SNPs in the vicinity of P2X7R, RANK/RANKL/OPG and Wnt Signalling Pathways and their Association with Bone Phenotypes in Academy Footballers

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Abstract

Context: Genotype plays an important role in influencing bone phenotypes, such as bone mineral density, but the role of genotype in determining responses of bone to exercise has yet to be elucidated.

Objective: To determine whether 10 SNPs associated with genes in the vicinity of P2X7R, RANK/RANKL/OPG and Wnt Signalling Pathways are associated with bone phenotypes in elite academy footballers (Soccer players) and to determine whether these genotypes are associated with training induced changes in bone.

Design, participants, and methods: 99 elite academy footballers volunteered to participate. Peripheral computed tomography of the tibia (4%, 14%, 38% and 66% sites) was performed immediately before and 12 weeks after an increase in football training volume. Genotypes were determined using proprietary fluorescence-based competitive allele-specific PCR assays.

Results: No significant genotype x time interactions were shown for any of the SNPs analysed ($P > 0.05$). A main effect of genotype was shown. SOST SNP rs1877632 (trabecular density), P2X7R SNPs rs1718119 (cortical thickness and CSA), rs3751143 (SSI, CSA, cortical CSA and periosteal circumference) RANK/RANKL/OPG SNPs rs9594738 (periosteal circumference), rs1021188 (cortical thickness and CSA) and rs9594759 (cortical density) were associated with bone phenotypes ($P < 0.05$).

Conclusions: No association was shown between P2X7R, RANK/RANKL/OPG and Wnt Signalling SNPs and a change in bone phenotypes following 12 weeks of increased training volume in elite academy footballers. However, SNPs were associated with bone phenotypes pre training. These data highlight the complexity of SNPs in the vicinity of the RANK/RANKL/OPG, P2X7R and Wnt metabolic regulatory pathways with bone phenotypes in elite academy footballers.

Introduction

Attaining a heightened bone mass in early adulthood is important for long-term bone health and the prevention of osteoporosis [1], which makes the adolescent population highly relevant for investigating how bone responds to exercise. The osteogenic effects of football are greater than in other sports [2;3], most likely due to the high magnitude, frequency and multi-directional nature of the movements that football training and match play necessitate [4]. Bone Mineral Content (BMC) [3;5], areal Bone Mineral Density (BMD) [6] and cortical cross sectional area (CSA), circumference and thickness [7], as well as bone strength [8], have all been shown to be increased in recreational football players compared to sedentary control populations. Bone adaptions have also been shown in the same cohort of adolescent elite footballers used in the present study after only 12 weeks of increased volume of football training [9].

Despite this, negative bone related responses to exercise have been shown in football players. Participation in unaccustomed exercise and rapid increases in training volume, for example, have been implicated in the development of stress fracture injury [10]. 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 [11].

There is a lack of information relating to the mechanisms that may regulate the individual adaptations that are caused by exercise participation. Genotype has been associated with osteoporosis [12], stress fracture injury [9;13] and bone turnover [14;15]. It has been suggested that genotype may mediate the bone response to exercise and may explain some of the variability observed in bone adaptations [16]. Despite evidence of genetic factors being

associated with bone phenotypes, little is known about how genotype mediates the bone response to training volume.

The aim of the present study was to investigate whether a genotype dependent change in bone phenotypes is evident in adolescent academy footballers following 12 weeks of increased football-specific training.

Method

Participants

First year, full-time male academy footballers (n=117) were recruited through previously established relationships with Nottingham Trent University and by word of mouth from five full-time football academies to form the Bone Adaptation in Academy Footballers cohort. 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 and being fully briefed and having the opportunity to ask questions, participants signed a statement of informed consent, completed a pre-scan screening form and completed a health screen questionnaire, which was scrutinised in order to confirm that they met the inclusion/exclusion criteria. Participants detailed their playing position, the age at which they first played competitive football and the amount of hours they spent training prior to full-time academy enrolment.

