carbohydrate |
| CI | Confidence Intervals |
| COL1A1 | Collagen type I Alpha 1 |

| | |
|---|---|
| COL5A1 | Collagen type V alpha 1 |
| CON | Control group |
| CSA | Cross Sectional Area |
| CT | Computerised Tomography |
| CTR | Calcitonin Receptor |
| β-CTX | Collagen Type 1 Cross-linked C-telopeptide |
| cyclic AMP | Cyclic Adenosine Monophosphate |
| DKK | Dickkopf |
| dNTPs | Deoxynucleotide Triphosphates |
| DPD | urinary free Deoxypyridinoline |
| DNA | Deoxyribonucleic Acid |
| Dsh | Dishevelled |
| DXA | Dual-Energy X-ray Absorptiometry |
| EDTA | Ethylenediaminetetraacetic Acid |
| EE | End of Exercise |
| ELISA | Enzyme Linked Immunosorbent Assay |
| ET | Endurance Trained |
| FH | Football and Hockey Players |
| FRET | Fluorescence Resonance Energy Transfer |
| FTA | Fast Technology for Analysis |
| FU1 | Follow up 24h |
| FU2 | Follow up 48h |
| FU3 | Follow up 72h |
| Fzd | Frizzled |
| EM | Leg Stress Fracture Excluding Metatarsals |
| GC | Vitamin D-binding protein gene |
| GOOD | Gothenburg Osteoporosis and Obesity Determinants |
| GSK-3β | Glycogen Synthase Kinase-3β |
| GWAS | Genome Wide Association Studies |

| | |
|---|---|
| HRpQCT | High Resolution Computed Tomography |
| HWE | Hardy-Weinberg Equilibrium |
| IGF-1 | Insulin-like Growth Factor 1 |
| IAT | Individual Anaerobic Threshold |
| ICTP | C-Terminal Telopeptide Type I Collagen |
| IFCC | International Federation of Clinical Chemistry |
| IL1 | Interleukin 1 |
| IL6 | Interleukin 6 |
| IL18 | Interleukin 18 |
| IOF | International Osteoporosis Foundation |
| IU | International Units |
| KASP | Kompetitive Allele Specific PCR |
| Lef | Lymphoid enhancing factor |
| LGC | Local Government Chemists |
| LRP5 | Low density Lipoprotein Receptor-related Protein 5 |
| LRP6 | Low density Lipoprotein Receptor-related Protein 6 |
| MAF | Minor Allele Frequency |
| MALDI-TOF MS | Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry |
| MAPK | Mitogen-activated protein kinases |
| MC | Multiple Stress Fracture Cases |
| M-CSF | Macrophage Colony-Stimulating Factor |
| MRI | Magnetic resonance imaging |
| MrOS | Osteoporotic Fractures in Men Study |
| $MgCl_2$ | Magnesium Chloride |
| mRNA | messenger Ribonucleic Acid |
| N/A | Not Applicable |
| NA | Non-Athletes |
| NF-kβ | Nuclear Factor Kappa β |
| NO | Nitric Oxide |

| | |
|---|---|
| NTX | Collagen type 1 cross-linked N-telopeptide |
| OC | Osteocalcin |
| OD | Optical Density |
| OPG | Osteoprotegerin |
| OR | Odds Ratio |
| Osx | Osterix |
| P | Post exercise |
| P1CP | Carboxy-terminal Propeptide of type 1 Procollagen |
| P1NP | Type 1 Procollagen N-terminal |
| P21 | Stress Fracture Pre 21 |
| P2X7R | Gene encoding P2X Purinoceptor 7 |
| PCR | Polymerase Chain Reaction |
| PGE2 | Prostaglandin E2 |
| PL | Placebo group |
| pQCT | peripheral Quantitative Computed Tomography |
| PTH | Parathyroid Hormone |
| PTHrP | Parathyroid hormone-related protein |
| PYD | Urinary total Pyridinoline |
| R | Runners |
| RA | Recreational Active |
| RANK | Receptor Activator of Nuclear Factor-$_K$B |
| RANKL | Receptor activator of nuclear factor-$_K$B ligand |
| RER | Respiratory Exchange Ratio |
| RFLP | Restriction Fragment Length Polymorphism |
| RPE | Rating of Perceived Exertion |
| rpm | Revolutions per minute |
| RS | Reference SNP ID |
| Runx2 | Runt-related transcription factor 2 |
| SFEA | Stress Fracture Elite Athlete |

| | |
|---|---|
| Sfrps | Secreted frizzled-related proteins |
| SPSS | Statistics Package for Social Science |
| SOST | Sclerostin encoding gene |
| SNP | Single nucleotide polymorphisms |
| SSI | Stress Strain Index |
| Tcf | T cell factor |
| TE | Tris-EDTA buffer |
| TGF-β | Transforming Growth Factor β |
| TNF | Tumor Necrosis Factors |
| TRACP5b | Tartrate-Resistant Acid Phosphatase 5b |
| vBMD | volumetric Bone Mineral Density |
| VDR | Vitamin D Receptor |
| $VCO_2$ | Carbon Dioxide Output |
| $VO_2$ | Oxygen Uptake |
| $\dot{V}O_{2max}$ | Maximal Oxygen Uptake |
| Vox | Voxel |
| VT | Ventilatory Threshold |
| WC | Whole Cohort |
| Wnt | Drosophila melanogaster wingless gene |

Manuscripts

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# Chapter 1.0. General Introduction

Bone is a dynamic tissue in a constant state of change as a result of several factors including, chemical triggers, growth factors and the stresses placed upon it as a result of muscular and mechanical loading. Bone structure and composition are primarily determined by basic multicellular units (BMU) comprised of osteoclasts, osteoblasts and osteocytes. In healthy bone, homeostasis and structural integrity are maintained through the balance of continuous bone resorption and bone formation by osteoclasts and osteoblasts (Zaidi, 2007). Disruptions to the balance of formation and resorption can lead to bone deviating from homeostasis and the generation of micro-damage forming on the bone surface (Chapurlat and Delmas 2009), which can ultimately lead to bone injury and disease, ranging from high bone mass disorders to osteoporosis (O'Brien *et al.,* 2005; Boyle *et al.,* 2003). Weight-bearing mechanical loading has been shown to cause an increase in bone remodelling above basal levels, in which both bone formation and resorption are altered (Scott *et al.*, 2010; 2011a; 2012a). These dynamic processes lead to loading specific changes in bone structure and integrity (Wolff's Law, Wolff 1892) allowing bone to adapt in accordance to the load that is applied to it.

The measurement of bone turnover by biochemical markers such as type 1 procollagen N-terminal (P1NP) and collagen type 1 cross-linked C-telopeptide (β-CTX), and of bone structure by methods such as Peripheral Quantitative Computed Tomography (pQCT) and High Resolution Computed Tomography (HRpQCT), are commonly used to assess the effectiveness of pharmaceuticals and exercise or dietary interventions on bone characteristics. Data from exercise intervention studies indicate that weight-bearing mechanical loading has a mainly anabolic effect on bone (Maimoun and Sultan, 2009). However, the magnitude of the adaptations vary depending on the exercise conducted (Greene *et al.,* 2012, Evans *et al.,* 2012) and the anatomical site measured (Evans *et al.,* 2012). Exercise resulting in a high magnitude of loading and irregular movement patterns is predominately shown to produce

greater osteogenic effects, in comparison to exercise eliciting low loading impacts and necessitating regular movement patterns (Greene *et al.,* 2012). This is exemplified by studies involving footballers and gymnasts demonstrating these athletes to have increases in a variety of bone characteristics including, size, density and thickness compared to swimmers and cyclists (Morgan *et al.,* 2011; Ferry *et al.,* 2012; Greene *et al.,* 2012; Heinonen *et al.,* 1993). It is still unclear whether there is a specific magnitude of loading that is required to elicit osteogenic effects. The bone response to a single bout of exercise shows a large degree of individual variability (Ratntalainen *et al.,* 2009; Kerschan-Schindl *et al.,* 2009), which might be due to a number of factors including: lack of experimental controls, the high degree of biological variability that can exist in some biochemical markers of bone turnover (Clowes *et al.,* 2002), feeding (Guillemant *et al.,* 2004), circadian rhythm (Fraser *et al.,* 2010) and sex hormones (Camacho and Kleerekoper, 2006). All of these factors have been shown to influence the concentration of biochemical markers of bone turnover at rest, and in response to exercise. Even after controlling for confounding variables (Scott *et al.,* 2010; 2011a; 2012a) large individual variability remains, suggesting intrinsic factors may be involved in the mediation of the response.

Despite the mainly anaerobic effects of exercise on bone, there is the possibility that exercise can contribute to short-term local bone loss and lead to bone injuries, such as stress fracture. Stress fracture injuries are overuse bone injuries that commonly occur in athletes and military personal, due to the cyclic, repetitive nature of their training (Warden *et al.,* 2006). The prevalence and frequency of injury depends on the sport played and the military population observed. Stress fractures account for 0.7%-20% of all athletic sports injuries (Bennell *et al.,* 1997; Fredericson *et al.,* 2006), while the percentage of military recruits suffering from stress fracture injury ranges from 5%-31% (Milgrom *et al.,* 1985; Armstrong *et al.,* 2004). Injury

can result in major consequences for athletes and military personnel including: significant discomfort, reduced performance, loss of valuable training time and loss of earnings. Stress fracture injuries are caused by mechanical loading that is applied in a rhythmic, repeated, sub-threshold manner (McBryde, 1985), although the exact pathophysiology is not fully understood (Warden *et al*., 2007). That said, inadequate bone remodelling (Schaffler *et al.,* 1990) and bone characteristics, such as low bone mineral density (BMD) (Wentz *et al.,* 2012) and lower cortical area (Popp *et al.,* 2009) have been associated with the development of stress fracture injury. Environmental factors including diet, training status, training environment, individual biomechanics and psychological factors have also been associated with stress fracture injury (Bennell *et al.,* 1999). In particular, it would seem that a rapid increase in training volume or the engagement in unaccustomed exercise is associated with stress fracture injury (Bennell *et al.,* 1999). This is supported by the observation of a relatively high proportion of stress fracture injuries in newly recruited military personal (Stroh Bach *et al.,* 2012)

Although the relative risk is unknown, a genetic contribution to stress fracture risk is likely, given the development of multiple stress fractures in the same individuals at various skeletal sites (Lambros and Alder, 1997), stress fracture injuries occurring in monozygotic twins (Singer *et al.,* 1990; Van Meensal and Peers 2010), high stress fracture recurrence rates (Gehrmann and Renard, 2006) and variability in stress fracture incidence in individuals who are exposed to comparable training loads (Giladi *et al.,* 1986). Despite this, there are very few published studies investigating genetic associations with stress fracture injury. Stress fracture candidate gene studies conducted hitherto have exclusively used military personnel (Korvala *et al.,* 2010; Yanovich *et al.,* 2011; Cosman *et al.,* 2013; Valimaki *et al.,* 2005; Chatzipapas *et al.,* 2009). While some genetic associations in genes such as, low density lipoprotein

receptor-related protein 5 (*LRP5*) and the vitamin D receptor (*VDR*) have been shown (Korvala *et al.,* 2010; Yanovich *et al.,* 2011; Chatzipapas *et al.,* 2009), others have failed to confirm these findings (Cosman *et al.,* 2013; Valimaki *et al.,* 2005). The small range of SNPs investigated, small cohorts used, and the lack of homogeneity in prior training history may be the reason for the disparities. The aetiology and genotypic determinants of stress fracture injury might be different between military personnel and elite athletes due to the different types of training that they perform.

Numerous large scale genome wide association studies (GWAS), investigating multiple bone phenotypes including, BMD, markers of bone turnover and osteoporotic fracture have been conducted (Paternoster *et al.,* 2010; Roshandel *et al.,* 2011; Medina-Gomez *et al.,* 2012; Zheng *et al.,* 2012). These studies have identified hundreds of specific genes and SNPs significantly associated with bone phenotypes. The genes and SNPs of significance vary depending on the population and anatomical site of the bone phenotype investigated. The genes and SNPs of greatest importance in relation to bone phenotypes have not been analysed for associations with stress fracture injury and, as such, the genes and SNPs that may increase susceptibility to stress fracture injury remain unclear. A direct or indirect association due to genes acting on bone phenotypic variants such as remodelling characteristics and structural adaptation may be evident given the previous genetic associations with these phenotypes (Kemp *et al.,* 2013; Dhamrait *et al.,* 2003; Roshandel *et al.,* 2010). There is an absence of any data investigating the association of genotype with stress fracture injury in elite athletes, prompting questions about how genotype may influence the pathophysiology of injury in this population.

In summary, stress fractures are a commonly suffered injury by otherwise healthy individuals that have potentially serious consequences. Since the knowledge base regarding the pathogenesis of stress fracture injury is currently limited, so too is our ability to prevent, manage and treat it. Knowledge of how genotype may increase susceptibility to stress fracture injury through alterations in bone metabolism and structure will have wide ranging implications for sports medicine and bone health. The athlete model may also inform knowledge in relation to the management of clinical bone disorders, dental implants and orthopaedic surgical procedures. The study of skeletal responses to exercise also have the benefit of pace, given that large changes in bone phenotypes can be shown following a short period of time (10-12 weeks). This would help in the examination of factors relating to bone diseases, such as osteoporosis. Thus, it would be useful to examine whether the specific SNPs associated with changes in bone phenotypes, such as BMD, cortical thickness and fracture risk are also associated with bone metabolism, structural changes in size, density and thickness and stress fracture risk in response to exercise.

Therefore, the aim of this thesis is to determine whether selected SNPs are associated with; 1) adaptations in bone structure as a result of increased training load, 2) stress fracture injury susceptibility in elite athletes and 3) bone metabolism following prolonged treadmill running.

These aims will be examined in 4 studies reported in Chapters 4,5, 6 and 7:

- Initially a methodological study was conducted in order to determine the most suitable method of sample collection for the subsequent experimental studies. The findings from this study are reported in Chapter 4.
- Study 2, reported in Chapter 5 investigated whether genotype is associated with bone structural alterations in full-time academy footballers following 12 weeks of increased training volume.

- Study 3, reported in Chapter 6 investigated if variations in genotype, previously associated with other bone phenotypes, were associated with stress fracture injury in elite athletes.
- Study 4, reported in Chapter 7 was an initial investigation in whether genetic factors were associated with the biochemical markers of bone resorption by measuring bone resorption before, immediately after (0h) and 24h,48h and 72h following 120 min of treadmill running.

# Chapter 2.0. Literature Review

## 2.1. Overview

Current literature has been reviewed relating to bone and the different phases that combine to make up the bone remodelling cycle. The positive and negative impact of mechanical loading on bone has been reviewed. The negative effects of mechanical loading on bone is discussed, including the suggested mechanisms and aetiology of stress fracture injury. This Chapter concludes by reviewing the literature relating to genetic susceptibility to bone phenotypic alterations.

## 2.2. Bone

Bone is a connective tissue, that has metabolic and structural functions. During bone developmental stages, such as bone growth, bone modelling occurs. In response to prolonged exercise or post-fracture, extensive bone remodelling takes place. During bone remodelling, woven bone contributes to the majority of the structural component of bone, which is replaced by stronger and more resilient lamellar bone as the bone develops. The axial and appendicular skeletal system provides structural support to the muscles, facilitates movement, and protects the vital organs.

Cortical and trabecular bone make-up the anatomical aspect of bone; cortical bone contributing up to 80% of the total skeleton (Arikoski *et al.*, 2002). Cortical bone is found on the bone surface and at the shaft of long bones (diaphysis), where it is strong and rigid allowing for mechanical loading and repeated strain. Trabecular bone is located primarily in the axial skeleton, at the ends of long bones and in the vertebrae; it is firm but appears to have a ‘spongy’ appearance. The external surface of the bone is covered by the periosteum, which contains osteoblasts and osteoclasts on its surface and is inter-connected with nerves,

lymphatic and blood vessels. The internal surface is covered by the endosteum containing osteoblasts and osteoclasts, which line the canals that navigate cortical bone. An overview of bone structure is shown in Figure 2.1. Bone also has metabolic and endocrine functions, including the facilitation of calcium and phosphate secretion that is regulated by calciotropic hormones (Guntur and Rosen, 2012). Protein and calcium are key components of bone homeostasis, playing important roles in bone metabolism. Decreased calcium circulating in blood lead to a sustained increase in parathyroid hormone (PTH) secretion, which over time, negatively impacts on bone mass due to an increase in bone turnover (Please see section 2.2.2.1.1.). Protein can have positive effects on calcium homeostasis through increased insulin-like growth factor 1 (IGF-1) concentrations (Schurch *et al.*, 1998), and negative effects through the generation of metabolic acidosis leading to an increase in bone resorption (Dawson-Hughes *et al.*, 2003).

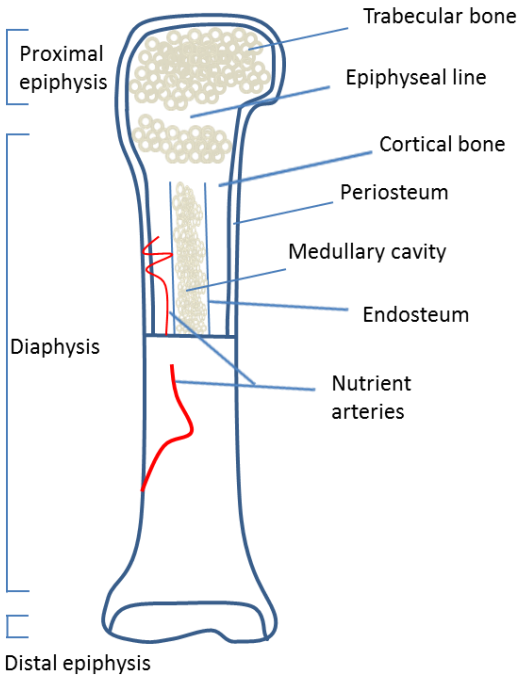


Figure 2.1. Structure of bone (adapted from Marieb and Hoehm, 2006).

The dynamic nature of bone is shown by the ability to adapt to both intrinsic and extrinsic factors, such as magnitude of mechanical loading, diet and hormonal fluctuations. Bone changes throughout the life span with peak mass and strength being reached in the early 20's and reducing thereafter (Figure 2.2.). Peak bone mass can be influenced by the level of mechanical loading, particularly during puberty (Bonnet and Ferrari, 2010). Genetic factors have been reported to largely explain (57%-92%) bone characteristics, such as bone mineral density (BMD) making it one of the most heritable human traits that has been identified (Slemenda *et al.*, 1991; Harris *et al.*, 1998). More recent findings incorporating GWAS (Zheng *et al.*, 2011; Hsu and Kiel, 2012) suggest that numerous complex gene interactions may occur.

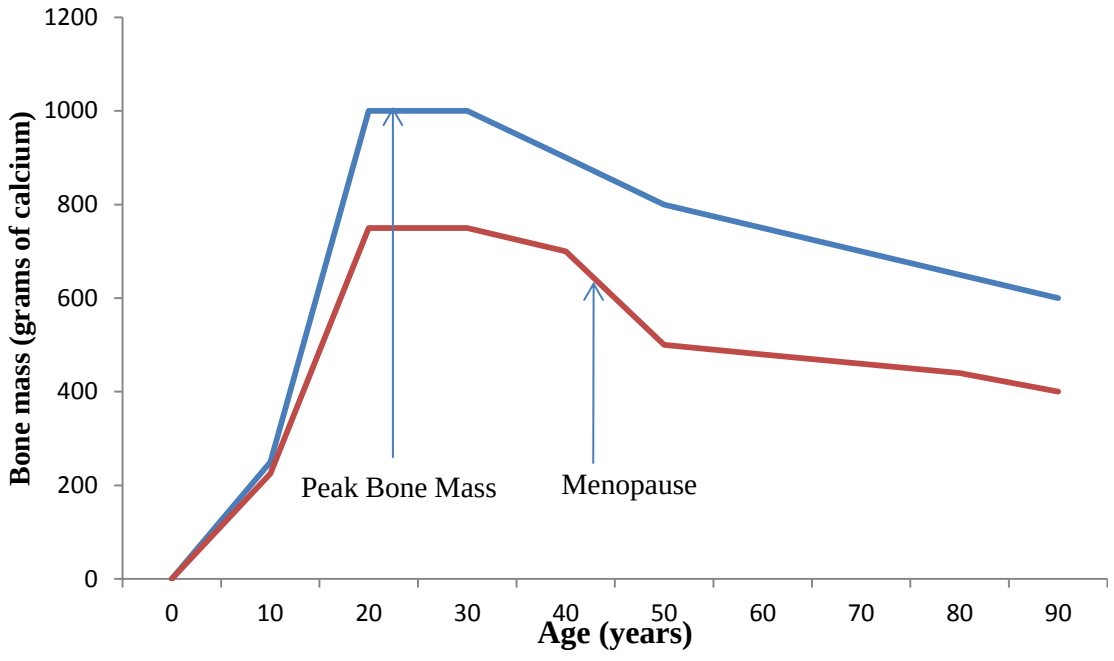


Figure 2.2. Age related bone mass accrual and loss. The blue line depicts males and the red line depicts females (Adapted from Bonnet and Ferrari, 2010).

### 2.2.1. Cells Involved in Bone Remodelling

The mechanisms of bone remodelling and maintenance are complex with many factors influencing activation and regulation. Molecular communication both between BMUs and with other cells in the bone marrow and on the bone surface, regulate bone remodelling in a targeted site specific manner. Growth factors (*e.g.*, transforming growth factor β (TGF-β)), hormones (*e.g.*, PTH), oestrogen, a variety of cytokines as well as diet, neural activation and mechanical loading are all known to influence bone remodelling (Figure 2.3.).

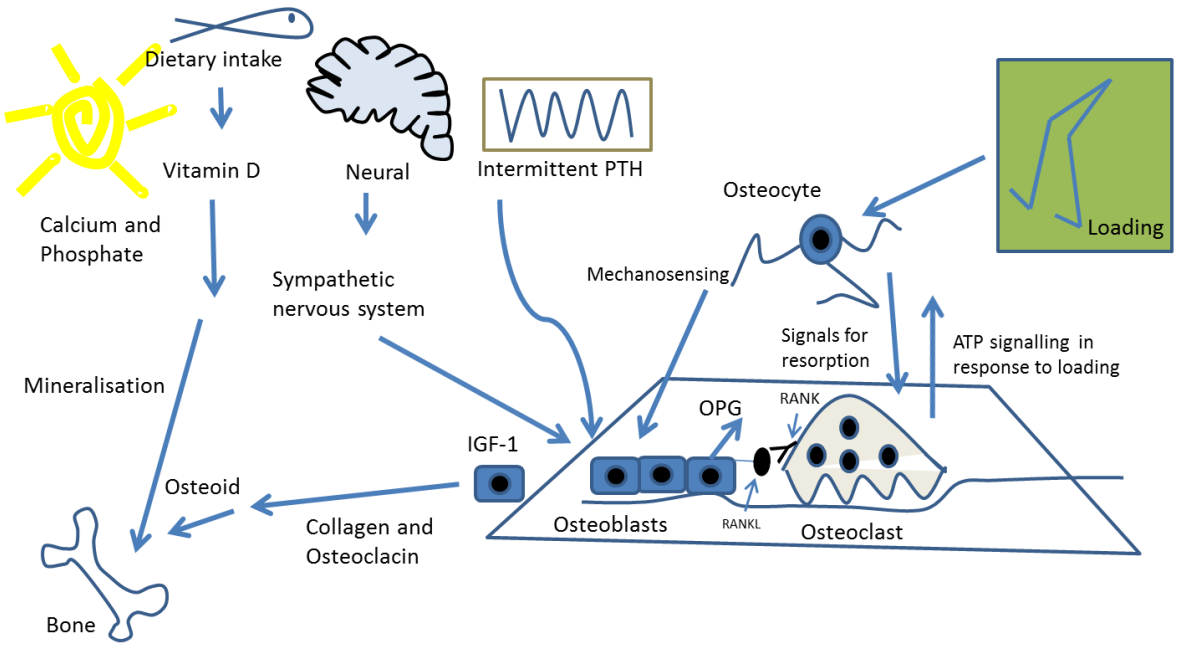


Figure 2.3. Diverse factors involved in the regulation of the skeleton (Adapted from Zaidi, 2007).

#### 2.2.1.1. Osteoblasts

Osteoblasts are the cells predominately involved in bone formation. They are derived through the proliferation and differentiation of mesenchymal stem cells when environmental conditions facilitate this process, such as the up-regulation of osteocalcin and type 1 collagen

genes (Kaveh *et al.,* 2011). Osteoblastogenesis is initially activated by the differentiation of mesenchymal cells into osteoblast precursors as a result of Runt-related transcription factor 2 (Runx2) and Osterix (Osx) expression (Camilleri and McDonold, 2006). This occurs as a result of increased expression of hormones and cytokines, including PTH, prostaglandin E2 and TGF-β and is mainly driven by *Wnt* proteins and bone morphogenetic proteins (BMPs) (Zaidi, 2007). Osteoblasts are mononuclear cells 20-30 μm in diameter that have a rough endoplasmic reticulum (Manolagas and Parfitt, 2010). After the formation of ossification centres, osteoblasts create new bone by the formation of non-mineralised matrix to form osteoid (Franz-Odendaal *et al.,* 2006) and govern mineralisation through the embedding of hydroxyapatite with collagen fibres (Manolagas, 2000). Following the bone formation process, 10-30% of osteoblasts are encased within the mineralised bone and become osteocytes, the remainder die via apoptosis or transform into bone lining cells (Manolagas 2000). Abnormalities that occur in bone remodelling can lead to inadequate mineralisation and increased risk of fragility fracture, bone deformation and osteopenia (Feng *et al.,* 2006).

#### 2.2.1.2. Osteocytes

Osteocytes are thought to act as the prominent bone cell mechanoreceptor, sensing the direction and magnitude of mechanical strain (Bonewald, 2007). They also have a regulatory role in osteoblast and osteoclast production and bone mineralisation (Bonewald, 2007). They are formed when mature osteoblasts become embedded in the bone matrix and transform into osteocytes. As they are formed, long dendritic branches are created as the cell changes shape and takes on a mechanoreceptor function (Vatsa *et al.,* 2008). Osteocytes are inter-connected to each other, to the bone-lining surface cells and to blood vessels through their dendrites, which are channelled within the canalicular system (Palumbo *et al.,* 1990). Damage directly

to the osteocyte or its canalicular system and/or osteocytes apoptosis are thought to be mechanisms by which osteocytes are able to recruit osteoblast and osteoclast progenitor cells, by way of chemical mediation, and activate bone turnover in a site specific manner (Lin *et al.,* 2009). Changes in fluid flow, stress, strain and pressure through the canalicular system can result in deformation of the osteocyte cell surface and is also thought to cause osteocyte apoptosis thus, initiating bone remodelling (Rochefort *et al.,* 2010). The central mechanism of how this occurs remains unclear, with many receptors being proposed to regulate this process (Please see section 2.3.).

#### 2.2.1.3. Osteoclasts

Mononuclear osteoclast precursors differentiate into multi-nucleated osteoclasts that are responsible for bone resorption (Suda *et al.,* 1992). The differentiation of osteoclast precursors into monocytes and subsequently mature multi-nucleated osteoclasts is regulated by osteoblast cells in the bone marrow that express receptor activator of nuclear factor-$_{K}$B ligand (*RANKL)* and macrophage colony-stimulating factor (M-CSF). Receptor activator of nuclear factor-$_{K}$B (*RANK)* binds with its ligand (*RANKL*) on the pre-osteoclast cell surface (Simonet *et al.,* 1997), in conjunction with M-CSF binding to c-Fms on the surface of the cell, leading to osteoclast differentiation. The importance of M-CSF is demonstranted by mutations in the C*sf1* gene, which leads to a lack of M-CSF expression, causing low BMD in mice (Kodama *et al.,* 1991). Both, the *RANK/RANKL/OPG* signalling pathway (Please see section 2.5.2.1.3.2.) and M-CSF expression are key in the volume and longevity of osteoclast cells. When osteoclasts bind to the bone surface a resorption compartment is formed into which hydrogen cation ions are secreted thus dissolving the inorganic component of bone and creating a cavity ready for subsequent bone formation (Martin and Sims, 2005). The

osteoclast cell has a ruffled border that is able to uptake adenosine triphosphate (ATP) driven from proton pumps, forming an acidic environment for mineral resorption. Osteoclast activity can be influenced by many factors incuding, mechanical loading, hormones, growth factors and ultimately genetics.

### 2.2.2. Bone Remodelling

Bone modelling occurs throughout growth. It is the process by which bone formation occurs without equivalent prior bone resorption thus, the skeleton acquires bone and through adaptations based on hormonal influences and the environmental strains applied to it, it's size and shape, are determined (Baron and Kneissel, 2013). Following puberty, bone modelling is terminated and the epiphyseal plate is closed. In contrast, bone remodelling adaptations occur in order to repair damage from habitual use, to react to fluctuations in cytokines and growth factors, and respond to hormonal alterations and mechanical loading (Zaidi, 2007). Bone remodelling (Figure 2.4.) refers to the cycle of events encompassing the recruitment of osteoclasts, followed by their resorption of old bone and the formation of new bone by osteoblasts. Bone is in a constant state of remodelling in order to preserve and maintain its structure and integrity. In 1892 the dynamic nature of bone was addressed for the first time; Wolff proposed that bone changed in shape and architecture in response to the stresses applied upon it (Wolff, 1892). The mechanostat theory (Frost, 1964) further developed this model by adding that bone is regulated in a proportional manner to modify the skeleton in accordance with forces applied to it. Bone remodelling occurs on numerous bone surfaces including periosteal, endosteal, cortical and trabecular remodelling. The rate of bone remodelling differs dependent on the composition of bone; for example, cortical remodelling occurs at a slower rate in comparison to trabecular (Manolagas, 2000; Parfitt, 1994). Osteoblastic bone formation, osteoclastic bone resorption and osteocytes are closely

integrated or coupled, as they act to maintain bone homeostasis throughout the life span. Mechanical support, regulation of mineral homeostasis and the repair of micro-damage all benefit from bone remodelling (Eriksen, 2010). The rate of remodelling and the number of remodelling sites are increased by various skeletal conditions, such as in the most common form of osteoporosis in which systematic alterations in hormone levels increase bone resorption (Boyce *et al.*, 2003). Absence or inadequacies in response to environmental stresses by BMU compromises bone integrity and can lead to the development of micro-damage to the bone architecture, local bone loss and ultimately bone injuries, such as stress fracture injury. Advances have been made in recent years in relation to the mechanisms responsible for regulating bone adaptations to mechanical loading (Baron and Kneissel, 2013).

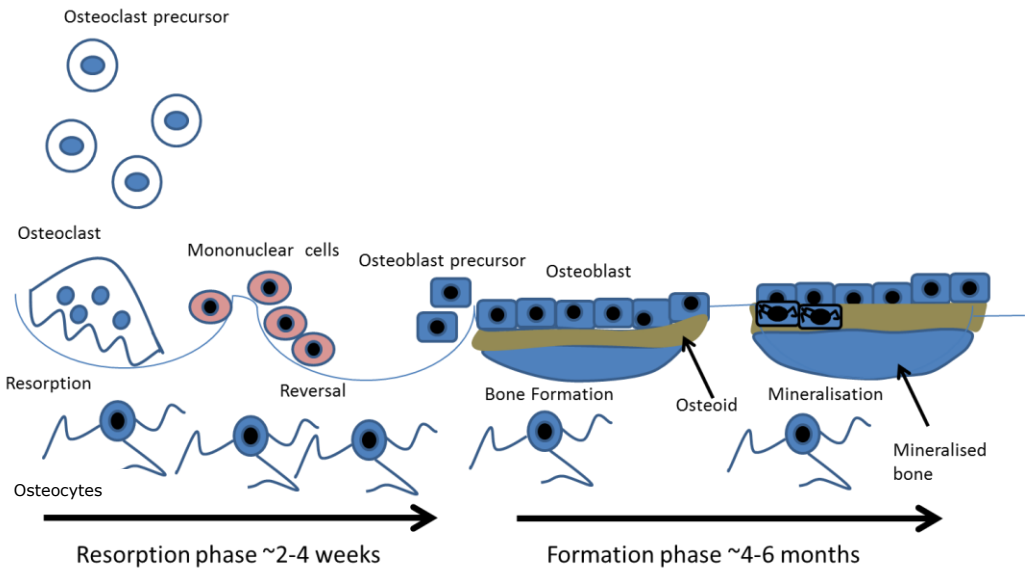


Figure 2.4. The Bone remodelling cycle. Bone remodelling has four main phases, resorption, reversal, formation and mineralisation. Its initiation causes osteoclast precursor recruitment and maturation into multinuclear cells, which are activated by RANK binding to its ligand. These cells attach to the bone surface, and with the secretion of enzyme cathepsin K, create an acidic environment in which the organic matrix of the bone is dissolved and a resorption pit is created. Osteoclasts disperse and mononuclear cells prime the surface of the bone ready for formation. Osteoblast precursors then mature into osteoblasts and create a non-mineralised matrix to form osteoid. This matrix is then mineralised under the mediation of osteoblasts. ~20% of the osteoblasts will become trapped in the bone matrix and change into osteocytes, while the remaining osteoblasts become bone lining cells.

#### 2.2.2.1. Factors that Influence Bone Remodelling

Bone remodelling and skeletal form are influenced by PTH (Poole and Reeve, 2005), TGF-β (Tang and Alliston 2012), vitamin D status (Reid *et al.,* 2014) and cytokines (such as interleukin(s)-1,-6,-11) (Steeve *et al.*, 2004). It has been established that bone homeostasis is maintained by the tightly sequenced recruitment, differentiation and maturation of BMUs. It is currently unclear as to the importance of the specific factors that control this process. Prominent factors will be reviewed in the following sections.

##### 2.2.2.1.1. PTH

PTH has a regulatory role in governing blood calcium homeostasis. Increased extracellular ionised calcium concentrations inhibit PTH secretion from the chief cells of the parathyroid gland, while decreases in circulating ionised calcium cause an increase in PTH (Poole and Reeve, 2005). When PTH is released in response to low blood ionised calcium it activates the PTH/PTHrP receptor, which is expressed by osteoblasts, osteocytes and bone lining cells (Poole and Reeve, 2005). This causes activation of G-protein signalling following the binding of ligands such as cyclic AMP (cAMP), and results in the mobilisation of intracellular calcium (Poole and Reeve, 2005). Although prominent in osteoclastic bone resorption activity, it has previously been thought that osteoclasts do not have PTH receptors and are regulated indirectly by osteoblast activation (Dempster *et al.*, 2005). Immunocytochemistry and protein expression studies have contested this view by showing that the resorptive function of osteoclasts is regulated by PTH in the absence of osteoblasts (Dempster *et al.*, 2005). PTH has many functions (Figure 2.5.) and directly influences osteoblast activity, indirectly influences bone homeostasis (by activation of IGF-1) and inhibits growth factor components such as sclerostin (Keller and Kneissel, 2005).

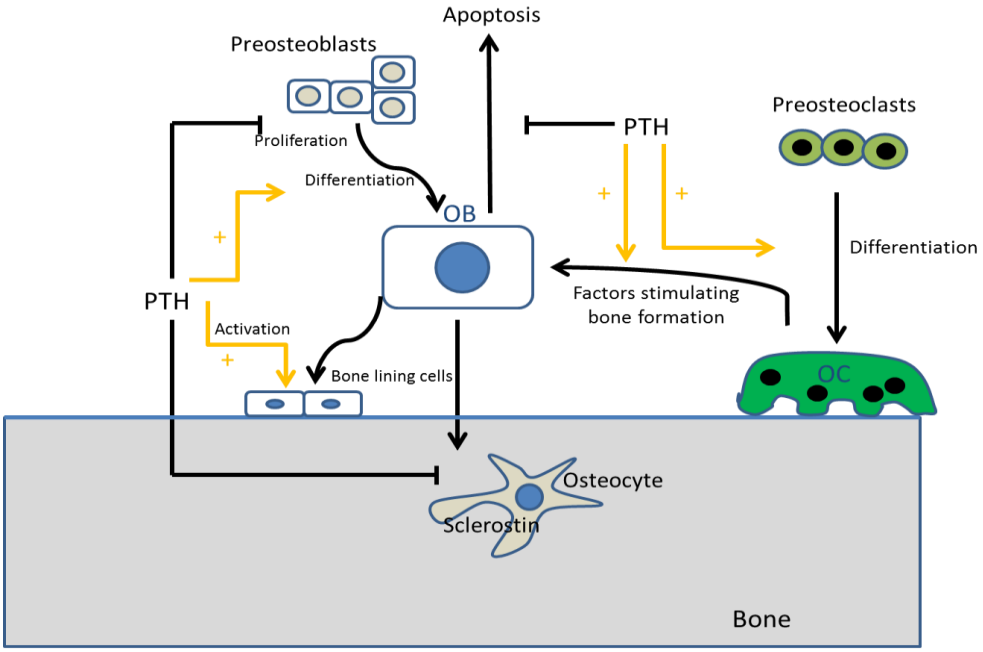


Figure 2.5. Aspects of PTHs role in bone metabolism (adapted from Khosla *et al.*, 2008). PTH increases activation and differentiation of osteoblasts (OB) while also decreasing apoptosis in osteoblasts, proliferation of pre-osteoblasts and activation of sclerostin. In osteoclasts (OC), PTH increases differentiation and has been suggested to increase osteoblast stimulating factors produced by osteoclasts.

PTH has contrasting effects on bone, dependent on the duration of exposure; intermittent PTH exposure inhibits osteoblast apoptosis causing an anabolic, osteogenic affect, while sustained increases have the opposite catabolic effects (Poole and Reeve, 2005). PTH administered intermittently has been shown to increase bone formation markers and bone resorption markers (albeit to a lesser extent) (Hodsman *et al.*, 2003), increase osteoblast number (Hodsman and Steer, 1993) and reduce osteoblast apoptosis (Jilka *et al.*, 1999), which is likely to be the result of PTH increasing differentiation, recruitment and proliferation of osteoblasts (Poole and Reeve, 2005). PTH also increases phosphate excretion and 1, 25 dihydroxyvitamin D formation in the kidney, increases intestinal calcium absorption and decreases sclerostin production, making it an important factor to the *Wnt* signalling pathway (Please see section 2.5.2.1.3.1.).

The mechanism by which continuous PTH secretion causes catabolic effects may be related to an increase in *RANKL* expression (Locklin *et al.,* 2003) and/or a reduction in *SOST* expression (Keller and Kneissel, 2005). PTH also appears to have divergent effects on cortical and trabecular bone. Mutations in the PTH receptor lead to large increases in PTH signalling resulting in a loss of cortical bone and increase in trabecular bone (Syme *et al.,* 2005). In addition, PTH receptor knockout mice have increased cortical and decreased trabecular bone mass (Lanske *et al.,* 1999). PTH is well established to have a major role in bone homoeostasis (Poole and Reeve, 2005, Locklin *et al.,* 2003), although the underlying mechanisms for these effects require further investigation.

#### 2.2.2.1.2. TGF-β

Transforming growth factor beta (TGF-β) has a critical role in cellular proliferation, differentiation and is thought to be an important factor in osteoclastogenesis (Tang *et al.,* 2009). TGF-β is integral to bone resorption since it directly enables osteoclast formation through the differentiation of osteoclast pre-cursors and facilitation of osteoclastogenesis by attracting osteoclast pre-cursors to the surface of the bone (Tang *et al.,* 2009). Conversely, indirect TGF-β activity has been shown to suppress osteoclast formation by inhibiting *RANKL* (Fox and Lovibond, 2005) and mutations in TGF-β contribute to the bone thickening disorder Camurati-Engelmann disease (Jansseus *et al.,* 2000). Despite the reported importance of TGF-β, it is unable to independently affect osteoclast formation (Fox and Lovibond, 2005) and requires crosstalk with its ligands, receptors and agonists to produce functional effects (Ikushima and Miyazono, 2012). TGF-β also has an important role in bone formation through the activation of bone matrix secreting cells, such as osteocalcin and the recruitment of osteoprogenitor cells (Tang and Alliston, 2012). The complexity of TGF-βs role in bone resorption and formation, is further highlighted by the seeming contrary effects

of up and down regulation of TGF-β being shown to lead to low bone mass disorders (Tang and Alliston, 2012).

#### 2.2.2.1.3. Vitamin D

Vitamin D and its analogs are well publicised for their importance in the maintenance of bone health (Reid *et al.,* 2014). The vitamin D endocrine system, which includes ergocalciferol (vitamin $D_2$), cholecalciferol (vitamin $D_3$) and vitamin D in its active form, (1,25-dihydroxyvitamin D (1,25-$(OH)_2D$)) has been shown to play a vital role in bone health (Holick, 1996) and to a lesser degree, has been associated with increased immune response and muscle strength and reduced proliferation of cancer cells (Haussler *et al.,* 1998). The association of vitamin D status with bone health is highlighted by deficiencies resulting in diseases such as Rickets and osteomalacia (Mawer *et al.,* 2001). Vitamin D, obtained from diet and sunlight, must undergo two hydroxylations before it is functional. Firstly, the liver converts vitamin D to 25-hydroxyvitamin D (25(OH)D) and secondly, the kidney hydroxylases 25(OH)D to form vitamin D in its active form, (1,25-$(OH)_2D$) both, 25(OH)D and 1,25$(OH)_2D$ are used as biomarkers of vitamin D status. 1,25-$(OH)_2D$ is important for calcium homeostasis, as low circulating blood ionised calcium concentrations lead to an increased release of PTH from the parathyroid gland, which in turn leads to hydroxylation at the kidney and activation of 1,25-$(OH)_2D$ causing an increase in calcium absorption (Thomas *et al.,* 1998). The importance of 1,25-$(OH)_2D$ has also been shown by its association with bone characteristics including BMD and fracture prevention in meta-analysis investigations (Bischoff-Ferrari *et al.,* 2005). Despite studies investigating vitamin D status (both, 1,25-$(OH)_2D$ and 25(OH)D) with diverse bone phenotypes, in a number of populations, doubt remains over the clinical significance and magnitude of the effects. Supplementation with

700 International units (IU) of vitamin D has been associated with increased plasma 1,25-$(OH)_2$D and 25(OH)D concentrations and also decreased fracture risk (Dawson-Hughes *et al.,* 1997). However, recent findings show that very high doses of cholecalciferol (500,000 IU) increase fracture risk (Sanders *et al.,* 2010). This discovery is controversial as it opposes the majority of the published literature in the area, albeit supplementing with smaller doses (for review see Holick, 2004). The reason for the controversial findings are likely to be due to the high dose of vitamin D supplementation given, which may have caused toxicity in the participants leading to hypercalcemia. Symptoms of hypercalcemia include muscle weakness, tiredness and loss of appetite (Vogiatzi *et al.,* 2014), which could all increase fracture risk. Environment variances such as low socioeconomic status (Jones *et al.,* 2004) have been associated with increased fracture prevalence.

1,25-$(OH)_2$D has previously been associated with the regulation of osteocalcin and an increase in bone resorption through the control of calcium homeostasis, low serum calcium leading to increased PTH secretion from the parathyroid gland in turn stimulating the hydroxylation (Staal *et al.,* 1998), which provides a more mechanistic explanation of the findings. Serum 25-(OH) D concentrations have been associated with many bone phenotypes, including lower BMD and fracture risk (Valimaki *et al.,* 2004). Ruohola *et al.,* (2005) showed that lower 1,25-$(OH)_2$D concentrations increased the likelihood of stress fracture injury in 800 military recruits over a 90 day period. Lappe *et al.* (2008) reported lower prevalence of stress fractures in 5021 female naval recruits who had been given 800IU of vitamin D supplementation together with 2000 mg calcium supplement prior to training. However, Lappe *et al.,* (2008) failed to report the type of vitamin D supplemented with ($D_2$ or $D_3$) and also failed to assess vitamin D status before or after supplementation. As these assessments were not made it is impossible to know the vitamin D status of the recruits

before the intervention, which is likely to have influenced the findings. As vitamin D was supplemented in combination with calcium it is also impossible to isolate the suggested benefits to vitamin D. Dietary vitamin D consumption has also been associated with increased BMD and reduced stress fracture incidence in a two-year prospective study involving competitive female cross-country runners (Nieves *et al.,* 2008). Although this study is well controlled in terms of the homogeneity of the population investigated (all female, competitive cross-country runners, aged 18-26 y), the mixed components of participants' dietary intake make it difficult to isolate vitamin D intake as the regulatory factor.

#### 2.2.2.1.4. Interleukins

IL1 is a pleiotropic cytokine that induces the expression of hematopoietic cytokines, such as IL6 and initiates a complex signalling cascade that includes the activation of RANK, thus facilitating osteoclast survival. The importance of IL1 is shown by IL1 receptor knockout mice being immune to ovariectomy related bone loss (Lorenzo *et al.,* 1998). IL6 stimulates mesenchymal progenitor cells to aid the differentiation of osteoblasts and also prolong osteoblast longevity. However, the main role of IL6 is in increasing osteoclastogenesis and subsequent bone resorption by facilitating interactions between osteoblasts and osteoclasts. IL1 and IL6 are interrelated and increase bone resorption primarily by controlling the expression of RANKL, with increased concentrations of IL1 and IL6 leading to a net increase (Steeve *et al.,* 2004). Prolonged running (>1 h) increases concentrations of circulating cytokines, including IL1 and IL6, which peak at the end of exercise (Scott *et al.,* 2011b, 2013a) and stay elevated for 1-5 days following prolonged exercise (ironman) (Neubauer *et al.,* 2008). Increases in IL1 and IL6 appear to be intensity and activity specific, with the largest increases seen in running (Fischer, 2006) and no change seen in cycling (Starkie *et al.,* 2005) or a knee-extensor activity (Steensberg *et al.,* 2002). The mechanisms as to why this

occurs are not clear, but is likely to be related to an immune response following local damage to the working muscle and involve the mechanotransductive response of bone to weight-bearing exercise.

#### 2.2.2.2. Neural Control

The bone remodelling process is mediated by neural activation of the hypothalamus, directly affecting BMUs, and by indirectly affecting hormonal signals from the pituitary (Zaidi, 2005). Leptin is thought to act as a prominent neural inhibitor of osteoblastic function (Elefteriou *et al.,* 2005) and leptin knockout mice have been shown to have a reduced sympathetic tone, which caused high bone mass and increased bone formation, whereas leptin infusion reduced bone mass in wild-type and leptin deficient mice (Ducy *et al.,* 2000). The neural mediation of bone phenotypes is demonstrated by high bone mass being shown in mice lacking dopamine, and mice given beta blockers also showing high bone mass and an increase in bone formation (Takeda *et al.,* 2002). Human studies have also shown beta blocker ingestion to reduce fracture risk by at least 10% (Yang *et al.,* 2012). Increased bone loss has been shown in humans with reflex sympathetic dystrophy, a disease causing abnormal activation of the sympathetic nervous system (Patel and Elefteriou, 2007). Conversely, an increased osteogenic response is seen in traumatic brain injury patients, which is thought to be due to a decrease in bone noradrenaline levels (Tam *et al.,* 2008). Despite the evidence for neural mediation of bone remodelling, the magnitude of the effect on bone in healthy individuals has not been fully elucidated.

## 2.3. Mechanical Loading

Exercise is known to affect bone as a result of three main actions: 1) as a direct result of mechanical loading that is detected by the mechanoreceptors and follows a signalling cascade

to ultimately induce bone remodelling; 2) muscular contractions applying stress and strain to the bone, which is then translated to the mechanoreceptors; 3) exercise induced changes in hormonal factors altering concentrations of circulating minerals that have a direct effect on bone turnover and an indirect effect through altered mineral homeostasis (Figure 2.6.).

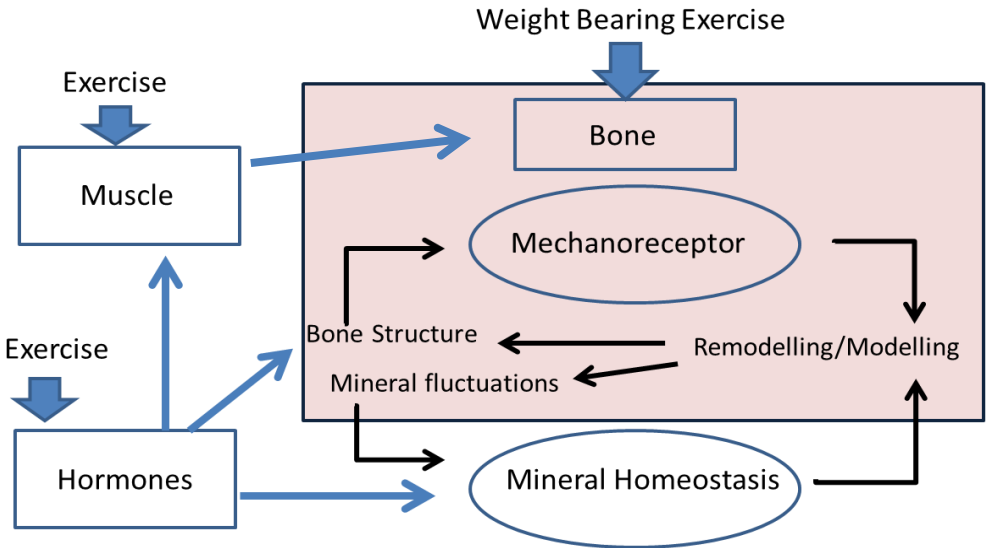


Figure 2.6. Schematic representation of the effect of weight bearing exercise on bone maintenance and adaptation. Weight-bearing exercise directly impacts upon bone, which is sensed by the osteocytes that then inform the mechanoreceptors to signal for a proportional remodelling response. Weight bearing exercise also causes the secretion of hormones that influence bone structural components, mineral expression and the muscular contractive process. Muscle contractions, caused by exercise; create strain on the bone structure, which is sensed in the same way as mechanical loading.

The intrinsic ability of bone to adapt based on the functional demands required of it was first documented by Wolf (1892). The mechanostat theory further built upon this theory (Explained in section 2.2.2.), suggesting that a large amount of stress and strain is placed upon bone will cause proportional bone adaptations, creating larger and stronger bones (Karlsson *et al.*, 1993) in an anatomical site specific manner (Kannus *et al.*, 1994). Equally, a lack of loading, such as that experienced during space flight (Carmelict *et al.*, 2001), prolonged bed rest (LeBlanc *et al.*, 1990; Nagaraja and Risin 2013) and simulated

microgravity environments (Blaber *et al.,* 2014) can cause an acceleration in bone loss. However, while loading has been shown to produce predominately anabolic effects upon bone, catabolic effects have been reported (Please see section 2.3.1.). The exact amount of loading required to achieve an anabolic effect is not well established, similarly it is not clear whether a threshold occurs upon which the anabolic effects are actioned. Pharmaceutical therapeutic interventions, such as the administration of denosumab, have focused on the inhibition of bone resorption (Bone *et al.,* 2011) or the promotion of bone formation (Padhi *et al.,* 2011) by targeting specific components of bone remodelling pathways in an attempt to delay the age related decline in BMD and onset of osteoporosis. However, these strategies fail to replicate the complex interactions between bone formation and resorption that mechanical loading initiates and are unable to regulate bone turnover in a site specific manner (Thompson *et al.,* 2012).

Mechanotransduction is the mechanism by which mechanical loading is sensed and converted into complex cellular interactions. The mechanism of how loading is initially sensed and eventually induces a structural bone adaptation is complex (Figure 2.7. provides a simplified explanation) and the specific mechanisms are not well established. Bone cells and their precursors are able to respond to both biological and physical factors in order to regulate bone turnover (Salter *et al.,*1997). Osteocytes, osteoblasts and mesenchymal cells are all mechanosensitive and able to recruit, proliferate and differentiate in response to various forms of loading (Scott *et al.,* 2008) to induce changes in bone remodelling (Robling and Turner, 2009). This is shown in mouse studies where the application of mechanical loading caused a reduction in osteoclast formation and reduced *RANKL* expression (Rubin *et al.,* 2000). There is debate as to the specific mechanisms involved in the mechanotransduction process. It is generally believed that osteocytes sense mechanical loading and initiate the

remodelling process due to their location within the bone matrix and their connection to the canalicular system (Bonewald, 2011). Despite being most prominently investigated in relation to their role in bone formation, it has been suggested that osteoblasts may also have a sensory role (Papachroni *et al.*, 2009). Osteocytes are able to sense the magnitude and specific location on the bone where the strains (mechanical, shear, pressure) are occurring through a complex network of receptor interactions (Bonewald, 2011). Mechanoreceptors located on the osteocyte dendrites release an electrical charge, and as a result of this, calcium channels are activated and nitric oxide and prostaglandins, such as E2, are stimulated enabling signals to be sent to the osteoblasts and osteoclasts on the bone surface (Cowin and Moss, 2001). The number of cycles, magnitude and frequency of loading affect the cellular response (Robling and Turner, 2009; Turner *et al.*, 1995). Mouse models show that β-catenin activation (Please see section 2.5.2.1.3.1.) occurs in osteocytes 60 min following loading; however β-catenin was only detectable on the bone's surface 24 hours following loading (Kramer *et al.*, 2010) suggesting that osteocytes are able to respond quicker than osteoblasts to a loading stimulus. Osteocytes may also respond indirectly to mechanical loading, as a result of an increase in extracellular fluid waves in the canalicular system (Turner, 2006).

Bone cell signalling can induce a biochemical response leading to bone remodelling by recruiting a variety of mechanisms including: ATP signalling, integrins, G-proteins, the cytoskeleton and ion channels. The extent of the role each plays in bone signalling is not fully understood (Bonnet and Ferrari, 2010, Figure 2.7.). There are many theories as to how, and which mechanoreceptors respond to signals from bone cells to cause the up/down regulation of pathways to induce bone remodelling. It may be that multiple mechanoreceptors are involved in this action with an overlap based on the type magnitude and frequency of the load. *Wnt* signalling may also regulate mechanical loading in osteoblasts (Robinson *et al.*,

2006). The mechanical strains created as a result of loading cause a rapid increase in β-catenin in the cytoplasm, which causes it to translocate to the nucleus and up-regulate *Wnt* target genes, causing increased *Wnt* secretion (Robling and Turner, 2009). *Wnt*-signalling also influences mechanical loading induced osteocyte activation (Bonewald and Johnson, 2008). *In vitro* investigations have shown that fluid flow shear stress results in increased phosphorylation of glycogen synthase kinase-3β (GSK-3β) resulting in a change in expression of β-catenin target genes known to be integral to the *Wnt* signalling pathway (Please see section 2.5.2.1.3.1.) (Bonewald and Johnson, 2008).

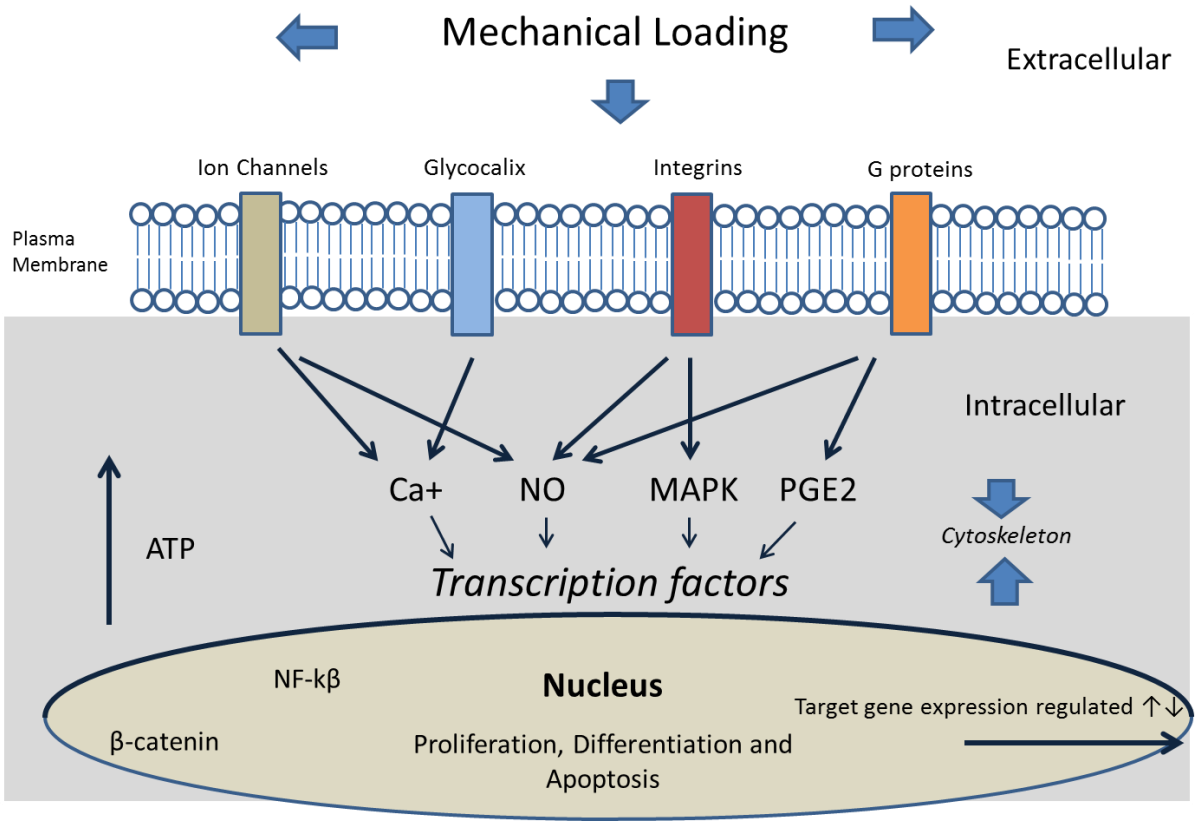


Figure 2.7. Selection of pathways demonstrating how mechanotransduction leads to bone cell response (Adapted from Bonnet and Ferrari, 2010). Osteocytes, osteoblasts and mesenchymal cells have the ability to act as sensors of various forms of loading (shear stress, pressure, tissue strain).There are various biological candidates to act as mechanoreceptors including but not limited to; adenosine 5'-triphosphate (ATP) being released by bone cells in response to mechanical loading, which is facilitated by the purinergic receptors (*P2X7R*) reviewed in section 5.4.3. Stretch activated ion channels that are activiated in response to membrane strain allow the influx of calcium, glycocalix on the cell surface which induces calcium influx

or increases cAMP as a result of fluid shear stress. Complex integrin-actin filaments activate signalling pathways such as MAPK as a result of cell-cell junction receptors sensing loading, and intracellular strain acts upon the cytoskeleton which effects binding to specific molecules and activates pathways known to induce G-protein signalling.

### 2.3.1. Metabolic Adaption of Bone to Exercise

A better understanding of the bone's response to mechanical loading may lead to the development of a clearer picture with regard to the pathophysiology and aetiology of bone injury and disease. Athletes are known to have a greater bone mass than non-athletes (Greene *et al.*, 2012), although longitudinal training intervention studies, examining the impact of different modes of exercise, have produced inconsistent outcomes (for review see Maimoun and Sultan, 2011). Examination of bone turnover and changes in bone structural adaptations as a result of exercise may lead to the discovery of mechanistic evidence that may be of benefit in the prevention and treatment of bone debilitating diseases and act as a preventative measure to minimise fracture incidence.

#### 2.3.1.1. Single Bout of Exercise

The response of bone to a single bout of exercise is not yet fully understood. Given the subtle changes to bone structure that result from a single bout of exercise, we are reliant upon measurement of biochemical markers of bone turnover before, during and after exercise to provide us with information relating to bone cellular activity.

#### 2.3.1.1.1. Biochemical Markers of Bone Turnover

There are a number of biochemical markers of bone turnover that are regularly used to monitor bone formation including type 1 procollagen N-terminal (P1NP), bone alkaline phosphatase (bone-ALP), osteocalcin (OC), carboxy-terminal propeptide of type 1 procollagen (P1CP). Other markers are used to monitor the bone resorption process and these include: collagen type 1 cross-linked C-telopeptide (β-CTX), collagen type 1 cross-linked N-telopeptide (NTX), urinary total pyridinoline (PYD), urinary free deoxypyridinoline (DPD), tartrate-resistant acid phosphatase 5b (TRACP5b) and C-Terminal Telopeptide Type I Collagen (ICTP). These are used in clinical settings to monitor the effectiveness of treatment for bone disease (Lee and Vasikaran, 2012) and to assess the bone response to exercise (Scott *et al.,* 2010; 2011a; 2012a). Through the measurement of blood and urine markers, such as proteins/peptides and by-products of bone formation and resorption, an acute indication of bone metabolic responses before structural adaptations occur can be detected. Currently, there is no consensus on which biochemical markers of bone turnover give the most accurate estimate of bone formation and resorption, which may be due to different bone markers giving insight to different aspects of the bone remodelling process. Although widely used (Table 2.1.), biochemical markers of bone turnover have shown inter-individual variability in response to exercise. The reason for the variability in the response may be due to the number of different biochemical markers of bone turnover used and differences in the methodologies of assessment (see Table 2.1. for examples of studies investigating the effects of a single bout of exercise on biochemical markers of bone turnover). Recommendations have been made by the International Osteoporosis Foundation (IOF) and the International Federation of Clinical Chemistry (IFCC) to standardise β-CTX as a marker of bone resorption and procollagen type 1 N-terminal propeptide (P1NP) as a marker of bone formation (Vasikaran *et al.,* 2011). The usefulness of these biochemical markers of bone turnover have been shown by evidence that

β-CTX can be used as predictor of fracture risk independent of BMD (Garnero *et al.,* 2003; Bauer *et al.,* 2009), is sensitive to exercise (Scott *et al.,* 2010; 2011a; 2012a) and pharmaceutical interventions (Garnero *et al.,* 1996; Sondergaard *et al.,* 2009).

A number of factors are known to influence the accurate measurement of the biochemical markers of bone turnover including; the timing of the sample collection, the preservative used for collection (Stokes *et al.,* 2011), the sample type (blood or urine) and analytical factors (postprandial hyperglycaemia, alterations in acid-base balance, blood flow and enzyme action (Clowes *et al.,* 2002; 2005). β-CTX is also influenced by renal function, food consumption and circadian variation (Fraser *et al.,* 2010; Brown *et al*., 2009). With appropriate control measures in place, urine and blood markers of bone turnover provide a useful measurement in which acute exercise-induced changes can be estimated. Through bone marker assessment, the biological rate and magnitude of osteoblastic bone formation and osteoclastic bone resorption can be assessed, providing a clinically relevant tool to assess the normal and pathologic responses of bone turnover.

Table 2.1. Studies investigating biochemical markers of bone turnover in response to a single bout of exercise.

| Study | Population | Exercise Protocol | Venepuncture | Markers of Bone Formation | Markers of Bone Resorption |
|---|---|---|---|---|---|
| Nishiyama *et al.,* 1988 | M 19; 20-24 9 Volleyball players | Treadmill running 30 mins 43-52% of individual maximum. | Base, EE, P 1 h. | OC increase EE (NA), P 1 h (A vs base and NA). A base significantly different from NA. No change in Bone-ALP. | n/a |
| Brahm *et al.,* 1996 | M RA 10; 22-53 F RA 10; 22-55 | 28 +-5 km M, 15 +-6 km F. | Base, FU1,FU2. | OC decrease FU1 in M. P1CP decrease in F FU1. No change in PTH or bone ALP. | ICTP increase in M FU2. |
| Thorsen *et al.,* 1997 | F 14; 24-26 | Outdoor running 50% $\dot{V}O_{2max}$ 45 min | Base, P 1h, FU1, FU3. | PICP decrease vs Base 1h. increase FU1 and FU3 vs base. PTH increase FU1, FU3 vs base. | ICTP increase FU1, FU3 vs Base. |
| Rong *et al.,* 1997 | M RA 8; 20-26 | Ergometer cycling; 45 min 55% $\dot{V}O_{2max}$, 15 min 85% $\dot{V}O_{2max}$. Resistance exercise, leg press 5 x 8 reps. | Base, EE, P 1h, P 4h FU1. | PTH increase EE (resistance exercise). OC no change. | ICTP no change. |
| Ashizawa *et al.,* 1998 | M 14; 24-25 | Standard resistance exercise protocol 3 x 10 sets of 7 manoeuvres. | Base, EE, FU1,FU2,FU3. | P1CP decrease FU1. Bone-ALP decrease FU2, FU3. No change in OC. | DPD decrease FU3. TRACP5b decrease FU1. |
| Crespo *et al.,* 1999 | M 11 ET F 7 ET; 22-33 | 42 km outdoor race. | Base, EE, FU1. | Bone-ALP increase EE,FU1 vs base | TRACP5b increase EE vs base, decrease FU1 vs base. |

| | | | | | |
|---|---|---|---|---|---|
| Zittermann *et al.,* 2002 | M athletes 18; 24-26 | 60 mins outdoor running 70% of maximum speed. | Base (pre feeding), P 3h. | P1CP decrease P 3 h. | B-CTX decrease P 3h. |
| Guillemant *et al.,* 2004 | 12 M ET; 23-37 | Ergometer cycling 80% $\dot{V}O_{2max}$. | 1h pre, 30 min pre, base, 30 min during, EE, P .5h, P 1h, P 2h. | No change in Bone-ALP | β-CTX increase P .5h, P 1h, P 2h vs base. |
| Whipple *et al.,* 2004 | M 9; 20-23 | Standard resistance exercise protocol 3 x 10 sets of 7 manoeuvres. | Base, EE, P 1h, P 8h, FU1,FU2. | Bone-ALP increase EE (vs con). P1CP no change | NTX decrease P1, P8, FU2 (vs Control). |
| Maimoun *et al.,* 2005 | M RA 11 W RA 10; 60-88 | Treadmill walking to exhaustion 8-12 mins. | Base,EE. | Increase in PTH EE. No change in Bone-ALP or OC. | No change in β-CTX. |
| Tosun *et al.*, 2006 | F S 9; 26-33 | Sub-maximal treadmill walking 30 mins, loaded (5kg) and un-loaded. | Base, EE, 15 min, FU1. | Bone-ALP decreased FU1 (un-loaded), increased FU1 (loaded). Increase in PTH EE (un-loaded). No change in OC, P1CP,PINP. | No change in β-CTX , ICTP. |
| Hermann *et al.,* 2007 | M 15 RA F 17 RA; 17-39 | 60 mins ergometer cycling 75%,95%,110% of AT. | Base, P 3h, FU1 | OC decrease in M at 75% (P 3h, FU1), increase at 95% (P 3h), 110% (P 3h). Decrease in F at 75% (P 3h, FU1), increase at 95% (FU1). P1NP decrease in M at 75% (P 3h, FU1), increase AT 110% (P 3h). Decrease in F at 75% (P 3h, FU1). | β-CTX increase in M at 95% (P 3 h) and 110% (P 3 h, FU1) AT. Decrease at 75% in M (FU1) and F (P 3 h). TRACP5b increase in F at 110% (P 3 h, FU1). |

| | | | | | |
|---|---|---|---|---|---|
| Pomerants *et al.,* 2008 | M 60; 10-18 | Ergometer cycling; Ramped protocol, 30 mins at 95% IVT. | Base, EE, P 30 min. | P1NP no change. | ICTP no change. |
| Rantalainen *et al.,* 2009 | M 15 various activity levels; 22-28 | Bilateral jumping to exhaustion 520-2278 range. | Base, EE, P 2h, FU1, FU2 | P1NP no change. | β-CTX increase FU2. |
| Kerschan-Schindl *et al.,* 2009 | M 16 F 2; 37-47 | Ultramarathon (mean 32h 55min). | Base, EE, FU3. | OC decrease EE vs base | β-CTX increase EE, FU2 vs base. |
| Scott *et al.,* 2010 | M 21 RA 10 ET; 23-34 | Treadmill running 65% $\dot{V}O_{2max}$ 60 min- 70% to exhaustion. | Base, 20min, 40min, 60min, Blood taken. EE, P 30 min, P 1 h, P 1.5 h, P 2 h, FU1, FU2,FU3,FU4. | P1NP and bone-ALP no change. OPG increase during, EE, P 2 h, FU1 vs BASE in RA and ET. PTH transient increase | β-CTX increase in RA and ET at FU1,FU2,FU3,FU4 vs Base. RA and ET accumulated increase vs CON. No diff RA vs ET |
| Lin *et al.,* 2011 | M 24; 18-19 | Plyometrics and interval running, under 10 mins per exercise. | Base, pre feeding; post feeding P 5 min EE, 15 min EE, P 1h, P 3h, P 6h, FU1, FU2,FU3. | OC increased P 5 min EE, P 1h (PL vs CON). | No change in TRAP. |
| Scott *et al.,* 2011a | M RA 10; 24-32 | 60 mins treadmill running at 55%,65%,75% $\dot{V}O_{2max}$. | Base, 20mins,40mins, EE, P 30 min, P 1 h, P 1.5 h, P 2 h, P 3 h FU1, FU2,FU3,FU4. | P1NP increased EE (pooled vs base). No change in Bone-ALP. PTH increase at 20 mins (75% vs base, 55%, 65%), 40 mins (75% vs base, 55%, 65%), EE (75% vs base, 55%, 65%). | β-CTX increase EE (75% vs 55%,65%), P 30 min (75% vs 55%), P 1h (75% vs 55%,65%) decrease P 3h (75 % vs 55%). β-CTX decrease vs base; EE, P 30 min, P 1h, P 2h, P 3h (55%). P 2h, P 3h (65%). |

| | | | | | |
|---|---|---|---|---|---|
| Rodgers *et al.*, 2011 | M RA 12 24-62 | Plyometerics and resistance exercise (fed and fasted) 3 x 10 reps of 6 standard manoeuvres. | Base, EE, P 1h, P 2h, FU1. | No change in OC or Bone-ALP. | β-CTX increased P 1h, FU2 (PL-Fed). No change in TRACP5b. |
| Scott *et al.*, 2012a | M RA 10 24±4 | 60 mins treadmill running at 65% $\dot{V}O_{2max}$ in fed and fasted states | Base (pre-feeding), 1 h pre, 30 min pre, pre exercise, 30 min, EE, P 1 h, P 2 h, P 3 h FU1, FU2,FU3,FU4. | Pooled PTH increase (EE vs base, FU1 vs base, base vs 1 h pre). Pooled OC decrease (30 min pre vs base, 30 min vs base). Pooled P1NP increase (30 mins vs base, P 3 h vs base). | β-CTX in fed decrease (1 h pre, 30 min pre and pre exercise vs base). β-CTX in fed increase (P 1 h vs base). β-CTX in fasted decrease (pre exercise vs base), increase at 30 min pre and pre exercise, 30 min and EE vs fed. β-CTX in fasted decrease at P 2 h and P 3 h vs base. No change in follow-up days. |

M = male; F= female; A= Athletes; NA= Non-Athletes; RA = recreational active; ET= endurance trained; AT = anaerobic threshold; BASE = pre; P= post; EE = end of exercise; FU1 = follow up 24h; FU2 = follow up 48h; FU3 = follow up 72h; PL= placebo group; CON= Control group;

#### 2.3.1.2. Mode of Exercise

The mode of exercise is an important factor in influencing the bone turnover response to a single bout of exercise, as it affects the type, magnitude, frequency and duration of loading. Different modes of habitual exercise, have been associated with divergent long-term effects on bone structure (Greene *et al.,* 2012; Nikander *et al.,* 2006; Nilsson *et al.,* 2012), suggesting that the bone response to a single bout of exercise is mode dependent. Resistance exercise has been associated with a decrease in bone resorption in the hours immediately following exercise (Ashizawa *et al.,* 1998; Rogers *et al.,* 2011) and up to 48h after exercise (Ashizawa *et al.,*1998) leading the authors to suggest that that a single exercise bout is sufficient to provide a large osteogenic stimulus. However, the only bone marker shown to decrease was TRACP5b, while other markers remained unchanged (β-CTX) (Ashizawa *et al.,* 1998; Rogers *et al.,* 2011). TRACP5b may reflect osteoclast number and not bone resorption (Alatalo *et al.,* 2000) casting doubt on the implication of these findings (Scott *et al.,* 2012b). It is difficult to define the effects of resistance exercise in comparison to other modes of exercise as the relative resistance and specific mode of resistance exercise differ among studies, which will have influenced the bone turnover response.

Studies investigating the effect of biochemical markers on bone turnover in response to endurance exercise, such as running and cycling, are more commonly investigated (Table 2.1.). Guillemant *et al.,* (2005) demonstrated that 60 min of high-intensity cycling (80% $\dot{V}O_{2max}$) increased β-CTX marker concentrations 30 min post exercise, which remained elevated until 2 hours after exercise. Bone-ALP was unchanged immediately after exercise, which was unsurprising as it is a marker of late stage mineralisation (Harris, 1990). In contrast, Scott *et al.* (2011a) discovered that β-CTX concentrations were in line with the

body's natural circadian rhythm (Fraser *et al.,* 2010) during 60 min running trials of variable intensity (55% and 65% $\dot{V}O_{2max}$), while concentrations of P1NP were increased. The decrease of β-CTX seen in cycling but not running is surprising, although the intensity of cycling was in excess of the running study, the loading experienced during running would have been far greater, causing an assumed greater osteogenic effect. Although, muscle contractions, shear stress and acidosis may be responsible for the osteogenic effects shown, current research suggests this would be insignificant in comparison to the affects of mechanical loading (Schipilow *et al.,* 2013). Further studies are needed to identify how exercise impacts on muscle and bone as a unit and to establish the modes of exercise during which muscle transfers loading to bone.

#### 2.3.1.3. Exercise Intensity

The effects of relative exercise intensity on bone turnover are not well understood. It is difficult to make comparisons between studies as a variety of measures have been used to define exercise intensity. These have included the percentage of maximal oxygen uptake (%$\dot{V}O_{2max}$; Scott *et al.,* 2010), the ventilatory threshold (VT; Maimoun *et al.,* 2006) and the individual anaerobic threshold (IAT; Hermann *et al*., 2007). Bone resorption has been reported to increase, decrease and be unaltered as a result of exercise (Table 2.1.), although the decrease attributed to the exercise in some studies may have been the natural circadian rhythm that β-CTX it is known to follow (Qvist *et al.,* 2002). Bone markers were not always measured in the days following exercise and this is a common limitation of studies, which is likely to have concealed any changes in bone turnover in the longer term. Scott *et al.,* (2011a), using a running exercise protocol, identified the differences in bone marker responses at three different exercise intensities (55%, 65% and 75% $\dot{V}O_{2max}$). Only the higher intensity exercise (75% $\dot{V}O_{2max}$) resulted in increased bone resorption (β-CTX) in the 60 min

following exercise but did not affect bone formation. The higher intensity trial would have caused a greater mechanical loading, which may be the reason for the association of mechanical loading with increased osteoclastogenesis (Li *et al.,* 2012). Despite being difficult to define due to intensity being quantified by different methods, it seems that higher intensity exercise produces an increased mechanical load, which translates into a detectable fluctuation in bone marker responses. However, the individual ground reaction forces created due to kinematic differences, make it difficult to directly associate higher intensity with increased loading.

#### 2.3.1.4. Exercise Duration

Short-duration exercise (*e.g.*, 8-12 min brisk walking; Maimoun *et al.,* 2005) appears to have little effect on bone turnover. Conversely, long duration exercise (*e.g.*, marathons or ultra-marathons) has been shown to suppress markers of bone formation (Brahm *et al.,* 1996; Kerschan-Schindl *et al.,* 2009; Malm *et al.,* 1993; Mouzopoulos *et al.,* 2007) and increase markers of bone resorption (Kerschan-Schindl *et al.,* 2009) immediately and up to 5 days-post exercise. The merits of these data are questionable, however, since the bone formation markers used in these studies (total osteocalcin) are not always indicative of bone accrual (Staal *et al.,* 1998) and the blood sample timings are not always consistent (occur at varying times due to differences in marathon finishing time). Whilst studies of this nature are informative of the bone marker response to sustained strenuous exercise, the lack of controls make it hard to compare studies (particularly those conducted in a field setting, under race conditions) as the timing of samples, exercise duration and relative intensity could not be controlled. In controlled laboratory conditions, Scott *et al.* (2010) showed increases in bone resorption when treadmill running for 1h at 70% $\dot{V}O_{2max}$ was followed by intermittent running to exhaustion, in comparison to a non-exercising Control group. 1h treadmill running

(Scott *et al.,* 2012a) at 70% $\dot{V}O_{2max}$ alone showed no difference in bone turnover markers suggesting that either the exhaustive element or the prolonged nature of running to exhaustion was a significant determinant in the increase in bone resorption.

#### 2.3.1.5. Training Status

Few recent studies have examined the effects of training status on bone turnover. Training status is difficult to assess as the definition of terms such as, recreationally active and elite, differs among studies. Most studies have compared the extremes of training status (athletes vs. sedentary Control group) (Herrmann *et al.,* 2007), meaning that the effects of different training volumes, intensities and durations on bone turnover remain relatively unknown. Scott *et al.* (2010) examined the response of bone turnover to strenuous exercise for 4 follow-up days after exercise in endurance-trained, recreational athletes and sedentary Controls. Baseline levels of bone markers did not differ between participants of different training status. No significant differences were also reported between recreationally-active and endurance-trained individuals in either bone resorption (β-CTX) or bone formation markers (P1NP, bone-ALP) after exhaustive exercise. These data are from an isolated sample of athletes who are likely to differ in terms of current and prior training histories, making it difficult to generalise the conclusions.

### 2.3.2. Structural Adaptation of Bone to Exercise

Long-term weight bearing exercise has a mainly anabolic effect on bone structure (Greene *et al.,* 2012; Weidauer *et al.,* 2012; Rantalainen *et al.,* 2011; Evans *et al.,* 2012; Wilks *et al.,* 2009; Nilsson *et al.,* 2012). Studies before 2007 have shown the anabolic properties of

exercise on bone in children and adolescents; with the majority of these studies using Dual-Energy X-ray Absorptiometry (DXA) as their method of bone assessment (for review see Hind and Burrows, 2007). DXA has limitations when scanning bone as it only scans in two dimensions and, therefore, has to make assumptions about the volumetric aspects of bone. These volumetric assumptions can lead to a measurement error of 5-8% in predicting volumetric bone density and geometry (Maghraoui and Roux, 2008). Peripheral Quantitative Computed Tomography (pQCT), High Resolution Computed Tomography (HRpQCT) and Micro-Computed Tomography (μCT) allow trabecular and cortical bone to be distinguished and accurate estimates of bone geometry to be assessed. The latter models also allow a 3D image of bone to be created facilitating the identification of the site specific location of bone adaptation (Nilsson *et al*., 2012; Schipilow *et al*., 2013; Ju *et al*., 2013).

The clearest example of exercise related adaptations are seen in unilateral sports where the dominant arm of tennis players is shown to have significantly increased bone mineral content (BMC) and cross-sectional area (CSA) in comparison to the non-dominant arm (Bass et al., 2002). Participation in physical activity and exercise interventions ($\geqslant$ 8 weeks) has been shown to increase bone strength, with findings being reported across a range of populations, including children/adolescents (Greene *et al*., 2012; Rantalainen *et al*., 2011), adults (Weidauer *et al*., 2012; Evans *et al*., 2012; Nilsson *et al*., 2012), athletes (Weidauer *et al*., 2012; Rantalainen *et al*., 2011; Nilsson *et al*., 2012) and retired athletes (Wilks *et al*., 2009). Changes have also been shown in a variety of different sports, including gymnastics (Greene *et al*., 2012), football (Weidauer *et al*., 2012; Rantalainen *et al*., 2011; Nilsson *et al*., 2012), running (Greene *et al*., 2012; Wilks *et al*., 2009; Weidauer *et al*., 2012) and resistance exercise (Rantalainen *et al*., 2011; Nilsson *et al*., 2012; Evans *et al*., 2012). However, the type, intensity and duration of exercise needed to bring about an osteogenic effect warrant further investigation.

#### 2.3.2.1. Mode

The mode of exercise has also been shown to mediate the osteogenic response, with significant differences shown in weight and non-weight bearing exercise (Greene *et al.*, 2012). Both weight and non-weight bearing exercise involves muscle contractions, which, along with gravitational loading, impact on bone to produce a net mechanical load. It was previously thought that the loading created by the muscular contractions could produce significant amounts of bone strain thus, causing an osteogenic effect (Burr *et al.*, 1996). This has led to the belief that sports of a minimal or non-weight bearing nature (*e.g.*, swimming, cycling) may be of benefit to bone health. However, recent reports using HR-pQCT have shown the effects of muscular strength to be minimal in comparison to mechanical loading in relation cortical thickness at the distal tibia (Schipilow *et al.*, 2013). This may explain why cross sectional studies investigating swimmers (Nikander *et al.*, 2006) and water polo players (Greene *et al.*, 2012) showed no differences in several bone characteristics including, tibial total cross sectional area, cortical density and cortical thickness when compared an inactive Control population. Conversely, gymnasts and track and field athletes had significantly greater bone characteristics including, significantly increased total CSA and trabecular density at the epiphysis and significantly increased cortical density and area at the diaphysis of the tibia, in comparison to water polo players and sedentary Control participants (Greene *et al.*, 2012), indicating that mechanical loading generated through impact is of paramount importance for bone accrual.

It has been shown that sports requiring a high magnitude of loading and irregular movement patterns consistently produce greater osteogenic effects than those eliciting low impacts and necessitating regular movement patterns (Greene *et al.*, 2012, Weidauer *et al.*, 2012, Rantalainen *et al.*, 2011, Nilsson *et al.*, 2012). Adult males who regularly participate in football were shown to have increased cortical BMD, cross sectional area, circumference and

thickness of the tibia in comparison to age matched participants who regularly take part in resistance training (Nilsson *et al.,* 2012). The anabolic bone response to football participation is thought to be due to the high magnitude, frequency and multiple directional movements that football training and match play necessitate (Vicente-Rodriguez *et al*., 2003). This is substantiated by Wilks *et al.* (2009), who showed running speed to be related to volumetric BMD and cortical area in groups of masters track athletes. pQCT measurements were taken from the tibia in sprinters, middle and long distance runners, race walkers and an age matched Control population. Bone characteristics were related to running speed in a descending order, sprinters being the largest followed by middle and long distance runners, race walkers and Control participants. Increases in bone strength (Ferry *et al.*, 2011), BMC (Morgan *et al.,* 2011), BMD (Ferry *et al.,* 2012), cortical CSA, circumference and thickness (Nilsson *et al.,* 2012) have been shown in recreational football players compared to sedentary Control populations in cross sectional studies. Prospective studies have also shown football participation to increase BMD (Helge *et al.*, 2010, Krustrup *et al.,* 2010) and BMC (Vicente-Rodriguez *et al.,* 2004). Despite this, stress related bone injuries occur in some elite footballers (Fredericson *et al.,* 2006), suggesting that intrinsic and extrinsic factors (Please see sections 2.5 and 2.6) can influence an individual's bone response.

#### 2.3.2.2. Duration and Intensity

Training duration and intensity are important factors when considering the osteogenic effects of habitual exercise on bone, as human studies using exercise interventions of various exercise durations and intensities have shown anabolic bone adaptation (Evans *et al.,* 2012, Lester *et al.,* 2009, Nilsson *et al.,* 2012). However, the specific effects that exercise intensity, in terms of magnitude of loading and cardiovascular strain, has on bone structure and health

are still not fully elucidated. Sports that require high intensity training and match play seem to correspond with greater bone characteristics in comparison to sports that do not have this type of intensive training routine (Greene *et al.,* 2012). It is uncertain whether different intensities of habitual exercise consisting of equal mode and duration cause divergent effects in bone response. Problems quantifying exercise intensity in a field setting and difficulties prescribing the same relative intensity for different individuals make the effects of exercise intensity challenging to explore. Despite this, it has been shown that site specific osteogenic effects occur following 13 weeks of aerobic or combined aerobic and resistance exercise in previously sedentary female volunteers (Evans *et al.,* 2013). Little is known, however, in relation to other forms of exercise and different populations.

The effects of exercise on bone are not always positive; volumetric BMD is either not affected by or has been negatively associated with habitual exercise (Nilsson *et al.,* 2012; Weidauer *et al.*, 2012, Rantalainen *et al.,* 2011). A reduction in exercise-induced remodelling may explain the high BMD values seen in sedentary groups in comparison to active populations. A sedentary lifestyle results in less remodelling cycles occurring, thus older and therefore more dense (Schoenau *et al.,* 2002) bone is more plentiful in an inactive population. Conversely, participation in physical activity with a greater magnitude of mechanical loading has been shown to cause damage to the bones micro-architecture resulting in the occurrence of stress fracture injuries (Bennell *et al.,* 1999). As bone remodelling increases, to repair the inflicted micro-damage, the bones cortical porosity increases (Wilks *et al.,* 2009) and density decreases, which can cause site specific weakness.

#### 2.3.2.3. Age

Habitual mechanical loading during pre-pubertal and pubertal stages has consistently been associated with current (Lorentzon *et al.,* 2005; Tobias *et al.,* 2007, Blinkley and Specker 2004) and future (Nilsson *et al.,* 2009) anabolic effects on bone characteristics. Physical activity during growth periods has been shown to increase bone accrual (Lorentzon *et al.,* 2005; Tobias *et al.,* 2007), cortical area (Lorentzon *et al.,* 2005) and BMD and work as a preventative mechanism against stress fracture injury (Tentorde *et al.,* 2013). Bone accrual gained by physical activity during growth periods has been preserved into adulthood even when regular exercise has been attenuated (Nilsson *et al.,* 2009; Tenforde and Fredericson 2011). Adults who ceased physical activity for an average of 6.5 years prior to bone assessment retained greater periosteal circumference and CSA than those who had never taken part in physical activity (Nilsson *et al.,* 2009). This evidence highlights puberty as a critical age in terms of long-term bone health.

#### 2.3.2.4. Training Status

Physical fitness has been shown to have an influence on bone characteristics and bone adaptation to physical activity. Low activity levels have been associated with lower cortical BMD, cross sectional area, circumference and thickness of the tibia when compared to habitually active populations regularly participating in weight bearing sports (Greene *et al.,* 2012; Nikander *et al.*, 2006; Nilsson *et al.*, 2012) and with increased risk of stress fracture injury in military recruits (Jones *et al.,* 2002). Conversely, well trained athletes in non-weight bearing sports, such as swimming and water polo have been shown to have a tibial total cross sectional area, cortical density and cortical thickness comparable to sedentary populations (Greene *et al.,* 2012; Nikander *et al.,* 2006). The effects of sudden increases in unaccustomed

exercise in already physically active participants are yet to be established despite being suggested as a key risk factor for stress fracture injury risk (Fredericson *et al.,* 2006).

#### 2.3.2.5. Site Specific Adaptations

Differences in bone size, density, thickness, and ultimately bone strength, are site specific and thus vary dependent on the scanned location (Nilsson *et al.,* 2012; Greene *et al.,* 2012). The inconsistencies in inter-study anatomical scan locations (*i.e.,* % of tibial length) make it difficult to compare studies that have measured different sites and assess if a hierarchical structure of sports associated with bone properties. Posterior, anterior, lateral and medial aspects of bone also show site specific differences in BMD (Rantalainen *et al.,* 2011; Evans *et al.,* 2012). These differences can be associated with the direction and magnitude in which the forces are applied (Nikander *et al.,* 2010). Females who participated in high impact (*e.g.,* jumping, hurdling) or intermittent high impact sports (*e.g.,* football, squash) had lower cortical volumetric BMD compared to sedentary Controls at the inner, outer and mid cortical tibia regions (Rantalainen *et al.,* 2011). As stress fractures commonly occur at the medial aspect of the tibia, information about this is pertinent. However, site specific analysis of pQCT results are not routinely conducted; therefore this area of research warrants further investigations.

The wide variety of populations used, and the range of scanning sites implemented make it difficult to compare studies, thus making accurate hierarchical assessments of osteogenic sporting activities very difficult to determine. While the adaptation of bone to suit its environment is key to bone health and injury avoidance, there is an important role for

exercise in the determination of long-term bone health. Knowledge of how exercise influences bone morphology in the short and long-term, and the mechanisms responsible for this will improve our understanding of how exercise influences bone turnover.

### 2.4. Stress Fracture Injury

Stress fracture injuries can be classified into two categories; insufficiency stress fracture and fatigue stress fracture. The former occurs as a consequence of pre-existing bone weakness and occurs when the bone is placed under ordinary strain. The latter is a result of the bone being placed under prolonged strain. Both types of stress fracture are the result of inadequacies in bone repair, leading to damage of the micro-architecture and the formation of micro-cracks (Pegrum *et al.,* 2012). Stress fractures differ from traumatic fractures due to the absence of above fracture threshold impact or loading causing the fracture. Stress fractures were first characterised in the 1800's and referred to as 'marching fractures' that manifested as foot pain and inflammation that occurred in soldiers following repetitive marching (Childers *et al.,* 1990). Theories have been proposed to explain the occurrence of stress fracture injury, from spasticity and spasm of the muscle (Deutschlander 1921) to impaired circulation (Solane 1936). The aetiology of stress fracture injury is not fully understood with multiple intrinsic and extrinsic risk factors being proposed (Table 2.2.). Stress fractures occur in the absence of an acute high magnitude traumatic impact and occur as a result of cumulatively repetitive below threshold loads encountered as a result of mechanical loading and/or muscular strain (Fyhrie *et al.,* 1998). Repetitive loading of sufficient magnitude can lead to damage or micro-cracks within the bone structure (Chapurlat and Delmas, 2009). If adequate rest periods are undertaken bone is able to self-repair or remodel, by the removal of old bone and the formation of new bone (Gefen and Neulander, 2007) (Figure 2.8.).

However, if adequate rest periods are not allowed and adequate bone repair is not under taken, more loading cycles occur and the micro-cracks can elongate and propagate, when more loading cycles are applied (Schaffler *et al.,* 1990). During the bone remodelling process, the porosity of the cortex increases, leading to weakening of bone due to secondary bone mineralisation taking place. This is closely followed by intra-cortical remodelling via osteoblastic activity, resulting in bone expansion at the expense of bone tissue volume (Eriksen and Langdahl, 1995). Under these conditions the bone is more liable to local stress and is at a greater risk of micro-damage accumulation and ultimately stress fracture injury (O'Brien *et al.,* 2005). Strength and stiffness of the bone can contribute to protection against crack propagation and ultimately fracture. Conversely, deficits in bone characteristics, such as low BMD (Wentz *et al.,* 2012), bone geometry and strength (Popp *et al.,* 2009) may increase the risk of stress fracture injury incidence. Micro-cracks may also have a protective function, as they allow for the dissipation of energy during the loading process (Dong *et al*., 2010). The process of bone remodelling to counteract micro-cracks is currently unclear, it has been suggested that two types of remodelling occur; background remodelling (occurring continually), and targeted remodelling (in response to micro-cracks) (Parfitt *et al.,* 1996). Studies conducted in rabbits have shown that resorption lacunae are present in areas subjected to micro-cracks, while no resorption occurs in unaffected areas (Hedgecock *et al.*, 2007), which substantiates this theory.

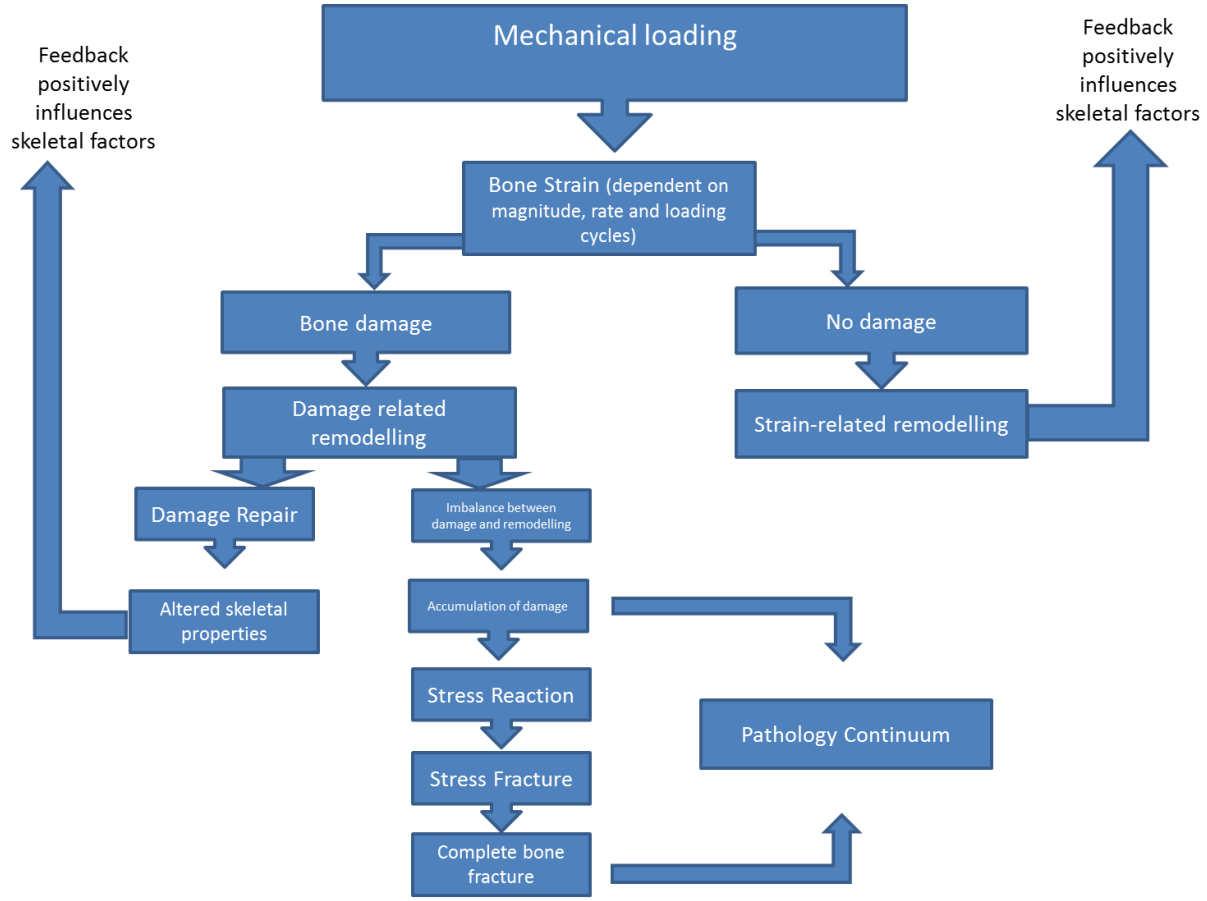


Figure 2.8. Schematic depicting how mechanical loading can lead to positive skeletal adaptations or fracture.

Stress fracture injuries are relatively common in elite athletes, accounting for up to 20% of all clinically reported sports injuries (Bennell *et al.*, 1996; Fredericson *et al.*, 2006). Stress fractures have been reported at multiple anatomical sites, although weight bearing bones are at a greater risk due to the pathophysiology of injury. With this in mind, it is not unexpected that the tibia is the most common site of stress fracture injury in athletic and military populations (Rauh *et al.*, 2006; Gaeta *et al.*, 2005; Arendt *et al.*, 2003). Metatarsal, pelvis, ulna, navicular, fibula, rib, neck of femur and lumbar spine are also sites in which stress fracture injuries have been reported (Iwamoto *et al.*, 2003), with the location of stress fracture seemingly common to particular sports. For example, stress fracture injury of the rib appears to occur almost exclusively in rowers, due to the unique motion that is involved in the stroke component of the rowing action (Dragoni *et al.*, 2007). Sprinters experience stress fracture

injuries most commonly in the metatarsal compared to middle and long distance runners who most regularly encounter tibial stress fractures; the differences are most likely due to the different loading patterns these sport necessitate (Bennell *et al.,* 1996). The causes of stress fracture are multi-facetted and arise from extrinsic influences on intrinsic processes relating to bone metabolism and overall health, which will be discussed in the following sub-sections.

### 2.4.1. Extrinsic Factors

A high volume of training is vital to stress fracture injury development, which is demonstrated by stress fracture occurrence being almost exclusively limited to elite athletes and military personal. The mode of exercise, and more specifically the magnitude and duration of loading patterns, are key to the development of stress fracture occurrence (Warden *et al.,* 2006). Unaccustomed training may result in a disruption of the bone remodelling balance and may alter the anatomical site loaded, along with a change in the number of remodelling cycles. A cause and effect theory of unaccustomed training leading to stress fracture incidence is difficult to prove due to the lack of data detailing the amount of athletes that do not suffer from a stress fracture injury after changes in their training protocol. Despite this, there are cases of athletes reporting changes in training prior to stress fracture injury. Also, there is a high stress fracture injury prevalence in newly inducted college athletes (Goldberg and Pecora, 1994) and military recruits (Milgrom *et al.,* 1985; Beck *et al.,* 1996).

### 2.4.2. Intrinsic Factors

The intrinsic factors implicated in stress fracture risk centre around metabolic, endocrine and genetic variations. Female athletes are at a greater risk of stress fracture in comparison to

males (Brunet *et al.*, 1990), possibly due to changes in hormonal status associated with heavy training that result in amenorrhea, which is common in female athletes (Nattiv *et al.,* 2007) and is often also associated with nutritional deficiency and low BMD (Loud *et al.,* 2005). These factors independently, or in combination, could lead to increased stress fracture injury susceptibility (Bennell *et al.,* 1999), although some reports suggest population specific factors may also be implicated (Warden *et al.,* 2006; McCormick *et al.,* 2012). Specific bone phenotypes have also been reported to be associated with stress fracture injury risk; cross sectional studies have shown that lower BMD (Wentz *et al.,* 2012; Pouilles *et al.,* 1989) and smaller cortical area (Popp *et al.,* 2009) are common in stress fracture sufferers. Prospective studies have also shown BMD to be important factors when considering stress fracture risk (Beck *et al.,* 2000; Lappe *et al.,* 2005). Despite being implicated in the pathophysiology of stress fracture injury (Pegrum *et al.,* 2012), the evidence for bone metabolism influencing stress fracture risk is not clear. Some studies have shown bone metabolism to be unrelated to stress fracture injury (Myburgh *et al.,* 1990; Bennell *et al.,* 1998; Valimaki *et al.,* 2005), although Murguia *et al.*(1988) did show a significant link between markers of bone resorption and stress fracture incidence. The reason for the lack of evidence may be the difficulty in determining bone metabolism in the days preceding stress fracture occurrence. As the majority of studies are carried out retrospectively, bone metabolism at the time of sampling could have little relation to bone metabolism at the time of actual stress fracture injury. Without conducting large, well-controlled, prospective studies regularly monitoring bone turnover and stress fracture injury incidence, the relationship between bone metabolism and stress fracture is likely to remain unproven.

Table 2.2. Examples of intrinsic and extrinsic risk factors associated with stress fracture injury.

| Risk Factor | Example References |
|---|---|
| *Intrinsic* | |
| Bone Phenotypes | |
| Bone Density | Wentz *et al.*, 2012 |
| Cortical Area | Popp *et al.*, 2009 |
| Bone Turnover | Murguia *et al.*, 1988 |
| Bone Mass | Warden *et al*., 2005 |
| Small Muscle Mass | Bennell *et al*., 1996 |
| Poor Flexibility | Hughes *et al*., 1984 |
| Prior Stress Fracture | Loud *et al.*, 2009 |
| Leg Length | Brunet *et al*., 1990; Bennell *et al*., 1996 |
| Female | Brunet *et al.*, 1990 |
| Eating Psychopathology | Duckham *et al.*, 2012 |
| Genetics | Chatzipapas *et al.*, 2009, Korvala *et al.*, 2010 |
| White Race | Brunet *et al.*, 1990 |
| Amenorrhea | Friedl *et al*., 1992, Duckham *et al.*, 2013 |
| | |
| *Extrinsic* | |
| Smoking | Reynolds *et al*., 1994 |
| Alcohol | Bennell *et al.*, 1996 |
| High Training Volume | Jones *et al*., 2002 |
| Muscle Fatigue | Bennell *et al*., 1996; Beck *et al*., 2000 |
| Unaccustomed Exercise | Goldberg and Pecora 1994 |
| Insufficient Prior Training | Gardner *et al.*, 1998 |
| High Magnitude Repetitive Exercise | Warden *et al.*, 2006 |
| Sport Played | Bennell *et al.*, 1999 |
| Calcium Intake | Nieves *et al*., 2010 |
| Vitamin D Intake | Lappe *et al*., 2008 |
| Physical Fitness | Jones *et al*., 2002 |
| Inappropriate Footwear | Finestone *et al.*, 1991 |
| Training Surface | Zahger *et al*., 1998 |

## 2.5. Genetics

### 2.5.1. Genetic Associations with Athletic Prowess, Physical Fitness and Health

The ‘‘nature or nurture’’ debate is commonly held in relation to sporting success and health attributes, with the inherited aspect of physical fitness, athletic prowess and health having been suggested for many years. Only in the last 10 years, with the advent of the human gene map for performance and health-related fitness phenotypes, and advances in genotyping

technology, have researchers been able to establish associations between an individual's genotype and their athletic ability, health and fitness (Bray *et al.,* 2009). It is now well established that both genotype and environmental factors influence physical traits, but the extent of the proportional contribution for distinct phenotypes remains a pertinent question. Currently the majority of research conducted centres around single SNPs. Gene-gene and gene-environment interactions are also thought to influence phenotypes, although this area of research is in its infancy. To date the predominant role of genetics in sport has been to try to determine or explain the performance capabilities of athletes. This is difficult to accomplish due to the multi-factorial nature of performance and the many intricate environmental variables involved. Physical fitness and numerous health related phenotypes have been demonstrated to have a large genetic element (Bouchard *et al.,* 1992). The identification of genes and SNPs that contribute to physical fitness has proved difficult, which may be due to each SNP contributing a small amount to the overall effect. The ever expanding human gene map for physical performance and health related phenotypes provides an example of this, currently containing 239 genes that are purported to influence fitness and health phenotypes (Roth *et al.,* 2011). The map includes various genes and a range of levels of investigation, including small cohorts from diverse populations, which may increase the risk of erroneous findings and could be the reason for the difficulty seen in replication. The lack of a quantifiable phenotype in some studies also raises questions in relation to the mechanistic role of how a particular gene influences health and fitness. Genetic susceptibility to injury risk may be easier to elucidate as less extrinsic variables are thought to be involved. This is an emerging area in sports medicine, as currently the overwhelming majority of the data on genetic associations with injury risk and adverse health conditions is focused on clinical populations, which are very rarely young and/or active.

### 2.5.2. Genetics and Injury

One of the major challenges for researchers investigating genetic associations with medical conditions, and an application of the recently completed human genome project, is to use the data collected to have a better understanding of the management and treatment of injury and assist with the prevention of such injuries and disorders. Information derived from genetic studies can be valuable in counteracting diseases, as seen by the recent development of pharmaceuticals as a result of genetic research (Padhi *et al.,* 2010). Several genes and SNPs may increase susceptibility to injury, but predisposition may not always lead to injury occurrence due to potential counteracting genes and the likely, polygenic nature of injury influencing gene expression. Epigenetic modification may also influence an individual's susceptibility to injury risk. In a sports medicine setting, very few injuries have been examined for genetic associations, although tendonopathy and concussion are two injuries that have been shown to have genetic associations. Variations in collagen type V alpha 1 (*COL5A1*) and tenaisan-C genes have been associated with tendonopathy (Mokone *et al.,* 2005, Mokone *et al.,* 2006), while concussion risk has been associated with apolipoprotein E in collegiate American footballers (Tierney *et al.,* 2010). Despite the large number of studies exploring genetic associations with bone phenotypes, there is a lack of literature investigating bone phenotypes in athletic populations.

#### 2.5.2.1. Genetics and Bone

It is now well established that genetic elements influence several bone phenotypes and are important in bone disorders, including osteoporosis (Paternoster *et al.,* 2010) and Paget's disease (Albagha *et al.,* 2010). Due to the prevalence of osteoporosis in the general population and the large economic cost to health providers, genetic studies investigating

osteoporosis risk factors are popular. Twin and family studies have shown that numerous bone phenotypes are highly genetic, namely; bone turnover (Garnero *et al.*, 1996), skeletal structure (Havill *et al.,* 2013) and fracture risk (Estrada *et al.,* 2012). The genes that are associated with different bone phenotypes are diverse as some phenotypes share genetic risk factors and some are unique to a particular phenotype. Both candidate gene and genome wide association studies (GWAS) have been used to try to determine genetic associations with bone phenotypes. The candidate gene approach relies on prior knowledge of the function of the SNP and knowledge of a biochemical pathway in which it can be hypothesised to affect the phenotype of interest. GWAS make no prior assumptions regarding the genes or SNPs of interest and utilise large sample sizes to detect modest effects. Few GWAS have been conducted in the sport and health setting, although GWAS have been extensively used for clinical exploration and can act as a hypothesis generation tool in order to select genes of interest for subsequent candidate gene studies. Due to the high number of SNPs studied in GWAS, a high level of significance is needed in order to allow for the multiple comparisons used in the analysis. This could lead to the suggestion that GWAS are too conservative and more targeted approaches would prevent type I errors. The level of significance needed also leads to the requirement for a large amount of participants in order to produce adequate power, which can increase the heterogeneity of the population, potentially leading to an increase in the amount of environmental confounding factors.

BMD is the phenotype predominately measured in genetic studies looking for associations with bone disease due to its relationship with fracture, ease of measurement and the evidence of a strong genetic component (Duncan and Brown, 2008). Currently 12 GWAS have detected 62 SNPs related to BMD at GWAS significance level ($p = 10x^{-8}$) (Estrada *et al.*, 2012), demonstrating the polygenic nature of BMD. The majority of genes identified by

GWAS have not been previously associated with bone phenotypes in candidate gene studies. A recent meta-analysis of GWAS consisting of 81,949 cases and 102,444 Controls showed SNPs within *RANK, RANKL, OPG, LRP5, SOST* and *Wnt* to be associated with BMD and osteoporosis (Estrada *et al.,* 2012). These findings have been replicated in other GWAS, suggesting genes involved in the *RANK/RANKL/OPG* signalling pathway (Paternoster *et al.,* 2010a; 2010b; Roshandel *et al.,* 2010; 2011) and *Wnt* signalling pathway (Medina-Gomez *et al.,* 2012; Zheng *et al.,* 2012) may be important in the mediation of bone phenotypes. Despite strong associations between BMD and risk of fracture (Leslie *et al.,* 2007), low BMD does not lead to fracture in all cases, leading to the hypothesis that other factors are involved in the pathophysiology of fracture.

#### 2.5.2.1.1. Genetics and Fracture

Fracture can be defined as a complete or incomplete break in bone as a result of an above threshold force and is a common injury encountered by a large proportion of the population (Donaldson *et al.,* 2008). Fracture risk can be heightened as a consequence of cumulative deterioration in bone strength and disturbances in bone remodelling (Nguyen *et al.,* 2007). Bone strength is multi-factorial; therefore BMD alone is not the only factor contributing to bone strength, suggesting other factors could lead to fracture. Fracture risk has a genetic element (Deng *et al.,* 2000; Zheng *et al.,* 2012) varying from 16%-46% depending on the type of fracture and the age of the population (Michaelsson *et al.,* 2005). The genetic component of fracture has been shown to decline with age (Michaelsson *et al.,* 2005), which coincides with increased susceptibility to falls through sarcopenia and impaired neuromuscular function (Gerontol *et al.,* 2012). As the majority of research into fracture risk is conducted in older individuals (50 y +) this may have underestimated the genetic

component. Many genes have been associated with fragility fracture at various sites, although no definitive genes or SNPs have been established. Several genes and SNPs have been associated with a variety of bone phenotypes and these findings have been replicated in various populations (Paternoster *et al.,* 2010a; 2010b; Roshandel *et al.,* 2010; 2011; Medina-Gomez *et al.,* 2012; Zheng *et al.,* 2012). As the genes and SNPs associated with bone phenotypes have been shown to vary, it is difficult to ascertain the genes associated with fracture preposition due to the complex nature of fracture. Certain genes may only be associated with fracture at specific anatomical locations due to the different compositions of trabecular and cortical bone at different anatomical sites. Studies to establish genes associated with fracture risk and bone phenotypes have mainly used post-menopausal women due to the large fracture prevalence in this population. Unfortunately, this population also has a large degree of heterogeneity due to the varied lifestyle factors, including previous and current habitual activity, which is known to influence bone phenotypes. Military studies also have a degree of heterogeneity, albeit to a lesser extent, due to the diverse background and range of fitness levels from which military personnel are recruited. In a young elite athlete population, the heterogeneity of environmental factors is reduced. Despite participation in different sports, training time is similar and it is likely that all elite athletes would have participated in organised sport from a young age (exercise during pubertal stage is important for bone accrual, please see section 2.3.2.2.) in order to attain elite status. These factors make elite athletes an excellent population in which to examine genetic susceptibility to bone injury. Genetic associations with bone injuries in athletic populations have not been studied to the extent of elderly or osteoporotic cohorts due to the relatively lower prevalence of common fractures in sport. Stress fractures are relatively common injuries in athletic populations (Bennell *et al.,* 1996; Fredericson *et al.,* 2006), but currently there is an absence of research into the genetic associations of their manifestation.

#### 2.5.2.1.2. Genetics and Stress Fracture

Despite a lack of studies into the genetic associations with stress fracture there is evidence to suggest that stress fracture injury susceptibility may have a genetic component. A potential genetic contribution to stress fracture injury risk is supported by findings on the development of multiple stress fractures at various skeletal sites (Lambros and Alder, 1997), comparable stress fracture injuries occurring in monozygotic twins (Singer *et al.*, 1990; Van Meensal and Peers, 2010), high stress fracture recurrence rates (Gehrmann and Renard, 2006) and variation in stress fracture incidence in military recruits subjected to comparable training loads (Giladi *et al.,* 1986). Recent candidate gene studies have sought to investigate the genetic component of stress fracture in military populations with varying results. Associations were shown for SNPs and haplotype blocks within the vitamin D receptor (Chatzipapas *et al.,* 2009; Korvala *et al.,* 2010) and the androgen receptor repeat sequence (Yanovich *et al.,* 2011), although no associations have also been shown for the same SNPs in similar military populations (Cosman *et al.,* 2013, Valimaki *et al.,* 2005). The reason for the disparity may be because of the range of SNPs analysed, small samples sizes (stress fracture cases) and, in some cases, the lack of a polygenic approach. Due to the development of stress fractures being associated with disturbances in bone remodelling (Warden *et al.,* 2006), SNPs repeatedly associated with bone phenotypes in large scale studies are needed. As all previous studies investigating stress fracture injury incidence have used military personnel, studies involving alternative cohorts (*e.g.,* athletes, dancers) would be useful to attain further knowledge on the aetiology of stress fracture injury.

Impact fractures, cause a complete or incomplete break in bone as result of an above threshold force being applied. The risk factors for impact fractures have some similarities to

stress fracture. Like impact fractures, a family history of a stress fracture is shown to increase risk (Loud *et al.*, 2007), as does low BMD (Popp *et al.*, 2009). However, risk factors such as age and body mass that have ambiguity in stress fracture prevalence are established risk factors for impact fractures (Nguyen *et al.*, 2007). The SNPs associated with fracture incidence are prime candidate genes to be associated with stress fracture incidence due to the overlapping elements of their pathophysiology. However, common osteoporotic fracture sites (*e.g.*, the hip and vertebrae) that are often studied in GWAS of BMD and fracture, are not common sites of stress fracture. Therefore, doubts remain as to whether the SNPs involved in stress fracture incidence are the same SNPs as those associated with other bone phenotypes. Relative risk of stress fracture injury is not known and the lack of empirical evidence for SNPs associated with stress fracture make the selection of SNPs challenging when GWAS cannot be utilised.

#### 2.5.2.1.3. Genetic Mediation of Bone

Despite no genetic markers being repeatedly associated with stress fracture incidence in athletes, many genes and SNPs are candidates for influencing stress fracture injury risk due to previously published literature on genetic associations with fracture, BMD and biochemical markers of bone turnover (Table 2.3.). The genes previously associated with bone phenotypes broadly fall into two main signalling pathways; *Wnt* signalling and *RANK/RANKL/OPG* signalling pathways.

##### 2.5.2.1.3.1. Wnt Signalling

*Wnt* signalling pathways work in a complex network of proteins involved in embryo development, cancer and physiological processing (Lie *et al.*, 2005). *Wnt* signalling acts through at least three distinct pathways, all of which are involved in bone homeostasis (Piters

*et al.,* 2008). These pathways regulate the differentiation and activation of bone cells, particularly osteoblasts and their precursors (Williams and Insogna, 2009). Increased *Wnt* signalling results in increased bone formation, whereas diminished *Wnt* signalling results in a decrease (Krishnan *et al.,* 2006). The three main pathways are the canonical pathway (Figure 2.9.), non-canonical planar cell polarity pathway, and the non-canonical *Wnt*/calcium pathway. All are initiated by *Wnt* ligands binding to a complex receptor composed of members of the frizzled gene family and low density lipoprotein receptor-related proteins (*LRP5* and *LRP6*). In the canonical pathway (Figure 2.9), *Wnt* binding to its ligands inhibits GSK-3β phosphorylation of β-catenin, allowing its accumulation in the cytoplasm. β-catenin then enters the nucleus where it can associate with the lymphoid enhancing factor (Lef)/T cell factor (Tcf) transcription factors to regulate gene expression (Case and Rubin, 2010). Both non-canonical pathways act without causing the accumulation of β-catenin. After initial activation, the non-canonical planar cell polarity pathway recruits the phosphoprotein Dishevelled (Dsh), which is ultimately involved with the regulation of the cytoskeleton. The non-canonical *Wnt*/calcium pathway also recruits Dsh and activates Dsh proteins causing downstream calcium release, which, in turn, activates calcineurin and CaMKII leading to an inhibition of the canonical pathway (Komiya and Habas, 2008). The *Wnt* signalling pathway can be inhibited by the binding of sclerostin (*SOST*) and Dickkopf (*DKK*) proteins to *LRP5* and *LRP6* thus preventing *Wnt* ligand association, which subsequently restricts downstream bone formation (Kawano and Kypta, 2003). *Wnt* signalling may also play a role in the regulatory response to mechanical loading (Robinson *et al.,* 2006). Fluid flow shear stress results in increased GSK-3β phosphorylation, β-catenin translocation and changes in expression of β-catenin target genes, such as *SOST* and *DKK* (Bonewald *et al.,* 2008).

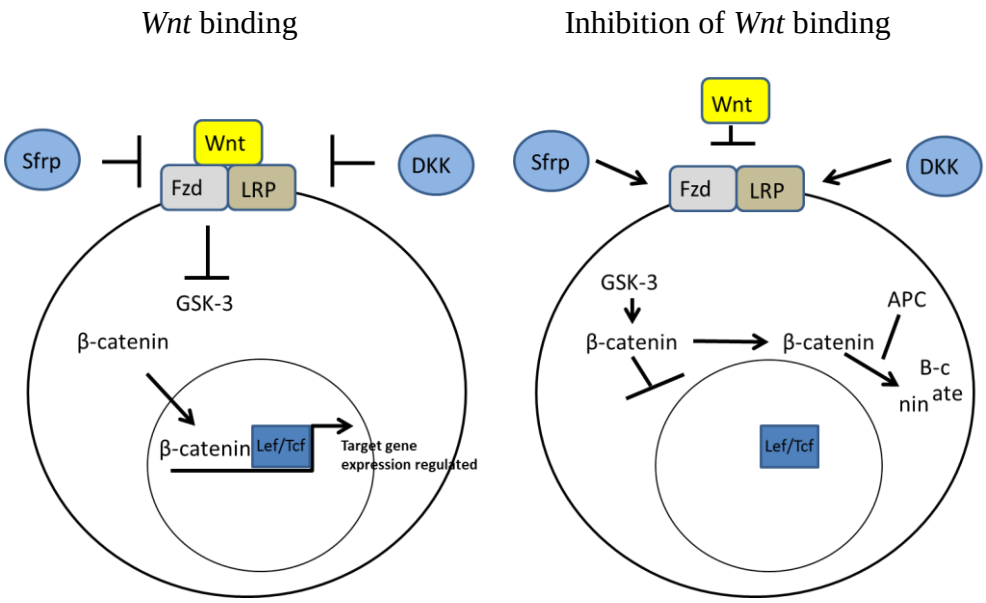


Figure 2.9. The canonical *Wnt* signalling pathway (adapted from Issack *et al.*, 2008). *Wnt* proteins bind to low-density lipoproteins receptor related proteins (LRP) and receptors made-up from the frizzled gene family. This occurs in the absence of secreted frizzled-related proteins (Sfrps) and Dickkopf (DKK) proteins which can bind to *Wnt* proteins and LRP, which effectively blocks *Wnt* signalling. When Wnt binding occurs, glycogen synthase kinase-3β (GSK-3) is inhibited and β-catenin is not phosphorylated. This allows β-catenin to bind with lymphoid enhancing factor (Lef)/T cell factor (Tcf) transcription factors to influence subsequent gene expression. In the absence of *Wnt* signalling, β-catenin is phosphorylated by GSK-3 and its degradation is facilitated by APC protein (APC).

Allelic variations in some of the genes involved in the *Wnt* signalling pathway (*LRP5/Wnt/SOST*) have been associated with bone phenotypic variations, including BMD and fracture risk (Table 2.3.) (Kato *et al.*, 2002; Medina-Gomez *et al.*, 2012; Zheng *et al.*, 2012; Balemans *et al.*, 2001).

Table 2.3. Single nucleotide polymorphisms (SNPs) selected for analysis into association with stress fracture injury in elite athletes. RS identification numbers are shown followed by location on the chromosome, the nearest gene and SNP name where applicable. Minor allele frequency (MAF) is also shown along with evidence of a metabolic function and evidence of a functional effect on bone. RS # written in **bold** were only genotyped in studies 2 and 4.

| RS # | location | Gene | SNP | MAF | Function | Bone related phenotype evidence |
|---|---|---|---|---|---|---|
| rs2230912 | 12q24.3 | *P2X7R* | Gln460Arg | 17% | Variant allele associated decrease in calcium influx (Cabrini et al., 2005). | Decrease in femoral neck BMD (Jørgensen et al., 2012) and osteoporosis (Wesselius et al., 2012). |
| rs208294 | 12q24.3 | *P2X7R* | His155Tyr | 43% | Variant allele associated with an increased receptor functionality and Calcium influx (Cabrini et al., 2005) | Decrease in femoral neck BMD (Wesselius et al., 2013) and aBMD in women (Hustead et al., 2012). Men homozygote for the variant allele show increases in BMD (Hustead et al., 2012). |
| rs1653624 | 12q24.3 | *P2X7R* | Ile568Asn | 1% | Variant allele shows decrease in *P2X7R* expression and cell trafficking and functionality (Wiley et al., 2003). | Variant allele shows increased fracture incidence (Ohlendorff et al., 2007). |
| **rs3751143** | 12q24.3 | *P2X7R* | Glu496Ala | 17% | Variant allele leads to impaired protein-protein interactions (Gu et al., 2001). Impairment of ATP-mediated immune responses and the release of *IL1* and *IL18* (Saunders et al., 2003). | Increased fracture incidence (Ohlendorff et al., 2007) and lower BMD (Wesselius et al., 2012, Husted et al., 2012). |
| **rs1718119** | 12q24.3 | *P2X7R* | Ala348Thr | 39% | Influences pore formation and variant allele shows an increased response to low concentrations of BzATP (Sun et al., 2010). | Increased BMD values (Ohlendorff et al., 2007) as well as reduced fracture risk (Jørgensen et al., 2012). Osteoporosis protection (Wesselius et al., 2012). |

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|---|---|---|---|---|---|---|
| rs3736228 | 11q13.4 | *LRP5* | *Ala1330Val* | 13% | *Ala1330Val* variant results in a functional difference of the *LRP5* protein, *Wnt*-signalling capacity decreased in the variant allele (Kiel et al., 2007). | Key determinant of bone mass (Gong et al., 2001). Activating point mutations result in high bone mass (Van Wesenbeeck et al., 2003). |
| rs1544410 | 12q13.11 | *VDR* | *BsmI* | 42% | Polymorphism function is largely unknown. However, regulation of vitamin status, protein-protein interactions and mediation of cell transcriptional factors are suggested functions (Uitterlinden et al., 2004). | Significantly associated with stress fracture risk (Chatzipapas et al., 2009). |
| rs731236 | 12q13.11 | *VDR* | *TaqI* | 43% | Polymorphism function is largely unknown. However, regulation of vitamin status, protein-protein interactions and mediation of cell transcriptional factors are suggested functions (Uitterlinden *et al*., 2004) | Significantly associated with fracture incidence individually (Nguyen *et al*., 2005) and in association with other VDR SNPs (Langdahl *et al*., 2000,Horst-Sikorska *et al*., 2005,2007) |
| rs7975232 | 12q13.11 | *VDR* | *ApaI* | 44% | Polymorphism function is largely unknown. However, regulation of vitamin status, protein-protein interactions and mediation of cell transcriptional factors are suggested functions (Uitterlinden *et al*., 2004) | Significantly associated with fracture incidence individually (Nguyen *et al*., 2005) and in association with other *VDR* SNPs (Langdahl *et al*., 2000,Horst-Sikorska *et al*., 2005,2007) |
| rs10735810 | 12q13.11 | *VDR* | *FokI* | 34% | Transcription activation characteristics of the *VDR* protein (Gross *et al*., 1998). | Significantly associated with fracture incidence individually (Gennari *et al*., 1999) and in association with other *VDR* SNPs (Langdahl *et al*., 2000, Quevedo *et al*., 2008). Significantly associated with stress fracture risk (Chatzipapas *et al*., 2009). |

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|---|---|---|---|---|---|---|
| rs1801197 | 7q21.3 | *CTR* | | 26% | Inhibits bone resorption and stimulates urinary calcium excretion (Nakamura *et al*., 2001). Functioning related to inhibition of parathyroid hormone (Garfia *et al*., 2000). | Minor allele C and with the *VDR* C-A haplotype associated with a decrease in stress fracture prevalence (Korvala *et al*., 2010) and BMD (Braga *et al*., 2002). |
| **rs1877632** | 17q11.2 | *SOST* | | 31% | Binds to *LRP5* and *LRP6* preventing binding with frizzled protein and *Wnt* signalling, and thereby reducing bone formation (Kneissel *et al*., 2009). | Homozygotes for the variant allele are associated with higher lumbar spine vBMD explaining 0.89% of differences seen in vBMD (Yerges *et al*., 2009). |
| **rs3018362** | 18q22.1 | *RANK* | *TNFRSF11A* | 42% | Integral to the production of NF-κB (*RANK*). Facilitates differentiation in osteoclasts (Boyle *et al.,* 2003). Mutations cause Paget disease (Albagha *et al.*, *2010*). Mechanism not well understood, duplication mutations in the TNFRSF11A gene over activate the pathway that promotes osteoclast formation (Boyle *et al.,* 2003). | Variant allele increases susceptibility to fractures (Styrkarsdottir *et al*., 2008), associated with BMD (Lui *et al*., 2010, Paternoster *et al*., 2010). |
| **rs1021188** | 13q14 | *RANKL* | *TNFRSF11* | 24% | Prominent in osteoclast differentiation (Boyle *et al.,* 2003). Exact function of the SNP is not known. | Associated with cortical bone density, endosteal circumference and cortical thickness. More prominent in males than females. Minor allele associated with higher circulating levels of free *RANKL* (Paternoster *et al*., 2010). |
| **rs9594738** | 13q14 | *RANKL* | *TNFRSF11* | 32% | Prominent in osteoclast differentiation (Boyle *et al.,* 2003) . Exact function of the SNP is not known. | Variant allele increases risk of low BMD and osteoporotic hip fracture (Styrkardottir *et al*., 2008, Paternoster *et al*., 2010). |

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|---|---|---|---|---|---|---|
| rs4355801 | 8q24 | *OPG* | *TNFRSF11B* | 30% | Osteoprotegerin (*OPG*) acts as a decoy receptor for RANKL inhibiting the binding process leading to the prevention of osteoclast precursor development into mature osteoclasts. Exact function of the SNP is not known. | Variant allele had an increased risk of osteoporosis and osteoporotic fractures (Styrkarsdottir *et al*., 2008). Lower BMD of the lumbar spine and for femoral neck (Richards *et al*., 2008). |
| rs7041 | 4q12-q13 | *GC* | *Glu416Asp* | 30% | Stimulation of osteoclast activity, associated with vitamin D level (Fang *et al*., 2009). | As part of a haplotype with the Thr420Lys, has been associated with lower BMD and increased fracture risk (Ezura *et al*., 2003, Lauridsen *et al*., 2004). Haplotype of Vitamin D binding protein (DBP) together with osteoporosis risk allele from *VDR* and or low calcium intake is associated with fracture risk (Fang *et al*., 2009). |
| rs4588 | 4q12-q14 | *GC* | *Thr420Lys* | 21% | Stimulation of osteoclast activity, associated with (25(OH)D) level (Fang *et al*., 2009). | As part of a haplotype with the *Glu416Asp*, *Thr420Lys* has been associated with lower BMD and increased fracture risk (Ezura *et al*., 2003, Lauridsen *et al*., 2004). Haplotype of *GC* together with osteoporosis risk allele from VDR and or low calcium intake is associated with fracture risk (Fang *et al*., 2009). |
| rs1800012 | 17q21.33 | *COL1A1* | | 11% | Increases in DNA–protein binding, transcription, and production of the *COLIA1* mRNA and protein are associated with *COLIA1* Sp1 SNP (Mann *et al*., 2001) | Associated with various bone phenotypes, including bone density, fragility fractures, postmenopausal bone loss, bone geometry , bone quality and bone mineralization (Ralston and Crombrugghe 2006) . Reduced yield strength of bone has been shown in heterozygotes for the variant allele (Uitterlinden *et al*., 1996). Homozygotes for the variant Sp1 SNP are associated with BMD and vertebral fractures (Ralston *et al*., 2006). Variant allele associated with a 3 fold increase in fracture incidence in pre-pubertal children (Blades *et al*., |

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|---|---|---|---|---|---|---|
| | | | | | | 2010) |
| rs16987491 | 19q13.41 | Kallikrein | *KLKN R35H* | 4% | Kinin agonists stimulate osteoclasts, however an exact role is yet to be defined (Lerner 1994). | Associated with BMD and stress fracture risk in unpublished data from collaborating group. |
| rs1799722 | 14q32.1 | Bradykinin receptor | *BDKRB2* | 37% | 9-amino acid peptide. Expressed by osteoblasts (Braun *et al*., 1996) | Associated with BMD and stress fracture risk in unpublished data from collaborating group. |
| SNPs genotyped in studies 2 and 4. | | | | | | |
| rs9594759 | 13q14 | *RANKL* | *TNFRSF11* | 42% | Prominent in osteoclast differentiation (Boyle *et al.*, 2003). Exact function of the SNP is not known. | Associated with bone turnover and BMD (Roshandel *et al*., 2010). Also, associated with BMD is in a separate cohort (Kemp *et al*., 2013). |
| rs2707466 | 7q12 | *Wnt16* | | 47% | *Wnt*16 acts as a ligand for members of the frizzled family of seven trans membrane receptors. Although the exact function of *Wnt*16 is not known, it is thought to be responsible for developmental signalling processes (Zheng *et al*., 2012). | Associated with cortical thickness in two separate cohorts and fracture risk (Zheng *et al*., 2012). |
| rs8065345 | 17q43 | *MAP3K14* | | 15% | The specific function is not known. However, MAP3K14 has been associated RANK activation (Malinin *et al*., 1997). | Associated with cortical area, cortical thickness. Also associated with lumbar spine BMD with BMD decreasing proportional to the number of risk allele carried (Roshandel *et al*., 2011). |
| rs13447445 | 7q22 | *IL6* | -174 G/C | 30% | -174 G/C is known to affect IL6 concentrations which are thought to | Risk of osteoporosis (Garnero *et al*., 2002, Ferrari *et al*., 2003, Mangana *et al*., 2008), |

| | | | |
|---|---|---|---|
| | | stimulate bone resorption through increased osteoclast differentiation (Fishman *et al*., 1998). | bone turnover (Fishman *et al*., 1998, Ferrari *et al*., 2001), BMD (Lorentzon *et al*., 2000, Garnero *et al*., 2002, Ferrari *et al*., 2003), fracture risk (Moffet *et al*., 2004) and bone accrual (Dhamrait *et al*., 2003) have all been associated with -174 G/C. |

#### 2.5.2.1.3.1.1. *Wnt*

19 *Wnt* ligands have currently been identified; some activate through the canonical pathway whereas others, such as *Wnt16,* use the non-canonical pathways (Garcia-Ibarbia *et al.,* 2013). Genetic association studies have recently shown variations in the gene that encodes *Wnt16* to be associated with BMD and wrist fracture (Medina-Gomez *et al.,* 2012; Zheng *et al.,* 2012), as well as osteoporosis (Estrada *et al.,* 2013). However, no associations with *Wnt16* were shown with hip fracture in a study investigating osteoporotic fracture cases (Garcia-Ibarbia *et al.,* 2013). The multitude of environmental factors that are associated with osteoporotic fracture (*e.g.,* activity levels, socioeconomic status; Jones *et al.,* 2004) and the decrease in genetic association with fracture and increased age (Michaelsson *et al.,* 2005) may be the reason for the disparities. A meta-analysis of 17 GWAS, consisting of 32,961 individuals, suggests that hundreds of variants with small effects, may contribute to BMD and potential fracture risk (Estrada *et al.,* 2012). Genes directly related to *Wnt* signalling were associated with BMD, as well as SNPs known to influence functions upstream of *Wnt* signalling (Estrada *et al.,* 2012).