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 (The cohort is described elsewhere, [17])

leaving a cohort of n=99 who completed both scans (Figure 1). The study conformed to Ionising Radiation (Medical Exposure) Regulations and was approved by the National Health Service Research Ethics Committee (reference 12/EM/0183).

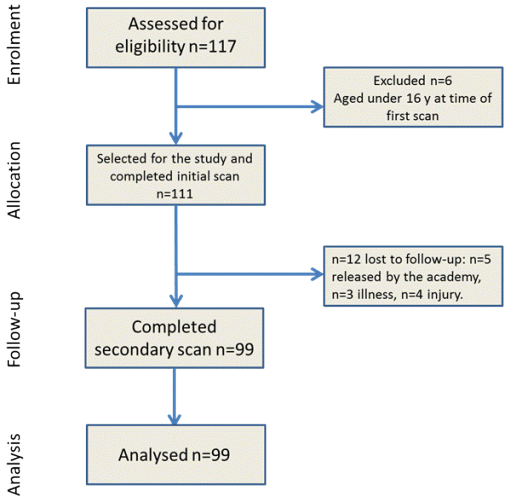

Figure 1. Academy footballers assessed and analysed.

Experimental Design

All participants were recently enrolled full-time academy football players. Participants were tested before an increase in training volume during the first week of pre-season training including height, body mass and bone phenotypes using pQCT. Participants then conducted 12 weeks of football specific training with their respective clubs, followed by a repeat of the measurements.

Procedures

Training Intervention

Academy footballers that were deemed of a suitable standard graduated through the academy to become first year scholars. All footballers were 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 were conducted by qualified coaches at the respective clubs.

pQCT

pQCT scans were conducted using an XCT 2000 (Stratec Medizintechnik, Pforzheim, Germany) to assess the bone phenotypes of the tibia of the dominant leg (the leg the participant most comfortably kicked a ball with). Before scanning commenced, the scanner was calibrated using a phantom of known density in accordance with manufacturer guidelines. pQCT has previously been shown to provide a reliable measurement of bone characteristics in humans by our wider research group (CV< 2% for total and Tb.Dn, and CV < 1% for Ct.Dn) [18]. 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. These sites are typically used to analyse trabecular and cortical phenotypes 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.

Bone Phenotypes

The following measures were analysed at each site of the tibia:
4%: total cross sectional area (Tot CSA, mm^2) and trabecular mineral density ($mg \cdot cm^3$).14% and 38%: Tot CSA, (mm^2), cortical CSA (mm^2), cortical mineral density ($mg \cdot cm^3$), cortical thickness (mm), periosteal circumference (mm) and stress strain index (SSI, mm^3). 66%: Tot CSA, (mm^2) and cortical mineral density ($mg \cdot cm^3$).

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 radiation exposure guidelines.

Genetic Procedures

Participants deposited saliva into a 5 mL collection tube that was subsequently mixed with 2 mL of preservative, in accordance with manufacturer guidelines (Norgen Biotek Corp, Saliva DNA Collection kit Thorold, Canada). Genomic deoxyribonucleic acid (DNA) extraction and analysis followed procedures outlined by Varley et al. [13]. In short, DNA was extracted in accordance with manufacturer guidelines and genotyped using proprietary fluorescence-based competitive allele-specific polymerase chain reaction assay. Researchers were blinded to the status of the genotyped individuals. The specific genes and SNPs selected for analysis were

based on previous findings related to genetic governance of bone phenotypes (BMD, osteoporosis and fragility fracture) and their locality to a bone regulatory pathway that might be responsible for any associations shown. SNPs in the vicinity of *RANK/RANKL/OPG*, *NF-κB* and *Wnt* signalling pathway, together with SNPs located in close proximity to the *P2X7R* and *IL6* genes were selected for genotyping. SNP were not selected based on them being representative of a haplotype within a gene.