#### 2.5.2.1.3.1.2. *SOST*

The *SOST* gene encodes sclerostin, a glycoprotein secreted primarily by osteocytes that inhibits *Wnt* signalling by binding to *Wnt* co-receptor *LRP5*, ultimately reducing bone formation (Williams and Insogna, 2009). Evidence has shown that serum sclerostin levels influence bone phenotypic variations (Kirmani *et al.,* 2012), leading to clinical trials being commissioned for the development of a sclerostin inhibitory drug as a therapy for the preservation of bone mass (Padhi *et al.,* 2010). Loss of function variations in *SOST* cause

high bone mass diseases such as Van Buchem's and sclerosteosis (Balemans *et al.,* 2001), characterised by an increase in bone formation and BMD (Van Lierop *et al.,* 2011). Variations in *SOST* SNPs have been associated with variations in BMD (Balemans *et al.,* 2001; Uitterlinden *et al.,* 2004). 0.89% of the variation in BMD at the lumbar spine was shown to be *SOST* SNP rs1877632 dependent (Yerges *et al.*, 2009). Allelic variation in *SOST* SNPs have also been related to serum sclerostin levels (Kuipers *et al.,* 2013), thus providing a potential mechanism for how it may influence bone phenotypes. Sclerostin concentrations have been theorised to be associated with mechanical loading (Fazeli *et al.,* 2013), making it a pertinent gene to explore in relation to elite athletes. Sclerostin levels have been shown to increase with high levels of habitual physical activity (Fazeli *et al.,* 2013), increase immediately following 120 min of treadmill running (Sale *et al.,* currently unpublished) and be greater in elite athletes competing in weight bearing sports in comparison to their non-weight bearing counterparts (Lombardi *et al.,* 2012). Conversely, increased physical activity was associated with lower sclerostin levels in humans (Amrein *et al.,* 2012), reduced *SOST* mRNA expression in the 24h post exercise (Robling *et al.,* 2007) and decreased *SOST* expression in animal models (Robling *et al.,* 2008). The disparity in the findings may be due to the intensity of mechanical loading, as the effects of high intensity loading (Fazeli *et al.,* 2013) were shown to differ from moderate intensity loading (Palombaro *et al.,* 2005). The time at which blood samples were taken may have also impacted upon the data, Sale *et al.* (currently unpublished) show that despite an initial significant increase from baseline following exercise, sclerostin levels returned to baseline 60 min post exercise. However, it should be noted that this was not compared to a non-exercising Control group and therefore, although currently unexplored, the possibility of variations in sclerostin due to natural circadian rhythm remains a possibility.

*SOST* expression/sclerostin regulation may be the mechanism by which bone formation is reduced during disuse. Unloading induces osteocyte and osteoblast apoptosis (Aguirre *et al.,* 2006) and inhibits osteoblast differentiation (Grano *et al.,* 2002). Deletion of the *SOST* gene caused insensitivity to unloading in murine models (Lin *et al.,* 2009). *SOST* expression changes in both loading and unloading conditions showing that *SOST* is regulated by the loading environment. The role of sclerostin involvement in mechanotransduction and ability to modulate anabolic effects of exercise is further established by sclerostin being inversely associated with BMD in non-athletes and positively associated with BMD in athletes (Fazeli *et al.,* 2013).

#### 2.5.2.1.3.1.3. *LRP5*

Low density lipoprotein receptor-related protein 5 (*LRP5*) is an integral requirement for mechanically induced bone formation through the *Wnt* signalling pathway. *LRP5* and *LRP6* act as co-receptors, binding to *Wnt* ligands to initiate the *Wnt* signalling pathway (Krishnan *et al.,* 2006). This initiation is inhibited by sclerostin acting as a decoy receptor and binding to *LRP5* and *LRP6,* preventing *Wnt* recruitment (Li *et al.,* 2005, Semenov *et al.,* 2005). *LRP5* mutations affect bone formation by osteoblasts, principally by altering *Wnt* signalling through the canonical β-catenin pathway (Ferrari *et al.,* 2005). Loss of function *LRP5* mice were characterised by low bone mass (Kayo *et al.,* 2002), bone weakening disorders (Kato *et al.,* 2002) and an inability to produce osteogenic responses to mechanical loading (Sawakami *et al.,* 2006). In contrast to this, mice possessing the gain of function alleles for *LRP5* have increased BMD, bone strength and resistance to fracture (Babij *et al.,* 2002). In humans, osteoporosis risk and fracture incidence have also been associated with allelic variation in SNPs in the proximity of the *LRP5* gene. This indicates that SNPs within the *LRP5* gene may

have a modulating role in the determination of bone phenotypes and supports the suggestion that *LRP5* and *Wnt* signalling are vital factors in mechanotransduction and bone accrual. Variations in *LRP5* SNPs may also mediate the bones response to physical activity as genotype has been shown to influence BMD at the lumbar spine in response to exercise. *Ala1330Val* is one of the most studied *LRP5* SNPs and has been associated with bone phenotypic responses, including peak bone mass (Saarinen *et al.,* 2007), osteoblast differentiation (Kato *et al.,* 2002), osteoblast/osteocyte apoptosis (Javaheri *et al.,* 2011) and BMD in a GWAS (Richards *et al.,* 2008). The rare allele of *LRP5* SNP *Ala1330Val* has been consistently associated with adverse bone phenotypes (van Meurs *et al.,* 2006, Saarinen *et al.,* 2007). Kiel *et al.* (2007) however, showed that individuals homozygous for the rare allele of *Ala1330Val* had increased BMD dependent on the amount of physical activity performed, whereas the opposite was apparent for common allele homozygotes whose BMD was inversely related to physical activity. Heterozygotes had a similar level of BMD irrespective of physical activity. This suggests that *Ala1330Val* may mediate the bone remodelling process in response to mechanical loading. Future studies need to replicate these findings as participant numbers for individuals homozygous for the rare allele were very low (n = 16). The amount and diversity of the phenotypes affected make it difficult to confirm the mechanism by which *Ala1330Val* may regulate bone phenotypes.

#### 2.5.2.1.3.2. RANK/RANKL/OPG

*RANK*, and its ligand, *RANKL*, members of the TNF superfamily, are integral to osteoclastogenesis (Boyce and Xing, 2008), as they stimulate osteoclast activation, formation and differentiation (Boyle *et al.,* 2003) (Figure 2.10.). *RANKL* is expressed on the surface of pre-osteoblastic stromal cells and binds to *RANK* on osteoclastic precursor cells. Macrophage

colony-stimulating factor (M-CSF) binding to the Fms receptor is needed for *RANKL-RANK* binding, as it mediates the amount of osteoclast precursor cells produced (Boyce and Xing, 2008). *RANKL* is essential for the differentiation of osteoclasts into multi-nucleated cells, their activation and longevity (Boyce and Xing, 2008). Osteoprotegerin (*OPG*) acts as a decoy receptor for *RANKL,* binding RANKL, leading to the prevention of osteoclast precursor development into mature osteoclasts, resulting in the subsequent attenuation of bone resorption (Boyle *et al.,* 2003). These factors in combination make the *RANK/RANKL/OPG* signalling pathway an important component in the regulation of bone turnover and of bone adaptation in response to exercise. Although the specific mechanisms of how SNPs within the *RANK/RANKL/OPG* signalling pathway regulate bone health remain unknown, several SNPs have been implicated in bone pathologies. BMD (Paternoster *et al.,* 2010; Styrkarsdottir *et al.,* 2008; Richards *et al.,* 2008), bone strength (Roshandel *et al*., 2011), bone turnover markers (Roshandel *et al.,* 2010) and osteoporotic fracture risk (Styrkarsdottir *et al.,* 2008) have all been associated with SNPs located in the proximity of the *RANK/RANKL/OPG* signalling pathway. Of the many SNPs associated, some have maintained association in replication cohorts and in various populations for BMD and fracture (Table 2.3.).

Osteoclast precursor differentiation (Boyce and Xing, 2008) is one mechanism of how SNPs from the *RANK/RANKL/OPG* signalling pathway regulate bone phenotypes, although due to the number of transcription factors and signalling pathways that are activated by *RANK/RANKL* interaction, the mechanisms are not well understood.

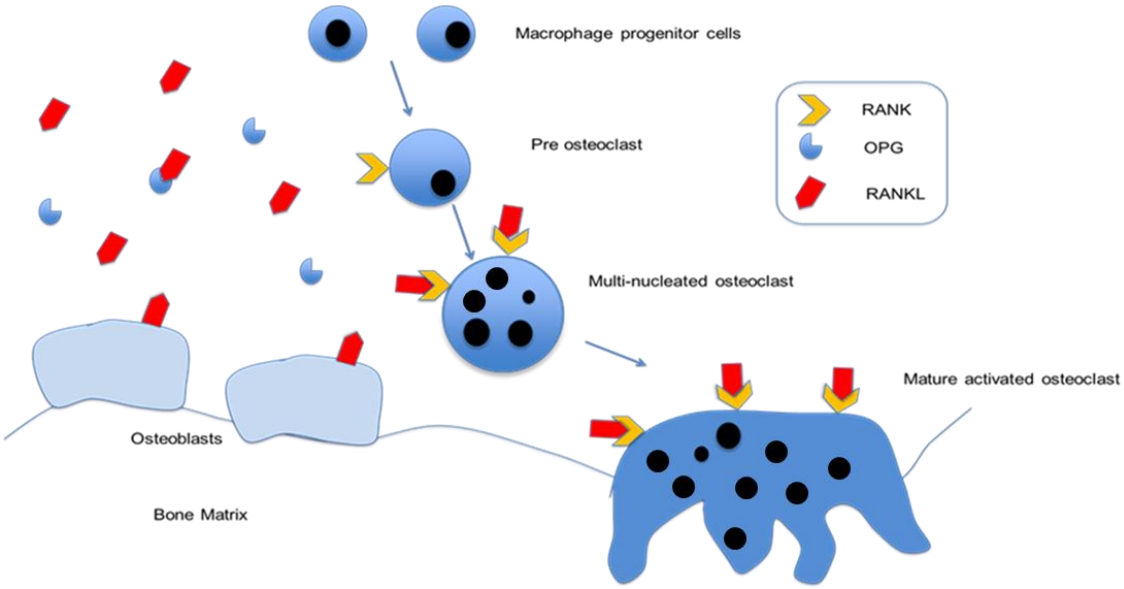


Figure 2.10. How the *RANK/RANKL/OPG* signalling pathway interacts in the differentiation of osteoclasts. Macrophage progenitor cells merge to become pre-fusion osteoclasts. Osteoblasts express RANKL, which binds to RANK on the cell surface, facilitating the formation of multi-nucleated osteoclasts. Osteoblasts can also express OPG, which binds to RANKL and inhibits pre-osteoclast differentiation.

#### 2.5.2.1.3.2.1. Vitamin D related genes

The genes that encode the vitamin D binding protein and the vitamin D receptor influence $1,25(OH)_2D$ status and therefore bone metabolism (Safadi *et al.*, 1999; Strugnell and Deluca 1997). The vitamin D endocrine system includes ergocalciferol (vitamin D 2), cholecalciferol (vitamin $D_3$), its active form, 1,25-dihydroxyvitamin D ($1,25-(OH)_2D$) and the Vitamin D Receptor (*VDR*) gene, of which there are four commonly studied SNPs; *BsmI*, *FokI*, *ApaI* and *TaqI* (Uitterlinden *et al.*, 2004).

##### 2.5.2.1.3.2.1.1. Vitamin D binding protein

The DBP binds to 25(OH)D and has been shown to prolong the circulating half-life of $1,25(OH)_2D$ in blood, regulate the delivery of 25(OH)D to target tissues and protect against

vitamin D deficiency in murine models (Safadi *et al.,* 1999). Conversely, *DBP* inhibits the activity of injected $1,25(OH)_2D_3$ in mice (Safadi *et al.,* 1999) causing doubt over its role. An inverse relationship between DBP concentrations and BMD has also been shown (Powe *et al.,* 2011). The reason for the contrasting findings may be due to functional vitamin D status being altered in individuals with similar 25(OH)D levels due to allelic differences in *DBP* SNPs. A variety of SNPs from the gene related to DBP (*GC*) have been associated with vitamin D concentrations and variations in bone phenotypes. The *GC* gene is important in the maintenance of bone homeostasis due to its known associations with BMD, bone metabolism (Eisman *et al.,* 1995) and its role in the downstream modulation of transcription factors related to calcium homeostasis (Strugnell and Deluca, 1997). Genetic variants in SNPs in the *GC* gene have also been associated with changes in $1,25(OH)_2D_3$ concentrations (Sinotte *et al.,* 2009; Carpenter *et al.,* 2013; Wang *et al.,* 2010) in a number of young adult populations (Gozdzik *et al.,* 2011) and osteoporosis risk in elderly Caucasians (Fang *et al.,* 2009).

#### 2.5.2.1.3.2.1.2. Vitamin D receptor

*VDR* is associated with $1,25(OH)_2D$ binding, which subsequently affects calcium absorption (Strugnell and Deluca 1997). Mutations in *VDR* cause vitamin D-resistant rickets, characterised by increased risk of fracture and genu varum (Kristjansson *et al.,* 1993), due to insufficient absorption of calcium and phosphate by the intestine rather than a direct influence on $1,25(OH)_2D_3$ concentrations (Ralston and de Crombrugghe, 2006). Although the *VDR* gene has been intensively studied, ambiguity exists in relation to its role within bone health and skeletal function. The inconsistencies could be due to a variety of factors including sample size, study cohort, failure to adequately control environmental factors and failure to sufficiently report the bone phenotype examined. McClung and Karl (2010) suggest that allelic variation in polymorphisms within the *VDR* gene can increase the concentration of

$1,25(OH)_2D$, which may facilitate bone health and therefore reduce the risk of bone injury. The mechanism by which this occurs is thought to be related to the binding of the *VDR* to $1,25(OH)_2D$, which induces absorption of calcium and phosphate for bone mineralisation and homeostatic metabolism (Korvala *et al.,* 2010). Many studies have shown *VDR* SNPs to be associated with aspects of bone health. *VDR* null mice have low bone mass characterised by hypocalcaemia, as well as elevated $1,25(OH)_2D_3$ levels (Malloy and Feldman, 2011). In humans, homozygotes for the F allele of the *FokI* SNP results in lower $1,25(OH)_2D_3$ concentrations in comparison to homozygotes of the f allele. This should be treated with caution, however, as the study participant numbers were low (n =81), particularly the ff group (n = 7). F allele homozygotes also exhibits a greater response to resistance training in terms of suppressed bone resorption (Tajima *et al.,* 2000). Similarly, the *BsmI* SNP has been associated with lower BMD (Thakkinstian *et al.,* 2004; Valdivielso *et al.,* 2006; Morrison *et al.,* 1994) and a haplotype block of *BsmI, ApaI* and *TaqI* has been associated with increased risk of osteoporosis (Thakkinstian *et al.,* 2004). *VDR* SNPs could also be involved in stress fracture prevalence as subtle changes in the bone structure can cause an area of weakness increasing susceptibility. Studies have suggested that SNPs within the *VDR* gene can cause a predisposition to stress fracture injury (Chatzipapas *et al.,* 2009; Korvala *et al.,* 2010). However, these studies have relied on relatively small cohorts (n=64 and n=192 respectively) therefore studies of replication cohorts need to be performed to substantiate these findings.

#### 2.5.2.1.3.2.2. *P2X7R*

The highly polymorphic purinergic *P2X7* receptors (*P2X7R*) are ligand-gated ion channels that are likely candidates to mediate bone phenotypic responses, given that previous studies have shown numerous functional SNPs cause differential functioning of osteoblasts,

osteoclasts and osteocytes (Gartland *et al.,* 2001; Ohlendorff *et al.,* 2007; Li *et al.,* 2005). ATP induced *P2X7R* activation causes distinct cellular alterations including, apoptosis and ultimately cell death (Ohlendorff *et al.,* 2007) as a result of membrane blebbing in osteoclasts (Panupinthu *et al.,* 2007) along with increased cell permeability in osteoclasts (Ohlendorff *et al.,* 2007) and osteoblasts (Garland *et al.,* 2001). *P2X7R* activation also stimulates the release of interleukin-1 alpha and interleukin-1 beta (Stokes *et al.,* 2010), which have been associated with increased IL-1 production and activation and subsequently with osteoclast formation and increases in bone resorption (Kim *et al.,* 2009). Studies using *P2X7R*-KO murine models have shown a decrease in bone mass (Ke *et al.,* 2005), a reduced inflammatory response (Labasi *et al.,* 2002) and a decrease in mechanical loading induced inter cell signalling (Li et al., 2005). The multitude of different *P2X7R* dependent cellular responses suggests a multi-dimensional role of *P2X7R* in bone remodelling. In response to mechanical loading, *P2X7Rs* are stimulated by extracellular ATP, which, in turn, activates osteoblasts causing increases in bone formation and induces apoptosis in osteoclasts, thus reducing bone resorption (Grol *et al.,* 2009). Bone cell differentiation and longevity, as well as mechanotransduction, may therefore have an element of *P2X7R* governance.

To date, 11 SNPs (gain and loss of function) within *P2X7R* have been associated with a functional effect on bone parameters. In humans, significant differences in bone loss (Jorgensen *et al.,* 2012; Gartland *et al.,* 2012), BMD at the hip and lumbar spine (Wesselius *et al.,* 2012; Gartland *et al.,* 2012), fracture risk (Ohlendorff *et al.,* 2007) and osteoporosis risk (Wesselius *et al.,* 2012; Husted *et al.,* 2012) have been attributed to various *P2X7R* SNPs (see Table 2.3.). SNPs within *P2X7R* can influence distinct functions, including pore formation and cell differentiation and longevity (Grol *et al.,* 2009). Several SNPs have been individually associated with various bone phenotypic alterations including lower BMD,

fracture risk and bone accrual (Wesselius *et al.*, 2012; Gartland *et al.*, 2012; Ohlendorff *et al*., 2007; Jorgensen *et al.,* 2012; Husted *et al.,* 2012). Due to the high level of linkage disequilibrium that exists, haplotype analysis has demonstrated the significance of several *P2X7R* SNPs with BMD, vertebral fracture and osteoporosis risk (Husted *et al.,* 2012; Jorgensen *et al.,* 2012).

### 2.6. Summary

Weight-bearing mechanical loading has a key role in the maintenance of bone health and can be used as a strategy to aid bone accrual. Despite this, negative effects of exercise on bone phenotypes have been reported. Due to the vast amount of data exemplifying the hereditary aspects of bone, genotype has been suggested as a mediator of bone responses. However, despite a large amount of research conducted into genetic susceptibility to adverse bone health, very little is known about the extent of the genetic mediation of bone turnover, bone accrual or stress fracture injury. The absence of any candidate SNP studies relating to stress fracture injury in elite athletes makes it unclear whether any genetic predisposition to such injuries occurs. The thesis will give further insight into this area.

# 3.0. General Methods

This Chapter describes the methodological development, biochemical and genetic analyses that were undertaken as part of this thesis. All studies had approval from the Nottingham Trent University Ethical Advisory Committee. Study 2 (Chapter 5) had dual approval by the Northampton National Research Ethics Service Committee and by the Nottingham Trent University Ethical Advisory Committee.

### 3.1. Methodological Development of Genotyping Method

In order to genotype a relatively large amount of samples (total number of samples collected n=640) for a substantial number of SNPs (total SNPs investigated n=26) in a suitable time frame, a cost effective, reliable, time effective, high throughput process was sought to ensure a realistic time frame was used for PhD completion.

Initially, restriction fragment length polymorphism (RFLP) was used. The first SNPs to be genotyped were Gln460Arg and Glu496Ala located on exon 13 within the *P2X7R*. Due to their close proximity, primers were designed that encompassed both restriction sites. 1µL of genomic DNA (~10ng), 0.5µL of forward primer (5'-AGACCTGCGATGGACTTCAC), 0.5µL of reverse primer (5'- GATTCTTGTGCCTCAGCCTC), 25µL of Mango Mix containing Mango *Taq* DNA polymerase, buffer, dNTPs and $MgCl_2$ (Bioline, London, UK) were mixed together with 23µL of water to form a 50µL reaction volume. The reaction was then denatured for 5 min at 94$^{o}$C, then thermocycled for 20 s at 94 $^{o}$C, for 5 s at 55 $^{o}$C and 60 s at 72 $^{o}$C, repeating 40 times, followed by a final 10 min at 72 $^{o}$C. Following PCR, the product was visualised via electrophoresis before subsequent restriction digest was carried out. AluI restriction enzyme (0.2µL) was added to 2µL of buffer, 10 µL of PCR product and 7.8µL of water, before reactions were incubated in a water bath for 2 hours at 37 $^{o}$C.

Following digestion, products were visualised on 3% agarose gel via electrophoresis. Despite the method being designed as a time effective way of genotyping, the immediacy of the bands made it difficult to visualise the results. Despite increasing the percentage of the gel, precipitation of the sample and experimenting with a polyacrylamide gel approach, results were not clearly visualised and consistent enough to rely on this method for the genotyping of a large cohort. As a consequence, genotyping using RFLP and restriction digest (using a single SNP) were investigated. This produced successful and reliable results and was used to genotype ~300 samples for Gln460Arg in the *P2X7R* gene. However, as this method was labour intensive and time consuming, it was decided that this would not be appropriate for the quantity of samples and number of SNPs that were identified to be genotyped in forthcoming studies.

Allele-specific PCR was considered and experimented with in order to find a more time efficient approach. This involved the design of a primer related to the polymorphic area and one mismatched primer. Each sample was amplified by PCR twice, once with the primer corresponding to the common allele and once with a primer corresponding to the variant allele. Visualisation of a band was taken as evidence of the allele being present; amplification of both samples related to heterozygotes while amplification on only one sample related to homozygotes for the variant or common allele. Despite this being a more time effective approach in comparison to RFLP, the need for two PCR reactions to be run per sample to define each allele limited the scope of this method for a large cohort.

In the continued effort to find a high throughput method of analysis Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) was investigated. This method is very high throughput with the potential of analysing 384 samples

in ~60 min, and can be used with the product resulting from mass extension PCR. Mass extension PCR comprises a single complementary base being added to the product in accordance with the sequence of the site of interest. MALDI-TOF MS is then used to determine the mass of the extended primer. As allelic variants have different molecular weights (*e.g.*, alleles A and T differ by 9Da whereas A and C differ by 24Da), the mass indicates the sequence and therefore the alleles present. The method comprised of: an initial PCR comprising of 1µL of genomic DNA (10ng), a biotinylated forward primer (5'-AGACCTGCGATGGACTTCAC) and reverse primer (5'-CAACCTCTGCCTCCCGGGTTC), 25µL of Mango Mix containing Mango *Taq* DNA polymerase, buffer, dNTPs and $MgCl_2$ (Bioline, London, UK) mixed together with 23µL of water to become a 50µL reaction volume. The reaction was then denatured for 5 min at 94$^{o}$C, then theromocycled for 30s at 68$^{o}$C, for 10s at 65$^{o}$C and 60s at 72$^{o}$C, repeating 35 times, followed by a final 10min at 72$^{o}$C. The resulting PCR product was then heated at 95$^{o}$C for 5min and then placed on ice. 40µL of PCR product was then added to 50µL of freshly washed streptavidin-coated magnetic beads, re-suspended in sodium chloride-sodium citrate buffer and mixed. The mixed solution was then placed in a magnetic stand and the supernatant was removed, the remaining beads attached to a single strand of DNA were then washed with double distilled water. Another PCR was then carried out: 1µL of PCR product was added to 1µL of each dNTP (100mM), 1µL of $MgCl_2$ (2mM), 2µL of buffer, 1µL of reverse primer that is aligned to the last base of the SNP (5'-CGCCTCCTTTCTAAGCAGCC), 0.1µL of Go Taq and 0.9µL of water to form a 10µL solution. The reaction was then denatured for 2min at 94$^{o}$C, then theromocycled for 10sec at 94$^{o}$C, for 60s at 55$^{o}$C and 60s at 72$^{o}$C, repeating 35 times, followed by a final 10min at 72$^{o}$C. Following PCR the product was heated at 90$^{o}$C for 1min and transferred onto ice before the mass extended supernatant was removed from the beads to be used in subsequent steps. The

mass extended product was desalted to remove impurities and added to 1µL of 3-hydroxypicolinic acid matrix, which was then spotted on a 384 well anchor chip. This was then analysed using MALDI-TOF MS (Bruker, Brenman, Germany).

Methodological problems were encountered, such as drying difficulties on the anchor chip and the peaks created on the MALDI-TOF output differed from the molecular weight expected (Figure 3.1.). The differing weights were presumed to be due to the presence of ion adducts which also carry a specific molecular weight. Due to the time constraints of the PhD process it was decided that this method development would not be practical given the applied focus of the programme, as such this method was not adopted.

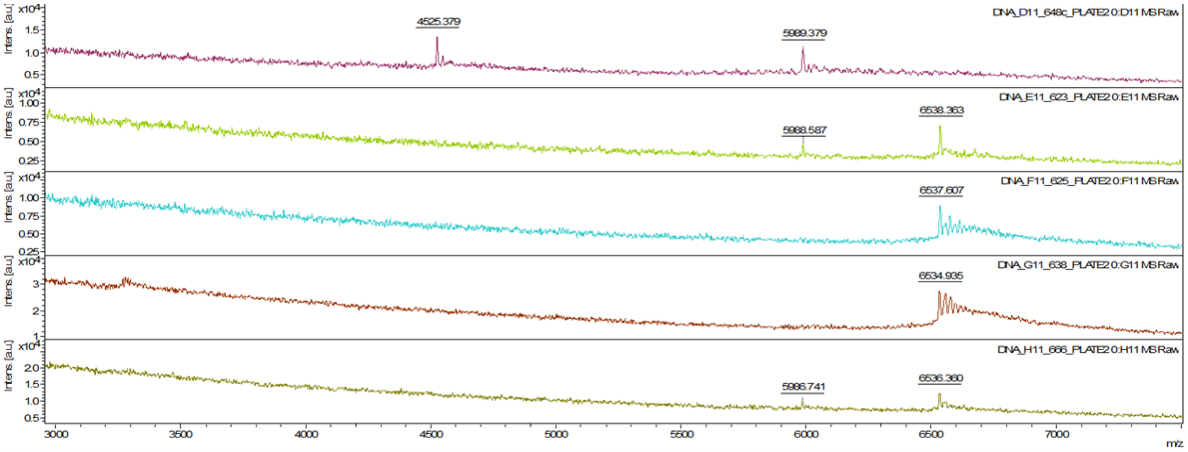

Figure 3.1. Graph output from the MALDI-TOF MS showing time (nanoseconds) and corresponding peak height.

As a time effective, cost effective method of analysis could not be found during the process of methodological development, RFLP methods of analysis were performed to ensure analysis was progressing. In conjunction with this, due to the cost and time efficiencies, samples were sent to LGC genomics for genotyping using Kompetitive Allele Specific PCR (KASP).

### 3.2. Saliva Collection

Four different DNA collection methods were investigated in Chapter 4 and based on the results shown, the following method was decided upon. Saliva was collected using commercially available saliva collection, preservation and isolation kits (Norgen Biotek Corp, Saliva DNA Collection kit Thorold, Canada). Participants were instructed not to eat, chew, drink or smoke 30 min prior to saliva collection. A water mouth rinse was also provided 10min prior to saliva collection. A collection funnel was mounted on the collection tube and the participant was advised to spit and/or dribble 2mL of saliva into the tube. The funnel was then discarded and the preservative ampoule was inserted into the collection tube and the screw cap connected. The tube was then shaken for 10 seconds to ensure the saliva and preservative ampoule were well mixed. The samples were then stored at room temperature and then subsequently frozen at -20ºC before further analysis.

#### 3.2.1. DNA Extraction

The collection tube was water incubated for 1h at 55$^{o}$C followed by gentle inversion and shaking. An aliquot of 500µL was then added to 10µL of proteinase K and mixed by vortex in a 1.5mL centrifugable tube before incubating for a further 30 min at 55$^{o}$C. An equal sample volume of isopropanol was added and mixed by inversion followed by 5min centrifugation at 13,000rpm. The supernatant was then removed and replaced by an equal sample volume of 70% ethanol followed by a final centrifugation for 1min at 13,000rpm. Following the removal of ethanol, each eppendorf was placed upside-down on a paper towel for >60min to remove any excess ethanol. Finally 100µL of TE buffer was added to rehydrate the DNA and was mixed by vortex for 10s and left overnight at room temperature to ensure complete rehydration. Samples were stored at -20$^{o}$C until subsequent analysis.

### 3.3. DNA Quantification

DNA was quantified using a NanoDrop spectrophotometer (NanoDrop technologies, Wilmington DE). The computer was switched on and the NanoDrop software uploaded. The nucleic acid option was selected and the spectrophotometer performed an initial clarification of wavelength. The pedestal arm was raised and 1.5µL of TE buffer was added to the spectrophotometer followed by a lowering of the pedestal arm. The 'blank' option was selected in order to run the blank baseline measurement. After this measurement was complete, the top and bottom of the measurement pedestal were wiped clean with a tissue. A sample volume of 1µL was then applied to the pedestal, the arm was lowered and the measure button was clicked. The subsequent display showed the concentration of DNA $ng \cdot \mu L^{-1}$ and quality was quantified using the OD260/OD280 and OD260/OD230 ratios as a measure of DNA purity against proteins (OD280) and salts and alcohol (OD230). This process was repeated for each sample with a 'blank' applied after every ten samples.

### 3.4. Database Coding

Each saliva sample collection tube (Study 2 and 3) or blood sample collection tube (Study 4) was encoded with a unique five digit code. Each code corresponded to a questionnaire (Study 3) or pQCT scan (Study 3). In Study 3, the page of the questionnaire representing the unique code was removed following data input and only the code was used in the remaining analysis ensuring complete anonymity for each participant.

### 3.5. Genotyping

Genotyping was conducted by two different methods:

Kompetitive Allele Specific PCR (KASP). DNA sample was mixed with target specific KASP forward and reverse primers. The two forward primers have competing tail sequences and a common reverse primer. KASP master mix containing two unique FRET cassettes that correspond to the tail sequences on each forward primer was then added. Following the initial period of denaturing the forward primer binds to the correct nucleotide primer, this enables the Taq polymerase to bind and elongate the strand. The common reverse primer then creates a complement to the opposite DNA strand. This creates an amplicon that incorporates the forward primer. A second round of PCR is then carried out in which the common reverse primer, which binds to the amplicon. During subsequent rounds of PCR, the FRET cassette gets incorporated into the amplicon; as the quencher is now removed the dye begins to fluoresce and increases in fluorescence as more copies are made. The level of fluorescence is then determined on a plate reader.

#### 3.5.1. *IL6* Genotyping

In studies 1 and 3 a 198 base pair fragment of the *IL6* gene was genotyped containing the *IL6* 174-G/C (rs1800795) SNP. This was amplified using PCR using 50µL reactions containing ~20ng of genomic DNA, 25µL of Mango Mix (Bioline, London, UK), 0.25µL of forward primer 5'- TGACTTCAGCTTTACTCTTTGT -3', 0.25µL of reverse primer 5'- CTGATTGGAAACCTTATTAAG-3' (Fernadez-Real *et al.*, 2000) and 23.5µL of ultra-pure water. After an initial denaturation at 94$^{o}$C for 10min, 35 cycles of 94$^{o}$C for 1min (denaturation), 55$^{o}$C for 35s (annealing) and 72$^{o}$C for 1min (extension) followed by 72$^{o}$C for 10min. 10µL of PCR product was added to 7.8µL nuclease free water, 2µL of buffer, 0.2µL

of SfaNI enzyme and 0.1µL of Bovine Serum Albumin (BSA) and digested for 37$^{o}$C for 4h. Following digestion the product was visualised via electrophoresis on a 2% agarose gel. Genotypes were identified according to their restriction sites. Homozygotes for the presence of the site (GG) elicited 140 and 58 bp products, heterozygotes (GC) 198, 140, 58 bp and homozygotes for the absence 198bp.

### 3.6. Statistical Methods

The statistics used are reported in the methodological section of each Chapter. P values of <0.05 were deemed significant.

# Chapter 4.0. Determination of the most Suitable Method for DNA Collection from Elite Athletes.

## 4.1. Introduction

Before starting the experimental trials a reliable, non-invasive and cost effective method of DNA collection needed to be confirmed to maximise the number of elite athletes that could be recruited. A high through-put, cost effective method in which DNA could be genotyped also needed to be selected in order to maximise the number of SNPs that could be genotyped.

The prevalence of stress fracture injury in athletes is 14 − 21% (Bennell *et al.*, 1996) and large cohorts are, therefore, recruited in order to identify any genetic associations. Elite athletes have demanding schedules involving high intensity training and match play, which often constitutes a high amount of travel. The method of DNA collection from this cohort needed to be reliable, robust and produce a high yield of genomic DNA as the opportunity to collect DNA from the same individual twice was unlikely. Blood drawing is the preferred method of collection due to high yield, reliability and the relative in-expense of collection and extraction procedures. However, collecting blood from a large cohort of athletes has a number of limitations: short-time periods for sample collection requiring numerous phlebotomists; trained phlebotomists need to be present for all samples, potentially eliminating athletes based outside of the researchers geographical area; aversion to needles may lead to participants refraining from participation; the storage conditions of blood are not conducive to transportation.

For these reasons, DNA collection via saliva and buccal cells has become popular in large epidemiologic studies, as it provides a reliable non-invasive method of DNA collection (Carlson *et al.*, 2004). Several studies have had success in obtaining DNA from mouthwash protocols (Andrisin *et al.*, 2002; Lum and Marchand 1998), cytobrushes (Satia-Abouta *et al.*, 2002), fast technology for analysis (FTA) cards (McClure *et al.*, 2009) and custom

manufactured saliva collection kits (Rogers *et al.,* 2007). As such, a pilot study was conducted to evaluate the DNA yield, purity and success of use in polymerase chain reaction (PCR) and restriction digest protocols of DNA extracted from blood, a mouthwash protocol, the Oragene saliva collection device (Oragene™ DNA collection kit, DNA Genotek, Inc., Ottawa, Ontario, Canada) and the Norgen saliva collection device (Norgen Biotek, Thorold, ON, Canada). In a previous study at Nottingham Trent University (Hennis *et al.,* unpublished observations), (FTA) cards (GE Healthcare, Buckinghamshire, UK) were used for DNA collection. However, inconsistences in DNA yield that have been previously been reported (Hanson *et al.,* 2007) were apparent. Due to these inconsistences in yield, cytobrushes (Steinburg *et al.,* 2002) and FTA cards were not evaluated in this study.

### 4.2. Method

Eight male participants volunteered to give a blood sample, which would be used as a marker to compare with the other methods. Each participant also gave 3 separate saliva samples which were collected by three separate means (Oragene, Norgen and a standard mouth wash protocol (Lum and Le Marchand 1998). Ethical approval was granted by the Nottingham Trent University Ethical Review committee.

Blood samples were collected via venepuncture, DNA was extracted in accordance with manufacturer guidelines using Wizard DNA extraction kits (Promega, USA). Saliva samples (Oragene, Norgen) were collected in accordance with manufacturer guidelines. The mouthwash protocol was carried out in line with previous literature (Lum and Le Marchand 1998). The Oragene and Norgen protocol used a custom made collection decive and required a preservation liquid to be mixed with the collected saliva, while the mouthwash protocol

involved rinsing the mouth with a branded mouthwash, and collection of the spittle. All saliva collected was stored at room temperature for 2d prior to analysis to replicate potential study conditions.

DNA yield and quality were assessed using a NanoDrop spectrophotometer (Nanodrop technologies, Wilmington DE). OD260/OD280 and OD260/OD230 ratios were assessed as a measure of DNA purity against proteins (OD280), salts and alcohol (OD230). Values above 1.6 for OD260/OD280 and close to 1 for OD260/OD230 were favoured.

After purity and quantification analysis, DNA was amplified for *ACE* genotype using PCR. Primers - 5'-CATCCTTTCTCCCATTTCTC-3; 5'-ATTTCAGAGCTGGAATAAAATT- 3'; 5'-TGGGATTACAGGCGTGATACAG- 3' were used (Evans *et al.,* 1994). The PCR conditions were as follows: initial denaturation at 94ºC for 5min, with 30 cycles of 94ºC for 60s, and 55ºC for 60s, followed by 55ºC for 5min. PCR product was visualised via electrophoresis using 2% agarose gel. *ACE* was used due to previous experience of successful genotyping of this SNP in previous research projects.

### 4.3. Results

The results of the four different DNA collection and analysis methods are shown in Table 4.1. Five DNA samples collected using a standard mouthwash protocol were unsuccessfully extracted.

All collection methods yielded mean 260/280 ratios above the preferred 1.6 value. The 260/230 ratios were also in the preferred range for blood, Oragene and Norgen methods. All

samples collected via blood, Oragene and Norgen methods were successfully visualised via electrophoresis.

Table 4.1. Comparative results of DNA quality and quantity between blood and saliva collection methods: Mean (range)

| Method of DNA collection | Blood | Oragene | Norgen | Mouthwash |
|---|---|---|---|---|
| Samples (n) | 8 | 8 | 8 | 8 |
| Successful samples(n) | 8 | 8 | 8 | 3 |
| Mean Concentration (ug·ml) | 56.8 (27.2-117.7) | 77.1 (35.7-120.4) | 94 (17.2-208.7) | 22.9(0-95.5) |
| Mean 260/280 nm ratio | 1.7 (1.5-2.0) | 1.7(1.4-2.0) | 1.7 (1.4-1.9) | 1.8(1.4-2.0) *3 samples |
| Mean 260/230 nm ratio | 0.9(0.4-1.1) | 0.9(0.6-1.1) | 0.8 (0.6-1.0) | 2.2 (0.7-3.1) *3 samples |
| Successful genotyping % | 100 | 100 | 100 | 12.5 |

### 4.4. Discussion

DNA concentration, purity and genotyping success for blood and the custom manufactured saliva collection devices are comparable to previous research (Hanson *et al.*, 2007; Rogers *et al.*, 2007). The failure of the mouthwash protocol to produce results may be due to the storage of samples at room temperature for 48h prior to DNA extraction. This may have been due to the absence of a preservative solution that is added to the Oragene and Norgen collection devices. The prolonged room temperature storage time was added to the standard protocol in order to replicate sample collection from participants, which may be sent via courier or collected away from the facilities needed for analyses.

The results showed that both Oragene and Norgen collection devices are equal to blood collection in terms of yield, purity and success genotyping. As both collection devices

performed similarly, the preferred collection device was determined by practical considerations such as cost and availability.

In summary, commercially manufactured saliva collection and extraction kits (Oragene, Norgen) are feasible non-invasive alternatives to blood for DNA collection that needs to be taken in a field setting, when time is at a premium and a sufficient number of specialist phlebotomists are not available. Saliva collection by Oragene or Norgen kits has the potential to maximise the number of participants recruited, while not compromising or reducing the quality of DNA.

Following this study, Norgen saliva collection kits were used in studies 2 and 3 (Chapter 5 and 6) in order to maximise the number of participants willing to take part in the studies without compromising on DNA quality and yield.

# Chapter 5.0. Genotype Dependent Changes in Bone Phenotypes in Academy Footballers

## 5.1. Introduction

Long-term weight bearing exercise has a primarily anabolic effect on bone phenotypes (Please see section 2.3.2.). Despite the consistent demonstration of the anabolic effects of weight-bearing exercise on bone, there remain some uncertainties over the level of stimulus needed to cause bone adaptations and the mechanisms by which these adaptations occur. Physical activity during growth periods has been shown to cause changes in many bone characteristics and act as a preventative mechanism against stress fracture injury (Please see section 2.3.2.3.), thus making the adolescent population relevant in investigations into bone response to exercise. The osteogenic effects of football are greater than in other sports (Ferry *et al.*, 2013; Ferry *et al.*, 2011; Morgan *et al.*, 2011; Mudd *et al.*, 2006; Creighton *et al.*, 2001), most likely due to the high magnitude, frequency and multi-directional nature of the movements that football training and match play necessitate (Vicente-Rodriguez *et al.*, 2003). BMC (Morgan *et al.*, 2011; Vicente-Rodriguez *et al.*, 2004), areal BMD (Ferry *et al.*, 2012; Helge *et al.*, 2010; Krustrup *et al.*, 2010), cortical CSA, circumference and thickness (Nilsson *et al.*, 2012), as well as bone strength (Ferry *et al.*, 2011) have all been shown to be increased in recreational football players compared to sedentary Control populations, in both prospective and cross sectional studies.

Despite mainly anabolic findings, negative outcomes as a result of weight-bearing exercise have been reported. Collegiate athletes (football, volleyball, cross country running) showed a reduced volumetric BMD at the tibia when compared to sedentary individuals (Weidauer *et al.*, 2012). Similarly, elite footballers showed lower cortical BMD at the tibia compared to swimmers (Schipilow *et al.*, 2013). Although sport selection bias could be evident due to the cross sectional nature of previous studies, one reason for the differences is thought to be due to increased bone remodelling, caused by loading, leading to a delay in secondary

mineralisation (Seeman and Delmas, 2006). Factors such as rapid increases in training volume have also been implicated in negative effects on bone phenotypes, including stress fracture injury (Bennell *et al.,* 1999). The reasons for exercise eliciting both positive and negative changes to bone structural properties are multi-faceted and are likely to involve the mode, intensity and volume of exercise, as well as various intrinsic and extrinsic factors (Please see sections 2.4.2. and 2.4.3.). Indeed, there is a lack of literature investigating the specific aspect of training that is associated with bone structural characteristics, as previous studies are either cross-sectional (Ferry *et al.,* 2012; Helge *et al.,* 2010; Krustrup *et al.,* 2010) or introduce an intervention that is unaccustomed and increases exercise volume, intensity and duration (Dhamrait *et al.,* 2003; Evans *et al.,* 2013).

Despite the wealth of cross sectional studies on the long-term bone adaptations in association with habitual participation in various sports, there is a lack of information relating to the mechanisms that may regulate these adaptations. Genotype has been associated with bone turnover (Garnero *et al.,* 1995; Roshandel *et al.,* 2010) and bone structural adaptations (Havill *et al.,* 2013). It has been suggested that genotype may mediate the bone response to exercise and may explain some of the variability observed in bone adaptations (Please see section 2.5.). Despite evidence of genetic factors being associated with bone phenotypes, little is known about how genotype mediates the bone responses to increased training load.

The aim of the present study is to investigate whether genotype is associated with phenotypic bone adaptations, quantified by pQCT, in adolescent academy footballers following 12 weeks of increased football-specific training.

## 5.2. Method

### 5.2.1. Participants

First year, full-time academy footballers (n=94) were recruited through previously established relationships with Nottingham Trent University and by word of mouth from five full-time football academies. Participants were deemed eligible for the study if they were aged ≥16 y, not currently taking any medication that influenced bone metabolism and had not received a joint replacement or prostheses. After reading the participant information sheet (Appendix 5.1.) and being fully briefed and having the opportunity to ask questions, participants signed a statement of informed consent (Appendix 5.2.), completed a pre-scan screening form (Appendix 5.3.) and completed a health screen questionnaire (Appendix 5.4.), which was scrutinised in order to confirm they met the inclusion/exclusion criteria. Participants detailed their playing position, age at which they first played competitive football and the amount of hours they spent training prior to full-time academy enrolment (Appendix 5.5.).

Following study completion, the respective coach and/or physiotherapist of the football club provided information related to each individual's training time, which included time missed as a result of injury for the previous 12 weeks. Fourteen players who received an initial scan were lost to the follow-up scan for a variety of reasons (Figure 5.1.), leaving a cohort of n=80 who completed both scans.

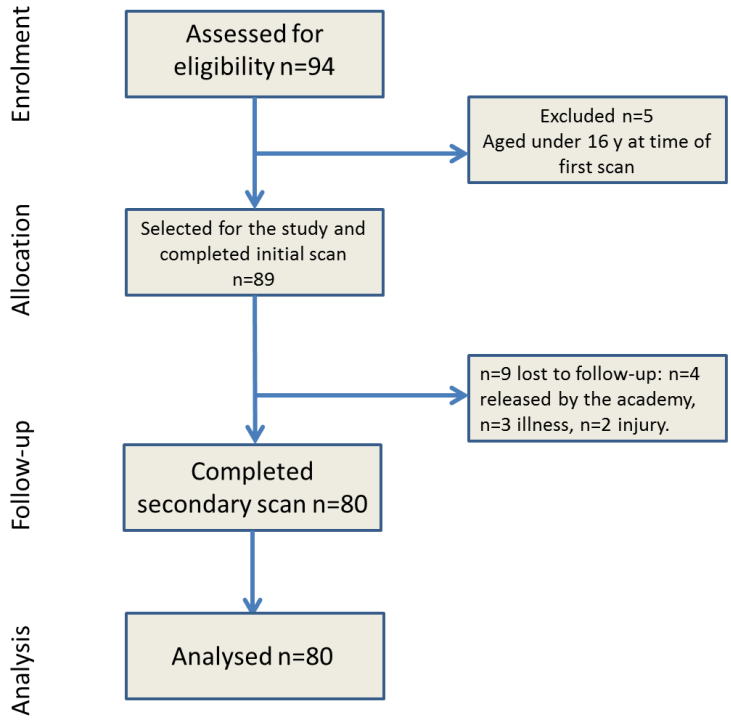


Figure 5.1. Academy footballers assessed and analysed depicted in a consort chart.

### 5.2.2. Experimental Design

All participants were recently enrolled full-time academy football players. Participants were tested for baseline variables during the first week of pre-season training including height, body mass and bone characteristics using pQCT. Participants then conducted 12weeks football specific training with their respective club followed by a repeat of the baseline assessments.

### 5.2.3. Procedures

#### 5.2.3.1. Anthropometrics

Height was measured to the nearest 0.2cm (Stadiometer, Seca, Hamburg, Germany). Participants removed footwear and stood flat footed with their heels against a back plate.

Body mass was recorded with participants wearing minimal clothing (shorts, t-shirt) to the nearest 0.05kg using electronic scales (Seca, Birmingham, U.K.).

#### 5.2.3.2. Training Intervention

Academy footballers that were deemed of a suitable standard graduated through the academy to become first year scholars. Although habitually accustomed to football training and match-play as part of their representation of the academy in younger age groups, the start of the study was timed to coincide with their first experience of full-time training. Football specific training (including, high intensity running drills, small-sided games and technique based drills) and match play was conducted by qualified coaches at the respective clubs.

#### 5.2.3.3. pQCT

pQCT scans were conducted using an XCT 2000 (Stratec Medizintechnik, Pforzheim, Germany) to assess the bone characteristics of the tibia of the dominant leg (leg the participant most comfortably kicks a ball with) (Figure 5.2.). Before scanning commenced the scanner was calibrated using a phantom of known density in accordance with manufacturer guidelines. The participant's tibial length was measured to the nearest 1 mm; defined as the midpoint of the medial malleolus to the medial aspect of the tibial plateau. The participants leg was then placed in the scanner with their foot secured in a purpose built attachment. The leg was aligned and a clamp was placed to the knee to reduce the possibility of artefacts by minimising any movement of the limb. The participant was instructed to remain as still as possible for the duration of the scan. Initially, a preliminary reference point locating scout-view scan was performed in the frontal plane to confirm the location of the middle of the distal end plate, which would act as a positioning line. Sectional images, 2 mm

thick were then obtained at the 4%, 14%, 38% and 66% sites of the tibia from this reference line with a voxel size set at 0.5mm for all measurements (Figure 5.4.). These sites are typically used to analyse trabecular and cortical characteristics of the tibia. A contour mode, with a threshold of 180mg·cm$^{-3}$, was used to separate soft tissue and bone. To analyse trabecular bone, a constant default threshold of 711mg·cm$^{-3}$ was used to identify and remove cortical bone. The integral XCT 2000 software (version 6.20A) was used to analyse the pQCT images.

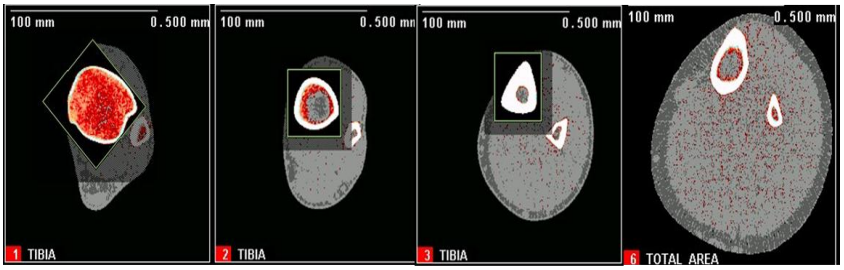


Figure 5.2. Example images from pQCT scanner at 4%, 14%, 38% and 66 % of tibial length.

### 5.2.3.3.1. Bone characteristics

The following measures were analysed at each site of the tibia:

4%: total cross sectional area (Tot CSA, mm$^2$) and trabecular mineral density (mg·cm$^{-3}$).14% and 38%: Tot CSA, (mm$^2$), cortical CSA (mm$^2$), cortical mineral density (mg·cm$^{-3}$), cortical thickness (mm), periosteal circumference (mm) and stress strain index (SSI,mm$^{-3}$). 66%: Tot CSA, (mm$^2$) and cortical mineral density (mg·cm$^{-3}$).

The SSI was determined using the bone threshold of 1200mg·cm$^{-3}$. SSI is the density-weighted polar section modulus of a cross-section and reflects the strength of the long bone

with respect to torsion (multi-plane bending), the formula for SSI calculation is shown in Figure 5.3.

$$SSI = \sum \frac{(d^2 \times A \times vBMDvox/vBMDmax)}{d_{max}}$$

Figure 5.3. Equation for the calculation of Strength Strain Index. A = CSA of the voxel, d = distance of the voxel from the centre of gravity, vBMDvox = vBMD in the voxel ($mg \cdot cm^3$), vBMDmax = maximum therorectical vBMD of human bone ($mg \cdot cm^3$) and $d_{max}$ = maximum distance of any of the voxels of the cortical CSA to the centre of gravity.

The same operator performed all pQCT measurements. If any movement artefacts (inaccuracies in the measurement caused by motion) were present following the scan the image was classed as invalid and a repeat measure was performed. If an artefact was present in the second image the participant was removed from the study in line with the radiation exposure guidelines.

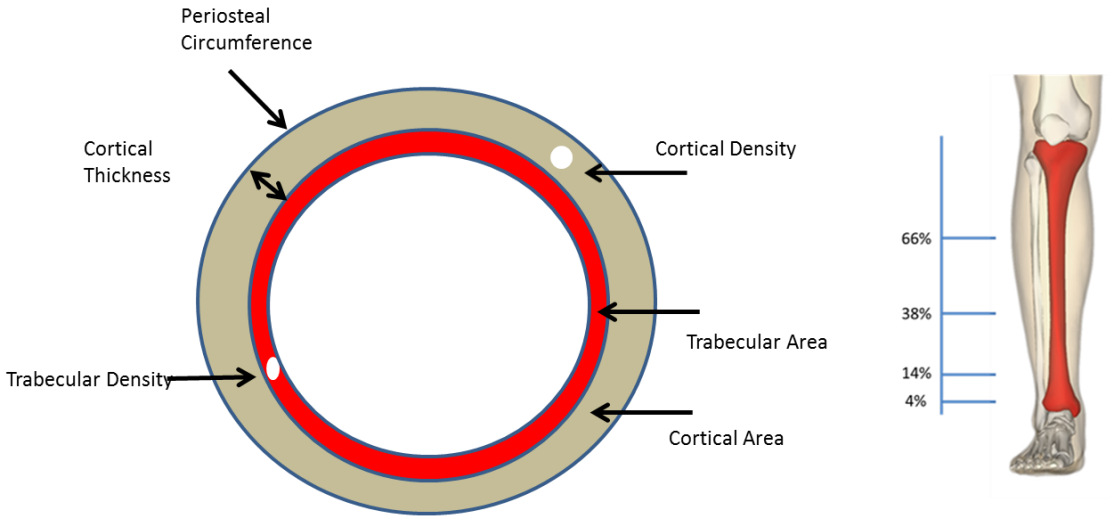


Figure 5.4. Scanned regions of the tibia in academy footballers.

### 5.2.4. Genetic Procedures

Methods of saliva sample collection, DNA extraction and genotyping are described in sections 3.4 – 3.8.

### 5.2.5. Statistical analysis

All data are presented as mean ± SD. Distributions of genotypes were tested for maintenance of Hardy-Weinberg equilibrium (HWE) using chi-squared. Paired sample t-tests were used to compare participant characteristics and bone characteristics at baseline and following the training period. Genotype related associations with bone phenotypes at baseline were analysed by one-way ANOVA. Repeated measures ANOVA was used to assess any bone phenotypic changes that occurred in relation to genotype as a result of the training period. P values of $<0.05$ were considered statistically significant. Multiple comparison 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. All statistical analysis was performed with Statistical Package for the Social Sciences (SPSS) version 13.0 (SPSS, Inc., Chicago, IL, USA).

## 5.3. Results

Eighty participants were available for the follow-up procedure (Figure 5.1.). All SNPs were in accordance with HWE, produced call rates $\geq$ 89% and had minor allele frequencies comparable to previous literature (Table 5.1.). Participants were made up from a variety of ethnicities (54 Caucasian, 14 Caucasian/black dual heritage, 7 black Caribbean, 4 black African and 1 Asian) and composed of differing playing positions (35 midfielders, 22 defenders, 16 forwards and 7 goalkeepers).

Table 5.1. SNPs for which academy footballers were genotyped, along with Hardy-Weinberg Equilibrium (HWE) P value, call rate % and minor allele frequency (MAF).

| | HW P-value | Call Rate % | MAF % |
|---|---|---|---|
| *RANKL* rs9594759 | 0.15 | 91 | 45 |
| *RANK* rs3018362 | 0.24 | 93 | 32 |
| *RANKL* rs1021188 | 0.39 | 96 | 25 |
| *P2X7* rs3751143 | 0.47 | 91 | 16 |
| *P2X7* rs1718119 | 0.82 | 95 | 42 |
| *Wnt16* rs2707466 | 0.16 | 90 | 50 |
| *RANKL* rs9594738 | 0.25 | 95 | 49 |
| *SOST* rs1877632 | 0.55 | 89 | 21 |
| *MP3K* rs8065345 | 0.82 | 95 | 15 |
| *IL6* rs13447445 | 0.06 | 98 | 42 |

### 5.3.1. Participant characteristics

Participants body mass significantly increased post intervention however, tibial length did not significantly change (Table 5.2). The amount of training hours per week significantly increased (106%) following full-time academy induction. Participant characteristics for each genotype are shown in Appendix 5.6.

Table 5.2. Characteristics of academy footballers analysed pre and post 12 wk of increased training volume: mean (SD). * denotes a significant difference (P<0.01).

| Characteristics (n=80) | Pre | Post | P value |
|---|---|---|---|
| Height (m) | 1.76±6.6 | 1.77±6.2 | >0.05 |
| Body Mass (kg) | 70.3±8.1 | 71.2±8.2 | <0.01* |
| Tibia length (mm) | 387.3±21.3 | 387.4±21.1 | >0.05 |
| Age when first played competitively (y) | 9.5±2.0 | N/A | N/A |
| Training (h/wk) | 5.8±2.3 | 11.9±1.3 | <0.01* |

### 5.3.2. Bone Response to Increased Training

Trabecular (4% of tibial length) and cortical density (14%, 38% of tibial length), cortical CSA (14%, 38% of tibial length), total CSA (66% of tibial length), cortical thickness (14%,

38% of tibial length) and SSI (14%, 38% of tibial length) significantly increased following 12 weekk of increased volume training (P<0.05) (Table 5.3.).

Table 5.3. Bone phenotypes (mean±SD) at 4%, 14%, 38% and 66% of the tibia measured prior to and following 12 wk football specific training. (CSA) = cross sectional area. A significance level of P<0.05 used. * was used to denote significance.

| | Football n=80 | | | |
|---|---|---|---|---|
| Bone Phenotype | Pre | Post | % Change | P value |
| 4% site | | | | |
| Trabecular Density (mg·cm$^3$) | 285.7±33.0 | 290.9±31.7 | 1.8↑ | <0.01* |
| Total CSA (mm$^2$) | 1334.1±149.7 | 1345.3±149.9 | 0.8↑ | 0.10 |
| 14% site | | | | |
| Cortical Density (mg·cm$^3$) | 1060.4±33.9 | 1066.5±30.4 | 0.6↑ | <0.01* |
| Total CSA (mm$^2$) | 570.1±80.4 | 572.5±80.0 | 0.4↑ | 0.09 |
| Cortical CSA (mm$^2$) | 214.1±23.9 | 216±23.2 | 0.9↑ | <0.01* |
| Cortical Thickness (mm) | 2.86±0.39 | 2.88±0.38 | 0.7↑ | 0.02* |
| Periosteal Circumference (mm) | 84.44±5.82 | 84.62±5.79 | 0.2↑ | 0.08 |
| SSI | 2038.2±368.6 | 2064.5±372.9 | 1.3↑ | 0.04* |
| 38% site | | | | |
| Cortical Density (mg·cm$^3$) | 1111.0±30.9 | 1116.6±27.6 | 0.5↑ | <0.01* |
| Total CSA (mm$^2$) | 487.4±62.5 | 488.3±61.3 | 0.2↑ | 0.29 |
| Cortical CSA (mm$^2$) | 357.2±41.6 | 360.3±40.6 | 0.9↑ | <0.01* |
| Cortical Thickness (mm) | 6.05±0.52 | 6.11±0.52 | 1.0↑ | <0.01* |
| Periosteal Circumference (mm) | 78.10±4.95 | 78.18±4.85 | 0.1↑ | 0.23 |
| SSI | 2055.8±399.5 | 2104.0±398.5 | 2.3↑ | <0.01* |
| 66% site | | | | |
| Cortical Density (mg·cm$^3$) | 839.5±115.2 | 838.7±106.8 | 0.1↓ | 0.84 |
| Total CSA (mm$^2$) | 1076.9±27.4 | 1082.3±25.5 | 0.5↑ | <0.01* |

### 5.3.3. Bone Phenotypes Genotype Associations

Of the 480 comparisons made, 15 significant associations were shown between SNPs in the proximity of the *RANK/RANKL/OPG, P2X7R* and *Wnt* signalling pathways and bone phenotypes were shown at baseline and post training, a genotype-dependent change bone phenotypic response to training was also shown (P<0.05). No significant associations were shown in *MAP3K14* rs8065345 SNP or *IL6* rs13447445 SNP (P<0.05).

5.3.3.1. *RANK/RANKL/OPG*

SNPs rs1021188, rs9594738 and rs9594759 within the *RANK/RANKL/OPG* signalling pathway were associated with bone phenotypes at baseline and post increased training ($P<0.05$, Table 5.4.). No genotype dependent differences were shown in relation to *RANK* SNP rs3018362. Despite evidence of various trends, no significant genotype x time interactions were shown ($P>0.05$).

Homozygotes for the T allele of rs9594738 was associated with cortical CSA showing a 10.2mm$^2$ (5.0%) and 16.5mm$^2$ (8.1%) decrease compared to homozygosity for the common allele and heterozygotes at baseline ($P>0.05$). Post training, homozygotes for the T allele had lower cortical CSA at the 14% (8.6mm$^2$, 4.0%; 16.6mm$^2$, 7.4%) tibial site ($P>0.05$).

Mean cortical CSA was significantly higher (13.3mm$^2$; 6%) in homozygotes for the common allele of *RANKL* SNP rs1021188 in comparison to the heterozygotes and homozygotes for the rare allele at baseline ($P <0.05$). Post training, decreases of 6.4% (13.7mm$^2$) and 6.7% (6.7mm) were shown in cortical CSA and cortical thickness at the 14% site when homozygosity for the common allele of rs1021188 was compared to heterozygotes and homozygosity for the rare allele ($P <0.05$).

Cortical density at the 66% site was also significantly less in homozygotes of the rare allele of *RANKL* SNPs rs9594759 post training in comparison to homozygotes of the common allele (-18.5 mg/cm$^3$, -1.7%) ($P <0.05$).

Table 5.4. Baseline and post training bone phenotypes in academy footballers compared by genotype. Bone phenotypes are shown for each SNP genotyped and comparisons between genotypes are assessed pre and post training. Comparisons between genotypes and the change in bone phenotype is also displayed. ***BOLD** depicts significance (P <0.05) and # depicts trends (P<0.10). P values of >0.05 are not reported.

| *RANKL* rs9594759 | Football n=73 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Pre | | | P value | Post | | | P value | P value |
| Genotype | CC = 18 | CT = 30 | TT =25 | Pre | CC = 18 | CT = 30 | TT =25 | Post | Change |
| 4% site | | | | | | | | | |
| Trabecular Density (mg·cm$^3$) | 282.2±32.8 | 290.5±30.6 | 290.2±34.6 | 0.66 | 286.1±33.7 | 296.0±28.8 | 295.7±32.0 | 0.52 | 0.63 |
| Total CSA (mm$^2$) | 1313.7±183.4 | 1360.1±134.6 | 1306.1±129.9 | 0.35 | 1316.0±177.9 | 1366.9±148.0 | 1319.9±122.4 | 0.39 | 0.80 |
| 14% site | | | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1056.4±32.2 | 1063.3±31.0 | 1063.9±35.2 | 0.73 | 1064.6±27.0 | 1069.1±30.5 | 1068.8±30.3 | 0.86 | 0.64 |
| Total CSA (mm$^2$) | 564.8±85.8 | 583.7±90.0 | 554.4±56.9 | 0.38 | 564.4±85.4 | 586.8±92.1 | 556.7±49.2 | 0.34 | 0.65 |
| Cortical CSA (mm$^2$) | 209.4±22.1 | 222.8±23.9 | 210.1±24.5 | 0.08 | 211.9±21.4 | 224.5±24.4 | 212.5±22.3 | 0.09 | 0.80 |
| Cortical Thickness (mm) | 2.81±0.39 | 2.94±0.37 | 2.84±0.43 | 0.46 | 2.84±0.37 | 2.96±0.40 | 2.86±0.37 | 0.50 | 0.80 |
| Periosteal Circumference (mm) | 84.02±6.35 | 85.41±6.43 | 83.36±4.18 | 0.41 | 83.99±6.32 | 85.63±6.58 | 83.56±3.70 | 0.37 | 0.64 |
| Stress Strain Index | 2021.9±376.0 | 2143.2±436.4 | 1947.2±231.6 | 0.14 | 2056.6±382.2 | 2160.5±441.3 | 1973.2±244.2 | 0.18 | 0.88 |
| 38% site | | | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1104.1±32.9 | 1114.3±26.0 | 1117.4±30.4 | 0.32 | 1109.5±30.1 | 1119.7±24.0 | 1123.4±25.1 | 0.22 | 0.95 |
| Total CSA (mm$^2$) | 488.8±55.0 | 501.2±71.3 | 474.4±54.2 | 0.29 | 488.7±53.4 | 503.3±69.3 | 474.8±52.4 | 0.22 | 0.57 |
| Cortical CSA (mm$^2$) | 345.86±35.6 | 372.0±46.5 | 350.4±36.6 | 0.13 | 358.6±35.6 | 375.0±45.1 | 352.3±36.4 | 0.10 | 0.34 |
| Cortical Thickness (mm) | 5.98±0.51 | 6.25±0.53 | 6.03±0.50 | 0.15 | 6.06±0.44 | 6.29±0.52 | 6.07±0.51 | 0.17 | 0.63 |
| Periosteal Circumference (mm) | 78.26±4.36 | 79.17±5.62 | 77.09±4.34 | 0.30 | 78.26±4.23 | 79.35±5.45 | 77.13±4.22 | 0.23 | 0.53 |
| Stress Strain Index | 2039.4±355.1 | 2162.8±471.4 | 1983.3±317.8 | 0.24 | 2107.0±356.8 | 2205.6±463.8 | 2025.8±337.8 | 0.25 | 0.72 |
| 66% site | | | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1065.5±25.8 | 1081.4±25.9 | 1083.6±26.3 | 0.06 | 1071.2±23.6 | 1085.8±25.1 | 1089.7±23.6 | **0.04** | 0.58 |
| Total CSA (mm$^2$) | 857.7±124.2 | 863.1±122.7 | 803.0±80.6 | 0.11 | 849.1±86.5 | 866.3±122.1 | 802.7±77.8 | 0.07 | 0.52 |

| *RANK* rs3018362 | Football n=74 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Pre | | | P value | Post | | | P value | P value |
| Genotype | AA =10 | AG=28 | GG=36 | Pre | AA =10 | AG=28 | GG=36 | Post | Change |
| 4% site | | | | | | | | | |
| Trabecular Density (mg·cm$^3$) | 238.4±34.4 | 284.6±29.3 | 289.8±34.9 | 0.77 | 290.2±29.8 | 288.8±30.2 | 295.0±33.0 | 0.72 | 0.53 |
| Total CSA (mm$^2$) | 1340.8±160.7 | 1337.9±121.4 | 1333.6±165.1 | 0.98 | 1333.4±155.4 | 1333.2±127.3 | 1355.3±162.5 | 0.82 | 0.10 |
| 14% site | | | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1051.1±21.7 | 1063.9±33.9 | 1061.0±32.9 | 0.56 | 1059.9±20.8 | 1069.7±31.8 | 1066.6±28.0 | 0.65 | 0.72 |
| Total CSA (mm$^2$) | 578.3±94.0 | 572.1±81.8 | 568.0±72.1 | 0.93 | 581.7±92.7 | 573.8±82.4 | 570.2±70.2 | 0.92 | 0.94 |
| Cortical CSA (mm$^2$) | 209.6±29.9 | 214.8±23.4 | 217.1±23.4 | 0.69 | 211.5±30.5 | 217.2±22.1 | 219.3±22.6 | 0.66 | 0.96 |
| Cortical Thickness (mm) | 2.75±0.34 | 2.86±0.39 | 2.91±0.41 | 0.53 | 2.77±0.34 | 2.89±0.39 | 2.93±0.39 | 0.49 | 0.87 |
| Periosteal Circumference (mm) | 85.0±6.83 | 84.60±5.78 | 84.32±5.34 | 0.94 | 85.26±6.72 | 84.72±5.82 | 84.49±5.22 | 0.93 | 0.92 |
| Stress Strain Index | 2076.3±474.6 | 2058.2±370.8 | 2037.6±342.0 | 0.95 | 2110.9±484.1 | 2074.6±348.4 | 2067.9±369.0 | 0.95 | 0.86 |
| 38% site | | | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1110.9±16.2 | 1110.2±33.7 | 1114.3±27.8 | 0.85 | 1115.3±18.0 | 1115.8±30.9 | 1120.6±23.6 | 0.72 | 0.73 |
| Total CSA (mm$^2$) | 485.5±70.6 | 490.6±61.1 | 489.6±59.9 | 0.97 | 488.4±72.1 | 492.4±60.3 | 489.0±57.1 | 0.97 | 0.31 |
| Cortical CSA (mm$^2$) | 356.8±53.5 | 362.7±37.8 | 359.5±41.0 | 0.92 | 360.4±53.6 | 365.9±37.8 | 361.3±40.0 | 0.89 | 0.24 |
| Cortical Thickness (mm) | 6.03±0.60 | 6.14±0.44 | 6.08±0.56 | 0.83 | 6.09±0.57 | 6.20±0.45 | 6.12±0.53 | 0.78 | 0.90 |
| Periosteal Circumference (mm) | 77.92±5.65 | 78.38±4.76 | 78.30±4.76 | 0.97 | 78.15±5.74 | 78.53±4.70 | 78.26±4.55 | 0.97 | 0.32 |
| Stress Strain Index | 2097.5±479.6 | 2059.1±413.2 | 2078.4±362.3 | 0.96 | 2131.5±484.5 | 2120.1±407.3 | 2120.2±371.3 | 0.99 | 0.72 |
| 66% site | | | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1072.2±17.2 | 1079.5±30.9 | 1078.1±24.5 | 0.75 | 1077.7±19.2 | 1083.6±29.6 | 1084.5±21.6 | 0.74 | 0.35 |
| Total CSA (mm$^2$) | 853.5±116.2 | 840.5±109.3 | 841.8±112.6 | 0.95 | 859.8±120.5 | 845.6±109.5 | 834.1±89.9 | 0.76 | 0.26 |

| *RANKL* rs1021188 | Football n=76 | | | | | | |
|---|---|---|---|---|---|---|---|
| | Pre | | P value | Post | | P value | P value |
| Genotype | CC/CT = 24 | GG = 52 | Pre | CC/CT = 24 | GG = 52 | Post | Change |
| 4% site | | | | | | | |
| Trabecular Density (mg·cm$^3$) | 285.5±30.2 | 290.0±36.7 | 0.58 | 291.0±36.7 | 294.5±34.8 | 0.64 | 0.55 |
| Total CSA (mm$^2$) | 1322.2±139.0 | 1354.5±160.8 | 0.37 | 1337.0±142.1 | 1347.5±158.8 | 0.77 | 0.11 |
| 14% site | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1060.6±33.9 | 1062.3±32.9 | 0.84 | 1066.2±30.6 | 1069.9±29.0 | 0.62 | 0.46 |
| Total CSA (mm$^2$) | 564.3±71.9 | 576.9±90.7 | 0.51 | 568.1±72.6 | 575.2±87.8 | 0.71 | 0.07 |
| Cortical CSA (mm$^2$) | 210.9±23.5 | 224.2±21.8 | **0.02** | 213.0±22.7 | 226.7±21.2 | **0.01** | 0.75 |
| Cortical Thickness (mm) | 2.82±0.38 | 2.99±0.37 | 0.07 | 2.84±0.37 | 3.03±0.35 | **0.04** | 0.36 |
| Periosteal Circumference (mm) | 84.05±5.24 | 84.90±6.55 | 0.54 | 84.33±5.28 | 84.79±6.37 | 0.74 | 0.07 |
| Stress Strain Index | 2008.6±358.6 | 2112.8±380.1 | 0.25 | 2043.6±365.7 | 2117.2±387.7 | 0.42 | 0.27 |
| 38% site | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1112.8±30.3 | 1111.4±30.0 | 0.85 | 1118.9±26.8 | 1115.7±26.6 | 0.62 | 0.31 |
| Total CSA (mm$^2$) | 480.5±59.3 | 501.5±65.9 | 0.17 | 481.0±57.4 | 503.6±64.6 | 0.13 | 0.40 |
| Cortical CSA (mm$^2$) | 352.9±39.2 | 371.8±43.1 | 0.06 | 356.0±38.4 | 374.2±42.9 | 0.07 | 0.51 |
| Cortical Thickness (mm) | 6.02±0.51 | 6.24±0.52 | 0.09 | 6.09±0.49 | 6.27±0.51 | 0.15 | 0.24 |
| Periosteal Circumference (mm) | 77.6±4.72 | 79.22±5.21 | 0.17 | 77.61±4.56 | 79.39±5.11 | 0.13 | 0.40 |
| Stress Strain Index | 2007.1±385.9 | 2180.4±397.9 | 0.08 | 2062.9±378.3 | 2211.8±426.3 | 0.13 | 0.37 |
| 66% site | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1078.0±27.5 | 1077.2±25.7 | 0.90 | 1084.0±25.3 | 1081.6±24.6 | 0.70 | 0.33 |
| Total CSA (mm$^2$) | 835.5±114.2 | 846.9±112.1 | 0.69 | 833.0±100.3 | 849.4±113.2 | 0.53 | 0.55 |

| *RANKL* rs9594738 | Football n=76 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Pre | | | P value | Post | | | P value | P value |
| Genotype | CC = 21 | CT = 32 | TT = 23 | Pre | CC = 21 | CT = 21 | TT = 23 | Post | Change |
| 4% site | | | | | | | | | |
| Trabecular Density (mg·cm$^3$) | 875.9±54.9 | 892.5±54.2 | 890.3±54.4 | 0.50 | 884.3±50.5 | 898.8±51.7 | 898.4±50.6 | 0.64 | 0.41 |
| Total CSA (mm$^2$) | 1325.2±175.7 | 1352.7±136.7 | 1308.8±134.9 | 0.54 | 1340.8±176.7 | 1350.0±145.3 | 1335.2±125.9 | 0.93 | 0.22 |
| 14% site | | | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1059.6±28.7 | 1062.9±35.9 | 1058.8±34.4 | 0.89 | 1068.1±21.4 | 1068.5±35.7 | 1063.8±28.5 | 0.84 | 0.55 |
| Total CSA (mm$^2$) | 571.2±88.5 | 573.8±82.4 | 555.2±59.1 | 0.66 | 572.8±91.3 | 575.5±82.7 | 559.4±52.3 | 0.74 | 0.76 |
| Cortical CSA (mm$^2$) | 214.7±19.9 | 221.0±24.8 | 204.5±21.9 | **0.03** | 215.5±19.6 | 223.5±24.2 | 206.9±19.9 | **0.02** | 0.37 |
| Cortical Thickness (mm) | 2.87±0.38 | 2.94±0.37 | 2.76±0.41 | 0.22 | 2.89±0.39 | 2.97±0.38 | 2.77±0.35 | 0.14 | 0.59 |
| Periosteal Circumference (mm) | 84.48±6.54 | 84.72±5.88 | 83.42±4.34 | 0.69 | 84.59±6.72 | 84.84±5.89 | 83.75±3.93 | 0.77 | 0.66 |
| Stress Strain Index | 2082.4±380.1 | 2091.4±404.7 | 1894.0±207.8 | 0.09 | 2122.4±397.0 | 2106.0±401.9 | 1921.9±218.1 | 0.11 | 0.72 |
| 38% site | | | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1108.2±26.7 | 1113.6±33.2 | 1113.5±29.1 | 0.79 | 1114.3±24.4 | 1118.6±30.4 | 1119.8±23.8 | 0.78 | 0.77 |
| Total CSA (mm$^2$) | 501.8±57.3 | 488.5±66.6 | 465.5±53.2 | 0.13 | 501.4±55.1 | 490.7±65.3 | 465.6±51.1 | 0.12 | 0.39 |
| Cortical CSA (mm$^2$) | 364.0±36.5 | 363.1±45.2 | 345.0±36.0 | 0.19 | 366.5±34.8 | 366.3±44.3 | 347.2±36.0 | 0.16 | 0.68 |
| Cortical Thickness (mm) | 6.01±0.52 | 6.18±0.54 | 6.00±0.47 | 0.43 | 6.11±0.44 | 6.23±0.53 | 6.05±0.49 | 0.41 | 0.99 |
| Periosteal Circumference (mm) | 79.29±4.54 | 78.28±5.26 | 76.37±4.25 | 0.13 | 79.27±4.35 | 78.36±5.14 | 76.39±4.11 | 0.11 | 0.37 |
| Stress Strain Index | 2137.5±362.9 | 2089.5±453.5 | 1907.9±281.6 | 0.11 | 2193.3±347.9 | 2129.7±446.8 | 1957.0±321.1 | 0.11 | 0.88 |
| 66% site | | | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1071.2±21.4 | 1080.4±31.6 | 1080.2±24.7 | 0.43 | 1076.8±18.8 | 1085.1±30.5 | 1086.5±21.5 | 0.38 | 0.65 |
| Total CSA (mm$^2$) | 869.6±120.0 | 840.8±114.9 | 794.1±82.0 | 0.07 | 860.6±86.6 | 844.6±115.4 | 793.3±79.0 | 0.059 | 0.41 |

| *P2X7R* rs3751143 | Football n=73 | | | | | | |
|---|---|---|---|---|---|---|---|
| | Pre | | P value | Post | | P value | P value |
| Genotype | CC/CT = 22 | TT = 51 | Pre | CC/CT = 22 | TT = 51 | Post | Change |
| 4% site | | | | | | | |
| Trabecular Density (mg·cm$^3$) | 281.6±35.3 | 289.6±33.0 | 0.33 | 288.1±33.8 | 294.0±32.1 | 0.45 | 0.21 |
| Total CSA (mm$^2$) | 1324.0±146.7 | 1340.3±155.8 | 0.67 | 1340.0±138.7 | 1345.8±160.3 | 0.88 | 0.47 |
| 14% site | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1057.0±33.8 | 1065.0±30.4 | 0.29 | 1063.8±32.3 | 1069.7±27.9 | 0.42 | 0.36 |
| Total CSA (mm$^2$) | 553.1±66.5 | 582.1±87.6 | 0.20 | 554.9±56.1 | 584.2±89.7 | 0.19 | 0.94 |
| Cortical CSA (mm$^2$) | 215.4±29.8 | 215.7±21.6 | 0.96 | 218.3±28.7 | 217.3±21.3 | 0.89 | 0.33 |
| Cortical Thickness (mm) | 2.94±0.51 | 2.84±0.34 | 0.38 | 2.96±0.47 | 2.86±0.35 | 0.34 | 0.79 |
| Periosteal Circumference (mm) | 83.23±4.92 | 85.30±6.30 | 0.20 | 83.40±4.22 | 85.44±6.45 | 0.21 | 0.89 |
| Stress Strain Index | 1959.0±251.3 | 2114.7±405.7 | 0.12 | 2000.4±298.4 | 2130.1±404.6 | 0.20 | 0.39 |
| 38% site | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1106.0±34.5 | 1115.4±26.9 | 0.22 | 1114.1±31.0 | 1119.5±25.0 | 0.41 | **0.04** |
| Total CSA (mm$^2$) | 485.5±61.1 | 497.6±63.9 | 0.34 | 480.3±57.3 | 499.1±63.4 | 0.28 | 0.40 |
| Cortical CSA (mm$^2$) | 354.5±43.6 | 364.1±40.3 | 0.38 | 356.4±43.1 | 367.2±39.6 | 0.31 | 0.24 |
| Cortical Thickness (mm) | 6.08±0.68 | 6.09±0.47 | 0.83 | 6.11±0.63 | 6.16±0.48 | 0.67 | 0.39 |
| Periosteal Circumference (mm) | 77.56±4.88 | 78.92±5.04 | 0.33 | 77.56±4.60 | 79.04±4.98 | 0.28 | 0.43 |
| Stress Strain Index | 1987.6±425.2 | 2134.6±425.5 | 0.17 | 2051.2±357.5 | 2175.0±417.2 | 0.26 | 0.41 |
| 66% site | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1074.0±31.5 | 1080.3±24.0 | 0.33 | 1080.6±28.3 | 1084.9±23.5 | 0.48 | 0.20 |
| Total CSA (mm$^2$) | 831.8±116.9 | 854.5±115.1 | 0.49 | 822.0±80.1 | 857.3±115.0 | 0.22 | 0.15 |

| *P2X7R* rs1718119 | Football n=76 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Pre | | | P value | Post | P value | | P value | P value |
| Genotype | CC = 25 | CT = 38 | TT =13 | Pre | CC = 25 | CT = 38 | TT =13 | Post | Change |
| 4% site | | | | | | | | | |
| Trabecular Density (mg·cm$^3$) | 290.6±37.5 | 283.9±31.6 | 286.9±30.0 | 0.74 | 296.2±35.9 | 289.2±30.5 | 290.1±30.0 | 0.69 | 0.52 |
| Total CSA (mm$^2$) | 1323.9±158.0 | 1330.4±140.5 | 1360.7±164.9 | 0.76 | 1333.2±158.2 | 1339±144.6 | 1364.7±160.4 | 0.82 | 0.96 |
| 14% site | | | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1062.5±27.6 | 1060.9±33.4 | 1068.4±33.4 | 0.77 | 1070.1±24.2 | 1065.7±31.1 | 1072.8±32.2 | 0.70 | 0.50 |
| Total CSA (mm$^2$) | 560.3±89.1 | 573.9±79.1 | 583.8±72.2 | 0.67 | 559.8±84.6 | 578.3±79.7 | 584.1±74.9 | 0.59 | 0.29 |
| Cortical CSA (mm$^2$) | 220.4±24.1 | 209.6±23.9 | 222.1±18.7 | 0.11 | 223.4±23.1 | 210.6±22.6 | 225.7±20.0 | **0.04** | 0.10 |
| Cortical Thickness (mm) | 3.0±0.41 | 2.77±0.39 | 2.92±0.28 | 0.08# | 3.03±0.39 | 2.78±0.37 | 2.98±0.30 | **0.02** | 0.09# |
| Periosteal Circumference (mm) | 83.67±6.56 | 84.74±5.59 | 85.50±5.31 | 0.63 | 83.65±6.26 | 85.06±5.63 | 85.51±5.50 | 0.55 | 0.29 |
| Stress Strain Index | 2045.0±366.5 | 2020.8±373.3 | 2159.3±356.8 | 0.53 | 2095.6±397.9 | 2045.0±373.4 | 2147.8±335.2 | 0.67 | 0.25 |
| 38% site | | | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1110.9±24.1 | 1113.9±34.1 | 1116.0±22.4 | 0.86 | 1116.2±20.6 | 1118.8±31.7 | 1121.4±19.4 | 0.85 | 0.95 |
| Total CSA (mm$^2$) | 494.64±75.4 | 484.1±56.8 | 499.2±54.1 | 0.69 | 495.0±72.1 | 485.9±57.4 | 499.0±52.1 | 0.75 | 0.63 |
| Cortical CSA (mm$^2$) | 366.4±47.5 | 351.1±37.3 | 372.9±31.7 | 0.15 | 369.1±45.8 | 353.5±36.8 | 376.8±33.1 | 0.12 | 0.55 |
| Cortical Thickness (mm) | 6.20±0.58 | 5.94±0.54 | 6.28±0.30 | **0.05** | 6.25±0.50 | 5.98±0.54 | 6.38±0.35 | **0.02** | 0.33 |
| Periosteal Circumference (mm) | 78.62±6.03 | 77.87±4.45 | 79.01±4.27 | 0.70 | 78.67±5.77 | 78.02±4.48 | 79.09±4.12 | 0.75 | 0.67 |
| Stress Strain Index | 2105.8±448.7 | 2025.8±373.3 | 2087.3±317.1 | 0.88 | 2161.9±463.1 | 2045.0±377.1 | 2171.7±328.0 | 0.67 | 0.26 |
| 66% site | | | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1076.4±19.7 | 1079.5±29.6 | 1081.0±25.6 | 0.85 | 1081.4±16.5 | 1085.0±28.0 | 1085.8±26.9 | 0.86 | 0.90 |
| Total CSA (mm$^2$) | 852.9±134.5 | 837.4±104.1 | 850.3±108.0 | 0.86 | 844.9±113.7 | 838.6±101.9 | 857.0±107.3 | 0.82 | 0.39 |

| *Wnt16* rs2707466 | Football n=72 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Pre | | | P value | Post | | | P value | P value |
| Genotype | AA = 21 | AG = 30 | GG = 21 | Pre | AA = 21 | AG = 30 | GG = 21 | Post | Change |
| 4% site | | | | | | | | | |
| Trabecular Density (mg·cm$^3$) | 293.8±30.0 | 286.0±30.8 | 278.8±33.8 | 0.32 | 298.8±27.6 | 289.0±29.8 | 287.2±33.2 | 0.40 | **0.01** |
| Total CSA (mm$^2$) | 1319.5±147.2 | 1357.9±145.0 | 1313.8±157.1 | 0.52 | 1329.9±146.9 | 1358.4±149.8 | 1330.7±155.7 | 0.74 | 0.60 |
| 14% site | | | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1054.3±39.7 | 1062.6±33.5 | 1069.0±22.2 | 0.35 | 1061.9±32.6 | 1069.4±33.4 | 1072.4±18.4 | 0.50 | 0.43 |
| Total CSA (mm$^2$) | 583.7±74.6 | 578.5±83.8 | 552.1±71.9 | 0.37 | 584.9±77.1 | 578.0±79.8 | 558.9±74.4 | 0.53 | 0.14 |
| Cortical CSA (mm$^2$) | 214.3±25.2 | 213.2±23.3 | 217.0±21.7 | 0.85 | 217.0±23.1 | 216.4±23.5 | 217.5±21.4 | 0.99 | 0.11 |
| Cortical Thickness (mm) | 2.82±0.42 | 2.81±0.32 | 2.96±0.38 | 0.32 | 2.86±0.41 | 2.85±0.31 | 2.94±0.37 | 0.65 | 0.06 |
| Periosteal Circumference (mm) | 85.57±5.41 | 85.06±5.96 | 83.13±5.38 | 0.36 | 85.55±5.59 | 85.04±5.68 | 83.63±5.54 | 0.52 | 0.14 |
| Stress Strain Index | 2101.0±350.0 | 2070.0±403.2 | 2001.7±327.5 | 0.67 | 2152.7±353.8 | 2105.4±418.0 | 1983.6±302.8 | 0.32 | 0.12 |
| 38% site | | | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1102.3±31.6 | 1114.4±32.4 | 1123.5±17.3 | 0.06 | 1108.4±27.9 | 1120.3±28.6 | 1127.9±15.5 | 0.05 | 0.71 |
| Total CSA (mm$^2$) | 505.9±54.5 | 492.4±71.8 | 471.9±47.7 | 0.20 | 507.6±55.1 | 491.2±67.9 | 474.8±48.9 | 0.21 | 0.14 |
| Cortical CSA (mm$^2$) | 367.1±28.2 | 358.9±45.1 | 354.6±37.1 | 0.61 | 370.0±37.3 | 362.2±44.4 | 356.4±36.6 | 0.56 | 0.45 |
| Cortical Thickness (mm) | 6.06±0.49 | 6.03±0.51 | 6.16±0.53 | 0.66 | 6.11±0.46 | 6.12±0.50 | 6.17±0.52 | 0.91 | 0.08 |
| Periosteal Circumference (mm) | 79.62±4.30 | 78.47±5.65 | 76.91±3.87 | 0.20 | 79.75±4.34 | 78.39±5.33 | 77.15±3.96 | 0.21 | 0.16 |
| Stress Strain Index | 2001.3±364.0 | 2067.5±454.4 | 1971.0±300.7 | 0.17 | 2238.7±355.3 | 2140.4±467.6 | 1997.0±285.9 | 0.14 | 0.29 |
| 66% site | | | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1071.8±25.8 | 1078.2±31.2 | 1084.3±18.3 | 0.35 | 1077.7±22.2 | 1083.9±30.7 | 1089.0±16.6 | 0.36 | 0.60 |
| Total CSA (mm$^2$) | 857.5±92.6 | 859.2±133.9 | 816.3±89.3 | 0.32 | 860.2±94.0 | 853.1±112.8 | 818.4±90.7 | 0.35 | 0.78 |

| *SOST* rs1877632 | Football n=71 | | | | | | |
|---|---|---|---|---|---|---|---|
| | Pre | | P value | Post | | P value | P value |
| Genotype | GG = 45 | GA/AA = 26 | Pre | GG = 45 | GA/AA = 26 | Post | Change |
| 4% site | | | | | | | |
| Trabecular Density (mg·cm$^3$) | 281.6±29.6 | 298.1±36.7 | **0.04** | 286.2±28.8 | 303.6±33.2 | **0.03** | 0.58 |
| Total CSA (mm$^2$) | 1333.6±163.0 | 1316.9±105.9 | 0.64 | 1347.0±162.2 | 1316.9±111.0 | 0.40 | 0.35 |
| 14% site | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1064.6±28.9 | 1058.2±32.5 | 0.41 | 1071.0±27.4 | 1061.8±28.3 | 0.20 | 0.27 |
| Total CSA (mm$^2$) | 571.9±84.8 | 562.7±67.9 | 0.64 | 573.7±83.7 | 565.8±68.4 | 0.68 | 0.70 |
| Cortical CSA (mm$^2$) | 214.4±20.1 | 216.3±30.1 | 0.75 | 216.0±19.9 | 219.4±28.8 | 0.55 | 0.19 |
| Cortical Thickness (mm) | 2.86±0.37 | 2.90±0.41 | 0.68 | 2.88±0.37 | 2.94±0.40 | 0.53 | 0.40 |
| Periosteal Circumference (mm) | 84.56±6.12 | 83.96±4.94 | 0.67 | 84.70±6.06 | 84.19±4.98 | 0.71 | 0.70 |
| Stress Strain Index | 2049.9±359.4 | 2041.0±392.7 | 0.92 | 2078.3±371.6 | 2059.2±393.2 | 0.84 | 0.72 |
| 38% site | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1113.5±27.0 | 1112.9±26.7 | 0.93 | 1118.9±24.0 | 1117.4±24.2 | 0.82 | 0.62 |
| Total CSA (mm$^2$) | 490.7±63.0 | 486.2±65.6 | 0.77 | 490.5±60.2 | 489.2±66.0 | 0.94 | 0.09 |
| Cortical CSA (mm$^2$) | 361.4±38.6 | 360.9±47.0 | 0.96 | 364.0±37.9 | 364.0±46.4 | 0.97 | 0.68 |
| Cortical Thickness (mm) | 6.11±0.45 | 6.15±0.61 | 0.76 | 6.17±0.43 | 6.19±0.60 | 0.86 | 0.50 |
| Periosteal Circumference (mm) | 78.37±4.95 | 78.0±5.27 | 0.74 | 78.37±4.73 | 78.24±5.29 | 0.92 | 0.09 |
| Stress Strain Index | 2063.7±387.6 | 2081.7±441.2 | 0.86 | 2122.4±401.4 | 2114.7±425.3 | 0.94 | 0.36 |
| 66% site | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1079.4±25.2 | 1078.0±23.7 | 0.59 | 1085.1±24.3 | 1082.4±21.9 | 0.65 | 0.38 |
| Total CSA (mm$^2$) | 846.1±119.2 | 830.6±106.7 | 0.83 | 841.9±103.8 | 834.9±108.4 | 0.79 | 0.34 |

| *MP3K* rs8065345 | Football n=76 | | | | | | |
|---|---|---|---|---|---|---|---|
| | Pre | | P value | Post | | P value | P value |
| Genotype | AA = 55 | AG/GG = 21 | Pre | AA = 55 | AG/GG = 21 | Post | Change |
| 4% site | | | | | | | |
| Trabecular Density (mg·cm$^3$) | 285.3±32.1 | 289.8±36.5 | 0.60 | 290.6±31.1 | 294.7±34.8 | 0.61 | 0.85 |
| Total CSA (mm$^2$) | 1339.0±142.8 | 1335.5±178.0 | 0.93 | 1345.1±144.7 | 1348.1±175.5 | 0.94 | 0.65 |
| 14% site | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1058.5±33.7 | 1064.8±32.0 | 0.46 | 1064.6±29.1 | 1071.5±32.0 | 0.37 | 0.85 |
| Total CSA (mm$^2$) | 567.4±72.9 | 581.2±102.6 | 0.51 | 569.8±74.3 | 582.5±97.9 | 0.55 | 0.74 |
| Cortical CSA (mm$^2$) | 214.3±25.7 | 216.6±18.8 | 0.70 | 216.3±25.1 | 219.0±18.0 | 0.66 | 0.80 |
| Cortical Thickness (mm) | 2.86±0.41 | 2.87±0.35 | 0.96 | 2.89±0.41 | 2.90±0.34 | 0.94 | 0.90 |
| Periosteal Circumference (mm) | 84.28±5.30 | 85.16±7.38 | 0.57 | 84.45±5.40 | 85.28±7.05 | 0.59 | 0.83 |
| Stress Strain Index | 2022.3±345.3 | 2120.9±425.5 | 0.30 | 2040.5±335.3 | 2164.6±460.0 | 0.20 | 0.38 |
| 38% site | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1111.0±32.3 | 1112.4±26.0 | 0.86 | 1116.4±29.3 | 1118.5±22.2 | 0.77 | 0.71 |
| Total CSA (mm$^2$) | 484.9±57.7 | 500.1±76.4 | 0.35 | 486.5±57.8 | 499.7±72.0 | 0.41 | 0.32 |
| Cortical CSA (mm$^2$) | 355.2±39.2 | 370.1±45.0 | 0.16 | 358.1±39.6 | 372.7±41.8 | 0.16 | 0.70 |
| Cortical Thickness (mm) | 6.03±0.56 | 6.23±0.48 | 0.15 | 6.08±0.56 | 6.28±0.36 | 0.14 | 0.99 |
| Periosteal Circumference (mm) | 77.92±4.60 | 79.05±6.01 | 0.38 | 78.06±4.59 | 79.05±5.66 | 0.43 | 0.37 |
| Stress Strain Index | 2038.6±356.3 | 2152.0±495.5 | 0.27 | 2084.1±350.8 | 2205.5±506.9 | 0.24 | 0.78 |
| 66% site | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1076.7±28.0 | 1078.3±25.4 | 0.82 | 1082.0±26.1 | 1084.8±23.3 | 0.67 | 0.46 |
| Total CSA (mm$^2$) | 831.1±101.7 | 871.0±144.8 | 0.18 | 834.5±102.1 | 859.4±119.0 | 0.37 | 0.86 |

| *IL6* rs13447445 | Football n=78 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Pre | | | P value | Post | | | P value | P value |
| Genotype | CC = 18 | CG = 30 | GG = 30 | Pre | CC = 18 | CG = 30 | GG = 30 | Post | Change |
| 4% site | | | | | | | | | |
| Trabecular Density (mg·cm$^3$) | 282.9±41.2 | 287.7±28.0 | 286.9±33.7 | 0.88 | 288.1±39.5 | 293.3±27.1 | 291.8±32.4 | 0.86 | 0.91 |
| Total CSA (mm$^2$) | 1347.6±153.6 | 1323.4±163.9 | 1326.8±131.8 | 0.85 | 1352.9±159.7 | 1331.7±157.4 | 1346.2±137.7 | 0.88 | 0.69 |
| 14% site | | | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1052.6±30.1 | 1060.5±31.2 | 1067.2±36.0 | 0.33 | 1057.3±29.2 | 1066.4±26.0 | 1073.2±34.0 | 0.22 | 0.91 |
| Total CSA (mm$^2$) | 548.0±68.7 | 577.7±92.7 | 575.3±75.2 | 0.43 | 550.1±72.0 | 579.9±88.7 | 578.3±76.6 | 0.41 | 0.97 |
| Cortical CSA (mm$^2$) | 214.6±28.3 | 211.1±22.6 | 217.4±22.5 | 0.59 | 216.5±27.9 | 214.2±21.4 | 218.6±22.7 | 0.77 | 0.28 |
| Cortical Thickness (mm) | 2.93±0.46 | 2.80±0.40 | 2.88±0.32 | 0.46 | 2.96±0.47 | 2.83±0.36 | 2.89±0.34 | 0.50 | 0.66 |
| Periosteal Circumference (mm) | 82.83±5.15 | 84.96±6.62 | 84.86±5.46 | 0.43 | 82.98±5.37 | 85.14±6.34 | 85.07±5.55 | 0.40 | 0.97 |
| Stress Strain Index | 1950.6±363.4 | 2058.9±396.8 | 2079.0±343.4 | 0.49 | 1986.0±389.4 | 2077.3±396.6 | 2105.9±354.8 | 0.56 | 0.88 |
| 38% site | | | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1111.4±28.5 | 1107.9±30.0 | 1115.5±33.2 | 0.63 | 1116.4±26.4 | 1113.2±26.2 | 1121.1±30.2 | 0.55 | 0.96 |
| Total CSA (mm$^2$) | 471.3±58.4 | 492.8±73.4 | 492.7±54.5 | 0.46 | 471.7±54.8 | 493.4±72.6 | 494.1±54.3 | 0.42 | 0.89 |
| Cortical CSA (mm$^2$) | 349.0±40.8 | 361.8±42.5 | 358.4±42.2 | 0.59 | 352.2±39.5 | 365.0±42.1 | 361.2±40.6 | 0.58 | 0.92 |
| Cortical Thickness (mm) | 6.05±0.65 | 6.09±0.39 | 6.02±0.64 | 0.86 | 6.18±0.60 | 6.17±0.38 | 6.07±0.60 | 0.76 | 0.82 |
| Periosteal Circumference (mm) | 76.82±4.73 | 78.49±5.77 | 78.57±4.33 | 0.45 | 76.87±4.44 | 78.55±5.70 | 76.68±4.30 | 0.42 | 0.91 |
| Stress Strain Index | 1947.7±362.3 | 2090.3±463.2 | 2091.7±362.4 | 0.42 | 2004.2±354.9 | 2151.3±478.3 | 2122.2±345.0 | 0.46 | 0.53 |
| 66% site | | | | | | | | | |
| Cortical Density (mg·cm$^3$) | 1072.9±27.4 | 1078.1±25.6 | 1079.8±29.0 | 0.70 | 1077.6±26.0 | 1083.9±23.1 | 1084.7±28.0 | 0.62 | 0.74 |
| Total CSA (mm$^2$) | 821.7±136.3 | 838.2±122.5 | 854.4±98.1 | 0.64 | 809.7±93.7 | 841.2±122.1 | 856.4±99.9 | 0.35 | 0.27 |

#### 5.3.3.2. *Wnt* Signalling

Trabecular density at the 4% site was associated with the rare allele of the *SOST* SNP rs1877632 ($P<0.05$). Carriers of at least one rare allele were shown to have a 16.5 $mg \cdot cm^3$ (5.9%) greater trabecular density when compared to homozygotes for the common allele prior to the start of 12 weeks increased training volume ($P<0.05$). Following training these differences in trabecular density at the 4% site remained (17.4$mg \cdot cm^3$, 6.1%) ($P<0.05$).

*Wnt16* SNP rs2707466 was associated with cortical density at the 38% site following training ($P<0.05$). Homozygotes of the C allele was associated with an increase (19.5$mg \cdot cm^3$, 1.8%) in comparison to homozygotes of the A allele (Table 5.4.). A significant time x genotype interaction effect was also shown for rs2707466 in relation to trabecular density at the 4% site and cortical thickness at the 14% site ($P<0.05$) (Table 5.4.). Homozygotes of the C allele showed a greater increase in trabecular density at the 4% site in comparison to other allele combinations ($P<0.05$) (Table 5.4.).

#### 5.3.3.3. *P2X7R*

*P2X7R* SNPs rs1718119 showed a significant association with cortical thickness at the 38% site ($P<0.05$) at the baseline scan. Homozygotes of the rare, gain of function allele was significantly associated with a 0.34mm (5.4%) increase in comparison to heterozygotes ($P<0.05$). Phenotypes assessed at the 12 weeks scan also showed a similar 0.40mm (6.3%) increase in cortical thickness at the 38% site in comparison to the rare allele of rs1718119. rs1718119 heterozygotes showed lower cortical thickness at the 14% site post training in comparison to homozygotes for the rare (0.2mm, 7.2%) and common (0.25mm, 9%) allele. A significantly lower cortical CSA (38% site) was also evident in heterozygotes when

compared to homozygotes for the rare (12.8mm$^2$, 6.1%) and common (15.1mm$^2$, 7.2%) allele ($P<0.05$). Despite no significant associations being shown at the pre or post scan measurements, heterozygosity and homozygosity for the rare allele of rs3751143 was shown to have a greater increase in cortical density at the 38% site (8.1mg·cm$^{-3}$ compared to 4.1mg·cm$^{-3}$, significant time x genotype interaction).

## 5.4. Discussion

This study shows, for the first time, that specific SNPs are associated with trabecular density, cortical thickness, cortical CSA and density of the tibia in adolescent, male, elite academy footballers. The associations of SNPs with distinct bone phenotypes at different tibial sites highlight the complexity of the genetic contribution to bone morphology. Cross-sectional studies in non-athletic adults have shown SNPs to have an association with bone phenotypes at rest (Havill *et al.,* 2013; Paternoster *et al.,* 2010; Styrkarsdottir *et al.,* 2008; Richards *et al.,* 2008). Similarly, studies conducted in military recruits have shown genotype to be associated with bone accrual in response to 10 weeks basic training (Dhamrait *et al.,* 2003).

### 5.4.1. *RANK/RANKL/OPG* signalling pathway

An association between SNP rs9594738 and bone size was shown, with homozygotes of the T allele associated with lower cortical CSA. It has previously been reported that the T allele was associated with lower BMD at the femur and lumbar spine (Kemp *et al.,* 2013; Styrkarsdottir *et al.,* 2008) and associated with a greater hip fracture risk (Zhang *et al.,* 2011). However, Guo *et al* (2012) reported beneficial effects of the T allele, showing it to be protective for osteoporotic hip fracture. The reason for the contrasting findings may be due to the ethnicity of the population, as the positive effects of the T allele have only been shown in

a Chinese population. As only 1% of the participants in the current study were from Asian heritage (self-reported), the present data follow previously published findings showing an association between the variant allele of rs9594738 and adverse bone phenotypes (Table 2.3.). Although there is no known mechanism for the effects of rs9594738 on bone remodelling and structural differences, the variance in genotype may inhibit *RANK - RANKL* binding and, therefore, influence osteoclast differentiation and activation, subsequently mediating bone resorption (Boyle *et al.,* 2003). rs9594738 may also have a functional role in bone homeostasis through its regulation of how other factors influence the *RANK/RANKL/OPG* signalling pathway. It has been suggested that allelic differences in the rs9594738 SNP may have a mediatory role in the process by which 1,25-$(OH)_2$D induces *RANKL* expression in osteoblast and osteoblast precursor cells (Yostovitz *et al.,* 2013). The key role of rs9594738 is further supported by the absence of linkage disequilibrium with other known functional *RANK/RANKL/OPG* SNPs. rs9594738 is located in a different haplotype block and has different transcription factor binding sites to other previously studied *RANK/RANKL/OPG* SNPs, meaning it is unlikely to act as a proxy for these SNPs, increasing the likelihood that its effects are divergent.

The minor allele of *RANKL* SNP rs1021188 was associated with lower cortical CSA and cortical thickness at 14% of the tibia pre and post increased training. A trend was also evident for time x genotype interaction, showing the minor allele to be associated with increased periosteal circumference and total CSA at the 14% tibial site (Table 5.4). These data are in accordance with large scale GWAS, reporting an association between the minor allele and lower cortical BMD (Paternoster *et al.,* 2010) and volumetric BMD (Paternoster *et al.,* 2013). The minor allele of rs1021188 has been associated a greater cortical porosity at the tibia (Paternoster *et al.,* 2013) and increased circulating free *RANKL* (Paternoster *et al.,* 2010),

possibly increasing osteoclastogenesis and bone resorption in carriers of the minor allele. Although speculative, the association of the minor allele of rs1021188 with lower cortical CSA and cortical thickness, could suggest an uncoupling of bone turnover resulting from increased bone resorption, providing a possible mechanistic explanation for the findings.

Homozygotes of the minor allele of *RANKL* SNP rs9594759 was associated with lower cortical density at the 66% site of the tibia following 12 weeks of increased volume of football training. As cortical density is a factor in bone strength, carriers of the minor allele might experience bone weakness and deficiencies that may lead to bone injury. The minor allele being associated with lower cortical density is contrary to previous research showing the minor allele of rs9594759 to be associated with greater BMD at the lumbar spine, hip and calcaneus (Styrkarsdottir *et al.,* 2008; Roshandel *et al.,* 2010). The reason for the difference in findings may be related to the age of the participants. Styrkarsdottir *et al.* (2008) and Roshandel *et al.* (2010) conducted studies demonstrating the positive effects of the minor allele in aged populations (mean age ~60 y, male and female). Previous studies have not alluded to the activity levels of their participants, although due to the age of the cohorts used it is likely that they are not currently elite athletes. The active status of the cohort in the present study may have also influenced the direction of the SNP's effect. This is speculative as gene-environment interactions are not well understood. Recent evidence suggests that the minor allele of rs9594759 is related to an impairment of neuromuscular function and muscular characteristics (Alfred *et al.,* 2013). It is possible that the effect of rs9594759 on bone is indirect and is due to impairment in muscular function that affects the amount of strain placed upon the bone. Bone and muscle act as a unit in the creation and application of strain, and this unit may have influenced the bone phenotypes measured in the present and in previous studies. Muscle is known to absorb some of the impact created by mechanical

loading and also exerts strain upon the bone during muscular contractions (Schoenau, 2005; Schipilow *et al.,* 2013), a deficiency in the muscles ability to do this may have resulted in the bone undergoing a higher degree of strain. The effect of the genotype may only convey subtle differences that are not shown in young elite athletes but over time may explain the greater BMD shown in the elderly participants investigated in previous studies (Styrkarsdottir *et al.,* 2008; Roshandel *et al.,* 2010).

### 5.4.2. *Wnt* Signalling

Following 12 weeks of increased training, cortical density, trabecular density and cortical thickness were associated with *Wnt16* SNP rs2707466. The *Wnt* signalling pathway is a major regulator of bone metabolism, having a significant role in the mediation of the differentiation and longevity of osteoblasts. The specific role of *Wnt* ligands and genes that are involved in the *Wnt* signalling process remain poorly understood. However, the influence of several SNPs on bone phenotypes (Zheng *et al.,* 2012; García-Ibarbia *et al.,* 2013) may be due to a mediating role in osteoblastic activity. *SOST* and *Wnt16* are two such genes that have previously been shown to influence bone phenotypes. *Wnt16* signals through the non-canonical pathway and the molecular mechanisms of its actions have been associated with stem cell regulation (Clements *et al.,* 2011). Following 12 weeks of increased training, homozygotes for the C allele was associated with greater cortical density at the 38% site of the tibia, while the change in trabecular density (4%) was also associated with homozygotes for the C allele. These data are in accordance with previous research showing the C allele to be associated with a bone phenotypes in two large samples in both young and old participants at the tibia and radius (Zheng *et al.,* 2012). A copy of the C allele has also been associated with a greater osteoporotic fracture risk (García-Ibarbia *et al.,* 2013). While homozygosity for

the C allele was associated with increased trabecular density in response to training, associations only existed in cortical density post training at the 38% site. Although, as the 38% site may be the most functionally relevant site in terms anatomical position of stress fracture injury (Wall and Feller, 2006), any changes shown at this site may be of the most clinical significance. The data related to BMD is equivocal, while the C allele has been associated with a lower BMD at the femoral neck and lumber spine (García-Ibarbia *et al.,* 2013), data from a larger, replication cohort showed no significant difference (Zheng *et al.,* 2012). The lack of agreement in the findings may highlight the site specific effects genotype has on bone composition and anatomical site. These data, taken together, substantiate the important role of *Wnt16* SNP rs2707466 in relation to bone phenotypes, particularly cortical thickness. As cortical thickness has previously been associated with stress related bone injuries (Newsham-West *et al.,* 2013), it could be suggested that *Wnt16* SNP rs2707466 genotype may have a role in the mediation of susceptibility to stress fracture injury. However, the magnitude of change in bone characteristics needed for clinical significance and clinical effects is not currently known.

The association of *SOST* rs1877632 with trabecular density, suggests that this SNP may mediate early bone remodelling in response to exercise. Expressed primarily in osteocytes, sclerostin has a key role in *Wnt* signalling as it acts as a negative regulator of bone formation (Krishnan *et al.,* 2006). The *SOST* gene has been associated with increased bone formation, BMD and increased trabecular bone mass in sclerostin null mice (Li *et al.,* 2008). In accordance with the present findings, carriers of the rare allele have previously been shown to display a greater BMD at the lumbar spine, an area made up of predominantly trabecular bone (Yerges *et al.,* 2009). Although the previous studies cohorts were elderly (mean age ~75 y) compared to the present population (mean age 16 y), the % difference was similar (5.9%

and 6.1% compared to 6.0% and 10.2%), showing the effect of the SNP may influence bone density in various populations. This suggests that the SNPs affect is demonstrated in early age and maintained throughout the lifespan, which may have implications for the early diagnosis of individuals at a heightened risk of bone disorders. As the bone mass and density achieved in childhood are predictive of bone mass and density in adulthood (Bonnet and Ferrari, 2010), it could be speculated that rs1877632 is an important determinant of these characteristics. If this association in confirmed, preventative measures, such as modification of training and exercise, could be put in place in order to maximise bone gain in those with unfavourable allelic variants.

### 5.4.3. *P2X7R*

Associations between the gain of function allele of rs1718119 and increased bone phenotypes were shown in academy footballers. Increased cortical thickness were shown at the 38% tibial site at baseline and following 12 weeks of increased volume training. Stress fracture injuries commonly occur in the vicinity of the 38% site of the tibia (Wall and Feller, 2006) and low CSA and cortical thickness are associated with stress fracture incidence (Popp *et al.,* 2009; Newsham-West *et al.,* 2013). This suggests that the rare allele of rs1718119 may provide a protective mechanism against stress fracture injury by increasing bone structural characteristics related to bone strength. Recent *in vivo* studies have shown variants in rs1718119 to be related to bone phenotypes. These include increased BMD in middle aged (≥50y) osteoporotic men and women (Wesselius *et al.,* 2012, Husted *et al.,* 2013) and a reduced susceptibility to vertebral fracture in post-menopausal women and osteoporotic men and women (Jorgenson *et al.,* 2012; Husted *et al.,* 2013). This is the first known study, however, to show associations between rs1718119 and bone geometry in a young, active

population. The rs1718119 SNP is located in transmembrane domain 2 of *P2X7R*; an allelic variation results in increased receptor functioning related to monocyte activation and increases in interleukin-1 alpha and beta release from monocytes and macrophages (Stokes *et al.,* 2010). The close proximity to a permeability gating region is demonstrated in the mediation of pore formation (Sun *et al.,* 2009) and increased permeability to $K^+$ and ethidium$^+$ in comparison to *P2X7R* wild-type mice (Stokes *et al.,* 2010). As well as the differences observed between homozygosity of the rare allele and heterozygotes, increased cortical thickness and CSA at the 14% site were also seen when comparing homozygotes of the common allele and heterozygotes. These differences are surprising as the rare allele would have been expected to show gain of function characteristics based on its known mechanistic function (Please see section 2.5.2.1.3.2.2.). It can be speculated that gene-gene and/or gene-environment interactions may have occured in which those homozygous for the common allele may be compensated for the loss of function by another SNP. It is difficult to substantiate this in the present study, but recent data in mice demonstrated gene-gene and gene-environment modulation related to exercise (Kelly *et al.,* 2014).

Homozygosity for the variant allele of SNP rs3751143 was associated with a greater cortical density at the 38% site of the tibia in response to 12 weeks of increased volume training. rs3751143 has a known cellular function and research has shown allelic variations to have bone phenotypic consequences (Wesselius *et al.,* 2012; Ohlendorff *et al.,* 2007). rs3751143 is associated with a loss of function when glutamic acid is substituted with alanine. Homozygosity for the variant allele of rs3751143 has been shown to cause a complete loss of receptor function, whereas heterozygotes have half of the receptor functionality (Gu *et al.,* 2002), meaning that a linear dose response relationship is evident. *In vitro,* rs3751143 variations are associated with osteoclast apoptosis (Ohlendorff *et al.,* 2007), reduced pore formation (Gu *et al.,* 2002) and a reduction in pro-inflammatory cytokine secretion (Sluyter

*et al.,* 2004). *In vivo* rs3751143 variants are associated with lower hip BMD (Husted *et al.,* 2013) and a greater risk of fracture (Wesselius *et al.,* 2012; Ohlendorff *et al.,* 2007), which are contrary to the greater bone strength shown in the present study. The reason for the differences may be related to the population assessed and/or the location of the bone phenotype. Previous studies assessed primarily elderly and osteoporotic patients (Husted *et al.,* 2013; Ohlendorff *et al.,* 2007) who have divergent bone characteristics and environmental stresses compared to the younger athletic cohort in the present study. Trabecular bone (hip, lumbar spine) were the main sites identified in previous studies, whereas the present study showed no association with trabecular BMD at the epiphysis of the tibia, while the rest of the measurements were taken on predominantly cortical bone.

The genotype dependent morphology of bone structural properties in the present study may have implications for bone health, and injury risk. Cortical bone size is an important factor in the determination of bone strength (Seeman and Delmas, 2006). Decreased bone size has been associated with increased fracture risk (Newsham-West *et al.,* 2013), while BMD is a commonly used predictor of fracture risk (Duncan and Brown, 2008). Decreased or inferior bone phenotypic responses to exercise training may reflect a delay in structural adaptations or may highlight an area of weakness in the bone remodelling response. This may be symptomatic of the early stages of bone injury or may reflect subtle bone weakness, which could potentially lead to bone disease in later life. The findings showing a genotype dependent response to football specific training may also have implications for weight-bearing exercise being used as a treatment and preventative measure for bone disease. The present study showed a genotype dependent response to increased training volume in *P2X7R* rs3751143 and *Wnt16* rs2707466 and that several bone phenotypes were associated with SNP related differences at baseline (Table 5.4.). Although the specific training stimulus each

individual encountered, in terms of intensity, was not known, these data suggest that there may be a genotype dependent bone phenotypic response to football training. This highlights that different directional and magnitudinal loads may occur in adolescent footballers encountering the same training volume. This supports the premise that individualised training regimes may be required in order to elicit positive bone adaptations in all.

Significant associations between genotype and participant characteristics were shown (Appendix 5.6.). Higher training hours were significantly associated with *RANK* rs3018362 prior to the intervention and *P2X7R* rs1718119 prior to and following the intervention. As previously reviewed (Please see section 2.3.1.), differences in training volume and type correspond to differences in bone phenotypes, although the associations were not related to corresponding bone phenotypic changes in the present study. Although body mass and training hours increased in the whole cohort after 12 weeks (Table 5.2.), no genetic associations were shown with any of the demographic data recorded (Appendix 5.6.). These results are not unexpected due to the large environmental influences on body mass and training volume and the highly polygenic nature of height (Yang *et al.*, 2010). Although some demographic differences are likely to be associated with genotype and may indirectly have an influence on bone phenotype, none of the SNPs investigated in the present study have been previously associated with the demographic data recorded. It is unclear how the demographic data impacts on, or mediates, the measured bone phenotypes. It could be speculated that as body mass and presumed muscle mass increase as a result of training, so too does the strain exerted on the bone through an increase in ground reaction forces and bone strain as a result of muscular contractions, although there are likely to be a vast amount of genes and SNPs that mediate this process. Gene-environment interactions remain a poorly understood area of investigation and warrant genome wide exploration in large, heterogeneous populations with

the use of bioinformatics resources to examine how various genetic and environmental interactions combine.

Overall, 12 weeks of increased volume football training showed an anabolic effect on numerous bone phenotypes at a range of tibial sites (Table 5.3.). The present study shows; 1) an anabolic effect from an increased training volume in an already habitually active cohort who have an history of football specific training; 2) bone morphological effects of increased training in elite adolescent footballers. These prospective data in adolescent footballers are in line with cross sectional (Morgan *et al.,* 2011; Ferry *et al.,* 2012; Nilsson *et al.,* 2012) and longitudinal (Helge *et al.,* 2010; Krustrup *et al.,* 2010; Vicente-Rodriguez *et al.,* 2004) studies showing the osteogenic effects of football specific training. The observed response is thought to be due to the high magnitude, irregular impacts that football specific training necessitates (Vicente-Rodriguez *et al.,* 2003). Although anabolic adaptations were evident across the group as a whole, a degree of individual response was evident, in keeping with previous data (Nilsson *et al.,* 2012; Krustrup *et al.,* 2010; Vicente-Rodriguez *et al.,* 2004).

Despite being the largest known study to undertake such an investigation, it is not without limitation. Ethnicity and specific training stimulus (academy players were from 5 different clubs, and played in numerous positions) were not controlled. However, the study cohort was homogenous; the participants were of equivalent age and all male, they had similar lifetime and recent training histories and environment variables, such as the time of year the scan took place and dietary habits (participants ate two meals per day together at respective club), were also comparable. As there was a 12 week time difference between the first and second scan, participant maturation may have influenced the findings. However, no significant differences occurred in tibial length, ensuring the same tibial site was being scanned during both visits.

Due to the relatively short follow-up period (12 weeks) maturation is unlikely to have influenced the findings (Meiring *et al.,* 2014). Despite significant genotype dependent differences observed in bone phenotypes, such as cortical thickness, CSA and density, it is unclear whether these differences are clinically significant in terms of heightened susceptibility to bone injury. Lower BMD (Wentz et al., 2012), cortical CSA (Popp *et al.,* 2009) and cortical thickness (Newsham-West *et al.,* 2013) have all previously been associated with stress fracture injury, although these associations were not made in adolescent footballers. As sports and activities have different levels of loading caused by differences in training and match-play, bone phenotypic differences have also been shown to occur (Please see section 2.3.2.). Therefore, an injury risk threshold for bone phenotypes is yet to be characterised in this specific population. Only the tibia was assessed in the present study and so, the bone changes shown cannot be generalised to changes in bone structure at other anatomical locations. However, by using pQCT, important differentiations between cortical and trabecular bone could be made, clarifying the specific impact football training has on the components of bone. Due to the number of comparisons made, there is a possibility that the findings occurred by chance, however as the majority of the results are in the same direction as previously published literature and a mechanistic explanation can be offered, the chances of a type II error are unlikely.

### 5.4.5. Conclusions

An increase in bone phenotypes following 12 weeks of increased volume football training is evident. Individual SNPs were associated with bone phenotypes at baseline, following the intervention and the change between pre and post measurements thus, highlighting the genotype dependent bone phenotypic response to the same training stimulus. These data add

to our understanding of the genetic contribution to bone phenotypic responses and morphology in response to mechanical loading. It also highlights possible mechanisms to which pharmaceutical interventions may be applied in the hope of strengthening bone to improve bone health.

# Chapter 6.0. Genetic Associations with Stress Fracture Injury in Elite Athletes

## 6.1. Introduction

Study 2 showed that specific SNPs were associated with bone phenotypes before and after increased training and, in some cases, were associated with the change in bone phenotypic responses to an increased training volume in elite adolescent footballers. It remains to be seen whether the genotype related associations shown are of clinical significance in relation to the manifestation of bone injury, such as stress fracture.

Stress fractures arise following the inability of bone to tolerate repeated mechanical loading and are characterised by damage to the bone micro-architecture (Warden *et al.,* 2006), thought to be caused by an imbalance in osteoblastic bone formation and osteoclastic bone resorption. The subsequent micro-damage results in a net bone loss and localised bone weakening, prompting stress fracture development (Fyhrie *et al.,* 1998). Stress fracture incidence in elite athletes and military recruits ranges from 14 to 21% (Lappe *et al.,* 2001; Bennell *et al.,* 1996) and most commonly manifests in the lower limbs (Gaeta *et al.,* 2005). Elite athletes and military recruits have an increased risk of stress fracture injury in comparison to the general population due to the high rate and amplitude of mechanical loading in their training.

Stress fracture injury in athletes is likely to have a complex aetiology involving numerous factors. For example, prior training (Tenforde *et al.,* 2013) and biomechanical variables (*e.g.,* running kinematics) (Milner *et al.,* 2006) are implicated in stress fracture risk. Susceptibility may also have a genetic component, supported by reports of monozygotic twins developing similar stress fracture injuries (Singer *et al.,* 1990; Van Meensal and Peers, 2010), multiple stress fractures occurring in the same individual (Lambros and Alder 1997), stress fractures in some individuals but not in others undertaking identical training protocols (Lappe *et al.,*

2001; Bennell *et al.*, 1996) and a family history of stress fracture injury acting as a risk factor (Loud *et al.,* 2007).

No published literature exists in relation to genetic associations with stress fracture injury in elite athletes. Genetic associations with stress fracture incidence have been investigated in military personnel using a variety of SNPs previously associated with receptors known to influence bone mineralisation (Garnero *et al.*, 1996), remodelling (Garnero *et al.,* 1996) and endocrine abnormalities (Beilin *et al.,* 2000). Associations were shown for SNPs and haplotype blocks within the vitamin D receptor (Chatzipapas *et al.,* 2009; Korvala *et al.,* 2010) and an androgen receptor repeat sequence (Yanovich *et al.,* 2011). Other studies have shown no associations for the same SNPs in other military populations (Cosman *et al.,* 2013; Valimaki *et al.,* 2005). The reason for the disparity may be due to the range of SNPs analysed and small numbers of stress fracture cases in some studies (*e.g.,* n=64, Chatzipapas *et al.,* 2009). Given that disturbance in bone remodelling and the inability of bone to withstand continued bouts of mechanical loading are implicated in the development of stress fracture injury (Warden *et al.,* 2006), SNPs consistently associated with these bone phenotypes in large-scale studies would appear worthy of focused study. As all previous studies have used military personnel, studies involving alternative cohorts with a similarly high incidence of stress fracture injury (*e.g.,* athletes) may provide insights into both the aetiology and genetic susceptibility to stress fracture injury.

Many genes and SNPs are candidates for influencing stress fracture injury risk from previously published literature on genetic associations with fracture, BMD and biochemical markers of bone turnover (Please see section 2.5.2.1.3.; Table 2.3.). Many SNPs are thought

to influence *Wnt* (Study 2 and please see section 2.5.2.1.3.1.) and *RANK/RANKL/OPG* (Study 2 and please see section 2.5.2.1.3.2.) signalling pathways .

Other less established genes/SNPs may also demonstrate genetic associations with stress fracture injury; these include *P2X7R* (Study 2 and Please see section 2.5.2.1.3.2.2.) and *VDR* (Please see section 2.5.2.1.3.2.1.2.), which have been shown to influence *RANKL* expression (Gartland *et al.*, 2003; Yoskovitz *et al.*, 2013). Amongst other hypothesised mechanisms, the kallikrein-kinin system has been shown to regulate bone remodelling (Lerner, 1994). Kinin agonists appear to increase bone resorption through the stimulation of osteoclasts enhancing mineral mobilisation and matrix degradation (Lerner, 1994). Functional variants of SNPs within this system have been associated with large decreases in bone remodelling *in vivo* and *in vitro* (Slim *et al.*, 2002).