Statistical analysis

All data are presented as mean ± 1SD. 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 phenotypes before and after an increase in training volume. 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. The SNPs selected have known mechanisms for an association with bone adaptation, and, therefore, no adjustment was made for multiple comparisons. Sample size was decided upon by the use of power calculation for ANOVA power model using an Alpha value of 0.05 and estimated root mean square standardized effect of 0.35. This produced a requirement of a minimum of 80 participants within two groups to achieve a power of 0.8. All statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS) version 21.0 (SPSS, Inc., Chicago, IL, USA).

Results

Ninety-nine participants were available for the follow-up procedure. All SNPs were in accordance with HWE, produced call rates $\geq 89\%$ and had minor allele frequencies comparable

to previous literature (Table 1). Participants were made up from a variety of ethnicities (64 Caucasian, 19 Caucasian/black dual heritage, 11 black Caribbean, 4 black African and 1 Asian) and were composed of differing playing positions (42 midfielders, 29 defenders, 19 forwards and 9 goalkeepers).

Table 1. SNPs for which academy footballers were genotyped, along with Hardy-Weinberg Equilibrium (HWE) P value and call rate %.

	HW P-value	Call Rate %
RANK rs3018362	0.28	89
RANKL rs9594759	0.11	92
RANKL rs9594738	0.27	95
RANKL rs1021188	0.43	96
P2X7 rs3751143	0.49	92
P2X7 rs1718119	0.83	95
Wnt16 rs2707466	0.28	90
SOST rs1877632	0.42	89
MP3K rs8065345	0.88	95
IL6 rs13447445	0.08	91

Participant characteristics

Body mass significantly increased after 12 weeks of increased training volume, although tibial length did not significantly change (Table 2). The amount of training hours per week significantly increased (106 %) following full-time academy induction.

Table 2. Characteristics of academy footballers analysed before and after 12 wk of increased training volume: mean ± 1SD. * denotes a significant difference ($P < 0.01$).

Characteristics (n=99)	Pre	Post	P value
Height (m)	1.76±0.56	1.77±0.62	0.27
Body Mass (kg)	70.1±8.5	71.4±8.7	<0.01*
Tibia length (mm)	387.3±21.3	387.6±20.8	0.41
Age when first played competitively (y)	9.4±1.4	N/A	N/A
Training (h/wk)	6.2±2.7	11.9±1.6	<0.01*

Bone Response to Increased Training

Trabecular (4 % of tibial length) and cortical (14 %, 38 % of tibial length) densities, 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 after 12 week of increased volume training ($P < 0.05$) (Varley et al., published in Int J Sports Med 2017; Table 3).

Table 3. Bone phenotypes (mean±1SD) at 4%, 14%, 38% and 66% of the tibia measured before and after 12 weeks of increased volume football specific training in elite footballers.

	Football n=99		
Bone Phenotype	Pre	Post	% change
4% site			
Trabecular Density (mg·cm^3)	284.2±31.1	289.6±31.1**	1.9↑
Total CSA (mm^2)	1338.3±149.7	1348.2±147.2	0.7↑
14% site			
Cortical Density (mg·cm^3)	1057.2±34.5	1066.9±32.7**	0.9↑
Total CSA (mm^2)	570.1±80.4	572.5±80.0	0.4↑
Cortical CSA (mm^2)	212.2±21.1	215.8±24.1**	1.7↑
Cortical Thickness (mm)	2.85±0.32	2.88±0.34*	1.1↑
Periosteal Circumference (mm)	84.3±5.5	84.5±5.4	0.2↑
SSI	2034.4±360.2	2061±377.2*	1.3↑
38% site			
Cortical Density (mg·cm^3)	1108.7±32.0	1115.2±29.9**	0.6↑
Total CSA (mm^2)	488.8±60.2	489.4±60.1	0.1↑