The present study will determine whether SNPs that have previously been associated with other bone phenotypes (Please see section 2.5.2.1.3.; Table 2.3.) are associated with the occurrence of stress fracture injury in elite athletes.

## 6.2. Method

### 6.2.1. Participants

518 elite athletes, 449 male and 69 female, were recruited from professional sports clubs and elite sporting associations based in North America and the United Kingdom to form the Stress Fracture in Elite Athletes (SFEA) cohort (see Table 6.1. for participant characteristics). Participating elite athletes competed in various sports including football, cricket, track and field, rowing, boxing, tennis, hockey and gymnastics. 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 they 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 (Appendix 6.1.). The stress fracture group was made up of athletes that were classified as having a radiologically confirmed stress fracture injury (*e.g.*, X-ray, MRI, CT). Athletes who reported stress fracture injuries without radiological confirmation were not included in 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. Case and Control athletes were present in each sport. Ethical approval was granted by the Nottingham Trent University Ethical Review Committee (Humans).

Data were sub-classified into male, female, cases of multiple stress fracture, invasion sports (football / hockey), cricketers, runners, stress fractures occurring before the age of 21 y and leg stress fractures, excluding the metatarsals, for the purposes of analyses. Sub-classifications were made due to the suspected sport specific aetiology of stress fracture, the assumed greater genetic element in cases of multiple stress fracture, the reportedly distinct anatomically specific mediation of bone by genotype, previously shown bone phenotypic sex specific associations with SNPs and the number of athletes in each sub-classification. Stress

fracture injury 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%).

Of the 518 athletes who volunteered to take part in the study, 17 were removed from the analysis due to inconclusive stress fracture diagnosis (*e.g.*, stress reactions, presumed stress fractures not radiologically confirmed). 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 to have never experienced a stress fracture or 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 cohort ($P<0.01$) (Table 6.1.). These differences remained significant along with height, body mass and hours training when only males were analysed ($P<0.01$). Of the other sub-classifications analysed, age (runners, cricketers, cases of multiple stress fracture, leg stress fractures excluding the metatarsals), height, (runners, cricketers, leg stress fractures excluding the metatarsals), weight (females, runners, cricketers, leg stress fractures excluding the metatarsals), BMI (cricketers, cases of multiple stress fracture, leg stress fractures excluding the metatarsals), age at elite status (female) and hours spent training per week (female) were significantly different when stress fracture cases were compared to Controls (Appendix 6.2.)

Table 6.1. Participant characteristics for stress fracture and non-stress fracture groups. A significance level of P<0.05 was used. P values of greater than 0.05 are stated as >0.05. * was used to denote significance.

| Characteristics | Stress fracture (n=125) | Non-stress fracture (n=376) | *P*-value |
|---|---|---|---|
| Age (y) | 27.7±7.5 | 24.4±5.4 | <0.01* |
| Age at stress fracture (y) | 19.9±3.9 | - | |
| 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 (y) | 18.2+4.2 | 17±2.2 | <0.01* |
| Training (h/wk) | 20±11.3 | 18.2±10.1 | 0.12 |
| Alcohol consumption (units/wk) | 5.2±6.9 | 4.1±6.1 | 0.15 |

### 6.2.2. Genetic Procedures

Methods of saliva sample collection, DNA extraction and genotyping are described in sections 3.4 – 3.8.

### 6.2.3. 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 (HWE) and differences in stress fracture occurance between genotypes. Odds ratios and corresponding 95% confidence intervals were calculated for stress fracture injury risk. 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. All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 13.0 (SPSS, Inc., Chicago, IL, USA).

## 6.3. Results

All SNPs were in accordance with HWE with the exception of rs4355801 (*OPG*), which produced call rates ≥ 91% and had minor allele frequencies comparable to previous literature (Table 6.2.). The method of genotyping is robust and has a high level of internal validation and reliability, making errors in genotyping an unlikely reason for the deviance.

Table 6.2. HWE P value, call rate and minor allele frequency (MAF) of SNPs analysed in stress fracture and non-stress fracture groups. A significance level of P<0.05 was used. * was used to denote significance.

| SNP | Gene | HW P-value | Call Rate % | MAF % |
|---|---|---|---|---|
| rs2230912 | *P2X7R* | 0.16 | 94.6 | 17 |
| rs208294 | *P2X7R* | 0.07 | 97 | 2 |
| rs1653624 | *P2X7R* | 0.53 | 96.5 | 3 |
| rs3751143 | *P2X7R* | 0.16 | 94 | 16 |
| rs1718119 | *P2X7R* | 0.1 | 91.3 | 40 |
| rs3736228 | *LRP5* | 0.93 | 96.5 | 15 |
| rs1544410 | *VDR* | 0.94 | 94.3 | 39 |
| rs731236 | *VDR* | 0.85 | 94.5 | 37 |
| rs7975232 | *VDR* | 0.09 | 94 | 47 |
| rs10735810 | *VDR* | 0.26 | 93.9 | 33 |
| rs1801197 | *CTR* | 0.39 | 94.6 | 28 |
| rs1877632 | *SOST* | 0.14 | 93.1 | 30 |
| rs3018362 | *RANK* | 0.53 | 95.8 | 35 |
| rs1021188 | *RANKL* | 0.11 | 96.2 | 18 |
| rs9594738 | *RANKL* | 0.24 | 96 | 42 |
| rs4355801 | *OPG* | ***0.04** | 95.6 | 41 |
| rs7041 | *GC* | 0.12 | 96.2 | 49 |
| rs4588 | *GC* | 0.79 | 95.8 | 28 |
| rs1800012 | *COL1A1* | 0.83 | 97 | 15 |
| rs16987491 | Kallikrein | 0.56 | 97.8 | 47 |
| rs1799722 | Bradykinin | 0.11 | 91.6 | 50 |
| rs3801387 | *Wnt16* | 0.71 | 95 | 28 |

Of the 533 comparisons made, 67 significant associations were shown (Table 6.3.). Of the 22 SNPs investigated, 12 were significantly associated with stress fracture injury in one or more of the classifications explored (Table 6.3.). Significant associations (P<0.05) with stress fracture injury were shown with *SOST*, *LRP5*, *P2X7R*, *RANK/RANKL/OPG*, Bradykinin and

*VDR* allele variations. No significant differences were seen in the other SNPs investigated (P>0.05) (Data shown in Appendix 6.3.). Odds ratio and 95% confidence intervals for all classifications are shown in Appendix 6.4.

### 6.3.1. *RANK/RANKL/OPG*

Four of the SNPs genotyped in close proximity to genes in the *RANK/RANKL/OPG* signalling pathway were associated with stress fracture injury occurrence in one or more sub-classifications (P<0.05). rs3018362 (*RANK*) was the most prominent; homozygotes for the rare allele combined with heterozygotes was significantly associated with a greater stress fracture risk in the whole cohort, males and runners (P<0.05). A copy of the variant *A* allele was also associated with stress fracture occurrence in the whole cohort, males and with stress fractures occurring before the age of 21 y (P<0.05).

Of the *RANKL* SNPs analysed, those homozygous for the rare allele of rs1021188 reported a greater occurrence of stress fracture injury in the whole cohort (OR 2.93; UCI 7.28- LCI 1.18), and in male (OR 3.38; UCI 9.35- LCI 1.22), multiple stress fracture (OR 3.28; UCI 10.11- LCI 1.06) and with stress fracture occurring before the age of 21y (OR 1.37; UCI 1.63- LCI 0.36) sub-classifications in comparison to the non-stress fracture Control groups (P<0.05). The rare allele frequency of rs1021188 was also associated with a greater stress fracture injury occurrence in football and hockey players (P<0.05). rs9594738 was associated with increased stress fracture occurrence in cricket players (homozygotes for the rare allele) and football/hockey player sub-classifications (homozygotes of the rare allele, allele frequency and rare allele combined with heterozygotes) (P<0.05). The rare allele combined with heterozygotes of *OPG* SNP rs4355801 was significantly associated with increased stress

fracture occurrence in runners and cases of multiple stress fracture when compared to homozygotes of the common allele, while the frequency of the rare allele was significantly increased in leg stress fracture (excluding metatarsal) and runners ($P<0.05$).

Exploratory analysis of allele combinations were conducted for multiple SNPs in close proximity to genes in the *RANK/RANKL/OPG* signalling pathway to examine how potential gene-gene interactions may affect stress fracture injury risk. However, due to the cohort size and in some cases the relatively low minor allele frequency, meaningful findings could not be deduced and therefore, no further analysis was undertaken.

### 6.3.2. *Wnt* Signalling

*SOST* SNP rs1877632 heterozygotes were associated with stress fracture risk in the whole cohort, cases of multiple stress fracture, football/hockey players, and runners when compared to homozygotes of the common allele ($P<0.05$). Significant associations were also shown when heterozygotes were combined with homozygotes of the rare allele and compared to homozygotes of the common allele in the same sub-classifications (with the exception of the running classification) ($P<0.05$). The frequency of the rare allele was significantly greater in stress fracture sufferers in the whole cohort, cases of multiple stress fracture and football/hockey stress fracture groups in comparison to non-stress fracture Control groups ($P<0.05$). The frequency of the rare allele of *LRP5* SNP rs3736228 was significantly associated with greater stress fracture occurrence in the running sub-classification ($P<0.05$).

### 6.3.3. *P2X7R*

Of the *P2X7R* SNPs only rs3751143 and rs1718119 were associated with stress fracture occurrence ($P<0.05$). Significant associations occurred across multiple sub-classifications (Table 6.3.). Greater stress fracture occurrence was detected when homozygotes for the rare allele were combined with heterozygotes of rs3751143 and compared to common allele homozygotes in the whole cohort and leg stress fracture (excluding metatarsal) sub-classifications ($P<0.05$). The frequency of the rare allele was also significantly greater in leg stress fracture (excluding metatarsal) and the cricketer stress fracture group in comparison to non-stress fracture Controls ($P<0.05$). Homozygosity for the variant, gain of function allele of rs1718119 was significantly associated with a reduced stress fracture risk in cases of multiple stress fracture and runners when compared to non-stress fracture Control groups ($P<0.05$). The gain of function allele of rs1718119 was also significantly greater in the non-stress fracture groups of both the multiple stress fracture and running cohorts ($P<0.05$).

### 6.3.4. Bradykinin

A significant association between stress fracture occurrence and the bradykinin 2 receptor insertion/deletion sequence rs1799722 was shown in the whole cohort, males, football/hockey players, females and stress fractures occurring before the age of 21 ($P<0.05$). Insertions (+9) were significantly associated with a greater occurrence of stress fracture risk in male, stress fractures occurring before the age of 21 y and football/hockey player sub-classifications ($P<0.05$). When insertions and insertion/deletions were combined, a significantly greater stress fracture risk was shown in the whole cohort, males and football/hockey players in comparison to those with the deletion sequence (-9) ($P<0.05$).

Table 6.3. Association of SNPs with stress fracture injury in elite athletes for the whole cohort, males, multiple stress fractures and leg stress fractures excluding metatarsal fractures. (Gene) = closest gene; (EA) = effect allele; (P) = P value; (Homo) = homozygote for the variant allele; (Combined with Heterozygote) = homozygote for the variant allele combine with heterozygote; (AF) = allele frequency; (-) = insufficient participants with variant allele to perform appropriate statistics. **BOLD** depicts significance (P<0.05).

| | | | | Whole cohort. *P* | | | Males. *P* | | | Multiple Stress Fractures. *P* | | | Leg excluding metatarsal. *P* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RS Number | Location | Gene | EA | Homo | Combined with Heterozygote | AF | Homo | Combined with Heterozygote | AF | Homo | Combined with Heterozygote | AF | Homo | Combined with Heterozygote | AF |
| rs2230912 | 12q24.3 | *P2x7* | G | 0.53 | 0.92 | 0.81 | 0.60 | 0.92 | 0.83 | 0.55 | 0.78 | 0.58 | 0.55 | 0.54 | 0.39 |
| rs208294 | 12q24.3 | *P2x7* | T | - | 0.32 | 0.10 | - | 0.52 | 0.21 | - | 0.70 | 0.37 | - | 0.39 | 0.16 |
| rs1653624 | 12q24.3 | *P2x7* | T | - | - | - | - | - | - | - | - | - | - | - | - |
| rs3751143 | 12q24.3 | *P2x7* | G | 0.12 | **0.05** | 0.06 | 0.27 | 0.81 | 0.08 | 0.66 | 0.62 | 0.45 | 0.08 | **0.02** | **0.02** |
| rs1718119 | 12q24.3 | *P2x7* | A | 0.34 | 0.60 | 0.18 | 0.63 | 0.67 | 0.34 | **0.04** | 0.11 | **0.01** | 0.07 | 0.91 | 0.26 |
| rs3736228 | 11q13.4 | *LRP5* | T | 0.46 | 0.27 | 0.44 | 0.82 | 0.73 | 0.75 | 0.10 | 0.11 | 0.47 | 0.82 | 0.59 | 0.52 |
| rs1544410 | 12q13.11 | *VDR* | b | 0.46 | 0.27 | 0.44 | 0.39 | 0.26 | 0.51 | 0.10 | 0.11 | 0.47 | **0.02** | **0.01** | **0.01** |
| rs731236 | 12q13.11 | *VDR* | t | 0.50 | 0.36 | 0.61 | 0.45 | 0.53 | 0.97 | **0.01** | **0.02** | 0.21 | **0.03** | **0.01** | **0.01** |
| rs7975232 | 12q13.11 | *VDR* | a | 0.62 | 0.54 | 0.92 | 0.39 | 0.28 | 0.64 | 0.50 | 0.97 | 0.52 | 0.17 | 0.17 | **0.04** |
| rs10735810 | 12q13.11 | *VDR* | f | 0.17 | 0.20 | **0.03** | 0.35 | 0.62 | 0.21 | **0.01** | **0.00** | **0.02** | 0.06 | 0.15 | **0.02** |
| rs1801197 | 7q21.3 | *CTR* | C | 0.72 | 0.74 | 0.48 | 0.57 | 0.53 | 0.30 | 0.99 | 0.88 | 0.87 | 0.69 | 0.84 | 0.57 |
| rs1877632 | 17q11.2 | *SOST* | G | **0.05** | **0.02** | **0.04** | 0.28 | 0.12 | 0.11 | **0.05** | **0.02** | **0.05** | 0.30 | 0.66 | 0.16 |
| rs3018362 | 18q22.1 | *RANK* | A | 0.08 | **0.05** | **0.00** | 0.07 | **0.04** | **0.00** | 0.17 | 0.14 | 0.38 | 0.68 | 0.43 | 0.35 |
| rs1021188 | 13q14 | *RANKL* | A | **0.02** | 0.98 | 0.27 | **0.03** | 0.85 | 0.21 | **0.01** | 0.31 | 0.98 | 0.13 | 0.49 | 0.96 |
| rs9594738 | 13q14 | *RANKL* | T | 0.26 | 0.26 | 0.67 | 0.32 | 0.18 | 0.39 | 0.74 | 0.81 | 0.86 | 0.99 | 0.92 | 0.93 |
| rs4355801 | 8q24 | *OPG* | G | 0.66 | 0.40 | 0.28 | 0.82 | 0.57 | 0.45 | 0.13 | **0.04** | 0.07 | 0.08 | 0.24 | **0.02** |
| rs7041 | 4q12-q13 | *DBP* | G | 0.50 | 0.99 | 0.84 | 0.71 | 0.84 | 0.74 | 0.53 | 0.32 | 0.21 | 0.53 | 0.68 | 0.79 |
| rs4588 | 4q12-q14 | *DBP* | A | 0.31 | 0.22 | 0.39 | 0.65 | 0.09 | 0.18 | 0.88 | 0.88 | 0.95 | 0.41 | 0.33 | 0.58 |
| rs1800012 | 17q21.33 | *COL1A1* | s | 0.38 | 0.93 | 0.57 | 0.67 | 0.96 | 0.81 | 0.33 | 0.61 | 0.94 | 0.52 | 0.99 | 0.71 |
| rs16987491 | 19q13.41 | Kallikrein | A | - | 0.23 | 0.64 | - | 0.20 | 0.63 | - | 0.76 | 0.92 | - | 0.19 | 0.65 |
| rs1799722 | 14q32.1 | Bradykinin | 9 | 0.06 | **0.04** | **0.00** | **0.01** | **0.03** | **0.00** | 0.47 | 0.35 | 0.72 | 0.26 | 0.54 | 0.14 |
| rs3801387 | 7q31.31 | *WNT16* | G | 0.30 | 0.45 | 0.14 | 0.30 | 0.29 | 0.09 | 0.62 | 0.40 | 0.31 | 0.39 | 0.93 | 0.62 |

| | | | Cricket | | | Football and hockey | | | Running | | | Female | | | Pre 21 stress fracture | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RS Number | Location | Gene | EA | Alone | Combined with Heterozygote | AF | Alone | Combined with Heterozygote | AF | Alone | Combined with Heterozygote | AF | Alone | Combined with Heterozygote | AF | Alone | Combined with Heterozygote | AF |
| rs2230912 | 12q24.3 | *P2x7* | G | 0.58 | 0.62 | 0.77 | 0.87 | 0.59 | 0.56 | - | 0.14 | 0.08 | - | 0.61 | 0.53 | 0.47 | 0.36 | 0.48 |
| rs208294 | 12q24.3 | *P2x7* | T | - | - | - | - | 0.12 | 0.10 | - | - | - | - | - | - | - | 0.39 | 0.32 |
| rs1653624 | 12q24.3 | *P2x7* | T | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| rs3751143 | 12q24.3 | *P2x7* | G | 0.27 | 0.16 | **0.03** | 0.25 | 0.40 | 0.72 | - | 0.42 | 0.31 | 0.70 | 0.42 | 0.95 | 0.65 | 0.55 | 0.39 |
| rs1718119 | 12q24.3 | *P2x7* | A | 0.95 | 0.80 | 0.88 | 0.98 | 0.88 | 0.94 | **0.03** | 0.36 | **0.00** | 0.61 | 0.95 | 0.57 | 0.95 | 0.78 | 0.85 |
| rs3736228 | 11q13.4 | *LRP5* | T | 0.55 | 0.49 | 0.45 | 0.89 | 0.68 | 0.61 | - | 0.29 | **0.02** | - | 0.73 | 0.90 | 0.70 | 0.67 | 0.75 |
| rs 1544410 | 12q13.11 | *VDR* | b | 0.33 | 0.09 | 0.16 | 0.29 | 0.14 | 0.12 | 0.90 | 0.96 | 0.69 | 0.79 | 0.84 | 0.54 | 0.22 | 0.10 | 0.18 |
| rs 731236 | 12q13.11 | *VDR* | t | 0.18 | 0.10 | **0.04** | 0.33 | 0.14 | 0.15 | 0.95 | 0.67 | 0.77 | 0.52 | 0.42 | 0.17 | 0.17 | 0.10 | 0.28 |
| rs 7975232 | 12q13.11 | *VDR* | a | 0.24 | 0.15 | **0.04** | 0.51 | 0.68 | 0.77 | 0.80 | 0.70 | 0.36 | 0.58 | 0.30 | 0.30 | 0.29 | 0.37 | 0.98 |
| rs 10735810 | 12q13.11 | *VDR* | f | 0.89 | 0.83 | 0.99 | 0.76 | 0.85 | 0.88 | 0.19 | 0.21 | **0.00** | 0.24 | 0.09 | **0.04** | 0.59 | 0.62 | 0.39 |
| rs1801197 | 7q21.3 | *CTR* | C | 0.90 | 0.95 | 0.86 | 0.21 | 0.29 | 0.66 | 0.36 | 0.23 | 0.23 | 0.63 | 0.46 | 0.54 | 0.50 | 0.86 | 0.48 |
| rs1877632 | 17q11.2 | *SOST* | G | 0.52 | 0.79 | 0.41 | **0.03** | **0.03** | **0.00** | **0.05** | 0.07 | 0.23 | 0.18 | 0.10 | 0.17 | 0.66 | 0.57 | 0.83 |
| rs3018362 | 18q22.1 | *RANK* | A | 0.33 | 0.14 | 0.07 | 0.06 | 0.40 | 0.81 | - | **0.01** | 0.10 | 0.96 | 0.94 | 0.82 | 0.16 | 0.09 | **0.03** |
| rs1021188 | 13q14 | *RANKL* | A | 0.28 | 0.97 | 0.43 | 0.11 | 0.14 | **0.03** | - | 0.15 | 0.14 | - | 0.69 | 0.84 | **0.04** | 0.16 | 0.14 |
| rs9594738 | 13q14 | *RANKL* | T | **0.05** | 0.06 | 0.43 | **0.04** | **0.01** | **0.02** | 0.55 | 0.30 | 0.13 | 0.45 | 0.90 | 0.49 | 0.45 | 0.27 | 0.51 |
| rs4355801 | 8q24 | *OPG* | G | 0.36 | 0.27 | 0.07 | 0.27 | 0.29 | 0.07 | 0.10 | **0.03** | **0.00** | 0.56 | 0.33 | 0.54 | 0.93 | 0.76 | 0.91 |
| rs7041 | 4q12-q13 | *DBP* | G | 0.16 | 0.58 | 0.62 | 0.20 | 0.10 | 0.64 | 0.40 | 0.96 | 0.21 | 0.73 | 0.54 | 0.87 | 0.44 | 0.66 | 0.83 |
| rs4588 | 4q12-q14 | *DBP* | A | 0.45 | 0.11 | 0.13 | 0.21 | 0.20 | 0.46 | - | 0.08 | 0.07 | - | 0.72 | 0.62 | 0.54 | 0.32 | 0.42 |
| rs1800012 | 17q21.33 | *COL1A1* | s | 0.37 | 0.83 | 0.45 | 0.41 | 0.40 | 0.56 | 0.27 | 0.25 | 0.26 | - | 0.40 | 0.13 | 0.29 | 0.71 | 0.34 |
| rs16987491 | 19q13.41 | Kallikrein | A | - | 0.74 | 0.33 | - | 0.67 | 0.28 | - | 0.41 | 0.68 | - | 0.97 | 0.98 | 0.10 | 0.54 | - |
| rs1799722 | 14q32.1 | Bradykinin | 9 | 0.28 | 0.89 | 0.24 | **0.00** | **0.00** | **0.00** | 0.48 | 0.30 | 0.38 | 0.69 | 0.88 | **0.04** | **0.05** | 0.09 | **0.01** |
| rs3801387 | 7q31.31 | *WNT16* | G | 0.65 | 0.55 | 0.77 | 0.30 | 0.08 | 0.20 | 0.62 | 0.54 | 0.13 | - | 0.60 | 0.74 | 0.56 | 0.42 | 0.25 |

The frequency of those with the insertion sequence was greater in the whole cohort, male, female, stress fractures occurring before the age of 21 and football/hockey player stress fracture groups in comparison to non-stress fracture Control groups ($P<0.05$).

### 6.3.5. *VDR*

A significant association between *VDR* SNP rs10735810 and increased occurrence of multiple stress fracture injury was shown in homozygotes of the rare f allele when compared to homozygotes of the common allele combined with heterozygotes ($P<0.05$). Stress fracture occurrence was also associated with the frequency of the rare allele in the following sub-classifications; cases of multiple stress fracture, male, leg stress fracture (excluding metatarsal) and runners ($P<0.05$). *VDR* SNP rs15444410 was significantly associated with leg stress fracture excluding metatarsal ($P<0.05$) with the frequency of the rare b allele was over-represented in stress fracture cases. Heterozygotes and heterozygotes combined with homozygotes of the rare allele, were associated with greater stress fracture occurrence ($P<0.05$). Carriers of the rare allele of rs731236 were associated with greater stress fracture occurrence in cases of multiple stress fractures and in leg fractures excluding the metatarsals ($P<0.05$). Conversely, the common allele of rs731236 was more frequent in the stress fracture group of the cricketer sub-classification in comparison to the non-stress fracture Control group ($P<0.05$). The rare allele frequency of rs7975232 was significantly associated with stress fracture occurrence in the cricket sub-classification ($P<0.05$). However, the opposite was true for the leg fractures (excluding the metatarsals) sub-classification, as the greater frequency of the common allele was associated with stress fracture injury risk ($P<0.05$).

## 6.4. Discussion

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 shows that SNPs associated with bone phenotypic alterations are associated with stress fracture injury in elite athletes. 12 novel SNPs, which have never been associated with stress fracture injury, in close proximity to *SOST*, *P2X7R*, *RANK*, *RANKL*, *OPG*, Bradykinin and *VDR* genes, were significantly associated with stress fracture injury in the whole cohort and various sub-classifications. The locality of the SNPs to major bone formation (*Wnt*) and bone resorption (*RANK/RANKL/OPG)* signalling pathways, together with some SNPs having a known functional effect on BMUs (*P2X7R*) may explain some of the contributory factors in the aetiology of stress fracture injury.

The association of different SNPs with distinct sub-classifications illustrates the complexity of stress fracture injury aetiology. Due to the diversity of the SNPs associated, the point at which the signalling pathway cascade is affected is still to be established. Although the exact mechanism of the association is awaiting discovery, alterations in bone remodelling and various mechanotransductive responses in bone to mechanical loading are two prominent theories for their effect. These findings add to previous research and meta-analyses that have shown these SNPs to be associated with other bone phenotypes (Table 2.3.).

### 6.4.1. *RANK/RANKL/OPG*

The association of the rare allele of *RANK* SNP rs3018362 with stress fracture injury risk adds to previous studies investigating the association of this SNP with other bone phenotypes. Individual studies and meta-analyses have shown the rare allele of SNP rs3018362 to be

associated with Paget's disease (Albagha *et al.,* 2005), and BMD at the tibia (Paternoster *et al.,* 2010), hip (Styrkarsdottir *et al.,* 2008; Liu *et al.,* 2010) lumbar spine (Liu *et al.,* 2010) and femoral neck (Shang *et al.,* 2013). Although rs3018362 has no known functional effect, the range of bone phenotypes it is associated with suggest it may impact on a range of different molecular mechanisms. The *RANK/RANKL/OPG* signalling pathway is known to have a key role in bone resorption as a result of its mediation of osteoclast functioning (Please see section 2.5.2.1.3.2.) and has been repeatability associated with BMD. Its important role is further emphasised by the association of the anti-RANKL drug denosumab with greater BMD and a reduction in fracture risk (Bone *et al.,* 2011). Although BMD is multi-factorial and is known to be influenced by factors such as age and diet, genetic variations in BMD determinates, such as cortical porosity, may be the reason for the associations shown (Paternoster *et al.,* 2010). As low BMD has been associated with stress fracture injury (Wentz *et al.,* 2012), this provides a plausible mechanism for the associations shown in the present study.

*RANKL* SNP rs1021188 was associated with increased stress fracture occurrence in several sub-classifications (Table 6.3.) and cortical CSA and thickness of the tibia in Study 2. These data provide a possible mechanism, in which reduced bone area and thickness may be associated with the pathophysiology of stress fracture. *RANKL* SNP rs1021188 has previously been associated with cortical porosity at the tibia in the GOOD cohort (Paternoster *et al.,* 2013), circulating free *RANKL,* and both cortical (Paternoster *et al.,* 2010) and volumetric BMD (Paternoster *et al.,* 2013) at the tibia in GWAS meta-analyses covering a range of populations. As circulating *RANKL* and cortical porosity were both associated with rs1021188 (Paternoster *et al.,* 2010), this suggests that mineralisation and bone resorption may be rs1021188 dependent.

Despite not being in linkage disequilibrium, high recombination rates have previously been shown between rs1021188 and rs9594738 (Paternoster *et al.,* 2010), suggesting information is being exchanged between the SNPs. However, rs1021188 and rs9594738 were associated with different stress fracture sub-classifications (football/hockey players the only common sub-classifications) (Table 6.3.) and were also associated with different bone phenotypes in Study 2. These data suggest that these SNPs may have loading specific effects on different aspects of bone, despite evidence of an interaction (Paternoster *et al.,* 2010). The association of rs9594738 with stress fracture injury in only cricket and football/hockey sub-classifications may reflect the loading patterns experienced in these sports. The high incidence of stress fracture injury in skeletal regions consisting of mainly trabecular bone in cricketers (lumbar spine) adds substance to this premise although, it should be acknowledged that the exact location of stress fracture injury in the lumbar spine was not known and the lumbar spine consists of both trabecular and cortical bone. *RANKL* SNP rs9594738 has previously been associated with phenotypes including tibial CSA (Study 2) and BMD (Styrkarsdottir *et al.,* 2008), while *OPG* SNP rs4355801 has been associated with osteoporotic fracture (Richards *et al.,* 2008). The present data showing these SNPs to be associated with stress fracture injury adds further evidence in support of their role in the regulation of bone phenotypic alterations and bone injury. As well as the differences in aetiology of injury between stress fracture and osteoporotic fracture, data from GWAS have shown bone phenotypic associations with rs4355801 and rs9594738 in areas mainly consisting of trabecular bone (Table 2.3.), whereas stress fractures occur predominantly in the tibial diaphysis and metatarsals (Iwamoto *et al.,* 2011), sites mainly comprised of cortical bone. The association of rs9594738 with stress fracture injury in cricketers, but not the whole cohort, may be explained by the frequency of stress fractures occurring at the lumbar spine (an area of mostly trabecular bone, caused by the unique nature of the cricket bowling action)

in cricketers (71% in the present study) in comparison to the whole cohort (33% in the present study). This suggests that rs9594738 may mediate the early stages of the mechanotranductive process predominantly acting upon trabecular bone.

The current findings in relation to stress fracture injury, together with the evidence from previous studies into other bone phenotypes, suggest that the SNPs analysed are important regulators of the *RANK/RANKL/OPG* signalling pathway (Please see section 2.5.2.1.3.2.). The variance in genotype might affect the binding process between *RANK* and *RANKL*, influencing osteoclast differentiation and activation (Boyle *et al.*, 2003); subsequently mediating bone resorption. The exact mechanism of how these SNPs regulate bone turnover, however, is not known. If bone formation fails to keep pace with bone resorption during periods of mechanical loading (training), bone loss and ultimately bone weakness are likely to occur, possibly increasing injury risk. This mechanism of injury seems plausible in the current elite athlete cohort, given the high amount of repetitive mechanical loading undertaken throughout training and match-play and the short periods of recovery between exercise sessions.

The influence of exercise on the *RANK/RANKL/OPG* signalling pathway, circulating *RANKL* and *OPG* concentrations and *RANK* density may also introduce confounding effects due to the athletic cohort used in the present study. Circulating free *RANKL* is notoriously difficult to measure (Hegedus *et al.*, 2002), whereas the influence of exercise on *OPG*, an analyte that is readily quantified, is more widely reported (Scott *et al.*, 2010; 2011a). The outcome of exercise on *OPG* concentrations is variable; concentrations of serum *OPG* have been shown to increase following running in recreationally active males (Scott *et al.*, 2010; 2011a) and are elevated in habitually active females (West *et al.*, 2009). Conversely, *OPG* concentrations

were not altered in obese males following a 6-month training programme (Davenport *et al.*, 2012). As the SFEA cohort was comprised of elite athletes from different sports, the different training regimes undertaken may have influenced *OPG* concentrations differently and, therefore, confounded any genetic associations. There is always the possibility that *OPG* fluctuations do not solely relate to effects on bone, since *OPG* is not bone specific and can be produced by muscle and endothelial cells (Collin-Osdoby, 2004), as well as osteoblasts that secrete *OPG* in response to exercise through inflammation or muscle damage.

### 6.4.2. *Wnt* Signalling

The *Wnt* signalling pathway is a predominant regulator of bone metabolism, having a particularly significant role in the mediation of the differentiation and longevity of osteoblasts thus affecting bone formation (Please see section 2.5.2.1.3.1.). The importance of *SOST* rs1877632 in stress fracture injury risk adds to data from Study 2 and previous research (Balemans *et al.,* 2001; Uitterlinden *et al.,* 2004; Yerges *et al.,* 2009) showing associations between this genotype and other bone phenotypes. While the rare allele was associated with stress fracture risk in the present study, an increase in trabecular density was shown in Study 2. The reason for these contrasting findings in relation to the directional influence of the *SOST* SNP may be due to the anatomical location of measurement. Study 2 only shows differences in trabecular density, while previous research has shown areas of predominantly trabecular bone (lumber spine) to be affected (Yerges *et al.,* 2009). As stress fractures most commonly occur in locations made up of cortical bone, it is not surprising that the present study data does not follow previous associations. Interestingly, in the cricket sub-classification, where a large proportion of stress fractures occur in the lumbar spine, no associations were shown. This further highlights the premise that SNPs may act on distinct

types of bone and have divergent effects on different bone compositions. The mechanisms of how the rare allele increases stress fracture risk is not known, however the variant allele has been associated with an increase in bone formation (Li *et al.,* 2008) thus, offering a potential mechanistic explanation. As sclerostin inhibits *Wnt* signalling and as result, reduces bone formation (Please see section 2.5.2.1.3.1.), it can be hypothesised that the rare allele of rs1877632 down regulates sclerostin expression. Athletes in weight bearing sports secrete higher levels of sclerostin in comparison to their non-weight bearing equivalents, suggesting there may be a mechanostransductive element of sclerostin mediation (Fazeli *et al.,* 2013). The reason for this is not known, but theories include; the result of a larger number of sclerostin expressing osteocytes in the bone of weight-bearing athletes due to their higher bone mineral content and increased sclerostin levels acting as a safety mechanism to limit 'excessive' increases in bone formation as the result of high volume loading (Fazeli *et al.,* 2013). Hitherto, the effect mechanical loading has on *SOST* expression has been equivocal (Lombardi *et al.,* 2012; Amrein *et al.,* 2012). The impact of high volume and high magnitude mechanical loading, resulting in increasing sclerostin concentrations, which will have been regularly experienced by elite athletes in the present study, combined with genetic variation in the *SOST* gene, could also be the reason for observed findings.

The rare allele of *LRP5* SNP rs3736228 was associated with the running sub-classification in the present study. Association studies between variants in rs3736228 SNP and bone phenotypes are common due to a role of rs3736228 in a range of bone phenotypes (Please see section 2.5.2.1.3.1.3.), including osteoblast differentiation (Kato *et al.,* 2002) and osteoblast/osteocyte apoptosis (Javaheri *et al.,* 2011). The functional effects of rs3736228 suggest the reason for the association with stress fracture injury risk might be due to inadequate bone formation, which could lead to long-term bone weakness.

### 6.4.3. *P2X7R*

The current data shows an association between stress fracture injury incidence and functional polymorphisms within *P2X7R*. The loss of function variant allele of rs3751143 was associated with stress fracture injury in the elite athlete cohort as a whole, cricketers and in stress fractures occurring in the leg (excluding metatarsals). The gain of function variant in rs1718119 was associated with a reduction in stress fracture risk in runners and cases of multiple stress fracture.

The association of rs3751143 allelic variants with stress fracture occurrence is supported by a known cellular function and previous research showing associations with bone phenotypes (Please see section 2.5.2.1.3.2.2.). The rs3751143 polymorphism is associated with a substantial loss of function when glutamic acid is substituted with alanine. *In vitro*, the variant allele of rs3751143 is associated with osteoclast apoptosis (Ohlendorff *et al.*, 2007), reduced pore formation (Gu *et al.*, 2002) and reductions in pro-inflammatory cytokine secretion (Sluyter *et al.*, 2004). *In vivo*, rs3751143 variants are associated with lower BMD (Husted *et al.*, 2013) and an increased risk of fracture (Wesselius *et al.*, 2012; Ohlendorff *et al.*, 2007). The present data, showing that the gain of function allele is associated with lower stress fracture injury incidence, are in accordance with data from the previous study showing that cortical thickness at the 38% site of the tibia is greatest in homozygotes for the rare allele. Recent studies have also shown variations in rs1718119 to be related to bone phenotypes (Wesselius *et al.*, 2012; Husted *et al.*, 2013; Jorgenson *et al.*, 2012).

Individual non-synonymous functional SNPs within the *P2X7R* regulate bone remodelling and the mechanisms by which this is mediated (Grol *et al.*, 2009). In response to mechanical loading, *P2X7Rs* are stimulated by extracellular ATP, which in activates osteoblasts causing

increases in bone formation and induces apoptosis in osteoclasts reducing bone resorption (Grol *et al.,* 2009). Thus, bone cell differentiation and longevity, as well as mechanotransduction, may have an element of *P2X7R* governance. rs1718119 and rs3751143 have previously been shown to have functional effects on both receptor functioning (Stokes *et al.,* 2010; Ohlendorff *et al.,* 2007) and human bone adaptations (Husted *et al.,* 2013). The rs1718119 polymorphism is located in the transmembrane domain 2 of *P2X7R*; a variation results in increased receptor functioning as a result of monocyte activation and increases in interleukin-1 alpha and interleukin-1 beta release from monocytes and macrophages (Stokes *et al.,* 2010). The close proximity of the variant to a permeability gating region is demonstrated in the mediation of pore formation (Sun *et al.,* 2009) and permeability to $K^+$ and ethidium$^+$ in excess of *P2X7R* wild-type mice (Stokes *et al.,* 2010). Homozygotes for the variant allele of rs3751143 have a complete loss of receptor function, whereas heterozygotes have half of the receptor functionality (Gu *et al.,* 2002), meaning that a linear dose response relationship is evident. The variant allele of rs3751143 was significantly over-represented in the elite athlete stress fracture cohort, and heterozygosity was also increased, although not significantly. The reason for this curious finding is not immediately apparent, although it could be hypothesised that another part of the receptor compensates for the loss of receptor function, thus preventing any adverse consequences and subsequent predisposition to stress fracture injury.

*P2X7R* is expressed in all bone cells, and for this reason the specific mechanism of how *P2X7R* influences stress fracture injury is difficult to determine and may be multi-factorial. The pathophysiology of stress fracture injury is related to repetitive loading cycles causing damage to bone micro-architecture. As a result of allelic variations in *P2X7R* SNPs, impairment in sensitivity to mechanical loading may cause genotype specific alterations in

mechanotransduction (Li *et al.,* 2005). *P2X7R* SNPs regulate suppression and activation of interleukin-1 beta secretion (Sluyter *et al.,* 2004), which increases/decreases pore formation (Gu *et al.,* 2002). Consequently, carriers of the rs1718119 and rs3751143 variant alleles suffer altered receptor surface expression (Sun *et al.,* 2010) leading to bone gain/loss (Ke *et al.,* 2003), which could also act as a mechanism for increasing susceptibility to stress fracture injury. Furthermore, higher interleukin-1 concentrations are likely in the cohort of the present study due to their activity status (Please see section 2.2.2.1.4.). The increase in osteoclastogenesis as a result of an increase in interleukin-1 secretion from continued training and match-play coupled with the increase in activation of interleukin-1, as a result of *P2X7R* genotype, may be the reason for the greater stress fracture injury susceptibility.

No significant associations were shown between the other *P2X7R* SNPs analysed and stress fracture injury despite previous evidence that they are associated with cell function and bone phenotypic changes (Stokes *et al* 2010; Husted *et al* 2013; Jorgensen *et al* 2012). The reason for the lack of association may be due to the use of aged or post-menopausal populations in previous studies, the anatomical site measured, or that the SNPs may act as a proxy for other, not studied, SNPs that directly regulate bone adaptation. Complete absence of *P2X7R* functionality has been shown in homozygotes for the variant allele of rs1653624 SNP (Wiley *et al.,* 2003) making it a pertinent SNP to investigate, despite its previously reported low minor allele frequency (Ohlendorff *et al.,* 2007). Homozygotes for the rare allele have been shown to have an greater vertebral fracture risk and an accelerated rate of bone loss in post-menopausal women (Ohlendorff *et al.,* 2007). Unfortunately, the lack of homozygosis for the variant allele in the present study (<1%) prevented statistical analysis from being performed.

### 6.4.4. Bradykinin

Stress fracture injury susceptibility was associated with the +9 variant of the *BK2R* in the cohort as a whole and in males, females, football/hockey players and those suffering from stress fractures before the age of 21. This is in contrast to murine models where increased bone mineral loss was shown to be associated with the -9 variant (Kakoki *et al.,* 2010).

The kallikrein-kinin system has been implicated in bone resorption through the stimulation of kininogen to form bradykinin. The metabolic effects of bradykinin are mediated through the osteoblast expressed (Kondo *et al.,* 2004) bradykinin receptors (Sharma *et al.,* 2006) and are thought to have an important role in bone resorption as a result of increased IL1 stimulation and resulting prostaglandin formation (Lerner, 1994). A functional SNP exists in the tissue kallikrein gene and heterozygosity leads to a considerable loss of function (Slim *et al.,* 2002). The 9 base pair repeat sequence of the gene encoding the bradykinin 2 receptors (*BK2R*) is associated with a significant increase in mRNA expression (Braun *et al.,* 1996) and skeletal muscle performance (Williams *et al.,* 2004).

*In vitro,* studies have shown *BK2R* to be involved in osteoblast synthesis causing the release of prostaglandin E2 and IL6 (Kondo *et al.,* 2004), which subsequently cause differentiation and stimulation of osteoclasts, leading to increased bone resorption (Lerner, 1994). Due to the high training loads exercised by the elite athletes in the present study causing heightened IL6 concentration (Please see section 2.2.2.1.4.), it can be hypothesised that this is amplified in +9 carriers, causing an increase in bone resorption. As IL6 has been shown to remain heightened 1-5 days following intense exercise (Neubauer *et al.,* 2008), adequate bone recovery time may not be allowed due to the nature of elite athlete training and match-play, which may lead to site specific bone weaknesses at locations under continued stress. This

may cause an increase in damage to the bones micro-architecture, and greater stress fracture risk, although future human studies investigating *BK2R* are required to confirm these findings.

### 6.4.5. VDR

*VDR* SNPs rs1544410, rs731236, rs7975232 and rs10735810 were significantly associated with stress fracture injury in specific sub-classifications. The role of vitamin D status and the *VDR* gene in bone homeostasis and stress fracture prevalence is a contentious issue (Please see section 2.5.2.1.3.2.1.2.). The present data in elite athletes showing the rare allele of rs1544410 and rs10735810 to be associated with stress fracture injury is in line with previous research in military personnel (Chatzipapas *et al.,* 2009). The confirmation of these SNPs role in stress fracture injury in a different population that necessitates different training demands underlines its importance in bone homeostasis and suggests the need for further investigation into the role of these SNPs in bone regulatory mechanisms.

The common allele of rs7975232 was associated with greater stress fracture susceptibility in the leg, excluding the metatarsal sub-classification, while the same allele was associated with a reduced risk of stress fracture in cricketers. Similarly, the rare allele of rs731236 was associated with stress fracture injury in the leg (excluding metatarsal) group and cases of multiple stress fracture, while also being associated with reduced stress fracture injury risk in cricketers. The high rate of stress fractures occurring at the lumbar spine in cricketers may be the reason for the differences shown and reflect diverse genetic mediation of different bone compositions. Previous studies have shown the rare allele of rs731236 (Nguyen *et al.,* 2005) to be associated with reductions in bone phenotypes consisting of mainly trabecular bone and

Study 2 also showed both trabecular and cortical bone, to be associated with different SNPs. As adaptations to loading in trabecular bone occur before cortical bone it may be that the SNPs associated with trabecular bone have a mechanism linked to the speed of mechanostanduction while the SNPs associated with greater cortical bone phenotypes may be linked to pathways that reduce excessive bone resorption. The reason for the conflicting findings may be due to the anatomical location of bone phenotype measurement and, in the present study, a potential difference in the aetiology of injury in different sports due to the specific actions some sports require.

Despite the significant associations in the present study, it is difficult to compare the findings to previous research due to the large amount of contrasting evidence. It seems that *VDR* SNPs have a role in stress fracture incidence in elite athletes, although the mechanisms of how susceptibility is affected remain unclear. The absence of any *VDR* SNPs consistently associated with bone phenotypes in GWAS studies suggest the *VDR* SNPs could be a proxy for other functional SNPs, and/or the associations are restricted to certain populations (Please see section 2.5.2.1.3.2.1.2.). Also, the majority of the candidate gene studies focusing on *VDR* can be characterised by relatively small cohorts, which is particularly the case in those examining the association with stress fracture incidence (n=64, Chatzipapas *et al.,* 2009; n=192, Korvala *et al.,* 2010), increasing the likelihood of erroneous findings.

### 6.4.6. Summary of Genetic Associations with Stress Fracture Injury

The number of SNPs associated with stress fracture injury highlights the polygenic nature of stress fracture injury and the multiple pathways that could be involved. The fact that SNPs are associated with varying population classifications outlines the influence of gene-

environment interactions on stress fracture injury and suggests that some SNPs may mediate specific compositions of bone.

GWAS have shown two main biological pathways to be related to bone mineral density and fracture risk (Estrada *et al.,* 2013); the *RANK/RANKL/OPG* signalling pathway and the *Wnt*-signalling pathway. The majority of SNPs associated with stress fracture incidence in the present study are either directly related to or have a downstream influence on these pathways, adding substantiation to their role as major regulatory pathways in bone health.

Even though stress fracture injuries cause significant discomfort, a loss of valuable training time and have a significant financial impact on the athlete and club, this is the first study to investigate genetic associations with stress fracture injury in an athlete cohort. The studies conducted previously have focused on military personnel (Korvala *et al.,* 2010; Cosman *et al.,* 2013). The challenging nature of recruiting elite athletes due to the lack of athlete availability, as a result of demanding training regimes and competition calendars, maybe a reason for the lack of published data. Stress fracture incidence in the present study (24.9% in the whole cohort) was higher than previously reported (0.5% in elite football players; Ekstrand and Torstveit 2010), although this might be the result of participation bias. The sports clubs, associations and individual athletes approached may have been more likely to volunteer if there was a history of stress fracture injury. The predominant site of stress fracture occurrence was the lower limb (56.8%), which is consistent with previously published data (Iwamoto *et al.,* 2011), although this is likely to be sport dependent.

### 6.4.7. Demographic Associations with Stress Fracture Injury

The stress fracture group were on average 3.3 y older at data collection (Table 6.3.), although it can be suggested 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 y. The whole cohort, male, and female stress fracture groups were also significantly older at the age of achieving elite status. It can be speculated that this may have been due to a stress fracture causing a prolonged absence from training, thus delaying their development. Alternatively, the earlier uptake of elite training during growth periods could have increased bone accrual, cortical geometry and BMD (Lorentzon *et al.,* 2005), which could have strengthened the bone and provided a preventative mechanism against stress fracture injury (Tenforde *et al.,* 2013). Although the precise stimulus needed to cause stress fracture injury is likely to be individual, increased training has been shown to increase stress fracture risk (Warden *et al.,* 2006) making it difficult to ascertain the reason for significant differences.

In the various sub-classifications, there was an association between stress fracture injury incidence and height, BMI and body mass, however, the direction of difference was sport dependent (*e.g.,* stress fractures were associated with heavier and taller cricketers but also associated with smaller and lighter runners; Appendix 6.2.). This may reflect a sport specific aetiology of injury due to the different loading cycles that specific sports necessitate. Stress fracture incidence in the present study was also associated with the number of weekly hours spent training in the male and female only cohorts (Appendix 6.2.). However, stress fracture incidence was associated with greater training hours in males and lower training hours in females. Both groups participated in a considerable amount of training per week (21.6±11.9 h and 14.4±6.6 h), although the lack of training load data make it difficult to confirm how

training hours affected stress fracture incidence in the present study. It is recommended that the influence of gene-environment interactions are explored in future studies.

### 6.4.8. Limitations

Although this is the largest study investigating genetic associations with stress fracture incidence to date, it is not without limitations. Whilst heterogeneity in sport type and training load are acknowledged as variable factors in the present study, it is currently unavoidable given the 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 large numbers of single sport groups in the 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, this is unlikely given that a stress fracture is a significant injury that causes a prolonged absence from training and competition (Ranson *et al.,* 2010). As such, is likely to be well recalled by the athlete. Although the results are in the same direction as previously published literature, the number of comparisons made suggest there is a possibility the findings occurred by chance.

### 6.4.9. Conclusion

In conclusion, stress fracture incidence in elite athletes was associated with 12 of the 22 SNPs investigated. These novel findings show SNPs previously associated with bone phenotypes were associated with stress fracture injury. These data suggest an important role for SNPs within the *RANK/RANKL/OPG* and *Wnt* signalling pathways in the regulation of bone strength and adaption to mechanical loading. However, stress fracture injury is certainly not a monogenic trait, and further gene-gene and gene-environment interactions need to be

explored. Further studies are needed to establish the underpinning mechanisms that explain how these SNPs are associated with stress fracture injury as it is not clear how allelic variations influence bone adaptations and subsequently escalate stress fracture risk.

# Chapter 7.0. A Preliminary Investigation into Genetic Associations with Bone Resorption Following Treadmill Running

## 7. 1. Introduction

Genetic associations with bone phenotypes in response to an increased training volume and several genetic associations with stress fracture injury occurrence were shown in studies 2 and 3. The influence of bone metabolism on bone accrual and bone health is well known. Therefore, genetic mediation of bone metabolism may provide a mechanistic explanation for the phenotypic differences shown in studies 2 and 3.

Despite the known positive outcomes of exercise on bone (Study 2), the mode, intensity and duration of exercise needed to promote osteogenic effects are not clearly defined (Please see section 2.3.1.1. and 2.3.2.). This may be due to bone remodelling being a complex process in which many molecular mechanisms remain poorly understood. Long-term weight-bearing exercise is associated with an osteogenic effect in excess of non-weight-bearing activities (Nikander *et al.,* 2006; Greene *et al.,* 2012). Sports which necessitate high impact loading, such as football and gymnastics, elicit a greater increase in bone strength, size and BMD in comparison to non-weight-bearing sports, such as swimming and water polo (Nikander *et al.,* 2006; Greene *et al.,* 2012). It remains to be seen whether the actual impact of the loading or the intracellular shear stress and pressure created as a result of forceful muscle contractions are the main drivers in this process. Recent data (Schipilow *et al.,* 2013) seems to suggest that the key factor in osteogenesis is the impact of loading, as the correlation between bone strength and muscular strength was shown to be minimal in comparison to mechanical loading.

To gain a better insight into the mechanisms of how exercise promotes adaptive processes in bone, single-bout exercise studies have been conducted. Studies conducted involving weight-bearing exercise, lasting in excess of 60 min and a relatively high percentage of $\dot{V}O_{2max}$ have

been shown to influence the bone remodelling cycle (Please see section 2.3.1.1.). Due to the subtle changes expected following a single-bout of exercise, biochemical markers of bone turnover are used to examine bone remodelling as an alternative to imaging and radiological scans that are commonly used to assess longitudinal changes. Markers of bone resorption have been demonstrated as an important tool in assessing the effectiveness of osteoporosis treatment (Garnero, 2008) and as a predictor of fragility fracture (Vasikaran *et al.,* 2011). Altered bone resorption activity can cause an uncoupling of the bone remodelling cycle, which is associated with bone micro-architectural damage and has been purported to lead to the manifestation of stress fracture injury (Warden *et al.,* 2006).

The degree of bone resorption following a period of prolonged mechanical loading may highlight subtle differences in the bone remodelling process, which may be undetectable in sedentary conditions. The detection of these differences may provide information in relation to the risk of injury and disease. Despite different bone turnover markers being used and disparities in the range of control measures undertaken, treadmill running of a relatively high intensity (≥70% $\dot{V}O_{2max}$) over a prolonged period (1h +) has been shown to affect bone turnover, favouring a net increase in bone resorption (Scott *et al.,* 2010; 2011a). Increased bone resorption in the elderly (~65y) has been related to fracture risk and osteoporosis (Vasikaran *et al.,* 2011). Conversely, increased bone resorption in adolescents, when at rest, has been positively associated with periosteal expansion and, therefore, increased bone diameter (Kemp *et al*., 2013).

Although the usefulness of biochemical markers of bone turnover have been regularly demonstrated (Please see section 2.3.1.1.1.), the response of bone to varying forms of exercise still remains equivocal. This may be due to the high degree of biological variability

that exists in the biochemical markers of bone turnover (Clowes *et al.,* 2002), including response to feeding (Guillemant *et al.,* 2004), circadian rhythm (Fraser *et al.,* 2010) and fluctuations based on sex hormone concentrations (Camacho and Kleerekoper, 2006). Despite this, studies controlling for confounding variables, and using IOF and IFCC recommended markers of bone turnover, have reported individual variation in biochemical markers of bone turnover (Scott *et al.,* 2010; 2011a; 2012a). Another potential reason for the individual variation in bone marker responses could be genotype. SNPs have been associated with bone phenotypes (Please see section 2.5.2.1.3.) and biochemical markers of bone turnover (Please see section 2.3.1.1.1.). Although genetic associations have been shown, the specific genes and SNPs that mediate the response and the magnitude of the genetic effect are not well established.

At present, no published data exists in relation to the role of genotype in explaining the variability in the bone resorption response to exercise. The present study will determine whether specific SNPs are associated with bone resorption (β-CTX) prior to, and following, 120 min of treadmill running and attempt to offer a mechanistic explanation to the findings in studies 2 and 3.

## 7.2. Methods

### 7.2.1. Participants

Healthy, male participants (n=42; Age, 23±4y; Height, 1.77±0.10m, Body Mass, 77.3±14.5 kg; $\dot{V}O_{2max}$, 51.6±5.9mL·kg$^{-1}$·min$^{-1}$) were recruited from within Nottingham Trent University by email, poster advertising and by word of mouth. Participants were recruited if they took

part in at least three exercise sessions per w, were non-smokers, aged 18-35y and not taking medication that influenced bone metabolism. Ethical approval was granted by the Nottingham Trent University Ethical Review Committee (Humans) and informed consent (Appendix 7.1.) and a health screen questionnaire (Appendix 7.2.) were completed prior to participation.

### 7.2.2. Design

Participants completed a familiarisation session during which they became accustomed with the trial procedures, followed by the determination of $\dot{V}O_{2max}$. Following the familiarisation session ( $\geq$ 7d), participants completed a seven day experimental trial consisting of a three day lead in period, an exercise intervention conducted on day four (D4) and three follow-up days (Figure 7.1.).

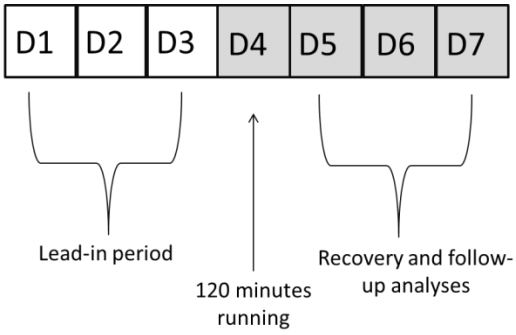


Figure 7.1. Timeline of 7 day experiemental trial for genetic associations with bone resorption following treadmill running. Adjoined boxes denote consecutive days (D1-D7).

### 7.3.1. Preliminary measures

#### 7.3.1.1. Sub-maximal Incremental Treadmill Test

To ensure participants were running at the same relative intensity (70% $\dot{V}O_{2max}$), participants completed a sub-maximal incremental treadmill test, consisting of a speed lactate test and a

$\dot{V}O_{2max}$ test (Jones and Doust, 1996) to determine the relationship between running speed and oxygen consumption.

#### 7.3.1.1.1. Speed Lactate

Both speed lactate and $\dot{V}O_{2max}$ tests were conducted on a motorised treadmill (HP Cosmos, Germany). The speed lactate test was conducted at a 1% gradient and consisted of three min stages with an initial running speed of $9km \cdot h^{-1}$. The treadmill speed was increased by $1km \cdot h^{-1}$ at the end of each three min stage. During the final min of each stage, expired air was collected using a Douglas Bag (Plysu Protection Systems, Milton Keynes, U.K.) for determination of oxygen consumption and carbon dioxide production. Heart rate (Polar, Finland) and rating of perceived exertion (RPE) (Borg, 1976) were also recorded in the final min of each stage. At the end of each three min stage, a finger prick blood sample was taken in order to analyse blood lactate concentration (Yellowstone Scientific Instruments, Big Sky, Montana). The test concluded when lactate concentrations were equal to, or in excess of $1mmol \cdot L^{-1}$ higher than the preceding sample. On test completion, the running speed at each stage was plotted against oxygen consumption ($mL \cdot kg \cdot^{-1} min^{-1}$) to determine the sub-maximal relationship between speed and oxygen consumption. The participant then had a 10 min rest period before the commencement of the $\dot{V}O_{2max}$ test.

#### 7.3.1.1.2. $\dot{V}O_{2max}$ testing procedure

The speed at which the $\dot{V}O_{2max}$ test was conducted was defined as the speed before the lactate increase was $1mmol \cdot L^{-1}$ higher than the preceding sample in the speed lactate test. The $\dot{V}O_{2max}$ test began at 0% gradient, which was increased by 1 % each min until volitional fatigue (Jones and Doust, 1996). Expired air was collected via Douglas Bag during the final

min of the test. On completion of the test, maximum heart rate was recorded and the expired air sample was analysed for oxygen consumption, carbon dioxide production and volume. Maximal oxygen uptake was determined and, by using the results from the speed lactate test, it was possible to equate the speed that would elicit the required percentage of maximal oxygen uptake to be used during the main trial.

#### 7.3.3.1.3. Analysis of Expired Air Samples

Expired air samples were analysed for oxygen consumption and carbon dioxide production using a paramagnetic oxygen analyser and an infra-red carbon dioxide analyser (Series 1400: Servomex, East Sussex, UK). Analysers were initially calibrated using known concentrations of nitrogen and an oxygen and carbon dioxide mixture. The volume of expired air was measured with a dry gas meter (Harvard Apparatus, Kent, UK) and corrected to standard temperature and pressure.

### 7.3.4. Experimental Trial

Participants completed a 3 day lead in period (D1-D3) in which they refrained from any prolonged (<15 min) or intense physical activity in order to provide adequate study control. On D4, participants arrived at the laboratory at 08:30 following an overnight fast. Measurements of resting expired air, height and weight were recorded, followed by a venous blood sample taken from the antecubital vein in the forearm. Participants rested in a supine position before the insertion of a venepuncture needle (Valu-set, Becton Dickinson, Plymouth, UK). Venous blood was collected using disposable syringes (Plastipak, Becton Dickinson UK) and immediately dispensed into blood collection tubes (Sarstedt, Leicester, U.K) coated with ethylenediaminetetraacetic acid (EDTA). Blood samples were spun for 10

min at 3000 rpm in a refrigerated centrifuge at 4ºC (AccuSpin 1R, Thermo Fisher Scientific Inc, Loughborough, UK). Following centrifugation, the plasma was dispensed using a Pasteur pipette into 1.5mL tubes, which were stored at -80ºC until further analysis. The participants commenced treadmill running (HP Cosmos, Germany) at 09:00am at speed equivalent to 70% $\dot{V}O_{2max}$ for 120 min. Heart rate and RPE were recorded at 10 min intervals, while 1 min expired air samples were collected using a Douglas bag at 18, 38, 58, 78, 98 and 118 min. $VO_2$ ($L \cdot min^{-1}$) and $VCO_2$ ($L \cdot min^{-1}$) were calculated, followed by the calculation of Respiratory Exchange Ratio (RER) ($VCO_2$/ $VO_2$), carbohydrate ($g \cdot min^{-1}$) and fat metabolism ($g \cdot min^{-1}$) (x = carbohydrate and y = fat metabolism, $VO_2 = 0.828\ x + 1.989\ y$, $VCO_2 = 0.828\ x + 1.419\ y$) and energy expenditure ($kJ \cdot min^{-1}$) (energy expenditure = [(y*39) + (x*17)]. During the run, 200mL of water was consumed every 20 min. Following run completion, participants remained in a fasted state and a further venous sample was taken. Body mass was also recorded and participants were given water equating to 150% of lost body mass. On completion of the final blood sample participants left the laboratory and consumed lunch.

Participants returned to the laboratory following an overnight fast on days 5, 6 and 7 (D5-D7) at 08:30 for further venous blood samples, which were prepared in the same way as previously described. Throughout the experimental trial participants recorded and maintained their dietary intake, physical activity, lifestyle activity and refrained from alcohol and caffeine comsumpton (D4 only) and prolonged (>15 min) or intense physical activity.

### 7.3.5. Biochemical Analysis of β-CTx

Quantification of β-CTx was determined using a commercially available enzyme linked immunosorbent assay (ELISA) (serum CrossLaps® ELISA Immuno Diagnostic Systems

[IDS]). The assay detects collagen type I fragments created as a result of bone resorption and uses monoclonal antibodies recognizing C-telopeptide fragments of collagen type I a1 chains containing the epitope Glu-Lys-Ala-His-Asp--Gly-Gly-Arg in an isomerised form (IDS, serum CrossLaps® ELISA manual). The ELISA was conducted in accordance to the following steps: 1) following preparation and equilibration of solutions at room temperature, 50µL of standards, controls and unknown samples were pipetted into the wells; 2) 150µL of the antibody solution was then added to each well before the immunostrips were covered with sealing tape and incubated for 120 min on plate mixing apparatus (Thermostar, BMG Labtech, Allmendgruen, Germany) at 300rpm and 21$^{o}$C; 3) Immunostrips were then taken washed with 300µL of washing buffer (this was repeated five times); 4) 100µL of the substrate solution was added to each well, sealed with sealing tape and mixed on plate mixing apparatus at 300rpm and 21$^{o}$C for 15min; 5) 100µL of stopping solution was added to each well; 6) finally, absorbance was measured at 450nm with 650nm as a reference on a plate reader (Elx800, BioTek Instruments, Vermont, USA). The ELISA had a detection limit of 0.020ng·mL$^{-1}$, with a reference range of 0.000-2.494ng·mL. To ensure reliable results were obtained 10% of the sample were duplicated. Inter-Assay and intra-Assay coefficient of variations were 2.5% and 1.8%.

### 7.3.6. Genotyping

DNA was isolated from whole blood samples in accordance with manufacturer guidelines using Wizard DNA extraction kits (Promega, USA). Quantification and analysis were conducted as outlined in section 3.5.

### 7.3.7. Statistical Analysis

All data are presented as mean ± SD. Distribution of genotypes was tested for maintenance of HWE using chi-squared. Paired sample t-tests were used to compare exercise related variables. Genotype related differences in β-CTX were analysed by t-test and one-way ANOVA, while area under the curve (AUC) was used to assess differences over the entire testing period. P values of <0.05 were considered statistically significant. All statistical analysis was performed with Statistical Package for the Social Sciences (SPSS) version 13.0 (SPSS, Inc., Chicago, IL, USA).

## 7.4. Results

### 7.4.1. Physiological Response to Treadmill Running

Based on 70% $\dot{V}O_{2max}$, the average running speed was 11.2±4.2km·h$^{-1}$. Participants body mass was reduced by an average of 1.8kg following run completion (post run body mass 75.5±9.6kg).

As expected, RER, carbohydrate metabolism, fat metabolism, energy expenditure, heart rate and RPE increased with the onset of exercise and remained increased in comparison to baseline, for the duration of the treadmill run (P<0.05) (Table 7.1.). However, no significant differences were shown between time points during the running protocol (P>0.05).