Cortical CSA (mm^2)	356.9±40.2	361.5±40.1**	1.3↑
Cortical Thickness (mm)	6.05±0.50	6.10±0.57**	0.8↑
Periosteal Circumference (mm)	78.0±5.1	78.3±4.6	0.4↑
SSI	2054.7±392.8	2101.9±396.1**	2.3↑
66% site			
Cortical Density ($mg \cdot cm^3$)	1074.7±27.4	1079.9±22.1**	0.5↑
Total CSA (mm^2)	9778.1±1072.5	9668.9±1081.2	1.1↓

(CSA) = cross sectional area. * was used to denote significance P<0.05; ** was used to denote significance P<0.01.

Bone Phenotype Genotype Associations

RANK/RANKL/OPG

No significant genotype by time interactions were shown for all *RANK/RANKL/OPG* analysed (P > 0.05). A main effect of genotype was shown for *RANK/RANKL/OPG* SNPs at baseline with periosteal circumference (rs9594738), cortical density (rs9594759), cortical CSA and thickness (rs1021188) (P < 0.05). Homozygotes for the variant T allele of *RANKL* SNP rs9594738 showed a smaller periosteal circumference compared to those homozygote for the common allele and heterozygotes at the 14% site (TT 83.5 ± 4.2 mm; CT 84.1 mm ± 5.9; CC 84.6 ± 6.2 mm; P = 0.05). No other bone phenotypes measured showed significant associations with rs9594738 (P > 0.05). Cortical CSA and thickness at the 14% tibial site were significantly higher (10.92 mm^2; 4.9 %; 0.17 mm; 6.1 %) in homozygotes for the common G allele of *RANKL* SNP rs1021188 (221.1 ± 25.4 mm^2; 2.97 ± 0.38 mm; P = 0.04) in comparison to the heterozygotes and homozygotes for the C allele combined (210.17 ± 24.3 mm^2; 2.80 ± 0.38 mm; P = 0.04). Cortical density at the 66% site was significantly less (15.5 $mg \cdot cm^3$, 1.4 %) in

homozygotes of the C allele of *RANKL* SNPs rs9594759 after training in comparison to homozygotes of the common allele (1069.0 ± 28.7 mg·cm3 compared to 1084.5 ± 24.4 mg·cm3) (P = 0.03). No significant genotype x time interactions were shown for any *RANK/RANKL/OPG* SNPs analysed (P > 0.05).

Wnt Signalling

No time by genotype interactions were shown with *SOST* SNP rs1877632 or *Wnt16* SNP rs2707466 (P > 0.05). *SOST* SNP rs1877632 showed a main effect of geneotype at baseline (P < 0.05). Carriers of at least one rare allele were shown to have a greater trabecular density at the 4% site when compared to homozygotes for the common allele (295.1 ± 34.4 mg·cm^3 in comparison to 280.9 ± 28.9 mg·cm^3; P = 0.05). *Wnt16* SNP rs2707466 was not associated with any bone phenotypes (P > 0.05).

P2X7R

No significant time by genotype interactions were shown for any of the *P2X7R* SNPs analysed (P > 0.05). A main effect of genotype was shown for *P2X7R* SNPs rs1718119 and rs3751143 at basline (P < 0.05). Homozygotes for the rare, gain of function T allele of *P2X7R* SNPs rs1718119 showed a greater cortical thickness at the 38% site (6.38 ± 0.34 mm, TT; 5.91 ± 0.59 mm CT; 6.22 ± 0.51 mm CC) compared to heterozygotes (P = 0.01). Cortical thickness at the 14% site of the tibia was also lower in heterozygotes (2.75 ± 0.38 mm) compared to C allele homozygotes (2.92 ± 0.27 mm; P = 0.03) and homozygotes for the T allele (2.97 ± 0.40 mm; P = 0.005). A significantly lower cortical CSA at the 14% site was also evident in heterozygotes (207.4 ± 25.8 mm^2) when compared to homozygotes for the rare (222.1 ± 17.9 mm^2; P = 0.03) and common allele (219.3 ± 24.4 mm^2; P = 0.01). SSI at the 14 % site was greater by 9.0 %

(187.6 mm^3) in homozygotes for the common T allele (2094.9 ± 390.5 mm^3) in comparison to those heterozygote and homozygote for the rare C allele (1907.3 ± 288.3 mm^3; P = 0.02).