Table 7.1. RER, carbohydrate metabolism, fat metabolism and energy expenditure before and during 120 min of treadmill running. * depicts P<0.05, ** depicts P<0.001.

| | Baseline | 18 min | 38 min | 58 min | 78 min | 98 min | 118 min |
|---|---|---|---|---|---|---|---|
| RER | 0.80±0.25 | 0.92±0.07* | 0.90±0.07 | 0.90±0.06 | 0.89±0.06 | 0.89±0.06 | 0.89±0.07 |
| CHO Metabolism ($g \cdot min^{-1}$) | 0.33±0.35 | 2.18±0.73** | 2.12±0.79 | 2.17±0.67 | 2.10±0.71 | 2.12±0.70 | 2.02±0.71 |
| FAT Metabolism ($g \cdot min^{-1}$) | 0.03±0.14 | 0.34±0.33** | 0.45±0.39 | 0.47±0.32 | 0.53±0.38 | 0.51±0.33 | 0.56±0.41 |
| Energy Expenditure ($kJ \cdot min^{-1}$) | 6.56±3.35 | 48.19±11.35** | 51.33±10.44 | 52.94±11.25 | 54.35±11.76 | 53.76±11.66 | 54.24±127 |

### 7.4.1.1. Glucose and Lactate

There was no effect of exercise on glucose concentrations at any time point following exercise. Lactate concentrations showed a significant increase from baseline immediately following exercise (P<0.05). Concentrations on the follow-up days showed no difference to baseline (P>0.05).

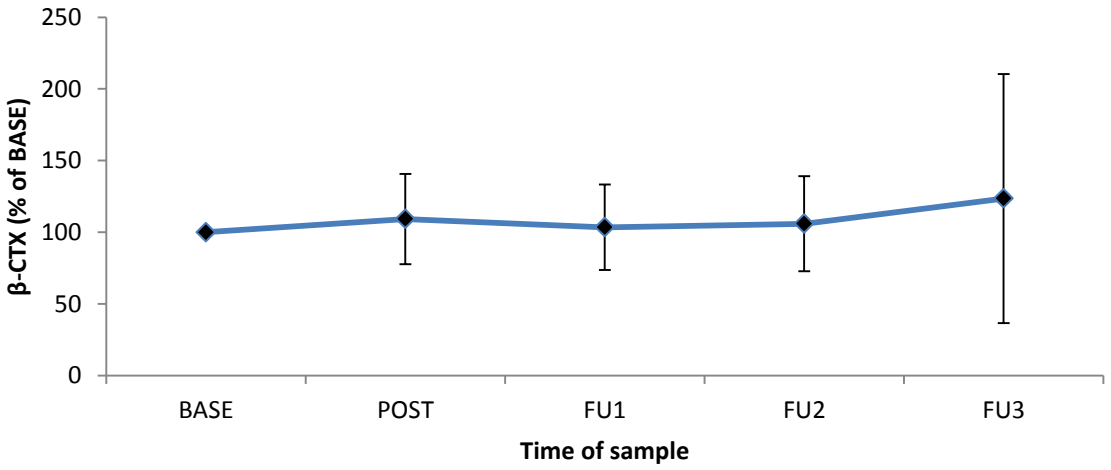


Figure 7.2. β-CTx concentrations for the entire group, expressed as a percentage of baseline (BASE) values immediately post exercise (POST) and in the 3 follow-up days (FU1-FU3).

#### 7.4.1.2. β-CTX

There were no significant differences in β-CTX in response to 120 min of treadmill running when absolute values were compared (BASE 0.67±0.30ng·mL$^{-1}$; POST 0.69±0.32ng·mL$^{-1}$; FU1 0.68±0.37ng·mL$^{-1}$; FU2 0.71±0.40ng·mL$^{-1}$; FU3 0.74±0.42ng·mL$^{-1}$) (P>0.05) or when calculated as a percentage change from baseline (P>0.05).

### 7.4.2. Genotype and β-CTX

All SNPs genotyped were in accordance with HWE (P>0.05), call rates and minor allele frequencies are shown in Table 7.2.

Table 7.2. SNPs for which the participants involved in 120 min of treadmill running were genotyped, along with HWE, P value, call rate % and minor allele frequency (MAF). P values >0.05 are not stated.

| | HWE P value | Call Rate % | MAF % |
|---|---|---|---|
| *RANKL* rs9594759 | 0.86 | 95 | 13 |
| *RANK* rs3018362 | 0.07 | 95 | 3 |
| *RANKL* rs1021188 | 0.29 | 95 | 3 |
| *P2X7* rs3751143 | 0.54 | 95 | 6 |
| *P2X7* rs1718119 | 0.34 | 93 | 10 |
| *RANKL* rs9594738 | 0.1 | 90 | 16 |
| *MP3K* rs8065345 | 0.51 | 95 | 8 |
| *IL6* rs13447445 | 0.35 | 93 | 15 |

Participants were grouped based on individual genotype for the purposes of analyses. Of the 190 comparisons made, no significant differences were shown in β-CTX when compared by genotype for time or genotype x time interactions (P<0.05) (Table 4.3.). Baseline β-CTX concentrations and AUC analysis also showed no association with genotype (P<0.05) (Table 4.3. and 4.4.).

Table 7.3. Absolute β-CTX concentrations for each genotype at the different time points measured before and following 120 min of treadmill running. P values <0.05 were deemed significant.

| *RANKL* rs9594759 | PRE | POST | FU1 | FU2 | FU3 | P Value Time | P Value Genotype x Time interaction |
|---|---|---|---|---|---|---|---|
| CC n=5 | 0.71±0.31 | 0.61±0.22 | 0.74±0.23 | 0.89±0.48 | 0.84±0.46 | | |
| CT n=19 | 0.69±0.31 | 0.70±0.31 | 0.69±0.29 | 0.72±0.36 | 0.73±0.43 | | |
| TT n=16 | 0.61±0.31 | 0.70±0.38 | 0.63±0.45 | 0.64±0.44 | 0.75±0.42 | | |
| P value | 0.68 | 0.86 | 0.81 | 0.48 | 0.82 | 0.23 | 0.62 |
| *RANK* rs3018362 | PRE | POST | FU1 | FU2 | FU3 | | |
| AA/AG n=22 | 0.69±0.28 | 0.66±0.33 | 0.68±0.36 | 0.74±0.37 | 0.74±0.32 | | |
| GG n=18 | 0.66±0.34 | 0.75±0.32 | 0.68±0.40 | 0.70±0.44 | 0.77±0.52 | | |
| P value | 0.76 | 0.39 | 0.99 | 0.78 | 0.64 | 0.49 | 0.78 |
| *RANKL* rs1021188 | PRE | POST | FU1 | FU2 | FU3 | | |
| CC/TT n=7 | 0.71±0.27 | 0.74±0.15 | 0.70±0.29 | 0.73±0.36 | 0.64±0.22 | | |
| TT n=33 | 0.66±0.32 | 0.67±0.34 | 0.68±0.39 | 0.71±0.42 | 0.74±0.42 | | |
| P value | 0.67 | 0.60 | 0.88 | 0.94 | 0.65 | 0.75 | 0.87 |
| *P2X7R* rs3751143 | PRE | POST | FU1 | FU2 | FU3 | | |
| TG/GG n=19 | 0.63±0.28 | 0.71±0.35 | 0.67±0.33 | 0.73±0.32 | 0.78±0.39 | | |
| TT n=21 | 0.70±0.34 | 0.66±0.29 | 0.70±0.42 | 0.74±0.46 | 0.68±0.40 | | |
| P value | 0.51 | 0.62 | 0.78 | 0.77 | 0.66 | 0.63 | 0.47 |
| *P2X7R* rs1718119 | PRE | POST | FU1 | FU2 | FU3 | | |
| CC n=14 | 0.62±0.28 | 0.70±0.37 | 0.60±0.33 | 0.62±0.39 | 0.73±0.41 | | |
| CT/TT n=25 | 0.71±0.32 | 0.70±0.31 | 0.74±0.40 | 0.78±0.41 | 0.76±0.45 | | |
| P value | 0.36 | 0.94 | 0.25 | 0.26 | 0.55 | 0.73 | 0.56 |
| *RANKL* rs9594738 | PRE | POST | FU1 | FU2 | FU3 | | |
| CC n=8 | 0.68±0.31 | 0.76±0.41 | 0.75±0.40 | 0.86±0.43 | 0.81±0.43 | | |
| CT n=24 | 0.64±0.33 | 0.64±0.30 | 0.65±0.37 | 0.65±0.41 | 0.66±0.41 | | |
| TT n=6 | 0.78±0.30 | 0.79±0.32 | 0.76±0.44 | 0.83±0.40 | 0.77±0.37 | | |
| P value | 0.65 | 0.50 | 0.74 | 0.37 | 0.51 | 0.76 | 0.89 |
| *MP3K* rs8065345 | PRE | POST | FU1 | FU2 | FU3 | | |
| AA n=24 | 0.62±0.27 | 0.64±0.31 | 0.61±0.34 | 0.64±0.36 | 0.74±0.41 | | |
| GA/GG n=16 | 0.76±0.35 | 0.79±0.33 | 0.80±0.41 | 0.83±0.44 | 0.77±0.45 | | |
| P value | 0.15 | 0.17 | 0.12 | 0.15 | 0.67 | 0.63 | 0.57 |
| *IL6* rs13447445 | PRE | POST | FU1 | FU2 | FU3 | | |
| CC n=6 | 0.58±0.26 | 0.64±0.32 | 0.65±0.40 | 0.71±0.43 | 0.88±0.48 | | |
| CG n=16 | 0.70±0.35 | 0.74±0.35 | 0.77±0.46 | 0.81±0.47 | 0.75±0.43 | | |
| GG n=18 | 0.65±0.31 | 0.64±0.31 | 0.61±0.29 | 0.64±0.35 | 0.63±0.35 | | |
| P value | 0.72 | 0.68 | 0.49 | 0.49 | 0.28 | 0.34 | 0.41 |

Table 7.4. Percentage change from baseline (100% = basline) β-CTX concentrations for each genotype at the different time points measured before and following 120 min of treadmill running. P values <0.05 were deemed significant.

| *RANKL* rs9594759 | POST | FU1 | FU2 | FU3 | P-Value AUC |
|---|---|---|---|---|---|
| CC n=5 | 90±25 | 98±33 | 120±30 | 128±81 | |
| CT n=19 | 107±33 | 106±30 | 108±39 | 126±74 | |
| TT n=16 | 119±32 | 103±32 | 100±29 | 151±119 | 0.91 |
| *RANK* rs3018362 | POST | FU1 | FU2 | FU3 | |
| AA/AG n=22 | 99±32 | 98±28 | 106±38 | 123±99 | |
| GG n=18 | 122±28 | 107±33 | 107±29 | 128±78 | 0.74 |
| *RANKL* rs1021188 | POST | FU1 | FU2 | FU3 | |
| CC/TT n=7 | 110±20 | 99±24 | 99.7±27 | 93.8±38 | |
| TT n=33 | 108±33 | 106±31 | 109±34 | 140±114 | 0.87 |
| *P2X7R* rs3751143 | POST | FU1 | FU2 | FU3 | |
| TG/GG n=19 | 114±29 | 109±32 | 110±38 | 135±105 | |
| TT n=21 | 103±33 | 100±28 | 104±28 | 111±70 | 0.24 |
| *P2X7R* rs1718119 | POST | FU1 | FU2 | FU3 | |
| CC n=14 | 117±36 | 100±40 | 95±33 | 112±51 | |
| CT/TT n=25 | 103±28 | 103±23 | 110±33 | 123±97 | 0.33 |
| *RANKL* rs9594738 | POST | FU1 | FU2 | FU3 | |
| CC n=8 | 115±41 | 108±31 | 126±27 | 143±100 | |
| CT n=24 | 108±31 | 105±33 | 100±37 | 112±91 | |
| TT n=6 | 100±16 | 93±18 | 104±13 | 103±40 | 0.39 |
| *MP3K* rs8065345 | POST | FU1 | FU2 | FU3 | |
| AA n=24 | 111±36 | 101±29 | 105±39 | 135±100 | |
| GA/GG n=16 | 108±26 | 105±32 | 108±26 | 111±70 | 0.82 |
| *IL6* rs13447445 | POST | FU1 | FU2 | FU3 | |
| CC n=6 | 117±35 | 110±35 | 121±41 | 169±105 | |
| CG n=16 | 109±27 | 109±24 | 117±35 | 127±106 | |
| GG n=18 | 106±35 | 98±34 | 95±26 | 102±59 | 0.14 |

## 7.5. Discussion

Despite the significant associations between genotype and bone phenotypes in studies 2 and 3 no associations between the SNPs genotyped and β-CTX were shown in response to 120 min of treadmill running at any of the time points measured. This is in contrast to previous cross-sectional studies that have shown various bone phenotypes (Zheng *et al.*, 2012; Lorentzon *et*

*al.,* 2000; Garnero *et al.,* 2002; Ferrari *et al.,* 2003), including β-CTX (Roshandel *et al.,* 2010; Kemp *et al.,* 2013), to be associated with genotype.

### 7.5.1. Genotype and β-CTX

Despite evidence that genotype is associated with bone turnover (Kemp *et al.,* 2013) and individual variability is shown in bone resorption following exercise (Scott *et al*., 2010; 2011a), this is the first study to assess the association of genotype with bone resorption following exercise. At present, no SNPs have been shown to have direct functional influence on β-CTX, for this reason the SNPs were selected for analysis based on a potential influence on bone resorption and/or a previously established association with bone phenotypes. The *RANK/RANKL/OPG* signalling pathway is known to have an important role in bone remodelling (Boyce *et al.,* 2003). SNPs thought to influence these pathways have previously been associated with bone phenotypes in response to increased training volume (Study 2), stress fracture injury (Study 3), BMD, bone mass, risk of fragility fracture and β-CTX concentrations (Paternoster *et al.,* 2010; Kemp *et al.,* 2013). For example, circulating RANKL and cortical porosity have both been associated with rs1021188 (Paternoster *et al.,* 2010), which suggests that mineralisation and bone resorption is rs1021188 dependent. Despite this, no associations were shown.

The reason for no association between the SNPs investigated and β-CTX may relate to the exercise component of the present study. Prolonged exercise has been shown to alter bone resorption from resting levels (Please see section 2.3.1.1.) and may have impacted on any genotype related differences due to the complex mechanisms by which weight-bearing exercise influences bone metabolism (Please see section 2.3.). Genotype was proposed to

partitailly explain the speed of the bone resorption response and the variance in β-CTX fluctuation (Kemp *et al.,* 2013). However, as bone remodelling involves several molecular mechanisms (Please see section 2.2.2.) numerous gene-gene and SNP-SNP interactions in addition to the SNPs studied may have influenced the β-CTX concentrations. This premise is supported by previous studies that have shown associations between genotype and β-CTX, using participants in a resting state (Roshandel *et al.,* 2010; Kemp *et al.,* 2013).

Another reason for the lack of association in the present study may also be related to the acute nature of the exercise (single bout) and the relatively short follow-up period (three days). The largest amount of variability in the present study was seen in FU3 (Figure 7.2.) suggesting that the effect of the intervention might take longer than three days to manifest in some participants. The three day follow-up period may have been insufficient to show any delayed fluctuations in bone resorption. It may be that to detect genotype specific effects, persistent, cumulative loading is required over a longer period of time. This type of loading has been shown to cause bone phenotypic structural adaptions (Dhamrait *et al.,* 2003; Evans *et al.,* 2013) and has also been associated with the pathophysiology of stress fracture injury (Warden *et al.,* 2006). However, the extension of the controlled follow-up period in a larger cohort would be challenging. As some of the SNPs genotyped have a known functional effect, it is surprising that no associations with bone resorption were shown. The reason for this may have been due to the differences being too subtle to detect or to confounding factors.

This is the only known study to investigate genetic associations with β-CTX, but is not without limitation. Due to the study being a preliminary investigation, only 42 participants were analysed. The addition of further participants may have increased the likelihood of detecting subtle differences in β-CTX variation in response to exercise. Future studies should

increase the sample size of the cohort however, it should be noted that the recruitment and administration of studies of this nature is diffuclt thus, making it challenging to a achevie a sample size of 100+. Only one biochemical marker of bone turnover (β-CTX) was chosen for analysis. This was decided based on the validity of the marker (IOF, IFCC) and previous associations between β-CTX and genotype (Kemp *et al.,* 2013). Other markers were not analysed due to a lack of evidence for a genetic association and financial constraints. As no measure of bone formation was analysed, it is impossible to draw conclusions over whether uncoupling of the bone remodelling response occurred. Gravitational forces, as well as muscular strain (Kohrt *et al.,* 2009) and gait characteristics (Martin and Marsh, 1992) can contribute to the magnitude of the impact received by the bone and the anatomical site to which the strain is applied. These factors were not determined in the present study, but may have influenced the individual variability in bone resorption shown. There was no change in β-CTX in response to 120 min of treadmill running at 70% of $\dot{V}O_{2max}$. Scott *et al,* (2011a) showed a 45% increase in β-CTX immediately following similar exercise procedures (1 h run, but followed by exercise to exhaustion) at a similar intensity (65-70% $\dot{V}O_{2max}$). The different assay methods for quantifying β-CTX, may be a reason for the lack of similarity in the results. Although no change in β-CTX was shown as a result of 120 min of treadmill running, β-CTX is known to have a distinct circadian rhythm (Fraser *et al.,* 2010). As such, the present results may have differed from a non-exercising Control population. However, as the *a priori* aim of the study was to assess if β-CTX concentrations are associated with genotype, a non-exercising Control population was not sought. Dietary intake in the days prior to the study and energy availability has also been associated with bone turnover (Ihle and Loucks, 2004). The type, intensity or lack of an exhaustive element to the current protocol may also be the reason for the differences shown between the current study and previous investigations.

### 7.5.2. Conclusion

Despite previous evidence (Please see section 2.5.2.1.), and data from studies 2 and 3 that bone phenotypes and bone resorption has a genetic component, in this exercise study, no significant associations were shown with SNPs previously implicated in bone phenotypes following 120 min of treadmill running. The reason for the lack of association may be due to an acute bout of exercise not being of sufficient intensity to induce a genotype specific effect and the size of the cohort not being sufficient to detect small effects.

# Chapter 8.0. General Discussion

The aim of this thesis was to investigate the effects of genotype on 1) adaptations in bone structure as a result of increased training load, 2) stress fracture injury susceptibility in elite athletes and 3) bone metabolism following prolonged mechanical loading. This thesis adds novel insights to the current knowledge base relating to the association of specific SNPs with stress fracture injury and bone phenotypic alterations and shows how genotype may be a mediating factor in the structural adaptations that occur to bone in response to exercise in elite athletes (As depicted in Table 8.1.; Figure 8.1.). A sudden increase in training volume, long-term habitual exercise and a single bout of treadmill running were investigated to explore the association of genotype with bone structural adaptions (increased training volume), incidence of stress fracture injury (long-term habitual exercise) and bone metabolism (single bout).

Genotype has previously been associated with altered bone remodelling and bone structural adaptations (Please see section 2.5.2.1.) (Table 8.1.). Additionally, a potential genetic contribution to stress fracture risk is supported by findings on the development of multiple stress fractures at various skeletal sites (Lambros and Alder, 1997), comparable stress fracture injuries occurring in monozygotic twins (Singer *et al.*, 1990; Van Meensal and Peers, 2010), high stress fracture recurrence rates (Gehrmann and Renard, 2006) and the variation in stress fracture incidence in participants with comparable training loads (Giladi *et al.*, 1986). However, the specific genes and SNPs associated with these phenotypes are not clearly defined. As athletes were used in studies 2 and 3, the level of remodelling experienced by these participants is likely to be in excess of the general public. Due to the high levels of bone remodelling, subtle genotype dependent bone alterations may be evident that would have been undetectable in other populations and may be indicative of underlining bone conditions.

Stress fracture injuries cause significant discomfort, result in a prolonged loss of training time (Ranson *et al.,* 2010) and can have a significant detrimental financial effect on the athlete and/or the club/organisation. Examples include; the stress fracture injuries which distrupted Paula Racliffe's running career causing her to miss and under perform at serveral major championships, prevented Jessica Ennis' participation in the 2008 Beijing Olympics, and ruled Tim Bresnan out of England's 2013 Ashes series against Australia. Despite this, no published research exists on how genotype may affect bone injury susceptibility in an elite athlete population. After investigating the genotype dependent association with bone phenotypes in both a laboratory based and an applied setting, several SNPs were associated with bone structural adaptations (Study 2) and stress fracture injury occurrence (Study 3). In particular, risk alleles in SNPs in close proximity to *RANKL*, *P2X7R* and *SOST* genes were associated with bone phenotypic differences and stress fracture injury incidence.

Table 8.1. An outline of previous research and data from the present thesis in relation to SNP associations with bone phenotypes. WC= Whole cohort, MC= Cases of multiple stress fracture, FH = Football/Hockey, P21 = Pre 21 stress fractures, C = Cricket, EM = Leg stress fracture excluding metatarsals, R = Running. Highlighted sections refer to the studies conducted in this thesis.

| SNP | Study | Population | Bone phenotypes involved |
|---|---|---|---|
| *RANKL* rs1021188 | | | |
| | Varley, Study 3 | Elite Athletes (n=518) WC, male, MC, FH and P21. | Stress Fracture injury |
| | Varley, Study 2 | Adolescent Academy Footballers (n=76) | Cortical thickness and CSA |
| | Paternoster *et al.*, 2010 | Meta-Analysis of ALSPAC, GOOD and MrOS Sweden cohorts (n=5789) | Cortical BMD<br>Cortical Thickness |
| | Paternoster *et al.*, 2010 | ALSPAC (n = 37) | *RANKL* concentration |
| | Paternoster *et al.*, 2013 | Meta-Analysis of ALSPAC, GOOD and MrOS Sweden cohorts (n=5789) | Cortical porosity<br>Cortical vBMD |
| *RANKL* rs9594738 | | | |
| | Varley, Study 3 | Elite Athletes (n=518) C and FH | Stress Fracture injury |
| | Varley, Study 2 | Adolescent Academy Footballers (n=76) | Cortical CSA |
| | Styrkarsdottir *et al.*, 2008 | Middle to old aged (59-85 yr) 5861 and replication cohorts of 4165 cohorts 2269, and 1491 | Lumbar spine BMD |
| | Zhang *et al.*, 2011 | 700 (~69 yr) (350 cases 350 Control) | Osteoporotic fracture |
| *P2X7R* rs3751143 | | | |
| | Varley, Study 3 | Elite Athletes (n=518) WC, EM and C | Stress Fracture injury |
| | Varley, Study 2 | Adolescent Academy Footballers (n=76) | Cortical BMD |
| | Husted *et al.*, 2013 | Men and women (n=574) (>59 yr) | BMD (hip in women and lumbar spine in men) |
| | Wesselius *et al.*, 2012 | Men and women (n=921) (≥50 yr) | BMD |
| | Ohlendorff *et al.*, 2007 | Postmenopausal women (n=1764) | Osteoporotic fracture, osteoclast |

| | | | |
|---|---|---|---|
| | | | apoptosis |
| *P2X7R* rs1718119 | | | |
| | Varley, Study 3 | Elite Athletes (n=518) MC and R | Stress Fracture |
| | Varley, Study 2 | Adolescent Academy Footballers (n=76) | Cortical CSA, Cortical Thickness |
| | Jorgenson *et al.*, 2012 | Women (45-58 y) (n=1694) | Osteoporotic fracture |
| | Husted *et al.*, 2013 | Men (n =120, 63 cases, 57 Control) (mean age 60.2 and 59.3) | Osteoporotic fracture, BMD |
| | Wesselius *et al.*, 2012 | Men and women (n=921) (≥50 yr) | BMD |
| *SOST* rs1877632 | | | |
| | Varley, Study 3 | Elite Athletes (n=518) WC, MC, FH and R | Stress Fracture injury |
| | Varley, Study 2 | Adolescent Academy Footballers (n=76) | Trabecular Density |
| | Zmuda *et al.*, 2011 | Male (~65 yr) (n=650) | Vertebral BMD |
| | Yerges *et al.*, 2009 | Male (+65) (n=862) | Vertebral BMD |

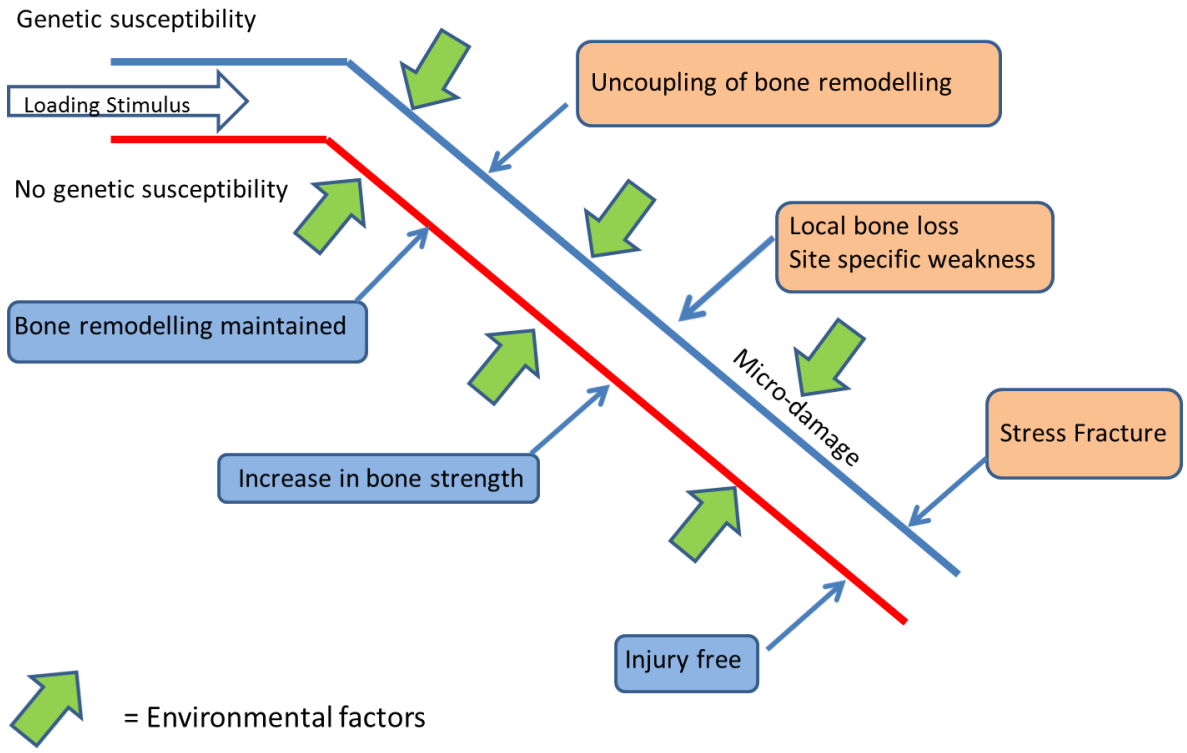


Figure 8.1. Simplified schematic of the process by which a stress fracture injury may occur in elite athletes based on genetic susceptibility. The red line indicates an individual without a genetic susceptibility to stress fracture injury. Orange boxes represent undesirable effects and blue boxes represent desirable effects. The bone remodelling cycle is maintained following a loading stimulus that results in a positive adaptation (increase in bone strength). The blue line indicates an individual with a genetic susceptibility to stress fracture injury where loading causes an uncoupling of the bone remodelling cycle, leading to site specific bone weakness, micro-damage and stress fracture injury. The green arrows indicate environmental factors (both intrinsic and extrinsic) that may interact with genetic factors and increase or decrease stress fracture risk.

## 8.1. Overview of Key Findings

Following 12weeks of increased volume football training, a number of phenotypes (trabecular density, cortical density, cortical CSA, cortical thickness, total CSA and SSI) were significantly greater in academy footballers at multiple tibial sites. SNPs in the proximity of genes in *P2X7R* and the *RANK/RANKL/OPG* signalling and *Wnt* signalling pathways were associated with bone phenotypes. Genotype dependent differences were shown before, after and as a result of 12 weeks increased volume football training in

adolescent academy footballers. Differences were shown at multiple tibial sites over a range of bone phenotypes. SNPs in close proximity to *SOST*, *P2X7R*, *RANK*, *RANKL*, *OPG*, Bradykinin and *VDR* genes were associated with stress fracture injury in various sub-classifications of elite athletes. Despite a large degree of individual variability, genotype was not associated with the bone resorption marker β-CTX prior to, immediately following or in the 3 d following 120min of treadmill running.

Of the SNPs analysed many were associated with specific bone structural phenotypes (Study 2) and stress fracture injury (Study 3). In Study 3, SNPs were associated with stress fracture injury in a range of different sporting sub-classifications suggesting that each SNP may be specific to a distinct aetiology of injury. The range of bone phenotypes and the different sites of the tibia that were associated with genotype suggest that different compositions of bone may be under different genetic mediation. Although previous studies have shown bone resorption (Roshandel *et al.,* 2011), and more specifically β-CTX, to be associated with SNPs in the proximity of the *RANK/RANKL/OPG* signalling pathway in a resting state (Kemp *et al.,* 2013), no significant associations were shown following exercise in the present thesis. This may indicate the role of specific SNPs in bone resorption is only detectable following repeated exercise bouts.

Individual SNPs from within *SOST*, *P2X7R* and *RANKL* genes were all associated with, variation in bone phenotypes and stress fracture injury incidence in elite athletes. This adds to previously published literature on non-athletes showing these genes to have a role in bone phenotypic adaptations.

### 8.2. *RANK/RANKL/OPG* Signalling Pathway

*RANKL* SNPs rs1021188 and rs9594738 were associated with cortical CSA, cortical thickness (rs1021188), cortical CSA (rs9594738) and incidence of stress fracture injury (Study 3). The minor C allele of rs1021188 was associated with 6% lower cortical CSA at baseline, and a 6.4% and 6.7% lower cortical thickness at baseline and following 12 weeks of increased training volume (Study 2). The C allele was also associated with greater stress fracture risk in the whole cohort and in males, cases of multiple stress fractures, football/hockey and stress fractures occurring pre-21 sub-classifications. The combination of with cortical CSA and thickness, together with greater stress fracture injury occurrence suggest a potential determinant in the pathophysiology of stress fracture injury. As cortical CSA and thickness are requisites of bone strength, it may be that decreases in these phenotypes leads to bone weakening, which might increase stress fracture injury risk. Despite showing associations with bone phenotype in academy footballers and stress fracture incidence in elite athletes, no significant associations between rs1021188 and the bone resorption marker β-CTX were shown in response to an initial investigation into the effect of 120 min of treadmill running (Study 4). This may indicate that multiple exercise bouts are required for the effects of genotype related associations to be detectable, or that the effect of an exercise bout on bone resorption may manifest beyond the 72-h follow-up period that was used in the present study. This premise is substantiated by the greatest amount of variability in β-CTX response shown in FU3 (Study 4). However, as Study 4 is a preliminary investigation it may have been underpowered to detect genotype dependent changes in β-CTX and therefore it cannot be concluded that the SNPs analysed are not associated with β-CTX. Previously, rs1021188 has been shown to be associated with cortical BMD and cortical thickness in a meta-analysis involving four large cohorts (Paternoster *et al.,* 2010). The association of cortical thickness is consistent with the current findings, although no

associations with cortical BMD were shown in the adolescent academy footballers, in Study 2. The reason for the contrasting findings may be due to the activity status and the rapid increase in training experienced by the academy footballers creating a distinct bone response. However, it is difficult to compare these findings to that of the meta-analysis as the activity levels in the cohorts studied were not provided.

Despite a functional role of these SNPs not being clearly defined, the key role of the *RANK/RANKL/OPG* signalling pathway in osteoclast differentiation and activation is widely reported (Boyle *et al.,* 2003). Although circulating free *RANKL* is notoriously difficult to measure (Hegedus *et al.,* 2002), the association of increased circulating free *RANKL* in those possessing the C allele of rs1021188 (Paternoster *et al.,* 2010) seems to support this SNPs role in the mediation of osteoclast activity. As an increase in *RANKL* would lead to increased bone resorption, a potential uncoupling of the bone remodelling cycle may have also occurred. Although speculative, the difference in bone resorption may have led to a net bone loss and could provide a pathophysiological cause of stress fracture development and decrease in cortical CSA and thickness. This mechanism of injury seems plausible in the current elite athlete cohort given the high amount of repetitive mechanical loading undertaken throughout training and match-play and the short periods of recovery between exercise sessions. It has also been suggested that allelic differences in the *RANK/RANKL/OPG* SNPs may have a mediatory role in the process by which vitamin D status induces *RANKL* expression in osteoblast and osteoblast precursor cells (Yoskovitz *et al.,* 2013), thus providing another possible mechanism of effect.

Although rs1021188 and rs9594738 are not in linkage disequilibrium, high recombination rates have been shown between these SNPs (Paternoster *et al.,* 2010). *RANKL* SNP

rs9594738 was associated with cortical CSA at the tibia and stress fracture occurrence in cricket and football/hockey player sub-classifications. The minor C allele was protective against stress fracture injury (OR 0.80, 95%CI: 0.30-2.17 in cricketers and OR 0.40, 95%CI: 0.83-0.19 in football/hockey players) and was associated with greater cortical CSA in adolescent footballers. These findings are in accordance with previous research showing the C allele to have a protective affect against osteoporotic fracture (Zhang *et al.,* 2011) and to be associated with greater BMD at the lumbar spine (Yoskovitz *et al.,* 2013).

The present results showing associations between SNPs in close proximity to genes in the *RANK/RANKL/OPG* signalling pathway and bone phenotypes in elite athletes are the first to show SNPs in this pathway to be associated with stress fracture injury risk and phenotypic adaptations as a result of exercise. Together, this underlines the importance of these SNPs in various populations in the maintenance of bone health and in the mediation of bone structural adaptations. These associations are important for sports medicine in helping to explain the aetiology of stress fracture incidence. The SNPs could be used as targets for further studies in the pursuit of pharmaceutical preventive interventions and therapeutic treatments in order accelerate recovery from stress fracture injury.

### 8.3. *SOST*

The minor allele of the *SOST* SNP rs1877632 was associated with increased trabecular BMD at the 4% site of the tibia (Study 2) and a greater stress fracture injury incidence in the whole cohort and in cases of multiple stress fracture, football/hockey players and running sub-classifications (Study 3). This is the first time that rs1877632 has been investigated in

association with stress fracture injury. The findings in relation to trabecular BMD are complemented by previous research in non-athletic populations (Yerges *et al.,* 2009), showing carriers of the rare allele to have a greater trabecular BMD in comparison to those carrying the common allele. Although the study by Yerges *et al* (2009) used an older population (mean age ~75y) compared to the present population (mean age 16y), the BMD based on allelic differences were similar (5.9% and 6.1% compared to 6% and 10.2%), showing that the effect of the SNP may be of influence across various populations. A greater BMD may confer an increase in bone strength and potentially provide a protective mechanism against stress fracture injury manifestation. However, the rare allele was also associated with stress fracture injury incidence in Study 2. Since greater BMD and stress fracture occurrence were associated with the rare allele, it could be speculated that a loading threshold may exist in which carriers of the rare allele may have augmented bone phenotypes in the short-term but if loading is sustained, a heighten long-term susceptibility to bone weakness occurs. This is due to the bone not being given a sufficient amount of time to recover and as a result increase in secondary mineralisation (Seeman, 2008). Secondary mineralisation may create a period of short-term bone weakness, as a result of heightened bone resorption and might thus, increase stress fracture injury susceptibility. However, no genetic associations with bone resorption were shown in Study 4. An alternative hypothesis may be that as stress fracture injuries do not commonly occur at the 4% site of the tibia (Green *et al.,* 1985), the mechanism by which greater trabecular density was shown in Study 2 and the occurrence of stress fracture injury (Study 3) may be different. Although interconnected, it is not uncommon for genotype (Kemp *et al.,* 2013), exercise (Wilks *et al.,* 2009; Schipilow *et al.,* 2013) and pharmaceutical interventions (Seeman *et al.,* 2010) to have divergent effects on trabecular and cortical bone. Trabecular bone phenotypes are less of a

determinant of bone strength relative to cortical bone (Martin, 1991), which may also explain the seemingly contrary findings.

### 8.4. *P2X7R*

Loss of function *P2X7R* SNP rs3751143 and gain of function *P2X7R* SNP rs1718119 were associated with bone phenotypic differences (Study 2) and stress fracture injury occurrence (Study 3). The rare allele of rs3751143 was associated with an increased cortical density in response to training at the 38% site of the tibia (8.1mg·cm$^{-3}$ compared to 4.1mg·cm$^{-3}$), in contrast to the findings of lower hip BMD by Husted *et al.* (2013) and an increased risk of osteoporotic fracture (Wesselius *et al.*, 2012; Ohlendorff *et al.*, 2007). The reason for the contradictory findings could relate to the population studied, the composition of bone analysed and the cross sectional nature of previous studies. The previous phenotypic associations have been shown in mainly elderly and osteoporotic patients (Husted *et al.*, 2013; Ohlendorff *et al.*, 2007) who have different bone phenotypes compared to the young athletic cohort used in the present study. Also, the location of previous associations has mainly been restricted to trabecular bone (hip, lumbar spine), while the present study showed no association with trabecular BMD at the epiphysis of the tibia.

In the present thesis, greater stress fracture injury occurrence was seen in the whole cohort and in cricketers and leg stress fracture sub-classifications (Study 3, Odds Ratios are shown in Appendix 6.4.). These findings may seem somewhat contradictory as BMD is a component of bone strength and associations between stress fracture injury and lower BMD have been shown in previous research (Wentz *et al.*, 2012), although the association of low BMD with stress fracture injury is far from established. As a result of the data in the present thesis it

could be speculated that cortical BMD may not be related to the pathophysiology of stress fracture injury and that other mechanisms may increase risk, such as lower cortical area (Popp *et al.,* 2009). The association of the rare allele of rs3751143 with stress fracture injury is also in accordance with data showing that bone size is an important factor in the determination of bone strength (Evans *et al.,* 2008). rs3751143 has been associated with osteoclast apoptosis (Ohlendorff *et al.,* 2007), reduced pore formation (Gu *et al.,* 2002) and reductions in pro-inflammatory cytokine secretion (Sluyter *et al.,* 2004). Homozygotes for the common allele would be expected to have a higher rate of bone resorption in comparison to those possessing an allelic variation, as new bone is less dense than old bone, the decreased rate of bone resorption may explain the rare allele being associated with cortical density in the present study. The findings in relation to stress fracture injury are more difficult to explain. Previous studies related to rs3751143 and bone phenotypes have investigated cohorts at risk of osteoporosis opposed to athletic populations. As the *P2X7* receptors are important factors in ATP signalling, as a result of mechanotransduction, the increased loading that is likely to have been experienced by the cohort in the present study may have caused different effects of the receptor. The influence of increased mechantransduction is unlikely to have altered the function of rs3751143, but repeated loading may have changed receptor functioning to facilitate this process.

Gain of function *P2X7R* SNP rs1718119 was associated with increased cortical thickness and CSA as well as lower stress fracture injury risk in multiple stress fracture cases and runners (Study 3). These data are in keeping with previous research showing the gain of function allele to be associated with protection against osteoporotic fracture (Jorgenson *et al.,* 2012; Husted *et al.,* 2013) and greater BMD (Wesselius *et al.,* 2012; Husted *et al.,* 2013). Although the sub-classifications were extremely homogeneous, both cases of multiple fracture and

running cohorts had relativity low sample sizes (particularly the running cohort), creating the need for replication cohorts. Also, those homozygous for the gain of function allele were unexpectedly associated with greater cortical thickness and CSA in comparison to heterozygotes; there was no difference between homozygotes for the rare or common allele. Thus, further investigations are required in large cohorts of athletes in order to replicate these findings.

### 8.5. Limitations

Whilst heterogeneity in traits such as lifestyle factors are acknowledged as variable factors in the present thesis, it is currently unavoidable given the size of the cohorts needed for each study. The specific limitations to each study are discussed in the relevant experimental Chapters of the thesis. Globally there is a need for studies examining genetic associations to have large participant numbers and be as homogenous as possible to reduce the impact of confounding environmental factors. Study 2 achieves this by containing a cohort of footballers aged 16 y with similar current and previous training histories. As exercise in pubertal stages is known to be associated with bone phenotypes in adulthood (Lorentzon *et al.,* 2007), this makes elite athletes a homogenous population and offers an advantage over studies using military recruits and the general population, who are likely to have vastly different physical activity histories. Similarly, in Study 3, despite being from different sports, elite athletes all have high training loads and it can be confidently presumed they have been participating from a young age in sporting pursuits in order to achieve elite status. Study 3 is the largest study to date examining the genetic associations with stress fracture injury occurrence and the only study to use a cohort of elite athletes. Ideally, a replication cohort would be sought to confirm or refute these findings, although this would be challenging in the

absence of a large-scale, multi-centred investigation, which was not possible in the current thesis. As the majority of the SNPs were selected on statistical results from GWAS, the functional effects of some SNPs is unknown, making it possible that these SNPs are in high linkage disequalilibrum with other SNPs that do cause a functional affect. Further research, both *in vivo* and *in vitro*, is needed including prospective studies investigating genetic associations with stress fracture injury in large heterogenic populations and knockout mice loading models exploring the mechanism of the SNPs that have been associated with stress fracture injury. Although the results are mainly in the same direction as previously published literature, the number of comparisons made suggest there is a possibility that some of the findings occurred by chance.

### 8.6. Conclusion

SNPs located near pathways known to influence bone remodelling were significantly associated with bone phenotypic alterations and stress fracture injury in elite adolescent footballers and elite athletes. These data, together with previously reported associations with other bone phenotypes, suggest an important role for these SNPs in the regulation of bone strength and the adaptation to mechanical loading. Stress fracture injuries are both common and incapacitating for athletes and military recruits, affecting large numbers of otherwise healthy and active individuals. The lack of understanding in relation to the aetiology and pathophysiology of injury also limits our capacity to prevent or treat sufferers. These novel findings are not only important for sports medicine in helping to explain the aetiology of stress fracture injury incidence, but also have implications for occupational health (For example, military populations) and orthopaedic surgery potentially leading to the development of individualised training and medicine based on genotype. Although stress

fracture injury occurs relatively infrequently in the general population, based on the aetiology of injury, some of the phenotypes associated with stress fracture may be common in osteoporotic fractures and other bone disorders. Therefore, the SNPs associated with both stress fracture and bone phenotypes may be pertinent in relation to common fracture and bone healing. However, stress fracture injury is not a monogenic trait and further gene-gene and gene-environment interactions need to be explored. The specific mechanisms of how these SNPs are associated with stress fracture injury are not clear, and further studies are needed to establish the underpinning factors of how these allelic variations influence bone adaptations and subsequently heighten stress fracture risk.

### 8.7. Future Investigations

This thesis expands the knowledge base on the role of genotype in bone adaptation and stress fracture injury. The association of common SNPs with bone structural differences and stress fracture injury may indicate that the effects of theses SNPs may be of clinical relevance, although the extent to which genotype governs this mediation remains unclear. Future investigations should include:

- The examination of stress fracture injury aetiology in sports that require irregular movement patterns (*e.g.*, football) and single plane movements (*e.g.* middle distance track running) would provide further understanding into the bone adaptations that occur as a result of specific training protocols and may give further insight into how gene-environment interactions occur.

- Gene-gene interactions exploring allele combinations is needed with larger cohorts are required. Bioinformatics expertise may also be needed to tease out complex and

hidden gene-gene interactions. Epigenetic factors, such as DNA methylation should be explored and may have the potential to influence the findings.

- The targeting of the specific SNPs shown to be associated with bone adaptations and stress fracture injury in the present thesis are needed both *in vivo* and *in vitro.* Investigating mechanotransduction and rate of bone remodelling over time to try and understand the mechanisms by which these SNPs are able to mediate the bone response to exercise.

- GWAS and prospective studies in elite athletes in order to replicate the current findings, although the limited population that is able to be called on in studies of this nature may restrict the cohort size. To overcome this issue it is likely that multi-centred investigations will be required.

- The genetic exploration of stress fracture injuries at specific anatomical sites as different compositions of bone may be under the control of different genetic mediators.

- A continuation of the prolonged treadmill running study (Study 4) with a larger cohort and the assessment of a greater number of biochemical markers of bone turnover to investigate genetic associations with net bone turnover.

# 9.0. References

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# 10.0 Appendices

## 5.1. Appendix. Participant information sheet: Genotype Dependent Changes in Bone Phenotypes in Academy Footballers

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# Participant Information Sheet

**Project Title** Genetic dependent changes in bone assessed by bone scan.

**Principal investigator: Ian Varley Nottingham Trent University**

We would like to invite you to volunteer in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. **Ian Varley will go through the information sheet with you and answer any questions you may have.** Please feel free to talk to others about the study if you wish. You have 4 weeks to make a decision on whether you would like to participate.
**Please ask if anything is not clear.**

**Project description** Increases in training cause the amount of loading placed on bone to increase. As a result of this, the amount of bone repair is likely to accelerate, possibly contributing to injuries, such as stress fractures. The current study looks to establish how different people's bones respond to football training.

**What is the purpose of the study?**

The study is part of a PhD thesis investigating genetic susceptibility to stress related bone injuries in elite athletes. The aim of the study is to investigate if there are any differences in bone after 12 weeks football training.

**Why have I been invited?**

As an academy footballer you partake in a high volume of training in a sport which has a relatively high prevalence of stress related bone injuries. At 16 years of age your training increases, this increase may cause variations in bone properties which may differ between individuals. We hope to recruit 120 academy footballers.

**Do I have to take part?**

It is up to you to decide to join the study. We will describe the study and go through this information sheet. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason.

**What will happen to me if I take part?**

We will ask you to visit Nottingham Trent University on two separate occasions separated by 12 weeks. Each visit will involve having a bone scan (this will be in the form of pQCT or peripheral quantitative computed tomography which is used for making measurements of bone mass and size), for each scan you will be required to sit stationary in a chair with your dominant leg inside the scanner for approximately 10 minutes. Giving the saliva sample will involve placing a small amount of saliva into a tube. Each visit will last a maximum of 30 minutes in total.

**Expenses and Payments**

You won't receive any payments for you participation in the study.

**What will I have to do?**

We will ask you to have two bone scans separated by 12 weeks (training as normal during the 12 weeks). These will take place at Nottingham Trent University, scans take approximately 10min and are completely pain free. The two scans that take place will be exactly the same. We will also ask you to provide a saliva sample.

**What are the possible disadvantages and risks of taking part?**

During the process of being scanned you will receive a small dose of radiation (<5 μSv) which is very small compared to other X-ray procedures and is the equivalent to the additional cosmic radiation dose received from a flight from the UK to Spain.

All genetic information will be linked anonymous, therefore you will not be informed of their specific genetic information.

**What are the possible benefits of taking part?**

We cannot promise the study will help you but the information we get from this study will help advance the pool of knowledge related to stress related bone injuries suffered by elite athletes. The results may also lead to the prevention and/or early diagnosis of bone injury.

**What happens when the research study stops?**

The information from the study will be fedback to your club as a whole. Unfortunately, individual data cannot be disclosed.

**What if relevant new information becomes available?**

If new information comes available that is applicable to the safety of the study we will inform you of this information. If the study is stopped for any reason, you to be informed with regard to the reasons.

**What will happen if I don't want to carry on with the study?**

You are free to withdraw from the study at any point. If you withdraw from the study, we will destroy all your identifiable samples and data.

**What if there is a problem?**

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions, tel: **0115 8483820**. If you remain unhappy and wish to complain formally, you can do this by contacting Nottingham Trent University's technical manager, Mark Cosgrove tel: **0115 8486691**, who is independent of the research program and will take you through the complaints procedure.

**Will my taking part in this study be kept confidential?**

Your data will be collected via information received from your bone scan and DNA extracted from your saliva sample. All information will be coded and stored securely and anonymously. All data will be used for analysis in the present study. All data will be retained for 5 years before being destroyed via incineration.

**Involvement of the General Practitioner/Family doctor (GP)**

You may wish is seek advice from your GP however, we will not inform your GP.

**What will happen to any samples I give?**

Your saliva sample will be collected in a coded tube; DNA will be extracted from the saliva. Saliva samples provided will be coded by way of a unique number which will not be traceable to you. Saliva and DNA samples will be stored in locked cabinets (Saliva) and freezers (DNA) with only the lead researcher, director of studies and senior technician having access. Your data from the bone scan will be stored on a password protected university computer.

You will not be able to be identified from the saliva or DNA sample.

Your samples will be stored for 5 year before destruction via incineration.

Your samples will be analysed within Nottingham Trent University.

In the future, your linked anonymous data and linked anonymous saliva/DNA samples may be shared with researchers outside the UK and outside the European Union. We would like to do this to ensure the best and widest use of the time you helped us conduct this project. You will not have any financial gain from your data being shared with other researchers.

If you do not wish for your data and samples to be shared, please let us know and indicate this on your consent form.

**Will any genetic tests be done?**

Your DNA will be analysed from the saliva sample you provide. Some research suggests the involvement individual variation in the bones response to exercise. The current study looks to establish the specific genetic differences that may alter the bones response to football training.

Your DNA will be analysed for a variety of genes associated with bone repair and growth.

The sample we wish you to provide and the subsequent DNA extracted will be coded and stored linked anonymously with the source of the DNA unknown to the experimenting team therefore, no individual feedback can be provided.

There is remote likelihood of any commercial significance arising from this study, you would not benefit financially if any commercial significance did arise.

**What will happen to the results of the research study?**

The results of the study will be fedback to your club and also published in a peer reviewed academic journal. Information will be provided as to the location of the publication when this information is known.

You will not be identified in any report or publication.

**Who is organising and funding the research?**

The research is funded by Nottingham Trent University.

**Who has reviewed the study?**

The research is looked at by an independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by (*will include when appropriate*)Research Ethics Committee.

***Further information and contact details***

**All participants will be given an information form and signed consent form.**

**I'm unsure whether to participant or not?**

Please take time to consider if you would like to participate. You have 4 weeks in which you may seek any advice you see fit. If you have any questions please don't hesitate to contact me on any of the methods listed below.

**Contact Details:**

Ian Varley, MRes
*Postgraduate Researcher*
Biomedical, Life and Health Sciences Research Centre
School of Science and Technology,
Nottingham Trent University,
Erasmus Darwin Building,
Clifton Lane,
Nottingham, UK.
NG11 8NS.
**Tel:** 0115 8483820
**Email:** Ian.Varley@ntu.ac.uk

Craig Sale, MSc, PhD
*Reader in Applied Physiology*
Biomedical, Life and Health Sciences Research Centre
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NG11 8NS.
**Tel:** 0115 8483505
**Email:** Craig.Sale@ntu.ac.uk

## 5.2. Appendix. Pre Scan Screening form: Genotype Dependent Changes in Bone Phenotypes in Academy Footballers

### Pre Scan Screening

Q1) Have you been subjected to any medical radiation exposures in the last 12 months? Y/N

If yes, please specify the number of scans, the type of scans and where they were performed.

_____
(Note for Operator: If the participant has received diagnostic x-ray exposures only (e.g. dental / medical x-rays) then they are free to participate, if they indicate that they have received radiotherapy or similar high dose treatments for cancer then they should not be allowed to participate.)

Q2) Have you been a volunteer for studies using the pQCT scanner at Nottingham Trent University in the last 12 months? Y/N

If yes, please specify the number of scans and the type of scans.

_____
(Note for Operator: If the participant has been scanned using the pQCT on 2 previous occasions in 12 months do not continue with the scan and contact RPS; other scans which have a higher effective dose should be discussed with RPS.)

Q3) Are you subjected to any other form of radiation exposure other than background (e.g at work)? If yes, please provide details.

_____
_____
(Note for Operator: Contact RPS before scanning if a participant answers yes to the above)

I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from Nottingham Trent University, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.

Participant:
Name _____
Date_____
Signature_____

Researcher taking consent:
Name _____
Date_____
Signature_____

**5.3. Appendix. Informed Consent: Genotype Dependent Changes in Bone Phenotypes in Academy Footballers**

# INFORMED CONSENT FORM

## Title of study: **Genotype dependent changes in bone phenotypes assessed via pQCT**

## Name of Principal Investigator: **Ian Varley**

Study number: **1**

REC approval number: **12/EM/0183**

Participant Name:……

Thank you for reading the information about our research project. If you would like to take part, please read and sign this form.

**Consent for the current study**

PLEASE INITIAL THE BOXES IF YOU AGREE WITH EACH SECTION:

| | |
|---|---|
| I have read the information sheet version ... dated __/__/__ for the above study and have been given a copy to keep. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. | ☐ |
| I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. | ☐ |
| I agree to give a sample of saliva for research in this study. I understand how the sample will be collected, that giving a sample for this research is voluntary and that I am free to withdraw my approval for use of the sample at any time. | ☐ |
| I agree to have a bone scan for research purposes. I understand what this will involve and that I will be exposed to a low level of radiation. | ☐ |
| I understand that all my data will be stored confidentially and that my anonymous data may be shared with other parties. | ☐ |
| I understand my training record may be accessed. | ☐ |

I agree to participate in this study.

| Participant: | Researcher taking consent: |
|---|---|
| Name _____ | Name _____ |
| Date_____ | Date_____ |
| Signature_____ | Signature_____ |

## 5.4. Appendix. Health Screen: Genotype Dependent Changes in Bone Phenotypes in Academy Footballers

HEALTH SCREEN

Name ....……

**Please complete this brief questionnaire to confirm fitness to participate:**

1.**At present,** do you have any health problem for which you are:

(a)on medication, prescribed or otherwise Yes ☐ No ☐

(b)attending your general practitioner Yes ☐ No ☐

(c)on a hospital waiting list Yes ☐ No ☐

2.**In the past two years,** have you had any illness which require you to:

(a)consult your GP Yes ☐ No ☐

(b)attend a hospital outpatient department Yes ☐ No ☐

(c)be admitted to hospital Yes ☐ No ☐

3.**Have you ever** had any of the following?

(a)Convulsions/epilepsy Yes ☐ No ☐

(b)Asthma Yes ☐ No ☐

(c)Eczema Yes ☐ No ☐

(d)Diabetes Yes ☐ No ☐

(e)A blood disorder Yes ☐ No ☐

(f)Head injury Yes ☐ No ☐

(g)Digestive problems Yes ☐ No ☐

(h)Heart problems Yes ☐ No ☐

(i)Problems with bones or joints Yes ☐ No ☐

(j)Disturbance of balance / coordination Yes ☐ No ☐

(k)Numbness in hands or feet Yes ☐ No ☐

(l)Disturbance of vision Yes ☐ No ☐

(m)Ear / hearing problems Yes ☐ No ☐

(n)Thyroid problems Yes ☐ No ☐

(o)Kidney or liver problems Yes ☐ No ☐

(p)Allergy to nuts, alcohol etc Yes ☐ No ☐

4.**Has any,** otherwise healthy, member of your family under the age of 35died suddenly during or soon after exercise? Yes ☐ No ☐

5.Are there any reasons why blood sampling may be difficult? Yes ☐ No ☐

6.Have you had a blood sample taken previously? Yes ☐ No ☐

7. Have you had a cold or flu or any flu like symptoms in the last month? Yes ☐ No ☐

**5.5. Appendix. Althletic Status Questionnaire: Genotype Dependent Changes in Bone Phenotypes in Academy Footballers**

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Nottingham Trent University, School of Science and Technology.

## Athletic status questionnaire

**Personal information:**

**Athlete ID number (*Researcher use only*):**

**Date of Birth (dd/mm/yyyy)** ☐ ☐ /☐ ☐ /☐ ☐ ☐ ☐ ☐

**Weight:** ☐ kgs OR ☐ stone ☐ lbs

**Height:** ☐ cms OR ☐ feet ☐ ins

**Which of the following best describes your ethnicity?**

☐ White ☐ Mixed white ☐ Black African

☐ Black Caribbean ☐ Asian ☐ Other

If other, please state…….

## Athletic Status:

### At what age did you first play competitive football?

### What is your regular playing position?
e.g. Central Defender or Goalkeeper

### How many hours a week do you spend training during a regular week?
Please take the time to think about this carefully

**5.6. Appendix. Characteristics of Academy Footballers Pre and Post 12 Weeks of Increased Training Volume Divided by Genotype**

Characteristics of academy footballers pre and post 12 weeks of increased training volume divided by genotype: mean ± SD. A significance level of P<0.05 used. P values are stated other that those <0.01. * was used to denote significance.

| *RANKL* rs9594759 | CC = 18 | CT = 30 | TT = 25 | P value | CC = 18 | CT = 30 | TT = 25 | P value | Interaction |
|---|---|---|---|---|---|---|---|---|---|
| | Pre | Pre | Pre | | Post | Post | Post | | |
| Height (m) | 1.76±5.14 | 1.77±6.93 | 1.74±6.66 | 0.60 | 1.76±5.19 | 1.78±6.83 | 1.74±6.52 | 0.91 | 0.16 |
| Weight (kg) | 69.7±5.7 | 71.9±8.7 | 68.8±7.2 | 0.30 | 70.5±6.2 | 73.0±8.6 | 69.4±7.3 | 0.22 | 0.83 |
| Training (h/wk) | 5.9±2.6 | 5.9±3.6 | 6.3±2.4 | 0.89 | 12.0±1.2 | 12.0±1.2 | 11.5±1.4 | 0.31 | 0.55 |
| Tibial length (mm) | 394.1±18.7 | 390.7±18.7 | 377.2±21.3 | 0.11 | 394.6±16.4 | 390.7±18.7 | 377.6±20.1 | 0.16 | 0.91 |
| Age at elite status (y) | 9.2±2.1 | 8.5±2.3 | 10.0±2.2 | 0.05 | | | | | |

| *RANK* rs3018362 | AA = 10 | AG = 28 | GG = 36 | P value | AA = 10 | AG = 28 | GG = 36 | P value | Interaction |
|---|---|---|---|---|---|---|---|---|---|
| | Pre | Pre | Pre | | Post | Post | Post | | |
| Height (m) | 1.75±7.12 | 1.77±6.65 | 1.77±6.51 | 0.65 | 1.76±6.94 | 1.77±6.16 | 1.77±6.57 | 0.73 | 0.54 |
| Weight (kg) | 69.9±8.6 | 70.7±8.9 | 70.3±6.0 | 0.96 | 70.0±9.2 | 72.0±8.5 | 71.2±6.4 | 0.76 | 0.43 |
| Training (h/wk) | 5.3±2.7 | 5.1±2.3 | 7.0±3.2 | 0.03 | 11.9±1.4 | 11.7±1.2 | 11.9±1.4 | 0.77 | 0.06 |
| Tibial length (mm) | 385.3±17.0 | 389.8±19.4 | 386.0±21.5 | 0.71 | 385.3±17.0 | 389.9±18.3 | 386.3±20.3 | 0.68 | 0.87 |
| Age at elite status (y) | 8.6±2.3 | 9.4±2.1 | 9.4±2.2 | 0.57 | | | | | |

| *RANKL* rs1021188 | CC/CT = 24 | GG = 52 | P value | CC/CT = 24 | GG = 52 | P value | Interaction | Interaction |
|---|---|---|---|---|---|---|---|---|
| | Pre | Pre | | Post | Post | | | |
| Height (m) | 1.78±6.25 | 1.75±6.88 | 0.12 | 1.79±6.31 | 1.76±6.48 | 0.11 | 0.93 | 0.16 |
| Weight (kg) | 72.2±7.3 | 69.2±7.6 | 0.12 | 73.0±7.7 | 70.2±7.7 | 0.15 | 0.79 | 0.39 |
| Training (h/wk) | 5.7±2.3 | 6.2±3.2 | 0.47 | 12.1±1.3 | 11.7±1.3 | 0.18 | 0.21 | 0.05 |
| Tibial length (mm) | 387.4±20.8 | 386.9±20.8 | 0.91 | 387.7±20.3 | 387.2±20.4 | 0.87 | 0.97 | 0.89 |
| Age at elite status (y) | 9.0±2.2 | 9.4±2.2 | 0.74 | | | | | |

| *RANKL* rs9594738 | CC = 21 | CT = 32 | TT = 23 | P value | CC = 21 | CT = 32 | TT = 23 | P value | Interaction |
|---|---|---|---|---|---|---|---|---|---|
| | Pre | Pre | Pre | | Post | Post | Post | | |
| Height (m) | 1.78±6.22 | 1.77±6.95 | 1.75±6.91 | 0.43 | 1.78±5.74 | 1.77±6.92 | 1.75±6.77 | 0.49 | 0.40 |
| Weight (kg) | 70.8±5.7 | 70.9±8.9 | 68.9±6.0 | 0.64 | 71.8±6.0 | 71.5±9.1 | 70.0±8.1 | 0.71 | 0.82 |
| Training (h/wk) | 5.5±2.4 | 6.3±3.6 | 6.9±8.3 | 0.63 | 12.1±1.2 | 11.8±1.3 | 11.5±1.3 | 0.36 | 0.34 |
| Tibial length (mm) | 397.6±16.9 | 385.5±18.9 | 378.9±22.3 | 0.01 | 397.6±16.9 | 385.7±19.3 | 379.2±22.1 | 0.02 | 0.59 |
| Age at elite status (y) | 9.2±1.9 | 9.0±2.4 | 9.8±2.2 | 0.36 | | | | | |

| *WNT16* rs2707466 | AA = 21 | AG = 30 | GG = 21 | P value | AA = 21 | AG = 30 | GG = 21 | P value | Interaction |
|---|---|---|---|---|---|---|---|---|---|
| | Pre | Pre | Pre | | Post | Post | Post | | |
| Height (m) | 1.75±6.23 | 1.76±6.38 | 1.77±7.84 | 0.54 | 1.76±5.84 | 1.77±6.13 | 1.78±7.74 | 0.56 | 0.44 |
| Weight (kg) | 70.2±6.2 | 70.7±8.3 | 70.2±7.8 | 0.97 | 70.1±6.4 | 71.1±8.2 | 72.7±8.1 | 0.60 | 0.01 |
| Training (h/wk) | 5.6±2.4 | 6.4±3.6 | 5.9±2.4 | 0.65 | 11.7±1.3 | 11.8±1.4 | 11.9±1.2 | 0.91 | 0.69 |
| Tibial length (mm) | 393.4±22.3 | 383.8±17.0 | 386.7±23.5 | 0.27 | 393.4±22.3 | 384.2±16.2 | 387.1±23.1 | 0.31 | 0.94 |
| Age at elite status (y) | 9.7±1.8 | 9.2±2.3 | 9.1±2.5 | 0.63 | | | | | |

| *MP3K* rs8065345 | AA = 55 | AG/GG = 21 | P value | AA = 55 | AG/GG = 21 | P value | Interaction |
|---|---|---|---|---|---|---|---|
| | Pre | Pre | | Post | Post | | |
| Height (m) | 1.76±6.85 | 1.75±6.58 | 0.51 | 1.77±6.61 | 1.76±6.54 | 0.41 | 0.30 |
| Weight (kg) | 70.3±7.5 | 70.2±8.5 | 0.98 | 71.5±7.7 | 70.2±8.7 | 0.53 | 0.07 |
| Training (h/wk) | 6.1±3.1 | 6.0±2.7 | 0.86 | 11.8±1.2 | 11.7±1.5 | 0.81 | 0.95 |
| Tibial length (mm) | 387.6±20.8 | 385.8±21.5 | 0.75 | 387.8±19.9 | 385.9±21.6 | 0.71 | 0.94 |
| Age at elite status (y) | 9.5±2.2 | 8.6±2.2 | 0.09 | | | | |

| *SOST* rs1877632 | GG = 45 | GA/AA = 26 | P value | GG = 45 | GA/AA = 26 | P value | Interaction |
|---|---|---|---|---|---|---|---|
| | Pre | Pre | | Post | Post | | |
| Height (m) | 1.76±7.63 | 1.76±6.18 | 0.86 | 1.77±7.12 | 1.77±6.94 | 0.78 | 0.52 |
| Weight (kg) | 71.3±7.6 | 69.2±7.6 | 0.28 | 72.5±7.6 | 69.6±7.6 | 0.13 | 0.26 |
| Training (h/wk) | 5.8±2.5 | 6.0±2.6 | 0.78 | 11.8±1.3 | 12.0±1.4 | 0.5 | 0.94 |
| Tibial length (mm) | 387.3±19.7 | 385.7±32.3 | 0.75 | 387.5±19.9 | 385.9±32.5 | 0.75 | 0.94 |
| Age at elite status (y) | 9.1±2.2 | 9.3±2.3 | 0.65 | | | | |

| *IL6* rs13447445 | CC = 18 | CG = 30 | GG = 30 | P value | CC = 18 | CG = 30 | GG = 30 | P value | Interaction |
|---|---|---|---|---|---|---|---|---|---|
| | Pre | Pre | Pre | | Post | Post | Post | | |
| Height (m) | 1.78±6.22 | 1.75±7.61 | 1.73±6.39 | 0.48 | 1.79±6.14 | 1.76±7.12 | 1.77±6.37 | 0.44 | 0.46 |
| Weight (kg) | 70.3±7.8 | 69.9±8.8 | 70.9±7.9 | 0.89 | 71.7±8.1 | 70.8±8.9 | 71.6±7.9 | 0.92 | 0.68 |
| Training (h/wk) | 5.7±2.6 | 6.4±2.6 | 6.0±3.5 | 0.75 | 12.1±1.5 | 11.6±1.3 | 11.8±1.2 | 0.47 | 0.51 |
| Tibial length (mm) | 386.4±17.7 | 384.1±23.0 | 391.0±21.3 | 0.45 | 386.4±17.7 | 384.6±22.4 | 391.4±21.6 | 0.51 | 0.94 |
| Age at elite status (y) | 10.1±2.2 | 9.0±2.2 | 9.0±2.3 | 0.22 | | | | | |

Characteristics of academy footballers pre and post 12 weeks of increased training volume divided by genotype: mean (SD). * denotes a significant difference ($P<0.05$).

## 6.1. Appendix. Informed Consent, Health Screen and Stress Fracture Questionnaire: Genetic Associations with Stress Fracture Injury

Nottingham Trent University, School of Science and Technology.

Informed Consent for the participation in the study entitled:

Genetic associations in stress fracture injury.

Researchers: Ian Varley, Dr Craig Sale and Dr David C. Hughes

### Background Information

Stress fractures are injuries to bone that are commonly suffered by athletes and military personnel who are performing strenuous weight bearing exercise. These injuries can cause significant discomfort, a loss of valuable training time and can have a significant financial impact. The exact reasons why people suffer stress fracture injury is not clear, although several predisposing mechanical, and environmental factors have been proposed. In addition, there is some indirect evidence which suggests the involvement of genetic factors in the development of stress fractures. Despite this there is relatively little published material that has addressed this issue. The current study looks to establish the specific genetic differences (polymorphisms) that mean that some individuals develop stress fractures whilst others with very similar activity patterns and training loads do not.

**Plan of Study**

Subjects are required to give a saliva sample. This will involve placing a small amount of saliva into a tube. In addition, you will be asked to complete a questionnaire asking you a few questions about whether or not you have suffered a stress fracture injury in the past. From the DNA sample we will determine selected genetic variations that might potentially be associated with the incidence of stress fracture injury.

Potential Risks of taking part in this study, include

There are ethical implications in discovering your own genetic profile; therefore, individuals will not be informed of their specific genetic information.

Yours Faithfully,

Ian Varley
ian.varley@ntu.ac.uk
07956346937
School of Science and Technology
Nottingham Trent University
Clifton campus
Nottingham

NOTTINGHAM TRENT UNIVERSITY

Statement of consent to participate in investigation entitled:

## Genetic Associations with Stress Fracture

I (subject name) ……. have read the information provided and agree to partake, in the proposed study. I am fully aware of the procedures to be carried out and have been informed in relation to the protocol. I agree to obey the universities regulations and the investigators instructions with regard to safety matters.

I am aware that I may withdraw my consent to participate in the research at any time without any obligation to explain why or any prejudice towards them.

I also understand that any personal information will not be passed on to any other parties.

I understand I will not be informed of any data concerning my specific genetic profile.

I have completed the health screening questionnaire and know of no other reasons, medical or otherwise, that will prevent me form taking part in this research.

Signed (Subject) ……. Date…….

Signed (Independent witness)……. Date…….

Signed (Primary researcher) ……. Date……

NOTTINGHAM TRENT UNIVERSITY

# Athletic Status and Stress Fracture Injury Questionnaire

***This questionnaire will only take you about 10 minutes to complete.***

Your answers to this questionnaire are strictly confidential and will not be seen by anyone other than the study investigators. The number on your saliva collection tube should be written in the box below. The questionnaire is completely anonymous and so please do not put your name anywhere on the form. Your information will only be identified by the unique number on your saliva collection pot.

Please be as honest, accurate and thorough as you can with your answers. If in doubt then please always give more information rather than less. If you would rather not answer a question please state **'no comment'** and if you do not know the answer to a question please state **'don't know'**. **Thank you for taking part.**

| **Please write Number (from saliva collection tube) in the box provided** |
| --- |
| |

**Personal information:**

**Date of Birth (dd/mm/yyyy)** ☐ ☐ /☐ ☐ /☐ ☐ ☐ ☐

**Sex** ☐ Male ☐ Female

**Height:** _____ cms OR _____ feet _____ ins

**Weight:** _____ kgs OR _____ stone _____ lbs

**Which of the following best describes your ethnicity?**

☐ White ☐ Mixed white ☐ Black African

☐ Black Caribbean ☐ Asian ☐ Other

If other, please state…….

Typical weekly alcohol intake:_____ (units / wk)
Standard Pint of beer (4%ABV) 2.3 units, Sprits 1.5 units, Glass of wine (standard 250ml) 3 units)

## Athletic Status:

**At what age did you first compete at an elite level?**

**What is your regular sport / playing position if applicable?**
e.g. marathon running/ NA OR basketball/ point guard

**How many hours a week do you spend training during a regular week?**
Please take the time to think about this carefully.

### How many years have you competed at National level?

☐ 0-3 years ☐ 4-6 years ☐ 7-9 years ☐ 10+ years

### How many times have you represented you country?

☐ 0-10 apps ☐ 11-20 apps ☐ 21-30 apps

☐ 31-40apps ☐ 41-50 apps ☐ 50+ apps

## Skeletal Injury:

### Have you ever broken or fractured a bone?

☐ Yes ☐ No

If yes, please give details of the bone/bones you broke and at what age you broke it.

| Age | Bone |
|---|---|
| 18 | e.g. Upper leg / femur |
| | |
| | |
| | |
| | |
| | |
| | |

### Have you ever been told that you have had a STRESS FRACTURE injury?

This might have been called a micro-fracture

☐ Yes ☐ No

**If yes, please give details of the bone/bones where the stress fracture occurred and at what age the fracture occurred. If no, please go to question 3.**

| Bone | Age | Date of injury | Recalled Weight at time of fracture |
|---|---|---|---|
| e.g. Upper leg / femur | 18 | 25/12/210 | 70kg |
| | | | |
| | | | |
| | | | |
| | | | |

**If you have had more than one stress fracture, please complete the following questions for each stress fracture occurrence. Further questions can be found on the continuation sheet at the back of the questionnaire.**

**FIRST Stress Fracture**

**If yes, was it confirmed by a bone scan?**
e.g. MRI scan, X-ray, CT scan, other radiological scan

☐ Yes ☐ No

How long did you have pain in the area of stress fracture before confirmation of stress fracture _____ (Weeks)

_____ (Months)

How long was it until the stress fracture stopped causing you pain _____ (weeks)_____ (months)

How long was it until the doctor advised it was healed_____ (weeks)_____ (months)

How long was it before you were able to get back to full training? _____ (weeks)_____ (months)

How was your stress fracture treated?

☐ **Rest**

If yes, how long did you rest for?

**Weeks**_____

**Months**_____

☐ **Operation**

If yes, how long after stress fracture?

**Weeks**_____

**Months**_____

Did you change your training in the 2 months before your stress fracture injury?
(if so, please provide details below)
_____

Were you taking any medication at the time of injury?
(if so, please provide details below)
_____

Have you ever had a recurrence (another stress fracture in exactly the same place)?

☐ Yes ☐ No

*Females Only:*

*Were you taking an oral contraceptive pill or did you have an implant at time of stress fracture?*

☐ *Yes* ☐ *No*

*At what age did your first menstrual period occur?: _____*

*Were you having regular menstrual periods at the time of your stress fracture?:* ☐ *Yes* ☐ *No*

*Had you been through the menopause at time of stress fracture?:*

☐ *Yes* ☐ *No*

---

**Have you ever suffered from prolonged shin pain during exercise that doesn't go away for several weeks?**

☐ Yes ☐ No

**Does anyone in your close family (Mother/Father/ Brother /Sister/Aunt/Uncle) suffer OSTEOPOROSIS OR FRAGILE bones?**

☐ Yes ☐ No ☐ Don't know

**Has anyone in your close family (Mother/Father/ Brother/Sister/Aunt/Uncle) ever had a STRESS FRACTURE?**

☐ Yes ☐ No ☐ Don't know

**6. Have you lived anywhere outside of the UK for more than 3 years?**

☐ Yes ☐ No

If yes, please state the city/town and countries.

Please provide more than one answer if appropriate

| Example: New York, United States |
| --- |
| …… |

NOTTINGHAM TRENT UNIVERSITY

Athletic status and stress fracture injury health screen

Number ....……

**Please complete this brief questionnaire to confirm fitness to participate:**

1.**At present,** do you have any health problem for which you are:

(a)on medication, prescribed or otherwise Yes ☐ No ☐

(b)attending your general practitioner Yes ☐ No ☐

(c)on a hospital waiting list Yes ☐ No ☐

2.**In the past two years,** have you had any illness which require you to:

(a)consult your GP Yes ☐ No ☐

(b)attend a hospital outpatient department Yes ☐ No ☐

(c)be admitted to hospital Yes ☐ No ☐

3.**Have you ever** had any of the following?

(a)Convulsions/epilepsy Yes ☐ No ☐

(b)Asthma Yes ☐ No ☐

(c)Eczema Yes ☐ No ☐

(d)Diabetes Yes ☐ No ☐

(e)A blood disorder Yes ☐ No ☐

(f)Head injury Yes ☐ No ☐

(g)Digestive problems Yes ☐ No ☐

(h)Heart problems Yes ☐ No ☐

(i)Problems with bones or joints Yes ☐ No ☐

(j)Disturbance of balance / coordination Yes ☐ No ☐

(k)Numbness in hands or feet Yes ☐ No ☐

(l)Disturbance of vision Yes ☐ No ☐

(m)Ear / hearing problems Yes ☐ No ☐

(n)Thyroid problems Yes ☐ No ☐

(o)Kidney or liver problems Yes ☐ No ☐

(p)Allergy to nuts, alcohol etc Yes ☐ No ☐

(q)Any problems affecting your nose e.g. recurrent nose bleeds Yes ☐ No ☐

(r)Any nasal fracture or deviated nasal septum Yes ☐ No ☐

4.**Has any,** otherwise healthy, member of your family under the age of 50 died suddenly during or soon after exercise? Yes ☐ No ☐

5.Have you ever smoked for a period of 5 years or longer ? Yes ☐ No ☐

6. Have you had a cold or flu or any flu like symptoms in the last month? Yes ☐ No ☐

Women only

7. Are you pregnant or trying to become pregnant? Yes ☐ No ☐

**If YES to any question, please describe briefly if you wish (eg to confirm problem was/is short-lived, insignificant or well controlled.)** .....

## 6.2. Appendix. Participant Characteristics for the Whole Cohort and each Stress Fracture Group Sub-Classification

Participant characteristics for each sub-classification. A significance level of P<0.05 was used. P values are stated other that those <0.01. * was used to denote significance.

Whole cohort

| Characteristics | Stress fracture (n=125) | Non-stress fracture (n=376) | *P*-value |
|---|---|---|---|
| Age (y) | 27.7±7.5 | 24.4±5.4 | <0.01* |
| Height (m) | 1.82±10 | 1.81±8.3 | 0.45 |
| Weight (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 (y) | 18.2±4.2 | 17±2.2 | <0.01* |
| Training (h/wk) | 20±11.3 | 18.2±10.1 | 0.12 |
| Alcohol consumption (units/wk) | 5.2±6.9 | 4.1±6.1 | 0.15 |

Male only

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

Female

| Characteristics | Stress fracture (n=41) | Non-stress fracture (n=27) | *P*-value |
|---|---|---|---|
| Age (y) | 29.4±9.4 | 26.2±4.1 | 0.06 |
| Height (m) | 1.69±7.4 | 1.69±7.7 | 0.78 |
| Weight (kg) | 56.5±5.8 | 63.5±7.6 | <0.01* |
| BMI | 16.8±1.6 | 18.2±3.5 | 0.07 |
| Age at elite (y) | 19.4±7.3 | 18.4±5.5 | <0.01* |
| Training (h/wk) | 14.4±6.6 | 16.9±2.4 | 0.05* |
| Alcohol consumption (units/wk) | 3.9±5.1 | 2.6±3.4 | 0.37 |

Runners

| | Stress fracture (n=27) | Non-stress fracture (n=35) | *P*-value |
|---|---|---|---|
| Age (y) | 32.8±9.7 | 26.1±6.3 | 0.03* |
| Height (m) | 1.73±8.8 | 1.78±9.7 | 0.03* |
| Weight (kg) | 61.8±10.3 | 68.6±11.2 | <0.01* |

| | | | |
|---|---|---|---|
| BMI | 17.9±2.5 | 19.1±2.4 | 0.06 |
| Age at elite (y) | 21.3±8.4 | 17.4±3.1 | 0.55 |
| Training (h/wk) | 16.0±6.3 | 14.4±7.4 | 0.34 |
| Alcohol consumption (units/wk) | 3.9±6.4 | 1.2±2.7 | 0.72 |

Cricket Players

| Characteristics | Stress fracture (n=42) | Non-stress fracture (n=113) | *P*-value |
|---|---|---|---|
| Age (y | 26.6±5.0 | 23.9±5.4 | <0.01* |
| Height (m) | 1.88±7.0 | 1.82±7.5 | <0.01* |
| Weight (kg) | 88.1±9.4 | 80.1±9.1 | <0.01* |
| BMI | 23.4±1.9 | 21.9±2.0 | 0.01* |
| Age at elite (y) | 17.8±1.6 | 17.3±2.2 | 0.23 |
| Training (h/wk) | 28.8±12.4 | 26.0±12.4 | 0.20 |
| Alcohol consumption (units/week) | 7.7±8.6 | 7.6±7.9 | 0.90 |

Football and Hockey Players

| | Stress fracture (n=36) | Non-stress fracture (n=208) | *P*-value |
|---|---|---|---|
| Age (y) | 24.8±5.8 | 24.5±5.5 | 0.75 |
| Height (m) | 1.82±8.2 | 1.81±8.1 | 0.30 |
| Weight (kg) | 79.8±10.4 | 78.1±10.0 | 0.36 |
| BMI | 21.9±2.1 | 21.6±2.1 | 0.47 |
| Age at elite (y) | 17.0±2.3 | 16.6±1.8 | 0.25 |
| Training (h/wk) | 12.7±4.2 | 13.1±4.1 | 0.64 |
| Alcohol consumption (units/wk) | 3.0±5.5 | 1.8±3.0 | 0.12 |

Multiple stress fractures (in comparison to non-stress fractures).

| Characteristics | Stress fracture (n=49) | *P*-value |
|---|---|---|
| Age (y) | 28.5±5.5 | <0.01* |
| Height (m) | 1.80±11.4 | 0.49 |
| Weight (kg) | 75.6±16.3 | 0.18 |
| BMI | 20.2±3.2 | <0.01* |
| Age at elite (y) | 18.2±5.1 | 0.91 |
| Training (h/wk) | 23.3±10.2 | 0.16 |
| Alcohol consumption (units/wk) | 3.9±4.7 | 0.82 |

Pre 21 stress fractures (in comparison to non-stress fractures).

| Characteristics | Stress fracture (n=80) | *P*-value |
|---|---|---|
| Age (y) | 25.2±5.3 | 0.29 |

| | | |
|---|---|---|
| Height (m) | 1.83±9.1 | 0.10 |
| Weight (kg) | 78.9±13.4 | 0.50 |
| BMI | 21.5±2.9 | 0.93 |
| Age at elite (y) | 16.7±2.1 | 0.22 |
| Training (h/wk) | 20.5±11.5 | 0.85 |
| Alcohol consumption (units/wk) | 4.7±7.1 | 0.49 |

Leg stress fractures excluding metatarsal (in comparison to non-stress fractures).

| Characteristics | Stress fracture (n=48) | *P*-value |
|---|---|---|
| Age (y) | 30.3±8.8 | <0.01* |
| Height (m) | 1.78±11.5 | <0.03* |
| Weight (kg) | 72.7±15.3 | <0.01* |
| BMI | 20.2±3.2 | <0.01* |
| Age at elite (y) | 19.2±6.4 | 0.55 |
| Training (h/wk) | 19.9±11.7 | 0.34 |
| Alcohol consumption (units/wk) | 4.5±6.5 | 0.72 |

## 6.3. Appendix. Genotype Distribution Between Stress Fracture Sufferers and Non-Stress Fracture Controls for the Whole Cohort and Sub-Classifications

Genotype distribution between stress fracture sufferers and non-stress fracture controls for the whole cohort and sub-classifications. (N/A) = insufficient participants with variant allele to perform appropriate statistics. P values <0.01 are reported as 0.00.

| | Stress fracture | Non-stress Fracture | Total | % | | Stress fracture | Non-stress Fracture | % |
|---|---|---|---|---|---|---|---|---|
| Whole Cohort | Genotype distribution | | | | | Allele frequency | | |
| VDR rs10735810 | | | | | | | | |
| FF | 49 | 169 | 218 | 0.46 | F | 148 | 483 | 0.69 |
| Ff | 50 | 145 | 195 | 0.41 | f | 90 | 221 | 0.31 |
| ff | 20 | 38 | 58 | 0.12 | Total | 238 | 704 | |
| Total | 119 | 352 | 471 | | | | | |
| | | p value | | | | | p value | |
| | | 0.17 | | | | | 0.03 | |
| P2X7 rs2230912 | | | | | | | | |
| AA | 78 | 243 | 321 | 0.68 | A | 193 | 593 | 0.83 |
| GA | 37 | 107 | 144 | 0.30 | G | 39 | 125 | 0.17 |
| GG | 1 | 9 | 10 | 0.02 | Total | 232 | 718 | |
| Total | 116 | 359 | 475 | | | | | |
| | | p value | | | | | p value | |
| | | 0.53 | | | | | 0.81 | |
| DBP rs4588 | | | | | | | | |
| AA | 8 | 29 | 37 | 0.08 | A | 71 | 193 | 0.27 |
| CA | 55 | 135 | 190 | 0.40 | C | 171 | 525 | 0.73 |
| CC | 58 | 195 | 253 | 0.53 | Total | 242 | 718 | |
| Total | 121 | 359 | 480 | | | | | |
| | | p value | | | | | p value | |
| | | 0.31 | | | | | 0.39 | |
| RANK rs3018362 | | | | | | | | |
| AA | 21 | 43 | 64 | 0.13 | A | 98 | 242 | 0.33 |
| GA | 56 | 156 | 212 | 0.44 | G | 138 | 482 | 0.67 |
| GG | 41 | 163 | 204 | 0.43 | Total | 236 | 724 | |
| Total | 118 | 362 | 480 | | | | | |
| | | p value | | | | | p value | |
| | | 0.09 | | | | | 0.01 | |
| OPG rs4355801 | | | | | | | | |
| AA | 43 | 138 | 181 | 0.38 | A | 139 | 432 | 0.60 |
| GA | 53 | 156 | 209 | 0.44 | G | 105 | 284 | 0.40 |
| GG | 26 | 64 | 90 | 0.19 | Total | 244 | 716 | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Total | 122 | 358 | 480 | | | | | |
| | | p value | | | | | p value | |
| | | 0.66 | | | | | 0.28 | |
| RANKL rs1021188 | | | | | | | | |
| AA | 10 | 10 | 20 | 0.04 | A | 48 | 122 | 0.17 |
| GA | 28 | 102 | 130 | 0.27 | G | 198 | 600 | 0.83 |
| GG | 85 | 249 | 334 | 0.69 | Total | 246 | 722 | |
| Total | 123 | 361 | 484 | | | | | |
| | | p value | | | | | p value | |
| | | 0.03 | | | | | 0.27 | |
| DBP rs7041 | | | | | | | | |
| GG | 29 | 98 | 127 | 0.26 | G | 119 | 361 | 0.49 |
| TG | 61 | 165 | 226 | 0.46 | T | 121 | 377 | 0.51 |
| TT | 30 | 106 | 136 | 0.28 | Total | 240 | 738 | |
| Total | 120 | 369 | 489 | | | | | |
| | | p value | | | | | p value | |
| | | 0.50 | | | | | 0.84 | |
| LRP5 rs3736228 | | | | | | | | |
| CC | 86 | 267 | 353 | 0.73 | C | 207 | 620 | 0.86 |
| TC | 35 | 86 | 121 | 0.25 | T | 39 | 102 | 0.14 |
| TT | 2 | 8 | 10 | 0.02 | Total | 246 | 722 | |
| Total | 123 | 361 | 484 | | | | | |
| | | p value | | | | | p value | |
| | | 0.56 | | | | | 0.44 | |
| VDR rs1544410 | | | | | | | | |
| Bb | 17 | 56 | 73 | 0.15 | B | 94 | 278 | 0.39 |
| Bb | 60 | 166 | 226 | 0.48 | B | 134 | 440 | 0.61 |
| BB | 37 | 137 | 174 | 0.37 | Total | 228 | 718 | |
| Total | 114 | 359 | 473 | | | | | |
| | | p value | | | | | p value | |
| | | 0.47 | | | | | 0.44 | |
| VDR rs731236 | | | | | | | | |
| Tt | 13 | 46 | 59 | 0.13 | t | 87 | 260 | 0.37 |
| Tt | 61 | 168 | 229 | 0.49 | T | 141 | 452 | 0.63 |
| TT | 40 | 142 | 182 | 0.39 | Total | 228 | 712 | |
| Total | 114 | 356 | 470 | | | | | |
| | | p value | | | | | p value | |
| | | 0.50 | | | | | 0.61 | |
| P2X7 rs1653624 | | | | | | | | |
| AA | 0 | 0 | 0 | 0.00 | A | 2 | 25 | 0.03 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| TA | 2 | 25 | 27 | 0.06 | T | 240 | 703 | 0.97 |
| TT | 119 | 339 | 458 | 0.94 | Total | 242 | 728 | |
| Total | 121 | 364 | 485 | | | | | |
| | | p value | | | | | p value | |
| | | N/A | | | | | N/A | |
| P2X7 rs1718119 | | | | | | | | |
| GG | 47 | 128 | 175 | 0.38 | G | 148 | 403 | 0.59 |
| GA | 54 | 147 | 201 | 0.44 | A | 84 | 275 | 0.41 |
| AA | 15 | 64 | 79 | 0.17 | Total | 232 | 678 | |
| Total | 116 | 339 | 455 | | | | | |
| | | p value | | | | | p value | |
| | | 0.35 | | | | | 0.18 | |
| VDR rs7975232 | | | | | | | | |
| aa | 31 | 102 | 133 | 0.28 | a | 127 | 378 | 0.53 |
| Aa | 65 | 174 | 239 | 0.50 | A | 113 | 332 | 0.47 |
| AA | 24 | 79 | 103 | 0.22 | Total | 240 | 710 | |
| Total | 120 | 355 | 475 | | | | | |
| | | p value | | | | | p value | |
| | | 0.62 | | | | | 0.92 | |
| COL1A1 rs1800012 | | | | | | | | |
| SS | 88 | 261 | 349 | 0.72 | S | 209 | 614 | 0.84 |
| Ss | 33 | 92 | 125 | 0.26 | s | 35 | 114 | 0.16 |
| ss | 1 | 11 | 12 | 0.02 | Total | 244 | 728 | |
| Total | 122 | 364 | 486 | | | | | |
| | | p value | | | | | p value | |
| | | 0.39 | | | | | 0.57 | |
| CTR rs1801197 | | | | | | | | |
| CC | 8 | 33 | 41 | 0.09 | C | 62 | 204 | 0.29 |
| TC | 46 | 138 | 184 | 0.39 | T | 172 | 510 | 0.71 |
| TT | 63 | 186 | 249 | 0.53 | Total | 234 | 714 | |
| Total | 117 | 357 | 474 | | | | | |
| | | p value | | | | | p value | |
| | | 0.72 | | | | | 0.48 | |
| WNT16 rs3801387 | | | | | | | | |
| AA | 60 | 188 | 248 | 0.52 | A | 168 | 517 | 0.73 |
| GA | 48 | 141 | 189 | 0.40 | G | 76 | 191 | 0.27 |
| GG | 14 | 25 | 39 | 0.08 | Total | 244 | 708 | |
| Total | 122 | 354 | 476 | | | | | |
| | | p value | | | | | p value | |
| | | 0.30 | | | | | 0.14 | |
| RANKL rs9594738 | | | | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC | 45 | 114 | 159 | 0.33 | C | 144 | 420 | 0.58 |
| TC | 54 | 192 | 246 | 0.51 | T | 98 | 302 | 0.42 |
| TT | 22 | 55 | 77 | 0.16 | Total | 242 | 722 | |
| Total | 121 | 361 | 482 | | | | | |
| | | p value | | | | | p value | |
| | | 0.27 | | | | | 0.67 | |

P2X7 rs208294

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC | 120 | 348 | 468 | 0.96 | C | 242 | 712 | 0.98 |
| TC | 2 | 16 | 18 | 0.04 | T | 2 | 18 | 0.02 |
| TT | 0 | 1 | 1 | 0.00 | Total | 244 | 730 | |
| Total | 122 | 365 | 487 | | | | | |
| | | p value | | | | | p value | |
| | | 0.32 | | | | | 0.10 | |

Bradykinin rs1799722

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 9 | 39 | 86 | 125 | 0.27 | 9 | 132 | 330 | 0.48 |
| 9/-9 | 54 | 158 | 212 | 0.46 | -9 | 98 | 356 | 0.52 |
| -9 | 22 | 99 | 121 | 0.26 | Total | 230 | 686 | |
| Total | 115 | 343 | 458 | | | | | |
| | | p value | | | | | p value | |
| | | 0.06 | | | | | 0.01 | |

Kallikrein rs16987491

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| AA | 0 | 0 | 0 | 0.00 | G | 226 | 694 | 0.97 |
| GA | 10 | 19 | 29 | 0.06 | A | 10 | 19 | 0.03 |
| GG | 113 | 347 | 460 | 0.94 | Total | 236 | 714 | |
| Total | 123 | 366 | 489 | | | | | |
| | | P value | | | | | p value | |
| | | 0.23 | | | | | 0.64 | |

P2X7 rs3751143

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| GG | 4 | 13 | 17 | 0.04 | G | 46 | 108 | 0.15 |
| TG | 38 | 82 | 120 | 0.25 | T | 188 | 604 | 0.85 |
| TT | 75 | 261 | 336 | 0.71 | Total | 234 | 712 | |
| Total | 117 | 356 | 473 | | | | | |
| | | p value | | | | | p value | |
| | | 0.13 | | | | | 0.05 | |

SOST rs1877632

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC | 50 | 191 | 241 | 0.50 | C | 159 | 513 | 0.71 |
| TC | 59 | 131 | 190 | 0.39 | T | 85 | 207 | 0.29 |
| TT | 13 | 38 | 51 | 0.11 | Total | 244 | 720 | |
| Total | 122 | 360 | 482 | | | | | |
| | | p value | | | | | p value | |
| | | 0.05 | | | | | 0.04 | |

| Male | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Stress fracture | Non-stress Fracture | Total | % | Allele | Stress fracture | Non-stress Fracture | % |
| | | Genotype distribution | | | | Allele frequency | | | |
| | FF | 42 | 150 | 192 | 0.47 | F | 120 | 429 | 0.69 |
| | Ff | 36 | 129 | 165 | 0.41 | f | 66 | 195 | 0.31 |
| | ff | 15 | 33 | 48 | 0.12 | Total | 186 | 624 | |
| | Total | 93 | 312 | 405 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.35 | | | | | 0.21 | |
| P2X7 rs2230912 | | | | | | | | | |
| | AA | 61 | 211 | 272 | 0.66 | A | 153 | 521 | 0.82 |
| | GA | 31 | 99 | 130 | 0.32 | G | 33 | 117 | 0.18 |
| | GG | 1 | 9 | 10 | 0.02 | Total | 186 | 638 | |
| | Total | 93 | 319 | 412 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.60 | | | | | 0.83 | |
| DBP rs4588 | | | | | | | | | |
| | AA | 7 | 27 | 34 | 0.08 | A | 61 | 176 | 0.27 |
| | CA | 47 | 122 | 169 | 0.41 | C | 131 | 466 | 0.73 |
| | CC | 42 | 172 | 214 | 0.51 | Total | 192 | 642 | |
| | Total | 96 | 321 | 417 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.16 | | | | | 0.18 | |
| RANK rs3018362 | | | | | | | | | |
| | AA | 18 | 39 | 57 | 0.14 | A | 80 | 214 | 0.33 |
| | GA | 44 | 136 | 180 | 0.43 | G | 108 | 430 | 0.67 |
| | GG | 32 | 147 | 179 | 0.43 | Total | 188 | 644 | |
| | Total | 94 | 322 | 416 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.07 | | | | | 0.00 | |
| OPG rs4355801 | | | | | | | | | |
| | AA | 35 | 125 | 160 | 0.39 | A | 112 | 384 | 0.61 |
| | GA | 42 | 134 | 176 | 0.43 | G | 80 | 246 | 0.39 |
| | GG | 19 | 56 | 75 | 0.18 | Total | 192 | 630 | |
| | Total | 96 | 315 | 411 | | | | | |
| | | | p value | | | | | p value | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 0.82 | | | | 0.46 | |

RANKL rs1021188

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | AA | 8 | 8 | 16 | 0.04 | A | 39 | 108 | 0.17 |
| | GA | 23 | 92 | 115 | 0.27 | G | 155 | 538 | 0.83 |
| | GG | 66 | 223 | 289 | 0.69 | Total | 194 | 646 | |
| | Total | 97 | 323 | 420 | | | | | |
| | | | p value | | | | p value | | |
| | | | 0.03 | | | | 0.21 | | |

DBP rs7041

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | GG | 21 | 84 | 105 | 0.25 | G | 88 | 313 | 0.48 |
| | TG | 46 | 145 | 191 | 0.45 | T | 100 | 339 | 0.52 |
| | TT | 27 | 97 | 124 | 0.30 | Total | 188 | 652 | |
| | Total | 94 | 326 | 420 | | | | | |
| | | | p value | | | | p value | | |
| | | | 0.71 | | | | 0.74 | | |

LRP5 rs3736228

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | CC | 71 | 243 | 314 | 0.75 | C | 167 | 561 | 0.87 |
| | TC | 25 | 75 | 100 | 0.24 | T | 27 | 85 | 0.13 |
| | TT | 1 | 5 | 6 | 0.01 | Total | 194 | 646 | |
| | Total | 97 | 323 | 420 | | | | | |
| | | | p value | | | | p value | | |
| | | | 0.82 | | | | 0.75 | | |

VDR rs1544410

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | bb | 13 | 52 | 65 | 0.16 | b | 74 | 250 | 0.39 |
| | Bb | 48 | 146 | 194 | 0.48 | B | 104 | 388 | 0.61 |
| | BB | 28 | 121 | 149 | 0.37 | Total | 178 | 638 | |
| | Total | 89 | 319 | 408 | | | | | |
| | | | p value | | | | p value | | |
| | | | 0.39 | | | | 0.51 | | |

VDR rs731236

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | tt | 9 | 43 | 52 | 0.13 | t | 66 | 235 | 0.37 |
| | Tt | 48 | 149 | 197 | 0.48 | T | 112 | 401 | 0.63 |
| | TT | 32 | 126 | 158 | 0.39 | Total | 178 | 636 | |
| | Total | 89 | 318 | 407 | | | | | |
| | | | p value | | | | p value | | |
| | | | 0.45 | | | | 0.97 | | |

P2X7 rs1653624

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | AA | 0 | 0 | 0 | 0.00 | A | 1 | 22 | 0.03 |
| | TA | 1 | 22 | 23 | 0.05 | T | 189 | 628 | 0.97 |
| | TT | 94 | 303 | 397 | 0.95 | Total | 190 | 650 | |
| | Total | 95 | 325 | 420 | | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | p value | | | | | p value | |
| | | | N/A | | | | | N/A | |
| P2X7 rs1718119 | | | | | | | | | |
| | GG | 36 | 112 | 148 | 0.38 | G | 113 | 354 | 0.59 |
| | GA | 41 | 130 | 171 | 0.44 | A | 69 | 250 | 0.41 |
| | AA | 14 | 60 | 74 | 0.19 | Total | 182 | 604 | |
| | Total | 91 | 302 | 393 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.62 | | | | | 0.34 | |
| VDR rs7975232 | | | | | | | | | |
| | aa | 23 | 94 | 117 | 0.29 | a | 99 | 339 | 0.54 |
| | Aa | 53 | 151 | 204 | 0.50 | A | 91 | 291 | 0.46 |
| | AA | 19 | 70 | 89 | 0.22 | Total | 190 | 630 | |
| | Total | 95 | 315 | 410 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.39 | | | | | 0.64 | |
| COL1A1 rs1800012 | | | | | | | | | |
| | SS | 70 | 237 | 307 | 0.73 | S | 165 | 553 | 0.85 |
| | Ss | 25 | 79 | 104 | 0.25 | s | 27 | 95 | 0.15 |
| | ss | 1 | 8 | 9 | 0.02 | Total | 192 | 648 | |
| | Total | 96 | 324 | 420 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.67 | | | | | 0.81 | |
| CTR rs1801197 | | | | | | | | | |
| | CC | 5 | 28 | 33 | 0.08 | C | 46 | 185 | 0.29 |
| | TC | 36 | 129 | 165 | 0.40 | T | 136 | 459 | 0.71 |
| | TT | 50 | 165 | 215 | 0.52 | Total | 182 | 644 | |
| | Total | 91 | 322 | 413 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.58 | | | | | 0.30 | |
| WNT16 rs3801387 | | | | | | | | | |
| | AA | 45 | 164 | 209 | 0.51 | A | 131 | 455 | 0.72 |
| | GA | 41 | 127 | 168 | 0.41 | G | 65 | 175 | 0.28 |
| | GG | 12 | 24 | 36 | 0.09 | Total | 196 | 630 | |
| | Total | 98 | 315 | 413 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.30 | | | | | 0.09 | |
| RANKL rs9594738 | | | | | | | | | |
| | CC | 37 | 101 | 138 | 0.33 | C | 116 | 370 | 0.58 |
| | TC | 42 | 168 | 210 | 0.51 | T | 74 | 268 | 0.42 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| TT | 16 | 50 | 66 | 0.16 | Total | 190 | 638 | |
| Total | 95 | 319 | 414 | | | | | |
| | | p value | | | | | p value | |
| | | 0.32 | | | | | 0.39 | |
| P2X7 rs208294 | | | | | | | | |
| CC | 94 | 312 | 406 | 0.96 | C | 190 | 638 | 0.98 |
| TC | 2 | 14 | 16 | 0.04 | T | 2 | 16 | 0.02 |
| TT | 0 | 1 | 1 | 0.00 | Total | 192 | 654 | |
| Total | 96 | 327 | 423 | | | | | |
| | | p value | | | | | p value | |
| | | 0.52 | | | | | 0.21 | |
| BRADYKININ rs1799722 | | | | | | | | |
| 9 | 33 | 75 | 108 | 0.27 | 9 | 111 | 289 | 0.48 |
| 9/-9 | 45 | 139 | 184 | 0.46 | -9 | 73 | 319 | 0.52 |
| -9 | 14 | 90 | 104 | 0.26 | Total | 184 | 608 | |
| Total | 92 | 304 | 396 | | | | | |
| | | p value | | | | | p value | |
| | | 0.01 | | | | | 0.00 | |
| KALLIKREIN rs16987491 | | | | | | | | |
| AA | 0 | 0 | 0 | 0.00 | A | 8 | 16 | 0.03 |
| GA | 8 | 16 | 24 | 0.06 | G | 184 | 636 | 0.97 |
| GG | 88 | 310 | 398 | 0.94 | Total | 192 | 652 | |
| Total | 96 | 326 | 422 | | | | | |
| | | p value | | | | | p value | |
| | | 0.20 | | | | | 0.63 | |
| P2X7 rs3751143 | | | | | | | | |
| GG | 4 | 12 | 16 | 0.04 | G | 34 | 89 | 0.14 |
| TG | 26 | 65 | 91 | 0.22 | T | 150 | 545 | 0.86 |
| TT | 62 | 240 | 302 | 0.74 | Total | 184 | 634 | |
| Total | 92 | 317 | 409 | | | | | |
| | | p value | | | | | p value | |
| | | 0.27 | | | | | 0.08 | |
| SOST rs1877632 | | | | | | | | |
| CC | 43 | 172 | 215 | 0.52 | C | 128 | 458 | 0.72 |
| TC | 42 | 114 | 156 | 0.38 | T | 64 | 180 | 0.28 |
| TT | 11 | 33 | 44 | 0.11 | Total | 192 | 638 | |
| Total | 96 | 319 | 415 | | | | | |
| | | p value | | | | | p value | |
| | | 0.28 | | | | | 0.11 | |

Cricket

| | Stress fracture | Non-stress Fracture | Total | % | Allele | Stress fracture | Non-stress Fracture | % |
|---|---|---|---|---|---|---|---|---|
| | Genotype distribution | | | | Allele frequency | | | |
| VDR rs10735810 | | | | | | | | |
| FF | 18 | 51 | 69 | 0.48 | F | 53 | 144 | 0.68 |
| Ff | 17 | 42 | 59 | 0.41 | f | 25 | 68 | 0.32 |
| ff | 4 | 13 | 17 | 0.12 | Total | 78 | 212 | |
| Total | 39 | 106 | 145 | | | | | |
| | | p value | | | | | p value | |
| | | 0.89 | | | | | 0.99 | |
| P2X7 rs2230912 | | | | | | | | |
| AA | 20 | 60 | 80 | 0.53 | A | 60 | 168 | 0.76 |
| GA | 20 | 48 | 68 | 0.45 | G | 20 | 52 | 0.24 |
| GG | 0 | 2 | 2 | 0.01 | Total | 80 | 220 | |
| Total | 40 | 110 | 150 | | | | | |
| | | p value | | | | | p value | |
| | | 0.58 | | | | | 0.77 | |
| DBP rs4588 | | | | | | | | |
| AA | 3 | 8 | 11 | 0.07 | A | 28 | 59 | 0.27 |
| CA | 22 | 43 | 65 | 0.43 | C | 54 | 161 | 0.73 |
| CC | 16 | 59 | 75 | 0.50 | Total | 82 | 220 | |
| Total | 41 | 110 | 151 | | | | | |
| | | p value | | | | | p value | |
| | | 0.25 | | | | | 0.13 | |
| RANK rs3018362 | | | | | | | | |
| AA | 9 | 18 | 27 | 0.18 | A | 38 | 82 | 0.37 |
| GA | 20 | 46 | 66 | 0.43 | G | 44 | 140 | 0.63 |
| GG | 12 | 47 | 59 | 0.39 | Total | 82 | 222 | |
| Total | 41 | 111 | 152 | | | | | |
| | | p value | | | | | p value | |
| | | 0.33 | | | | | 0.08 | |
| OPG rs4355801 | | | | | | | | |
| AA | 13 | 47 | 60 | 0.40 | A | 44 | 138 | 0.63 |
| GA | 18 | 44 | 62 | 0.41 | G | 38 | 80 | 0.37 |
| GG | 10 | 18 | 28 | 0.19 | Total | 82 | 218 | |
| Total | 41 | 109 | 150 | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | p value | | | | p value | |
| | | | 0.36 | | | | 0.07 | |
| RANKL rs1021188 | | | | | | | | |
| | AA | 4 | 4 | 8 | 0.05 | A | 16 | 36 | 0.16 |
| | GA | 8 | 28 | 36 | 0.23 | G | 68 | 190 | 0.84 |
| | GG | 30 | 81 | 111 | 0.72 | Total | 84 | 226 | |
| | Total | 42 | 113 | 155 | | | | | |
| | | | p value | | | | p value | |
| | | | 0.28 | | | | 0.43 | |
| DBP rs7041 | | | | | | | | |
| | GG | 8 | 35 | 43 | 0.28 | G | 42 | 122 | 0.54 |
| | TG | 26 | 52 | 78 | 0.51 | T | 40 | 104 | 0.46 |
| | TT | 7 | 26 | 33 | 0.21 | Total | 82 | 226 | |
| | Total | 41 | 113 | 154 | | | | | |
| | | | p value | | | | p value | |
| | | | 0.16 | | | | 0.62 | |
| LRP5 rs3736228 | | | | | | | | |
| | CC | 29 | 83 | 112 | 0.74 | C | 71 | 192 | 0.87 |
| | TC | 13 | 26 | 39 | 0.26 | T | 13 | 28 | 0.13 |
| | TT | 0 | 1 | 1 | 0.01 | Total | 84 | 220 | |
| | Total | 42 | 110 | 152 | | | | | |
| | | | p value | | | | p value | |
| | | | 0.55 | | | | 0.45 | |
| VDR rs1544410 | | | | | | | | |
| | bb | 5 | 23 | 28 | 0.19 | b | 26 | 100 | 0.45 |
| | Bb | 16 | 54 | 70 | 0.47 | B | 48 | 122 | 0.55 |
| | BB | 16 | 34 | 50 | 0.34 | Total | 74 | 222 | |
| | Total | 37 | 111 | 148 | | | | | |
| | | | p value | | | | p value | |
| | | | 0.33 | | | | 0.09 | |
| VDR rs731236 | | | | | | | | |
| | tt | 3 | 17 | 20 | 0.14 | t | 24 | 89 | 0.42 |
| | Tt | 18 | 55 | 73 | 0.50 | T | 56 | 125 | 0.58 |
| | TT | 19 | 35 | 54 | 0.37 | Total | 80 | 214 | |
| | Total | 40 | 107 | 147 | | | | | |
| | | | p value | | | | p value | |
| | | | 0.18 | | | | 0.04 | |
| P2X7 rs1653624 | | | | | | | | |
| | AA | 0 | 0 | 0 | 0.00 | A | 0 | 8 | 0.04 |
| | TA | 0 | 8 | 8 | 0.05 | T | 84 | 218 | 0.96 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| TT | 42 | 105 | 147 | 0.95 | Total | 84 | 226 | |
| Total | 42 | 113 | 155 | | | | | |
| | | p value | | | | | p value | |
| | | N/A | | | | | N/A | |

P2X7 rs1718119

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| GG | 16 | 39 | 55 | 0.37 | G | 49 | 125 | 0.59 |
| GA | 17 | 47 | 64 | 0.44 | A | 33 | 87 | 0.41 |
| AA | 8 | 20 | 28 | 0.19 | Total | 82 | 212 | |
| Total | 41 | 106 | 147 | | | | | |
| | | p value | | | | | p value | |
| | | 0.95 | | | | | 0.88 | |

VDR rs7975232

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| aa | 8 | 34 | 42 | 0.28 | a | 37 | 121 | 0.56 |
| Aa | 21 | 53 | 74 | 0.50 | A | 45 | 95 | 0.44 |
| AA | 12 | 21 | 33 | 0.22 | Total | 82 | 216 | |
| Total | 41 | 108 | 149 | | | | | |
| | | p value | | | | | p value | |
| | | 0.24 | | | | | 0.04 | |

COL1A1 rs1800012

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| SS | 30 | 78 | 108 | 0.70 | S | 72 | 185 | 0.83 |
| Ss | 12 | 29 | 41 | 0.27 | s | 12 | 39 | 0.17 |
| ss | 0 | 5 | 5 | 0.03 | Total | 84 | 224 | |
| Total | 42 | 112 | 154 | | | | | |
| | | p value | | | | | p value | |
| | | 0.37 | | | | | 0.45 | |

CTR rs1801197

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC | 4 | 14 | 18 | 0.12 | C | 23 | 67 | 0.30 |
| TC | 15 | 39 | 54 | 0.35 | T | 57 | 159 | 0.70 |
| TT | 21 | 60 | 81 | 0.53 | Total | 80 | 226 | |
| Total | 40 | 113 | 153 | | | | | |
| | | p value | | | | | p value | |
| | | 0.90 | | | | | 0.86 | |

WNT16 rs3801387

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| AA | 22 | 53 | 75 | 0.50 | A | 59 | 155 | 0.70 |
| GA | 15 | 49 | 64 | 0.42 | G | 23 | 65 | 0.30 |
| GG | 4 | 8 | 12 | 0.08 | Total | 82 | 220 | |
| Total | 41 | 110 | 151 | | | | | |
| | | p value | | | | | p value | |
| | | 0.65 | | | | | 0.77 | |

RANKL rs9594738

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC | 15 | 24 | 39 | 0.26 | C | 45 | 113 | 0.52 |
| TC | 15 | 65 | 80 | 0.54 | T | 35 | 105 | 0.48 |
| TT | 10 | 20 | 30 | 0.20 | Total | 80 | 218 | |
| Total | 40 | 109 | 149 | | | | | |
| | | p value | | | | | p value | |
| | | 0.05 | | | | | 0.43 | |

P2X7 rs208294

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC | 41 | 109 | 150 | 0.97 | C | 83 | 221 | 0.99 |
| TC | 1 | 3 | 4 | 0.03 | T | 1 | 3 | 0.01 |
| TT | 0 | 0 | 0 | 0.00 | Total | 84 | 224 | |
| Total | 42 | 112 | 154 | | | | | |
| | | p value | | | | | p value | |
| | | 0.92 | | | | | 0.91 | |

BRADYKININ rs1799722

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 9 | 11 | 28 | 39 | 0.26 | 9 | 46 | 108 | 0.50 |
| 9/-9 | 24 | 52 | 76 | 0.51 | -9 | 36 | 110 | 0.50 |
| -9 | 6 | 29 | 35 | 0.23 | Total | 82 | 218 | |
| Total | 41 | 109 | 150 | | | | | |
| | | p value | | | | | p value | |
| | | 0.28 | | | | | 0.24 | |

KALLIKREIN rs16987491

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| AA | 0 | 0 | 0 | 0.00 | A | 3 | 4 | 0.02 |
| GA | 3 | 4 | 7 | 0.05 | G | 79 | 218 | 0.98 |
| GG | 38 | 107 | 145 | 0.95 | Total | 82 | 222 | |
| Total | 41 | 111 | 152 | | | | | |
| | | p value | | | | | p value | |
| | | 0.33 | | | | | 0.74 | |

P2X7 rs3751143

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| GG | 3 | 3 | 6 | 0.04 | G | 15 | 24 | 0.11 |
| TG | 9 | 18 | 27 | 0.19 | T | 63 | 188 | 0.89 |
| TT | 27 | 85 | 112 | 0.77 | Total | 78 | 212 | |
| Total | 39 | 106 | 145 | | | | | |
| | | p value | | | | | p value | |
| | | 0.27 | | | | | 0.03 | |

SOST rs1877632

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC | 22 | 56 | 78 | 0.51 | C | 62 | 156 | 0.70 |
| TC | 18 | 44 | 62 | 0.40 | T | 22 | 68 | 0.30 |
| TT | 2 | 12 | 14 | 0.09 | | 84 | 224 | |
| Total | 42 | 112 | 154 | | Total | | | |
| | | p value | | | | | p value | |
| | | 0.52 | | | | | 0.41 | |

Multiple stress fractures

| | Stress fracture | Non-stress Fracture | Total | % | Allele | Stress fracture | Non-stress Fracture | % |
|---|---|---|---|---|---|---|---|---|
| | Genotype distribution | | | | Allele frequency | | | |
| VDR rs10735810 | | | | | | | | |
| FF | 14 | 169 | 183 | 0.46 | F | 49 | 483 | 0.69 |
| Ff | 21 | 145 | 166 | 0.42 | f | 45 | 221 | 0.31 |
| Ff | 12 | 38 | 50 | 0.13 | Total | 94 | 704 | |
| Total | 47 | 352 | 399 | | | | | |
| | | p value | | | | | p value | |
| | | 0.01 | | | | | 0.02 | |
| P2X7 rs2230912 | | | | | | | | |
| AA | 32 | 243 | 275 | 0.68 | A | 78 | 593 | 0.83 |
| GA | 14 | 107 | 121 | 0.30 | G | 14 | 125 | 0.17 |
| GG | 0 | 9 | 9 | 0.02 | Total | 92 | 718 | |
| Total | 46 | 359 | 405 | | | | | |
| | | p value | | | | | p value | |
| | | 0.55 | | | | | 0.58 | |
| DBP rs4588 | | | | | | | | |
| AA | 3 | 29 | 32 | 0.08 | A | 25 | 193 | 0.27 |
| CA | 19 | 135 | 154 | 0.38 | C | 69 | 525 | 0.73 |
| CC | 25 | 195 | 220 | 0.54 | Total | 94 | 718 | |
| Total | 47 | 359 | 406 | | | | | |
| | | p value | | | | | p value | |
| | | 0.88 | | | | | 0.95 | |
| RANK rs3018362 | | | | | | | | |
| AA | 4 | 43 | 47 | 0.12 | A | 34 | 242 | 0.33 |
| GA | 26 | 156 | 182 | 0.45 | G | 56 | 482 | 0.67 |
| GG | 15 | 163 | 178 | 0.44 | Total | 90 | 724 | |
| Total | 45 | 362 | 407 | | | | | |
| | | p value | | | | | p value | |
| | | 0.17 | | | | | 0.38 | |
| OPG rs4355801 | | | | | | | | |
| AA | 11 | 138 | 149 | 0.37 | A | 48 | 432 | 0.60 |
| GA | 26 | 156 | 182 | 0.45 | G | 46 | 284 | 0.40 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| GG | 10 | 64 | 74 | 0.18 | Total | 94 | 716 | |
| Total | 47 | 358 | 405 | | | | | |
| | | p value | | | | | p value | |
| | | 0.13 | | | | | 0.07 | |

RANKL rs1021188

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| AA | 5 | 10 | 15 | 0.04 | A | 17 | 122 | 0.17 |
| GA | 7 | 102 | 109 | 0.27 | G | 83 | 600 | 0.83 |
| GG | 38 | 249 | 287 | 0.70 | Total | 100 | 722 | |
| Total | 50 | 361 | 411 | | | | | |
| | | p value | | | | | p value | |
| | | 0.01 | | | | | 0.98 | |

DBP rs7041

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| GG | 15 | 98 | 113 | 0.27 | G | 51 | 361 | 0.49 |
| TG | 21 | 165 | 186 | 0.45 | T | 41 | 377 | 0.51 |
| TT | 10 | 106 | 116 | 0.28 | Total | 92 | 738 | |
| Total | 46 | 369 | 415 | | | | | |
| | | p value | | | | | p value | |
| | | 0.53 | | | | | 0.21 | |

LRP5 rs3736228

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC | 35 | 267 | 302 | 0.74 | C | 81 | 620 | 0.86 |
| TC | 11 | 86 | 97 | 0.24 | T | 15 | 102 | 0.14 |
| TT | 2 | 8 | 10 | 0.02 | Total | 96 | 722 | |
| Total | 48 | 361 | 409 | | | | | |
| | | p value | | | | | p value | |
| | | 0.71 | | | | | 0.67 | |

VDR rs1544410

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| bb | 5 | 56 | 61 | 0.15 | b | 39 | 278 | 0.39 |
| Bb | 29 | 166 | 195 | 0.48 | B | 53 | 440 | 0.61 |
| BB | 12 | 137 | 149 | 0.37 | Total | 92 | 718 | |
| Total | 46 | 359 | 405 | | | | | |
| | | p value | | | | | p value | |
| | | 0.10 | | | | | 0.47 | |

VDR rs731236

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| tt | 3 | 46 | 49 | 0.12 | t | 37 | 260 | 0.37 |
| Tt | 31 | 168 | 199 | 0.50 | T | 49 | 452 | 0.63 |
| TT | 9 | 142 | 151 | 0.38 | Total | 86 | 712 | |
| Total | 43 | 356 | 399 | | | | | |
| | | p value | | | | | p value | |
| | | 0.01 | | | | | 0.21 | |

P2X7 rs1653624

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| AA | 0 | 0 | 0 | 0.00 | A | 1 | 25 | 0.03 |
| TA | 1 | 25 | 26 | 0.06 | T | 95 | 703 | 0.97 |
| TT | 47 | 339 | 386 | 0.94 | Total | 96 | 728 | |
| Total | 48 | 364 | 412 | | | | | |
| | | p value | | | | | p value | |
| | | N/A | | | | | N/A | |

P2X7 rs1718119

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| GG | 23 | 128 | 151 | 0.39 | G | 67 | 403 | 0.59 |
| GA | 21 | 147 | 168 | 0.44 | A | 25 | 275 | 0.41 |
| AA | 2 | 64 | 66 | 0.17 | Total | 92 | 678 | |
| Total | 46 | 339 | 385 | | | | | |
| | | p value | | | | | p value | |
| | | 0.04 | | | | | 0.01 | |

VDR rs7975232

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| aa | 13 | 102 | 115 | 0.29 | a | 52 | 378 | 0.53 |
| Aa | 26 | 174 | 200 | 0.50 | A | 40 | 332 | 0.47 |
| AA | 7 | 79 | 86 | 0.21 | Total | 92 | 710 | |
| Total | 46 | 355 | 401 | | | | | |
| | | p value | | | | | p value | |
| | | 0.50 | | | | | 0.53 | |

COL1A1 rs1800012

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| SS | 32 | 261 | 293 | 0.71 | S | 79 | 614 | 0.84 |
| Ss | 15 | 92 | 107 | 0.26 | s | 15 | 114 | 0.16 |
| ss | 0 | 11 | 11 | 0.03 | Total | 94 | 728 | |
| Total | 47 | 364 | 411 | | | | | |
| | | p value | | | | | p value | |
| | | 0.33 | | | | | 0.94 | |

CTR rs1801197

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC | 4 | 33 | 37 | 0.09 | C | 25 | 204 | 0.29 |
| TC | 17 | 138 | 155 | 0.39 | T | 65 | 510 | 0.71 |
| TT | 24 | 186 | 210 | 0.52 | Total | 90 | 714 | |
| Total | 45 | 357 | 402 | | | | | |
| | | p value | | | | | p value | |
| | | 0.99 | | | | | 0.87 | |

WNT16 rs3801387

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Allele | | | |
| AA | 28 | 188 | 216 | 0.54 | feq | 73 | 517 | 0.73 |
| GA | 17 | 141 | 158 | 0.39 | G | 21 | 191 | 0.27 |
| GG | 2 | 25 | 27 | 0.07 | Total | 94 | 708 | |
| Total | 47 | 354 | 401 | | | | | |
| | | p value | | | | | p value | |
| | | 0.62 | | | | | 0.31 | |

RANKL rs9594738

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC | 16 | 114 | 130 | 0.32 | C | 55 | 420 | 0.58 |
| TC | 23 | 192 | 215 | 0.53 | T | 41 | 302 | 0.42 |
| TT | 9 | 55 | 64 | 0.16 | Total | 96 | 722 | |
| Total | 48 | 361 | 409 | | | | | |
| | | p value | | | | | p value | |
| | | 0.74 | | | | | 0.86 | |

P2X7 rs208294

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC | 47 | 348 | 395 | 0.96 | C | 95 | 712 | 0.98 |
| TC | 1 | 16 | 17 | 0.04 | T | 1 | 18 | 0.02 |
| TT | 0 | 1 | 1 | 0.00 | Total | 96 | 730 | |
| Total | 48 | 365 | 413 | | | | | |
| | | p value | | | | | p value | |
| | | 0.70 | | | | | 0.37 | |

BRADYKININ rs1799722

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 9 | 10 | 86 | 96 | 0.25 | 9 | 45 | 330 | 0.48 |
| 9/-9 | 25 | 158 | 183 | 0.47 | -9 | 45 | 356 | 0.52 |
| -9 | 10 | 99 | 109 | 0.28 | Total | 90 | 686 | |
| Total | 45 | 343 | 388 | | | | | |
| | | p value | | | | | p value | |
| | | 0.47 | | | | | 0.72 | |

KALLIKREIN rs16987491

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| AA | 0 | 0 | 0 | 0.00 | A | 3 | 19 | 0.03 |
| GA | 3 | 19 | 22 | 0.05 | G | 93 | 694 | 0.97 |
| GG | 45 | 347 | 392 | 0.95 | Total | 96 | 713 | |
| Total | 48 | 366 | 414 | | | | | |
| | | p value | | | | | p value | |
| | | 0.76 | | | | | 0.92 | |

P2X7 rs3751143

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| GG | 2 | 8 | 10 | 0.02 | G | 17 | 112 | 0.16 |
| TG | 13 | 96 | 109 | 0.27 | T | 75 | 604 | 0.84 |
| TT | 31 | 254 | 285 | 0.71 | Total | 92 | 716 | |
| Total | 46 | 358 | 404 | | | | | |
| | | p value | | | | | p value | |
| | | 0.66 | | | | | 0.45 | |

SOST rs1877632

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC | 15 | 175 | 190 | 0.46 | C | 58 | 493 | 0.68 |
| TC | 28 | 143 | 171 | 0.42 | T | 40 | 229 | 0.32 |
| TT | 6 | 43 | 49 | 0.12 | Total | 98 | 722 | |
| Total | 49 | 361 | 410 | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | p value | | | | p value | |
| | | | 0.05 | | | | 0.05 | |

Stress fracture
pre 21 years

| | Stress fracture | Non-stress Fracture | Total | % | Allele | Stress fracture | Non-stress Fracture | % |
|---|---|---|---|---|---|---|---|---|
| | Genotype distribution | | | | Allele frequency | | | |
| VDR rs10735810 | | | | | | | | |
| FF | 35 | 169 | 204 | 0.47 | F | 102 | 483 | 0.69 |
| Ff | 32 | 145 | 177 | 0.41 | f | 54 | 221 | 0.31 |
| ff | 11 | 38 | 49 | 0.11 | Total | 156 | 704 | |
| Total | 78 | 352 | 430 | | | | | |
| | | p value | | | | | p value | |
| | | 0.69 | | | | | 0.39 | |
| P2X7 rs2230912 | | | | | | | | |
| AA | 46 | 243 | 289 | 0.67 | A | 119 | 593 | 0.83 |
| GA | 27 | 107 | 134 | 0.31 | G | 29 | 125 | 0.17 |
| GG | 1 | 9 | 10 | 0.02 | Total | 148 | 718 | |
| Total | 74 | 359 | 433 | | | | | |
| | | p value | | | | | p value | |
| | | 0.47 | | | | | 0.48 | |
| DBP rs4588 | | | | | | | | |
| AA | 6 | 29 | 35 | 0.08 | A | 47 | 193 | 0.27 |
| CA | 35 | 135 | 170 | 0.39 | C | 111 | 525 | 0.73 |
| CC | 38 | 195 | 233 | 0.53 | Total | 158 | 718 | |
| Total | 79 | 359 | 438 | | | | | |
| | | p value | | | | | p value | |
| | | 0.54 | | | | | 0.42 | |
| RANK rs3018362 | | | | | | | | |
| AA | 14 | 43 | 57 | 0.13 | A | 65 | 242 | 0.33 |
| GA | 37 | 156 | 193 | 0.44 | G | 91 | 482 | 0.67 |
| GG | 27 | 163 | 190 | 0.43 | Total | 156 | 724 | |
| Total | 78 | 362 | 440 | | | | | |
| | | p value | | | | | p value | |
| | | 0.16 | | | | | 0.03 | |
| OPG rs4355801 | | | | | | | | |
| AA | 30 | 138 | 168 | 0.38 | A | 96 | 432 | 0.60 |
| GA | 36 | 156 | 192 | 0.44 | G | 62 | 284 | 0.40 |
| GG | 13 | 64 | 77 | 0.18 | Total | 158 | 716 | |
| Total | 79 | 358 | 437 | | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | p value | | | | | p value |
| | | | | 0.93 | | | | | 0.91 |
| RANKL rs1021188 | | | | | | | | | |
| | AA | 7 | 10 | 17 | 0.04 | A | 34 | 122 | 0.17 |
| | GA | 20 | 102 | 122 | 0.28 | G | 126 | 600 | 0.83 |
| | GG | 53 | 249 | 302 | 0.68 | Total | 160 | 722 | |
| | Total | 80 | 361 | 441 | | | | | |
| | | | | p value | | | | | p value |
| | | | | 0.04 | | | | | 0.14 |
| DBP rs7041 | | | | | | | | | |
| | GG | 17 | 98 | 115 | 0.26 | G | 75 | 361 | 0.49 |
| | TG | 41 | 165 | 206 | 0.46 | T | 81 | 377 | 0.51 |
| | TT | 20 | 106 | 126 | 0.28 | Total | 156 | 738 | |
| | Total | 78 | 369 | 447 | | | | | |
| | | | | p value | | | | | p value |
| | | | | 0.43 | | | | | 0.83 |
| LRP5 rs3736228 | | | | | | | | | |
| | CC | 57 | 267 | 324 | 0.73 | C | 136 | 620 | 0.86 |
| | TC | 22 | 86 | 108 | 0.24 | T | 24 | 102 | 0.14 |
| | TT | 1 | 8 | 9 | 0.02 | Total | 160 | 722 | |
| | Total | 80 | 361 | 441 | | | | | |
| | | | | p value | | | | | p value |
| | | | | 0.70 | | | | | 0.75 |
| VDR rs1544410 | | | | | | | | | |
| | bb | 12 | 56 | 68 | 0.16 | b | 66 | 278 | 0.39 |
| | Bb | 42 | 166 | 208 | 0.48 | B | 84 | 440 | 0.61 |
| | BB | 21 | 137 | 158 | 0.36 | Total | 150 | 718 | |
| | Total | 75 | 359 | 434 | | | | | |
| | | | | p value | | | | | p value |
| | | | | 0.22 | | | | | 0.18 |
| VDR rs731236 | | | | | | | | | |
| | tt | 8 | 46 | 54 | 0.13 | t | 58 | 260 | 0.37 |
| | Tt | 42 | 168 | 210 | 0.49 | T | 84 | 452 | 0.63 |
| | TT | 21 | 142 | 163 | 0.38 | Total | 142 | 712 | |
| | Total | 71 | 356 | 427 | | | | | |
| | | | | p value | | | | | p value |
| | | | | 0.17 | | | | | 0.28 |
| P2X7 rs1653624 | | | | | | | | | |
| | AA | 0 | 0 | 0 | 0.00 | A | 1 | 25 | 0.03 |
| | TA | 1 | 25 | 26 | 0.06 | T | 157 | 703 | 0.97 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| TT | 78 | 339 | 417 | 0.94 | Total | 158 | 728 | |
| Total | 79 | 364 | 443 | | | | | |
| | | p value | | | | | p value | |
| | | N/A | | | | | N/A | |