Homozygotes of the T allele of rs3751143 had number of phenotypes that were greater when compared to CC and CT variations combined; SSI (14 %, 2094.9±390.5 compared to 1907.3±288.3, P = 0.03; 38 %, 2131.8±405.7 compared to 1947.1±323.6, P = 0.04), total CSA (14 %, 579.7±84.3 compared to 543.8±65.5, P = 0.05) and periosteal circumference (14 %, 85.14±6.07 compared to 82.52±4.90, P = 0.05).

No significant associations were shown with bone phenotypes in relation to *MP3K* rs8065345 and *IL6* rs13447445 SNPs (P > 0.05).

Discussion

No time by genotype associations were shown in any of bone phenotypes assessed. It could be suggested that the intervention period may have been too short to show genotype dependent adaptations. The time scale for bone remodelling has yet to be characterised in an adolescent population following an increase in training volume, however, and previous studies have shown bone phenotypic adaptions [19] and genotype dependent adaptations [20] after a similar intervention period. The lack of time by genotype association may also be due to the academy footballers already being habituated to the type of exercise undertaken. There is a lack of literature investigating the influence of training and genotype and training on bone structural phenotypes. The research that does exist introduces an unaccustomed intervention that increases exercise volume, intensity and duration [20]. As the participants in the present study had all participated in football training and match-play for a number of years, the influence of genotype on change in bone phenotype could have already occurred.

There were, however, some novel main effects of genotype in association with bone phenotypes in the current study. Herein we have shown that six SNPs within the vicinity of three major bone metabolic regulatory pathways, namely the *RANK/RANKL/OPG, Wnt* signalling and purinergic signalling (*P2X7R)* pathways were associated with tibial phenotypes in elite adolescent, male, academy footballers.

RANK/RANKL/OPG signalling pathway

The T allele of SNP rs9594738 was associated with lower cortical CSA in elite adolescent footballers. Although there is no current mechanistic explanation for the associations shown, it could be suggested that the variance in genotype may inhibit *RANK* - *RANKL* binding and, therefore, influence osteoclast differentiation and activation, subsequently mediating bone resorption [21]. It has also 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 precursor cells [22]. 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. It has previously been reported that the T allele was associated with lower BMD at the femur and lumbar spine of adolescents and elderly Scandinavian [23] and Australian participants [16]. Guo *et al.*, [24] reported beneficial effects of the T allele, however, showing it to be protective against osteoporotic hip fracture. The reason for the contrasting findings may be due to the ethnicity of the populations studied, as the positive effects of the T allele have only been shown in

Chinese participants. Only 1% of the participants in the current study were from Asian heritage, thus showing an association between the variant allele of rs9594738 and adverse bone phenotypes in predominantly Caucasian participants.

The minor allele of the *RANKL* SNP rs1021188 was associated with lower cortical CSA and cortical thickness at the 14% site of the tibia. The minor allele of rs1021188 has been associated with a greater cortical porosity at the tibia [25] and increased circulating free *RANKL* [12], possibly increasing osteoclastogenesis and bone resorption in carriers of the minor allele. Although speculative, the associations shown in the present study, could suggest an uncoupling of bone turnover resulting from increased bone resorption, providing a possible mechanistic explanation for the findings. These data are in accordance with data showing the minor allele to be associated with stress fracture injury in elite athletes [13] and GWAS, reporting an association with lower cortical BMD [12] and volumetric BMD [25].