P2X7 rs1718119

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| GG | 27 | 128 | 155 | 0.37 | G | 88 | 403 | 0.59 |
| GA | 34 | 147 | 181 | 0.44 | A | 62 | 275 | 0.41 |
| AA | 14 | 64 | 78 | 0.19 | Total | 150 | 678 | |
| Total | 75 | 339 | 414 | | | | | |
| | | p value | | | | | p value | |
| | | 0.95 | | | | | 0.84 | |

VDR rs7975232

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| aa | 19 | 102 | 121 | 0.28 | a | 85 | 378 | 0.53 |
| Aa | 47 | 174 | 221 | 0.51 | A | 75 | 332 | 0.47 |
| AA | 14 | 79 | 93 | 0.21 | Total | 160 | 710 | |
| Total | 80 | 355 | 435 | | | | | |
| | | p value | | | | | p value | |
| | | 0.29 | | | | | 0.98 | |

COL1A1 rs1800012

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| SS | 59 | 261 | 320 | 0.72 | S | 139 | 614 | 0.84 |
| Ss | 21 | 92 | 113 | 0.25 | s | 21 | 114 | 0.16 |
| ss | 0 | 11 | 11 | 0.02 | Total | 160 | 728 | |
| Total | 80 | 364 | 444 | | | | | |
| | | p value | | | | | p value | |
| | | 0.29 | | | | | 0.38 | |

CTR rs1801197

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC | 4 | 33 | 37 | 0.09 | C | 40 | 204 | 0.29 |
| TC | 32 | 138 | 170 | 0.39 | T | 114 | 510 | 0.71 |
| TT | 41 | 186 | 227 | 0.52 | Total | 154 | 714 | |
| Total | 77 | 357 | 434 | | | | | |
| | | p value | | | | | p value | |
| | | 0.50 | | | | | 0.48 | |

WNT16 rs3801387

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| AA | 38 | 188 | 226 | 0.52 | A | 109 | 517 | 0.73 |
| GA | 33 | 141 | 174 | 0.40 | G | 49 | 191 | 0.27 |
| GG | 8 | 25 | 33 | 0.08 | Total | 158 | 708 | |
| Total | 79 | 354 | 433 | | | | | |
| | | p value | | | | | p value | |
| | | 0.56 | | | | | 0.25 | |

RANKL rs9594738

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC | 30 | 114 | 144 | 0.33 | C | 96 | 420 | 0.58 |
| TC | 36 | 192 | 228 | 0.52 | T | 62 | 302 | 0.42 |
| TT | 13 | 55 | 68 | 0.15 | Total | 158 | 722 | |
| Total | 79 | 361 | 440 | | | | | |
| | | p value | | | | | p value | |
| | | 0.45 | | | | | 0.51 | |

P2X7 rs208294

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC | 78 | 348 | 426 | 0.96 | C | 158 | 712 | 0.98 |
| TC | 2 | 16 | 18 | 0.04 | T | 2 | 18 | 0.02 |
| TT | 0 | 1 | 1 | 0.00 | Total | 160 | 730 | |
| Total | 80 | 365 | 445 | | | | | |
| | | p value | | | | | p value | |
| | | 0.66 | | | | | 0.32 | |

BRADYKININ rs1799722

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 9 | 26 | 86 | 112 | 0.27 | 9 | 89 | 330 | 0.48 |
| 9/-9 | 37 | 158 | 195 | 0.47 | -9 | 61 | 356 | 0.52 |
| -9 | 12 | 99 | 111 | 0.27 | Total | 150 | 686 | |
| Total | 75 | 343 | 418 | | | | | |
| | | p value | | | | | p value | |
| | | 0.05 | | | | | 0.01 | |

KALLIKREIN rs16987491

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| AA | 0 | 0 | 0 | 0.00 | A | 8 | 19 | 0.03 |
| GA | 8 | 19 | 27 | 0.06 | G | 152 | 713 | 0.97 |
| GG | 72 | 347 | 419 | 0.94 | Total | 160 | 732 | |
| Total | 80 | 366 | 446 | | | | | |
| | | p value | | | | | p value | |
| | | 0.10 | | | | | 0.54 | |

P2X7 rs3751143

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| GG | 3 | 8 | 11 | 0.03 | G | 28 | 112 | 0.16 |
| TG | 22 | 96 | 118 | 0.27 | T | 126 | 604 | 0.84 |
| TT | 52 | 254 | 306 | 0.70 | Total | 154 | 716 | |
| Total | 77 | 358 | 435 | | | | | |
| | | p value | | | | | p value | |
| | | 0.65 | | | | | 0.39 | |

SOST rs1877632

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC | 36 | 175 | 211 | 0.48 | C | 108 | 493 | 0.68 |
| TC | 36 | 143 | 179 | 0.41 | T | 52 | 229 | 0.32 |
| TT | 8 | 43 | 51 | 0.12 | Total | 160 | 722 | |
| Total | 80 | 361 | 441 | | | | | |
| | | p value | | | | | p value | |
| | | 0.66 | | | | | 0.83 | |

Female

| | | Stress fracture | Non-stress Fracture | Total | % | | Stress fracture | Non-stress Fracture | % |
|---|---|---|---|---|---|---|---|---|---|
| | | Genotype distribution | | | | Allele frequency | | | |
| VDR rs10735810 | | | | | | | | | |
| | FF | 7 | 19 | 26 | 0.39 | F | 28 | 54 | 0.68 |
| | Ff | 14 | 16 | 30 | 0.45 | f | 24 | 26 | 0.33 |
| | ff | 5 | 5 | 10 | 0.15 | Total | 52 | 80 | |
| | Total | 26 | 40 | 66 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.24 | | | | | 0.04 | |
| P2X7 rs2230912 | | | | | | | | | |
| | AA | 17 | 31 | 48 | 0.77 | A | 40 | 70 | 0.90 |
| | GA | 6 | 8 | 14 | 0.23 | G | 6 | 8 | 0.10 |
| | GG | 0 | 0 | 0 | 0.00 | Total | 46 | 78 | |
| | Total | 23 | 39 | 62 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.61 | | | | | 0.53 | |
| DBP rs4588 | | | | | | | | | |
| | AA | 1 | 2 | 3 | 0.05 | A | 10 | 17 | 0.23 |
| | CA | 8 | 13 | 21 | 0.34 | C | 40 | 57 | 0.77 |
| | CC | 16 | 22 | 38 | 0.61 | Total | 50 | 74 | |
| | Total | 25 | 37 | 62 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.93 | | | | | 0.62 | |
| RANK rs3018362 | | | | | | | | | |
| | AA | 3 | 4 | 7 | 0.11 | A | 18 | 28 | 0.36 |
| | GA | 12 | 20 | 32 | 0.51 | G | 30 | 50 | 0.64 |
| | GG | 9 | 15 | 24 | 0.38 | Total | 48 | 78 | |
| | Total | 24 | 39 | 63 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.96 | | | | | 0.82 | |
| OPG rs4355801 | | | | | | | | | |
| | AA | 8 | 12 | 20 | 0.30 | A | 27 | 46 | 0.56 |
| | GA | 11 | 22 | 33 | 0.49 | G | 25 | 36 | 0.44 |
| | GG | 7 | 7 | 14 | 0.21 | Total | 52 | 82 | |
| | Total | 26 | 41 | 67 | | | | | |
| | | | p value | | | | | p value | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 0.56 | | | | 0.54 | |
| RANKL rs1021188 | | | | | | | | |
| | AA | 2 | 2 | 4 | 0.06 | A | 9 | 14 | 0.18 |
| | GA | 5 | 10 | 15 | 0.23 | G | 43 | 62 | 0.82 |
| | GG | 19 | 26 | 45 | 0.70 | Total | 52 | 76 | |
| | Total | 26 | 38 | 64 | | | | | |
| | | | p value | | | | p value | | |
| | | | 0.77 | | | | 0.84 | | |
| DBP rs7041 | | | | | | | | |
| | GG | 8 | 14 | 22 | 0.33 | G | 31 | 48 | 0.59 |
| | TG | 15 | 20 | 35 | 0.52 | T | 21 | 34 | 0.41 |
| | TT | 3 | 7 | 10 | 0.15 | Total | 52 | 82 | |
| | Total | 26 | 41 | 67 | | | | | |
| | | | p value | | | | p value | | |
| | | | 0.73 | | | | 0.87 | | |
| LRP5 rs3736228 | | | | | | | | |
| | CC | 15 | 24 | 39 | 0.61 | C | 40 | 59 | 0.78 |
| | TC | 10 | 11 | 21 | 0.33 | T | 12 | 17 | 0.22 |
| | TT | 1 | 3 | 4 | 0.06 | Total | 52 | 76 | |
| | Total | 26 | 38 | 64 | | | | | |
| | | | p value | | | | p value | | |
| | | | 0.64 | | | | 0.90 | | |
| VDR rs1544410 | | | | | | | | |
| | bb | 4 | 4 | 8 | 0.13 | b | 20 | 28 | 0.36 |
| | Bb | 12 | 20 | 32 | 0.50 | B | 30 | 50 | 0.64 |
| | BB | 9 | 15 | 24 | 0.38 | Total | 50 | 78 | |
| | Total | 25 | 39 | 64 | | | | | |
| | | | p value | | | | p value | | |
| | | | 0.79 | | | | 0.55 | | |
| VDR rs731236 | | | | | | | | |
| | tt | 4 | 3 | 7 | 0.11 | t | 21 | 25 | 0.33 |
| | Tt | 13 | 19 | 32 | 0.51 | T | 29 | 51 | 0.67 |
| | TT | 8 | 16 | 24 | 0.38 | Total | 50 | 76 | |
| | Total | 25 | 38 | 63 | | | | | |
| | | | p value | | | | p value | | |
| | | | 0.52 | | | | 0.17 | | |
| P2X7 rs1653624 | | | | | | | | |
| | AA | 0 | 0 | 0 | 0.00 | A | 1 | 3 | 0.04 |
| | TA | 1 | 3 | 4 | 0.06 | T | 51 | 75 | 0.96 |
| | TT | 25 | 36 | 61 | 0.94 | Total | 52 | 78 | |
| | Total | 26 | 39 | 65 | | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | p value | | | | | p value |
| | | | | N/A | | | | | N/A |
| P2X7 rs1718119 | | | | | | | | | |
| | GG | 11 | 16 | 27 | 0.44 | G | 35 | 49 | 0.66 |
| | GA | 13 | 17 | 30 | 0.48 | A | 15 | 25 | 0.34 |
| | AA | 1 | 4 | 5 | 0.08 | Total | 50 | 74 | |
| | Total | 25 | 37 | 62 | | | | | |
| | | | | p value | | | | | p value |
| | | | | 0.61 | | | | | 0.57 |
| VDR rs7975232 | | | | | | | | | |
| | aa | 8 | 8 | 16 | 0.25 | a | 28 | 38 | 0.49 |
| | Aa | 12 | 22 | 34 | 0.53 | A | 22 | 40 | 0.51 |
| | AA | 5 | 9 | 14 | 0.22 | Total | 50 | 78 | |
| | Total | 25 | 39 | 64 | | | | | |
| | | | | p value | | | | | p value |
| | | | | 0.58 | | | | | 0.30 |
| COL1A1 rs1800012 | | | | | | | | | |
| | SS | 18 | 23 | 41 | 0.63 | S | 44 | 59 | 0.76 |
| | Ss | 8 | 13 | 21 | 0.32 | s | 8 | 19 | 0.24 |
| | ss | 0 | 3 | 3 | 0.05 | Total | 52 | 78 | |
| | Total | 26 | 39 | 65 | | | | | |
| | | | | p value | | | | | p value |
| | | | | 0.32 | | | | | 0.13 |
| CTR rs1801197 | | | | | | | | | |
| | CC | 3 | 5 | 8 | 0.13 | C | 16 | 20 | 0.27 |
| | TC | 10 | 10 | 20 | 0.32 | T | 36 | 54 | 0.73 |
| | TT | 13 | 22 | 35 | 0.56 | Total | 52 | 74 | |
| | Total | 26 | 37 | 63 | | | | | |
| | | | | p value | | | | | p value |
| | | | | 0.63 | | | | | 0.54 |
| WNT16 rs3801387 | | | | | | | | | |
| | AA | 17 | 23 | 40 | 0.62 | A | 41 | 60 | 0.77 |
| | GA | 7 | 14 | 21 | 0.32 | G | 11 | 18 | 0.23 |
| | GG | 2 | 2 | 4 | 0.06 | Total | 52 | 78 | |
| | Total | 26 | 39 | 65 | | | | | |
| | | | | p value | | | | | p value |
| | | | | 0.72 | | | | | 0.74 |
| RANKL rs9594738 | | | | | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC | 8 | 12 | 20 | 0.30 | C | 28 | 48 | 0.59 |
| TC | 12 | 24 | 36 | 0.54 | T | 24 | 34 | 0.41 |
| TT | 6 | 5 | 11 | 0.16 | Total | 52 | 82 | |
| Total | 26 | 41 | 67 | | | | | |
| | | p value | | | | | p value | |
| | | 0.45 | | | | | 0.49 | |

P2X7 rs208294

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC | 26 | 35 | 61 | 0.97 | C | 52 | 72 | 0.97 |
| TC | 0 | 2 | 2 | 0.03 | T | 0 | 2 | 0.03 |
| TT | 0 | 0 | 0 | 0.00 | Total | 52 | 74 | |
| Total | 26 | 37 | 63 | | | | | |
| | | p value | | | | | p value | |
| | | 0.23 | | | | | 0.23 | |

BRADYKININ rs1799722

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 9 | 7 | 10 | 17 | 0.27 | 9 | 24 | 39 | 0.51 |
| 9/-9 | 10 | 19 | 29 | 0.46 | -9 | 26 | 37 | 0.49 |
| -9 | 8 | 9 | 17 | 0.27 | Total | 50 | 76 | |
| Total | 25 | 38 | 63 | | | | | |
| | | p value | | | | | p value | |
| | | 0.69 | | | | | 0.64 | |

KALLIKREIN rs16987491

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| AA | 0 | 0 | 0 | 0.00 | A | 2 | 3 | 0.04 |
| GA | 2 | 3 | 5 | 0.08 | G | 52 | 75 | 0.96 |
| GG | 25 | 36 | 61 | 0.92 | Total | 54 | 78 | |
| Total | 27 | 39 | 66 | | | | | |
| | | p value | | | | | p value | |
| | | 0.97 | | | | | 0.98 | |

P2X7 rs3751143

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| GG | 0 | 1 | 1 | 0.02 | G | 12 | 19 | 0.24 |
| TG | 12 | 17 | 29 | 0.45 | T | 38 | 59 | 0.76 |
| TT | 13 | 21 | 34 | 0.53 | Total | 50 | 78 | |
| Total | 25 | 39 | 64 | | | | | |
| | | p value | | | | | p value | |
| | | 0.70 | | | | | 0.95 | |

SOST rs1877632

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC | 7 | 18 | 25 | 0.39 | C | 31 | 52 | 0.68 |
| TC | 17 | 16 | 33 | 0.52 | T | 21 | 24 | 0.32 |
| TT | 2 | 4 | 6 | 0.09 | Total | 52 | 76 | |
| Total | 26 | 38 | 64 | | | | | |
| | | p value | | | | | p value | |
| | | 0.18 | | | | | 0.17 | |

leg - ex metatarsal

| | | Stress fracture | Non-stress Fracture | Total | % | | Stress fracture | Non-stress Fracture | % |
|---|---|---|---|---|---|---|---|---|---|
| | | Genotype distribution | | | | Allele frequency | | | |
| VDR rs10735810 | | | | | | | | | |
| | FF | 14 | 169 | 183 | 0.47 | F | 44 | 483 | 0.69 |
| | Ff | 16 | 145 | 161 | 0.41 | f | 34 | 221 | 0.31 |
| | ff | 9 | 38 | 47 | 0.12 | Total | 78 | 704 | |
| | Total | 39 | 352 | 391 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.06 | | | | | 0.02 | |
| P2X7 rs2230912 | | | | | | | | | |
| | AA | 29 | 243 | 272 | 0.68 | A | 69 | 593 | 0.83 |
| | GA | 11 | 107 | 118 | 0.30 | G | 11 | 125 | 0.17 |
| | GG | 0 | 9 | 9 | 0.02 | Total | 80 | 718 | |
| | Total | 40 | 359 | 399 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.55 | | | | | 0.39 | |
| DBP rs4588 | | | | | | | | | |
| | AA | 2 | 29 | 31 | 0.08 | A | 22 | 193 | 0.27 |
| | CA | 18 | 135 | 153 | 0.39 | C | 52 | 525 | 0.73 |
| | CC | 17 | 195 | 212 | 0.54 | Total | 74 | 718 | |
| | Total | 37 | 359 | 396 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.41 | | | | | 0.58 | |
| RANK rs3018362 | | | | | | | | | |
| | AA | 6 | 43 | 49 | 0.12 | A | 30 | 242 | 0.33 |
| | GA | 18 | 156 | 174 | 0.43 | G | 48 | 482 | 0.67 |
| | GG | 15 | 163 | 178 | 0.44 | Total | 78 | 724 | |
| | Total | 39 | 362 | 401 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.68 | | | | | 0.35 | |
| OPG rs4355801 | | | | | | | | | |
| | AA | 8 | 138 | 146 | 0.37 | A | 37 | 432 | 0.60 |
| | GA | 21 | 156 | 177 | 0.45 | G | 41 | 284 | 0.40 |
| | GG | 10 | 64 | 74 | 0.19 | Total | 78 | 716 | |
| | Total | 39 | 358 | 397 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.08 | | | | | 0.02 | |

RANKL rs1021188

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| AA | 3 | 10 | 13 | 0.03 | A | 13 | 122 | 0.17 |
| GA | 7 | 102 | 109 | 0.27 | G | 65 | 600 | 0.83 |
| GG | 29 | 249 | 278 | 0.70 | Total | 78 | 722 | |
| Total | 39 | 361 | 400 | | | | | |
| | | p value | | | | | p value | |
| | | 0.13 | | | | | 0.96 | |

DBP rs7041

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| GG | 8 | 98 | 106 | 0.26 | G | 37 | 361 | 0.49 |
| TG | 21 | 165 | 186 | 0.46 | T | 41 | 377 | 0.51 |
| TT | 10 | 106 | 116 | 0.28 | Total | 78 | 738 | |
| Total | 39 | 369 | 408 | | | | | |
| | | p value | | | | | p value | |
| | | 0.53 | | | | | 0.79 | |

LRP5 rs3736228

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC | 27 | 267 | 294 | 0.74 | C | 65 | 620 | 0.86 |
| TC | 11 | 86 | 97 | 0.24 | T | 13 | 102 | 0.14 |
| TT | 1 | 8 | 9 | 0.02 | Total | 78 | 722 | |
| Total | 39 | 361 | 400 | | | | | |
| | | p value | | | | | p value | |
| | | 0.82 | | | | | 0.52 | |

VDR rs1544410

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| bb | 8 | 56 | 64 | 0.16 | b | 40 | 278 | 0.39 |
| Bb | 24 | 166 | 190 | 0.48 | B | 36 | 440 | 0.61 |
| BB | 6 | 137 | 143 | 0.36 | Total | 76 | 718 | |
| Total | 38 | 359 | 397 | | | | | |
| | | p value | | | | | p value | |
| | | 0.02 | | | | | 0.01 | |

VDR rs731236

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| tt | 7 | 46 | 53 | 0.13 | t | 39 | 260 | 0.37 |
| Tt | 25 | 168 | 193 | 0.49 | T | 39 | 452 | 0.63 |
| TT | 7 | 142 | 149 | 0.38 | Total | 78 | 712 | |
| Total | 39 | 356 | 395 | | | | | |
| | | p value | | | | | p value | |
| | | 0.03 | | | | | 0.01 | |

P2X7 rs1653624

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| AA | 0 | 0 | 0 | 0.00 | A | 1 | 25 | 0.03 |
| TA | 1 | 25 | 26 | 0.06 | T | 77 | 703 | 0.97 |
| TT | 38 | 339 | 377 | 0.94 | Total | 78 | 728 | |
| Total | 39 | 364 | 403 | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | p value | | | | p value | |
| | | | N/A | | | | N/A | |
| P2X7 rs1718119 | | | | | | | | |
| | GG | 14 | 128 | 142 | 0.38 | G | 50 | 403 | 0.59 |
| | GA | 22 | 147 | 169 | 0.45 | A | 26 | 275 | 0.41 |
| | AA | 2 | 64 | 66 | 0.18 | Total | 76 | 678 | |
| | Total | 38 | 339 | 377 | | | | | |
| | | | p value | | | | p value | |
| | | | 0.07 | | | | 0.26 | |
| VDR rs7975232 | | | | | | | | |
| | aa | 15 | 102 | 117 | 0.30 | a | 49 | 378 | 0.53 |
| | Aa | 19 | 174 | 193 | 0.49 | A | 27 | 332 | 0.47 |
| | AA | 4 | 79 | 83 | 0.21 | Total | 76 | 710 | |
| | Total | 38 | 355 | 393 | | | | | |
| | | | p value | | | | p value | |
| | | | 0.17 | | | | 0.04 | |
| COL1A1 rs1800012 | | | | | | | | |
| | SS | 28 | 261 | 289 | 0.72 | S | 67 | 614 | 0.84 |
| | Ss | 11 | 92 | 103 | 0.26 | s | 11 | 114 | 0.16 |
| | ss | 0 | 11 | 11 | 0.03 | Total | 78 | 728 | |
| | Total | 39 | 364 | 403 | | | | | |
| | | | p value | | | | p value | |
| | | | 0.52 | | | | 0.71 | |
| CTR rs1801197 | | | | | | | | |
| | CC | 2 | 33 | 35 | 0.09 | C | 20 | 204 | 0.29 |
| | TC | 16 | 138 | 154 | 0.39 | T | 58 | 510 | 0.71 |
| | TT | 21 | 186 | 207 | 0.52 | Total | 78 | 714 | |
| | Total | 39 | 357 | 396 | | | | | |
| | | | p value | | | | p value | |
| | | | 0.69 | | | | 0.57 | |
| WNT16 rs3801387 | | | | | | | | |
| | AA | 21 | 188 | 209 | 0.53 | A | 55 | 517 | 0.73 |
| | GA | 13 | 141 | 154 | 0.39 | G | 23 | 191 | 0.27 |
| | GG | 5 | 25 | 30 | 0.08 | Total | 78 | 708 | |
| | Total | 39 | 354 | 393 | | | | | |
| | | | p value | | | | p value | |
| | | | 0.39 | | | | 0.62 | |
| RANKL rs9594738 | | | | | | | | |
| | CC | 12 | 114 | 126 | 0.32 | C | 45 | 420 | 0.58 |
| | TC | 21 | 192 | 213 | 0.53 | T | 33 | 302 | 0.42 |
| | TT | 6 | 55 | 61 | 0.15 | Total | 78 | 722 | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Total | 39 | 361 | 400 | | | | | |
| | | p value | | | | | p value | |
| | | 0.99 | | | | | 0.93 | |
| P2X7 rs208294 | | | | | | | | |
| CC | 39 | 348 | 387 | 0.96 | C | 78 | 712 | 0.98 |
| TC | 0 | 16 | 16 | 0.04 | T | 0 | 18 | 0.02 |
| TT | 0 | 1 | 1 | 0.00 | Total | 78 | 730 | |
| Total | 39 | 365 | 404 | | | | | |
| | | p value | | | | | p value | |
| | | 0.39 | | | | | 0.16 | |
| BRADYKININ rs1799722 | | | | | | | | |
| 9 | 11 | 86 | 97 | 0.26 | 9 | 42 | 330 | 0.48 |
| 9/-9 | 20 | 158 | 178 | 0.47 | -9 | 32 | 356 | 0.52 |
| -9 | 6 | 99 | 105 | 0.28 | Total | 74 | 686 | |
| Total | 37 | 343 | 380 | | | | | |
| | | p value | | | | | p value | |
| | | 0.26 | | | | | 0.14 | |
| KALLIKREIN rs16987491 | | | | | | | | |
| AA | 0 | 0 | 0 | 0.00 | A | 4 | 19 | 0.03 |
| GA | 4 | 19 | 23 | 0.06 | G | 74 | 713 | 0.97 |
| GG | 35 | 347 | 382 | 0.94 | Total | 78 | 732 | |
| Total | 39 | 366 | 405 | | | | | |
| | | p value | | | | | p value | |
| | | 0.19 | | | | | 0.65 | |
| P2X7 rs3751143 | | | | | | | | |
| GG | 2 | 13 | 15 | 0.04 | G | 18 | 108 | 0.15 |
| TG | 14 | 82 | 96 | 0.24 | T | 54 | 604 | 0.85 |
| TT | 20 | 261 | 281 | 0.72 | Total | 72 | 712 | |
| Total | 36 | 356 | 392 | | | | | |
| | | p value | | | | | p value | |
| | | 0.08 | | | | | 0.02 | |
| SOST rs1877632 | | | | | | | | |
| CC | 16 | 191 | 207 | 0.52 | C | 50 | 513 | 0.71 |
| TC | 18 | 131 | 149 | 0.37 | T | 28 | 207 | 0.29 |
| TT | 5 | 38 | 43 | 0.11 | Total | 78 | 720 | |
| Total | 39 | 360 | 399 | | | | | |
| | | p value | | | | | p value | |
| | | 0.36 | | | | | 0.16 | |

Runners

| | Stress fracture | Non-stress Fracture | Total | % | | Stress fracture | Non-stress Fracture | % |
|---|---|---|---|---|---|---|---|---|
| | Genotype distribution | | | | Allele frequency | | | |
| VDR rs10735810 | | | | | | | | |
| FF | 11 | 13 | 24 | 0.39 | F | 37 | 38 | 0.70 |
| Ff | 15 | 12 | 27 | 0.44 | f | 31 | 16 | 0.30 |
| ff | 8 | 2 | 10 | 0.16 | Total | 68 | 54 | |
| Total | 34 | 27 | 61 | | | | | |
| | p value | | | | | p value | | |
| | 0.19 | | | | | 0.00 | | |
| P2X7 rs2230912 | | | | | | | | |
| AA | 24 | 16 | 40 | 0.67 | A | 56 | 44 | 0.79 |
| GA | 8 | 12 | 20 | 0.33 | G | 8 | 12 | 0.21 |
| GG | 0 | 0 | 0 | 0.00 | Total | 64 | 56 | |
| Total | 32 | 28 | 60 | | | | | |
| | p value | | | | | p value | | |
| | 0.14 | | | | | 0.08 | | |
| DBP rs4588 | | | | | | | | |
| AA | 2 | 1 | 3 | 0.05 | A | 11 | 14 | 0.26 |
| CA | 7 | 12 | 19 | 0.31 | C | 57 | 40 | 0.74 |
| CC | 25 | 14 | 39 | 0.64 | Total | 68 | 54 | |
| Total | 34 | 27 | 61 | | | | | |
| | p value | | | | | p value | | |
| | 0.14 | | | | | 0.07 | | |
| RANK rs3018362 | | | | | | | | |
| AA | 4 | 1 | 5 | 0.08 | A | 29 | 16 | 0.30 |
| GA | 21 | 14 | 35 | 0.58 | G | 37 | 38 | 0.70 |
| GG | 8 | 12 | 20 | 0.33 | Total | 66 | 54 | |
| Total | 33 | 27 | 60 | | | | | |
| | p value | | | | | p value | | |
| | 0.18 | | | | | 0.01 | | |
| OPG rs4355801 | | | | | | | | |
| AA | 9 | 10 | 19 | 0.31 | A | 33 | 35 | 0.65 |
| GA | 15 | 15 | 30 | 0.49 | G | 35 | 19 | 0.35 |
| GG | 10 | 2 | 12 | 0.20 | Total | 68 | 54 | |
| Total | 34 | 27 | 61 | | | | | |
| | p value | | | | | p value | | |
| | 0.10 | | | | | 0.00 | | |

RANKL rs1021188

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| AA | 2 | 1 | 3 | 0.05 | A | 9 | 11 | 0.20 |
| GA | 5 | 9 | 14 | 0.23 | G | 59 | 43 | 0.80 |
| GG | 27 | 17 | 44 | 0.72 | Total | 68 | 54 | |
| Total | 34 | 27 | 61 | | | | | |
| | | p value | | | | | p value | |
| | | 0.22 | | | | | 0.14 | |

DBP rs7041

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| GG | 15 | 8 | 23 | 0.37 | G | 44 | 32 | 0.57 |
| TG | 14 | 16 | 30 | 0.48 | T | 24 | 24 | 0.43 |
| TT | 5 | 4 | 9 | 0.15 | Total | 68 | 56 | |
| Total | 34 | 28 | 62 | | | | | |
| | | p value | | | | | p value | |
| | | 0.40 | | | | | 0.21 | |

LRP5 rs3736228

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC | 23 | 22 | 45 | 0.74 | C | 56 | 49 | 0.91 |
| TC | 10 | 5 | 15 | 0.25 | T | 12 | 5 | 0.09 |
| TT | 1 | 0 | 1 | 0.02 | Total | 68 | 54 | |
| Total | 34 | 27 | 61 | | | | | |
| | | p value | | | | | p value | |
| | | 0.38 | | | | | 0.02 | |

VDR rs1544410

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| bb | 5 | 3 | 8 | 0.13 | b | 26 | 20 | 0.37 |
| Bb | 16 | 14 | 30 | 0.50 | B | 40 | 34 | 0.63 |
| BB | 12 | 10 | 22 | 0.37 | Total | 66 | 54 | |
| Total | 33 | 27 | 60 | | | | | |
| | | p value | | | | | p value | |
| | | 0.90 | | | | | 0.69 | |

VDR rs731236

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| tt | 5 | 4 | 9 | 0.15 | t | 26 | 22 | 0.39 |
| Tt | 16 | 14 | 30 | 0.51 | T | 36 | 34 | 0.61 |
| TT | 10 | 10 | 20 | 0.34 | Total | 62 | 56 | |
| Total | 31 | 28 | 59 | | | | | |
| | | p value | | | | | p value | |
| | | 0.96 | | | | | 0.67 | |

P2X7 rs1653624

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| AA | 0 | 0 | 0 | 0.00 | A | 1 | 1 | 0.02 |
| TA | 1 | 1 | 2 | 0.03 | T | 65 | 55 | 0.98 |
| TT | 32 | 27 | 59 | 0.97 | Total | 66 | 56 | |
| Total | 33 | 28 | 61 | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | p value | | | | p value | |
| | | | N/A | | | | N/A | |
| P2X7 rs1718119 | | | | | | | | |
| | GG | 13 | 7 | 20 | 0.33 | G | 46 | 26 | 0.50 |
| | GA | 20 | 12 | 32 | 0.53 | A | 22 | 26 | 0.50 |
| | AA | 1 | 7 | 8 | 0.13 | Total | 68 | 52 | |
| | Total | 34 | 26 | 60 | | | | | |
| | | | p value | | | | p value | |
| | | | 0.03 | | | | 0.00 | |
| VDR rs7975232 | | | | | | | | |
| | aa | 11 | 8 | 19 | 0.32 | a | 38 | 29 | 0.54 |
| | Aa | 16 | 13 | 29 | 0.49 | A | 26 | 25 | 0.46 |
| | AA | 5 | 6 | 11 | 0.19 | Total | 64 | 54 | |
| | Total | 32 | 27 | 59 | | | | | |
| | | | p value | | | | p value | |
| | | | 0.80 | | | | 0.36 | |
| COL1A1 rs1800012 | | | | | | | | |
| | SS | 26 | 17 | 43 | 0.70 | S | 59 | 44 | 0.81 |
| | Ss | 7 | 10 | 17 | 0.28 | s | 9 | 10 | 0.19 |
| | ss | 1 | 0 | 1 | 0.02 | Total | 68 | 54 | |
| | Total | 34 | 27 | 61 | | | | | |
| | | | p value | | | | p value | |
| | | | 0.27 | | | | 0.26 | |
| CTR rs1801197 | | | | | | | | |
| | CC | 2 | 1 | 3 | 0.05 | C | 14 | 15 | 0.28 |
| | TC | 10 | 13 | 23 | 0.38 | T | 52 | 39 | 0.72 |
| | TT | 21 | 13 | 34 | 0.57 | Total | 66 | 54 | |
| | Total | 33 | 27 | 60 | | | | | |
| | | | p value | | | | p value | |
| | | | 0.36 | | | | 0.23 | |
| WNT16 rs3801387 | | | | | | | | |
| | AA | 18 | 17 | 35 | 0.56 | A | 47 | 43 | 0.77 |
| | GA | 11 | 9 | 20 | 0.32 | G | 21 | 13 | 0.23 |
| | GG | 5 | 2 | 7 | 0.11 | Total | 68 | 56 | |
| | Total | 34 | 28 | 62 | | | | | |
| | | | p value | | | | p value | |
| | | | 0.62 | | | | 0.13 | |
| RANKL rs9594738 | | | | | | | | |
| | CC | 7 | 9 | 16 | 0.26 | C | 34 | 33 | 0.59 |
| | TC | 20 | 15 | 35 | 0.56 | T | 34 | 23 | 0.41 |
| | TT | 7 | 4 | 11 | 0.18 | Total | 68 | 56 | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Total | 34 | 28 | 62 | | | | | |
| | | p value | | | | | p value | |
| | | 0.55 | | | | | 0.13 | |
| P2X7 rs208294 | | | | | | | | |
| CC | 33 | 27 | 60 | 1.00 | C | 66 | 54 | 1.00 |
| TC | 0 | 0 | 0 | 0.00 | T | 0 | 0 | 0.00 |
| TT | 0 | 0 | 0 | 0.00 | Total | 66 | 54 | |
| Total | 33 | 27 | 60 | | | | | |
| | | p value | | | | | p value | |
| | | n/a | | | | | n/a | |
| BRADYKININ rs1799722 | | | | | | | | |
| 9 | 9 | 9 | 18 | 0.35 | 9 | 35 | 26 | 0.62 |
| 9/-9 | 17 | 8 | 25 | 0.48 | -9 | 27 | 16 | 0.38 |
| -9 | 5 | 4 | 9 | 0.17 | Total | 62 | 42 | |
| Total | 31 | 21 | 52 | | | | | |
| | | p value | | | | | p value | |
| | | 0.48 | | | | | 0.38 | |
| KALLIKREIN rs16987491 | | | | | | | | |
| AA | 0 | 0 | 0 | 0.00 | A | 3 | 1 | 0.04 |
| GA | 3 | 1 | 4 | 0.06 | G | 67 | 53 | 0.96 |
| GG | 32 | 26 | 58 | 0.94 | Total | 70 | 54 | |
| Total | 35 | 27 | 62 | | | | | |
| | | p value | | | | | p value | |
| | | 0.44 | | | | | 0.68 | |
| P2X7 rs3751143 | | | | | | | | |
| GG | 1 | 1 | 2 | 0.03 | G | 14 | 9 | 0.16 |
| TG | 12 | 7 | 19 | 0.31 | T | 54 | 47 | 0.84 |
| TT | 21 | 20 | 41 | 0.66 | Total | 68 | 56 | |
| Total | 34 | 28 | 62 | | | | | |
| | | p value | | | | | p value | |
| | | 0.68 | | | | | 0.31 | |
| SOST rs1877632 | | | | | | | | |
| CC | 10 | 14 | 24 | 0.39 | C | 42 | 37 | 0.69 |
| TC | 22 | 9 | 31 | 0.51 | T | 26 | 17 | 0.31 |
| TT | 2 | 4 | 6 | 0.10 | Total | 68 | 54 | |
| Total | 34 | 27 | 61 | | | | | |
| | | p value | | | | | p value | |
| | | 0.05 | | | | | 0.23 | |

Football/hockey

| | | Stress fracture | Non-stress Fracture | Total | % | | Stress fracture | Non-stress Fracture | % |
|---|---|---|---|---|---|---|---|---|---|
| | | Genotype distribution | | | | Allele frequency | | | |
| VDR rs10735810 | | | | | | | | | |
| | FF | 17 | 97 | 114 | 0.50 | F | 46 | 275 | 0.71 |
| | Ff | 12 | 81 | 93 | 0.41 | f | 20 | 115 | 0.29 |
| | ff | 4 | 17 | 21 | 0.09 | Total | 66 | 390 | |
| | Total | 33 | 195 | 228 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.76 | | | | | 0.88 | |
| P2X7 rs2230912 | | | | | | | | | |
| | AA | 27 | 148 | 175 | 0.76 | A | 60 | 338 | 0.86 |
| | GA | 6 | 42 | 48 | 0.21 | G | 8 | 56 | 0.14 |
| | GG | 1 | 7 | 8 | 0.03 | Total | 68 | 394 | |
| | Total | 34 | 197 | 231 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.87 | | | | | 0.56 | |
| DBP rs4588 | | | | | | | | | |
| | AA | 2 | 19 | 21 | 0.09 | A | 22 | 109 | 0.28 |
| | CA | 18 | 71 | 89 | 0.38 | C | 48 | 287 | 0.72 |
| | CC | 15 | 108 | 123 | 0.53 | Total | 70 | 396 | |
| | Total | 35 | 198 | 233 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.21 | | | | | 0.46 | |
| RANK rs3018362 | | | | | | | | | |
| | AA | 7 | 20 | 27 | 0.12 | A | 22 | 124 | 0.31 |
| | GA | 8 | 84 | 92 | 0.39 | G | 46 | 276 | 0.69 |
| | GG | 19 | 96 | 115 | 0.49 | Total | 68 | 400 | |
| | Total | 34 | 200 | 234 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.06 | | | | | 0.81 | |
| OPG rs4355801 | | | | | | | | | |
| | AA | 18 | 74 | 92 | 0.40 | A | 49 | 233 | 0.59 |
| | GA | 13 | 85 | 98 | 0.42 | G | 21 | 159 | 0.41 |
| | GG | 4 | 37 | 41 | 0.18 | Total | 70 | 392 | |
| | Total | 35 | 196 | 231 | | | | | |
| | | | p value | | | | | p value | |
| | | | 0.27 | | | | | 0.07 | |

RANKL rs1021188

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| AA | 3 | 5 | 8 | 0.03 | A | 18 | 66 | 0.17 |
| GA | 12 | 56 | 68 | 0.30 | G | 50 | 326 | 0.83 |
| GG | 19 | 135 | 154 | 0.67 | Total | 68 | 392 | |
| Total | 34 | 196 | 230 | | | | | |
| | | p value | | | | | p value | |
| | | 0.11 | | | | | 0.03 | |

DBP rs7041

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| GG | 4 | 45 | 49 | 0.21 | G | 21 | 178 | 0.44 |
| TG | 13 | 88 | 101 | 0.43 | T | 45 | 224 | 0.56 |
| TT | 16 | 68 | 84 | 0.36 | Total | 66 | 402 | |
| Total | 33 | 201 | 234 | | | | | |
| | | p value | | | | | p value | |
| | | 0.20 | | | | | 0.40 | |

LRP5 rs3736228

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CC | 26 | 145 | 171 | 0.73 | C | 59 | 338 | 0.85 |
| TC | 7 | 48 | 55 | 0.24 | T | 9 | 62 | 0.16 |
| TT | 1 | 7 | 8 | 0.03 | Total | 68 | 400 | |
| Total | 34 | 200 | 234 | | | | | |
| | | p value | | | | | p value | |
| | | 0.89 | | | | | 0.61 | |

VDR rs1544410

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| bb | 6 | 30 | 36 | 0.16 | b | 30 | 145 | 0.37 |
| Bb | 18 | 85 | 103 | 0.45 | B | 36 | 249 | 0.63 |
| BB | 9 | 82 | 91 | 0.40 | Total | 66 | 394 | |
| Total | 33 | 197 | 230 | | | | | |
| | | p value | | | | | p value | |
| | | 0.29 | | | | | 0.15 | |

VDR rs731236

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| tt | 5 | 25 | 30 | 0.13 | t | 27 | 137 | 0.35 |
| Tt | 17 | 87 | 104 | 0.46 | T | 35 | 257 | 0.65 |
| TT | 9 | 85 | 94 | 0.41 | Total | 62 | 394 | |
| Total | 31 | 197 | 228 | | | | | |
| | | p value | | | | | p value | |
| | | 0.33 | | | | | 0.15 | |

P2X7 rs1653624

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| AA | 0 | 0 | 0 | 0.00 | A | 0 | 16 | 0.04 |
| TA | 0 | 16 | 16 | 0.07 | T | 68 | 384 | 0.96 |
| TT | 34 | 184 | 218 | 0.93 | Total | 68 | 400 | |
| Total | 34 | 200 | 234 | | | | | |
| | | p value | | | | | p value | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | N/A | | | | N/A | |
| P2X7 rs1718119 | | | | | | | | |
| | GG | 12 | 69 | 81 | 0.38 | G | 37 | 219 | 0.59 |
| | GA | 13 | 81 | 94 | 0.44 | A | 25 | 151 | 0.41 |
| | AA | 6 | 35 | 41 | 0.19 | Total | 62 | 370 | |
| | Total | 31 | 185 | 216 | | | | | |
| | | | p value | | | | p value | | |
| | | | 0.98 | | | | 0.94 | | |
| VDR rs7975232 | | | | | | | | |
| | aa | 9 | 57 | 66 | 0.29 | a | 38 | 206 | 0.53 |
| | Aa | 20 | 92 | 112 | 0.48 | A | 32 | 186 | 0.47 |
| | AA | 6 | 47 | 53 | 0.23 | Total | 70 | 392 | |
| | Total | 35 | 196 | 231 | | | | | |
| | | | p value | | | | p value | | |
| | | | 0.51 | | | | 0.77 | | |
| COL1A1 rs1800012 | | | | | | | | |
| | SS | 24 | 152 | 176 | 0.74 | S | 59 | 350 | 0.87 |
| | Ss | 11 | 46 | 57 | 0.24 | s | 11 | 54 | 0.13 |
| | ss | 0 | 4 | 4 | 0.02 | Total | 70 | 404 | |
| | Total | 35 | 202 | 237 | | | | | |
| | | | p value | | | | p value | | |
| | | | 0.41 | | | | 0.56 | | |
| CTR rs1801197 | | | | | | | | |
| | CC | 1 | 16 | 17 | 0.08 | C | 19 | 105 | 0.27 |
| | TC | 17 | 73 | 90 | 0.40 | T | 45 | 281 | 0.73 |
| | TT | 14 | 104 | 118 | 0.52 | Total | 64 | 386 | |
| | Total | 32 | 193 | 225 | | | | | |
| | | | p value | | | | p value | | |
| | | | 0.21 | | | | 0.66 | | |
| WNT16 rs3801387 | | | | | | | | |
| | AA | 15 | 105 | 120 | 0.53 | A | 45 | 282 | 0.73 |
| | GA | 15 | 72 | 87 | 0.38 | G | 25 | 102 | 0.27 |
| | GG | 5 | 15 | 20 | 0.09 | Total | 70 | 384 | |
| | Total | 35 | 192 | 227 | | | | | |
| | | | p value | | | | p value | | |
| | | | 0.30 | | | | 0.20 | | |
| RANKL rs9594738 | | | | | | | | |
| | CC | 20 | 69 | 89 | 0.38 | C | 52 | 240 | 0.60 |
| | TC | 12 | 102 | 114 | 0.49 | T | 18 | 158 | 0.40 |
| | TT | 3 | 28 | 31 | 0.13 | Total | 70 | 398 | |
| | Total | 35 | 199 | 234 | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | p value | | | | | p value |
| | | | 0.04 | | | | | 0.02 |
| P2X7 rs208294 | | | | | | | | |
| | CC | 35 | 188 | 223 | 0.94 | C | 70 | 389 | 0.96 |
| | TC | 0 | 13 | 13 | 0.05 | T | 0 | 15 | 0.04 |
| | TT | 0 | 1 | 1 | 0.00 | Total | 70 | 404 | |
| | Total | 35 | 202 | 237 | | | | | |
| | | | p value | | | | | p value |
| | | | 0.12 | | | | | 0.10 |
| BRADYKININ rs1799722 | | | | | | | | |
| | 9 | 17 | 45 | 62 | 0.28 | 9 | 45 | 176 | 0.46 |
| | 9/-9 | 11 | 86 | 97 | 0.43 | -9 | 21 | 204 | 0.54 |
| | -9 | 5 | 59 | 64 | 0.29 | Total | 66 | 380 | |
| | Total | 33 | 190 | 223 | | | | | |
| | | | p value | | | | | p value |
| | | | 0.00 | | | | | 0.00 |
| KALLIKREIN rs16987491 | | | | | | | | |
| | AA | 0 | 0 | 0 | 0.00 | A | 5 | 13 | 0.04 |
| | GA | 4 | 13 | 17 | 0.07 | G | 66 | 395 | 0.96 |
| | GG | 31 | 191 | 222 | 0.93 | Total | 70 | 408 | |
| | | 35 | 204 | 239 | | | | | |
| | | | p value | | | | | p value |
| | | | 0.28 | | | | | 0.67 |
| P2X7 rs3751143 | | | | | | | | |
| | GG | 0 | 8 | 8 | 0.03 | G | 12 | 68 | 0.17 |
| | TG | 12 | 52 | 64 | 0.28 | T | 52 | 330 | 0.83 |
| | TT | 20 | 139 | 159 | 0.69 | Total | 64 | 398 | |
| | Total | 32 | 199 | 231 | | | | | |
| | | | p value | | | | | p value |
| | | | 0.25 | | | | | 0.72 |
| SOST rs1877632 | | | | | | | | |
| | CC | 11 | 105 | 116 | 0.51 | C | 36 | 281 | 0.72 |
| | TC | 14 | 71 | 85 | 0.37 | T | 30 | 111 | 0.28 |
| | TT | 8 | 20 | 28 | 0.12 | Total | 66 | 392 | |
| | Total | 33 | 196 | 229 | | | | | |
| | | | p value | | | | | p value |
| | | | 0.03 | | | | | 0.00 |

## 6.4 Appendix. Odds Ratio and 95% Confidence Intervals for the Association of Each SNP with Stress Fracture Injury

Whole Cohort

| SNP | Odds ratio | 95% CI upper | 95% CI lower |
|---|---|---|---|
| rs2230912 | 1.02 | 1.59 | 0.65 |
| rs1653624 | N/A | N/A | N/A |
| rs208294 | N/A | N/A | N/A |
| rs1718119 | 1.57 | 3.01 | 0.81 |
| rs3751143 | 1.54 | 2.40 | 0.99 |
| rs1021188 | 2.93 | 7.28 | 1.18 |
| rs9594738 | 1.01 | 1.85 | 0.55 |
| rs3018362 | 1.94 | 3.62 | 1.04 |
| rs4355801 | 1.30 | 2.31 | 0.74 |
| rs1544410 | 1.12 | 2.16 | 0.59 |
| rs731236 | 1.00 | 2.04 | 0.49 |
| rs10735810 | 1.82 | 3.40 | 0.97 |
| rs7975232 | 1.00 | 1.84 | 0.54 |
| rs4588 | 0.93 | 2.14 | 0.40 |
| rs7041 | 1.05 | 1.87 | 0.59 |
| rs1800012 | 0.98 | 1.55 | 0.62 |
| rs1801197 | 0.72 | 1.63 | 0.31 |
| rs3736228 | 1.22 | 1.92 | 0.78 |
| rs1877632 | 1.31 | 2.64 | 0.65 |
| rs3801387 | 1.75 | 3.59 | 0.86 |
| rs1799722 | 2.04 | 3.71 | 1.12 |
| rs16987491 | 1.62 | 3.58 | 0.73 |

Male

| SNP | Odds ratio | 95% CI upper | 95% CI lower |
|---|---|---|---|
| rs2230912 | 1.04 | 1.70 | 0.64 |
| rs1653624 | N/A | N/A | N/A |
| rs208294 | N/A | N/A | N/A |
| rs1718119 | 1.38 | 2.75 | 0.69 |
| rs3751143 | 1.51 | 2.50 | 0.91 |
| rs1021188 | 3.38 | 9.35 | 1.22 |
| rs9594738 | 0.87 | 1.72 | 0.44 |
| rs3018362 | 2.12 | 4.17 | 1.08 |
| rs4355801 | 1.21 | 2.30 | 0.64 |
| rs1544410 | 1.08 | 2.25 | 0.52 |
| rs731236 | 0.82 | 1.86 | 0.36 |
| rs10735810 | 1.62 | 3.27 | 0.81 |
| rs7975232 | 1.11 | 2.19 | 0.56 |
| rs4588 | 1.06 | 2.60 | 0.43 |
| rs7041 | 0.90 | 1.70 | 0.47 |

| | | | |
|---|---|---|---|
| rs1800012 | 1.01 | 1.69 | 0.61 |
| rs1801197 | 0.59 | 1.61 | 0.22 |
| rs3736228 | 1.11 | 1.86 | 0.66 |
| rs1877632 | 1.33 | 2.85 | 0.62 |
| rs3801387 | 1.82 | 3.93 | 0.85 |
| rs1799722 | 2.83 | 5.67 | 1.41 |
| rs16987491 | 1.76 | 4.25 | 0.73 |

Female

| SNP | Odds ratio | 95% CI upper | 95% CI lower |
|---|---|---|---|
| rs2230912 | 1.37 | 4.60 | 0.41 |
| rs1653624 | N/A | N/A | N/A |
| rs208294 | N/A | 4.93 | 0.33 |
| rs1718119 | 0.97 | 2.70 | 0.35 |
| rs3751143 | 1.08 | 2.94 | 0.39 |
| rs1021188 | 0.80 | 2.41 | 0.26 |
| rs9594738 | 1.80 | 7.96 | 0.41 |
| rs3018362 | 1.04 | 2.97 | 0.37 |
| rs4355801 | 1.50 | 5.95 | 0.38 |
| rs1544410 | 1.11 | 3.15 | 0.39 |
| rs731236 | 1.55 | 4.46 | 0.54 |
| rs10735810 | 0.37 | 1.67 | 0.08 |
| rs7975232 | 1.80 | 7.81 | 0.41 |
| rs4588 | 0.83 | 2.35 | 0.29 |
| rs7041 | 1.17 | 3.35 | 0.41 |
| rs1800012 | 0.64 | 1.82 | 0.22 |
| rs1801197 | 1.47 | 4.03 | 0.53 |
| rs3736228 | 1.26 | 3.49 | 0.45 |
| rs1877632 | 2.44 | 7.16 | 0.83 |
| rs3801387 | 0.76 | 2.13 | 0.27 |
| rs1799722 | 1.27 | 6.17 | 0.15 |
| rs16987491 | N/A | N/A | N/A |

Runners

| SNP | Odds ratio | 95% CI upper | 95% CI lower |
|---|---|---|---|
| rs2230912 | 0.44 | 1.33 | 0.15 |
| rs1653624 | N/A | N/A | N/A |
| rs208294 | N/A | N/A | N/A |
| rs1718119 | 1.68 | 5.09 | 0.55 |
| rs3751143 | 1.55 | 4.52 | 0.53 |
| rs1021188 | 0.44 | 1.38 | 0.14 |
| rs9594738 | 1.83 | 5.76 | 0.58 |
| rs3018362 | 2.50 | 7.51 | 0.83 |
| rs4355801 | 1.63 | 4.87 | 0.55 |
| rs1544410 | 1.03 | 2.96 | 0.36 |
| rs731236 | 1.17 | 3.43 | 0.40 |

| | | | |
|---|---|---|---|
| rs10735810 | 1.94 | 5.51 | 0.68 |
| rs7975232 | 0.61 | 2.71 | 0.14 |
| rs4588 | 0.39 | 1.13 | 0.13 |
| rs7041 | 0.51 | 1.47 | 0.17 |
| rs1800012 | 1.91 | 5.82 | 0.63 |
| rs1801197 | 0.53 | 1.49 | 0.19 |
| rs3736228 | 2.10 | 7.04 | 0.63 |
| rs1877632 | 2.58 | 7.43 | 0.90 |
| rs3801387 | 1.37 | 3.79 | 0.50 |
| rs1799722 | 0.55 | 1.74 | 0.17 |
| rs16987491 | N/A | N/A | N/A |

Football/Hockey Players

| SNP | Odds ratio | 95% CI upper | 95% CI lower |
|---|---|---|---|
| rs2230912 | 0.78 | 1.91 | 0.32 |
| rs1653624 | N/A | N/A | N/A |
| rs208294 | N/A | N/A | N/A |
| rs1718119 | 1.01 | 2.93 | 0.35 |
| rs3751143 | 1.39 | 3.02 | 0.64 |
| rs1021188 | 1.75 | 3.67 | 0.83 |
| rs9594738 | 0.40 | 0.83 | 0.19 |
| rs3018362 | 1.77 | 4.77 | 0.66 |
| rs4355801 | 0.57 | 1.18 | 0.28 |
| rs1544410 | 1.82 | 5.55 | 0.60 |
| rs731236 | 1.89 | 6.15 | 0.58 |
| rs10735810 | 0.93 | 1.95 | 0.45 |
| rs7975232 | 0.81 | 2.44 | 0.27 |
| rs4588 | 1.60 | 3.31 | 0.77 |
| rs7041 | 0.54 | 1.14 | 0.26 |
| rs1800012 | 1.39 | 3.05 | 0.64 |
| rs1801197 | 1.50 | 3.19 | 0.71 |
| rs3736228 | 0.81 | 1.90 | 0.35 |
| rs1877632 | 3.82 | 10.68 | 1.36 |
| rs3801387 | 2.33 | 7.35 | 0.74 |
| rs1799722 | 4.46 | 13.00 | 1.53 |
| rs16987491 | N/A | N/A | N/A |

Multiple stress fracture

| SNP | Odds ratio | 95% CI upper | 95% CI lower |
|---|---|---|---|
| rs2230912 | 0.92 | 1.78 | 0.47 |
| rs1653624 | N/A | N/A | N/A |
| rs208294 | N/A | N/A | N/A |
| rs1718119 | 1.65 | 3.06 | 0.89 |
| rs3751143 | 1.18 | 2.28 | 0.61 |
| rs1021188 | 3.28 | 10.11 | 1.06 |
| rs9594738 | 1.17 | 2.80 | 0.48 |

| | | | |
|---|---|---|---|
| rs3018362 | 1.64 | 3.15 | 0.85 |
| rs4355801 | 1.96 | 4.85 | 0.79 |
| rs1544410 | 1.02 | 3.03 | 0.34 |
| rs731236 | 2.51 | 5.39 | 1.17 |
| rs10735810 | 3.81 | 8.90 | 1.63 |
| rs7975232 | 0.70 | 1.82 | 0.26 |
| rs4588 | 1.05 | 1.92 | 0.57 |
| rs7041 | 1.62 | 3.78 | 0.70 |
| rs1800012 | 1.19 | 2.29 | 0.62 |
| rs1801197 | 0.95 | 1.77 | 0.51 |
| rs3736228 | 1.06 | 2.08 | 0.54 |
| rs1877632 | 2.01 | 5.51 | 0.73 |
| rs3801387 | 0.77 | 1.43 | 0.41 |
| rs1799722 | 1.15 | 2.90 | 0.46 |
| rs16987491 | 1.22 | 4.28 | 0.35 |

Cricket Players

| | Odds ratio | 95% CI upper | 95% CI lower |
|---|---|---|---|
| rs2230912 | 1.20 | 2.48 | 0.58 |
| rs1653624 | N/A | N/A | N/A |
| rs208294 | N/A | N/A | N/A |
| rs1718119 | 1.03 | 2.80 | 0.38 |
| rs3751143 | 1.80 | 4.13 | 0.78 |
| rs1021188 | 1.41 | 3.07 | 0.65 |
| rs9594738 | 0.80 | 2.17 | 0.30 |
| rs3018362 | 1.96 | 5.44 | 0.71 |
| rs4355801 | 2.01 | 5.39 | 0.75 |
| rs1544410 | 0.46 | 1.44 | 0.15 |
| rs731236 | 0.54 | 1.13 | 0.26 |
| rs10735810 | 1.08 | 2.26 | 0.52 |
| rs7975232 | 2.43 | 6.92 | 0.85 |
| rs4588 | 1.81 | 3.75 | 0.87 |
| rs7041 | 1.18 | 3.66 | 0.38 |
| rs1800012 | 1.09 | 2.38 | 0.50 |
| rs1801197 | 1.02 | 2.11 | 0.50 |
| rs3736228 | 1.38 | 3.02 | 0.63 |
| rs1877632 | 0.91 | 1.85 | 0.45 |
| rs3801387 | 0.80 | 1.65 | 0.39 |
| rs1799722 | 0.53 | 1.62 | 0.17 |
| rs16987491 | N/A | N/A | N/A |

Leg excluding metatarsal

| | Odds ratio | 95% CI upper | 95% CI lower |
|---|---|---|---|
| rs2230912 | 0.79 | 1.65 | 0.38 |
| rs1653624 | N/A | N/A | N/A |
| rs208294 | N/A | N/A | N/A |

| | | | |
|---|---|---|---|
| rs1718119 | 1.17 | 2.36 | 0.58 |
| rs3751143 | 2.20 | 4.42 | 1.09 |
| rs1021188 | 1.37 | 1.63 | 0.36 |
| rs9594738 | 1.04 | 2.91 | 0.37 |
| rs3018362 | 1.52 | 4.14 | 0.56 |
| rs4355801 | 2.70 | 7.15 | 1.02 |
| rs1544410 | 3.26 | 9.83 | 1.08 |
| rs731236 | 3.09 | 9.27 | 1.03 |
| rs10735810 | 2.86 | 7.09 | 1.15 |
| rs7975232 | 0.62 | 1.23 | 0.31 |
| rs4588 | 1.40 | 2.76 | 0.71 |
| rs7041 | 0.87 | 2.28 | 0.33 |
| rs1800012 | 1.00 | 2.07 | 0.48 |
| rs1801197 | 0.93 | 1.81 | 0.48 |
| rs3736228 | 1.26 | 2.59 | 0.61 |
| rs1877632 | 1.57 | 4.55 | 0.54 |
| rs3801387 | 1.79 | 5.17 | 0.62 |
| rs1799722 | 2.11 | 5.95 | 0.75 |
| rs16987491 | 2.09 | 6.48 | 0.67 |

N/A depicts insufficient population number for analysis.

**7.1. Appendix. Informed Consent: Genetic Associations with Bone Turnover Following 120 minutes of Treadmill Running**

NOTTINGHAM TRENT UNIVERSITY

## Statement of consent to participate in the investigation entitled:

# Genetic associations with bone turnover following 120 minutes of treadmill running

Researchers: Mr Ian Varley, Dr Craig Sale

I (subject name) …… have read the information provided and agree to partake, as a subject in the proposed research entitled: Genetic associations with bone turnover following 120 minutes of treadmill running. I am fully aware of the procedures to be carried out and have been informed of any risks that they may present. I agree to obey the Universities regulations and the investigators instructions regarding safety matters.

I am aware that I may withdraw my consent to participate in the research at any time without any obligation to explain why or without any prejudice towards me.

I also understand that any personal information regarding myself will not be passed to any other parties.

I have completed the health screening questionnaire and know of no other reasons, medical or otherwise, that will prevent me from partaking in this research.

Signed (Subject)……. Date …….

Signed (Independent Witness)……. Date…….

Signed (Primary Researcher) ……. Date…….

## 7.2. Appendix. Health Screen: Genetic Associations with Bone Turnover Following 120 minutes of Treadmill Running

HEALTH SCREEN

Name .....……

**Please complete this brief questionnaire to confirm fitness to participate:**

1. **At present,** do you have any health problem for which you are:

(a) on medication, prescribed or otherwise Yes ☐ No ☐

(b) attending your general practitioner Yes ☐ No ☐

(c) on a hospital waiting list Yes ☐ No ☐

2. **In the past two years,** have you had any illness which require you to:

(a) consult your GP Yes ☐ No ☐

(b) attend a hospital outpatient department Yes ☐ No ☐

(c) be admitted to hospital Yes ☐ No ☐

3. **Have you ever** had any of the following?

(a) Convulsions/epilepsy Yes ☐ No ☐

(b) Asthma Yes ☐ No ☐

(c) Eczema Yes ☐ No ☐

(d) Diabetes Yes ☐ No ☐

(e) A blood disorder Yes ☐ No ☐

(f) Head injury Yes ☐ No ☐

(g) Digestive problems Yes ☐ No ☐

(h) Heart problems Yes ☐ No ☐

(i) Problems with bones or joints Yes ☐ No ☐

(j) Disturbance of balance / coordination Yes ☐ No ☐

(k) Numbness in hands or feet Yes ☐ No ☐

(l) Disturbance of vision Yes ☐ No ☐

(m) Ear / hearing problems Yes ☐ No ☐

(n) Thyroid problems Yes ☐ No ☐

(o) Kidney or liver problems Yes ☐ No ☐

(p) Allergy to nuts, alcohol etc Yes ☐ No ☐

4. **Has any,** otherwise healthy, member of your family under the age of 35 died suddenly during or soon after exercise? Yes ☐ No ☐

5. Are there any reasons why blood sampling may be difficult? Yes ☐ No ☐

6. Have you had a blood sample taken previously? Yes ☐ No ☐

7. Have you had a cold or flu or any flu like symptoms in the last month? Yes ☐ No ☐

Women only

**8. Are you pregnant or trying to become pregnant? Yes ☐ No ☐**

**If YES to any question, please describe briefly if you wish (eg to confirm problem was/is short-lived, insignificant or well controlled.)**

**.....**……
…….……