Homozygosity for the minor allele of *RANKL* SNP rs9594759 was associated with lower cortical density at the 66% site of the tibia. Recent evidence suggests that the minor allele of rs9594759 is related to an impairment of neuromuscular function and muscular characteristics [26]. Muscle is known to absorb some of the impact created by mechanical loading and also exerts strain upon the bone during muscular contractions [27;28]. Moreover, pleiotropic effects have recently been shown with BMD and lean mass [29]. A deficiency in the muscles ability to absorb load may have resulted in the bone undergoing a higher degree of strain and influenced the bone phenotypes measured in this and previous studies. These data are in contrast to previous research showing the minor allele to be associated with greater BMD at the lumbar spine, hip and calcaneus [15;23]. The reason for the difference in findings may be related to the differing ages of the participants across studies; with Styrkarsdottir *et al.* [23] and

Roshandel *et al.* [15] demonstrating their positive effects of the minor allele in aged populations (mean age ~60 y, male and female). In addition, the training and performance status of the cohort might have influenced the direction of the SNP's effect in the present study, although the potential gene-environment interactions are not well understood.

Wnt Signalling

The association of *SOST* rs1877632 with trabecular density, suggests that this SNP may mediate the early bone remodelling process. Expressed primarily in osteocytes, sclerostin has a key role in *Wnt* signalling as it acts as a negative regulator of bone formation [30]. Sclerostin null mice were shown to have increased bone formation, BMD and increased trabecular bone mass in comparison to their wild-type littermates [31]. In accordance with the present findings, carriers of the rare allele have previously been shown to display a greater BMD at the lumbar spine [32], albeit in elderly (mean age ~75 y) participants. That said, the similar percentage differences shown between the two studies (present study differences 4.8% and 5.1% compared to 6.0% in Yerges *et al.*, [32]) might suggest that the SNPs affect is demonstrated in early age and maintained throughout the lifespan. If confirmed in further large scale studies, this might have implications for the early diagnosis of individuals at a heightened risk of bone disorders.

We have previously shown that homozygosity for the C allele is associated with stress fracture injury incidence (Varley et al., under review). This seems to oppose the present findings as stress fracture incidence has been associated with a decreased BMD [33]. It can be speculated that Homozygosity for the C allele may have augmented bone phenotypes in the short-term, but, if loading is sustained, a heightened long-term susceptibility to bone weakness occurs as a result of increased secondary mineralisation [34]. An alternative hypothesis may be that as stress fracture injuries do not commonly occur at the 4% site of the tibia [35], the mechanism

by which greater trabecular density was shown and the occurrence of stress fracture injury could be different. Although interconnected, it is not uncommon for genotype [16], exercise [28;36] and pharmaceutical interventions [37] to have divergent effects on trabecular and cortical bone. Trabecular bone phenotypes are less of a determinant of bone strength relative to cortical bone [38], which may also explain the seemingly contrary findings.

P2X7R

Increased cortical density and thickness were associated with the rare allele of rs1718119 at the 14% and 38% tibial sites. These data support previous research showing that the rare A allele is associated with stress fracture injury in elite athletes and military personnel [9]. Stress fracture injuries commonly occur in the vicinity of the 38% site of the tibia [39] and low CSA and cortical thickness are associated with stress fracture incidence [40;41]. This suggests that the rare allele of rs1718119 may provide a protective mechanism against stress fracture injury by increasing bone structural phenotypes related to bone strength. *In vivo* studies have shown variants in rs1718119 to be related to increased BMD in middle aged (≥50y) osteoporotic men and women [42;43] and to reduced susceptibility to vertebral fracture in post-menopausal women [44] and osteoporotic men and women [43]. This is the first study, however, to show associations between rs1718119 and bone geometry in a young, active population. An allelic variation of rs1718119 results in increased receptor functioning related to monocyte activation and increased in interleukin-1 alpha and beta release from monocytes and macrophages [45]. The close proximity to a permeability gating region is demonstrated in the mediation of pore formation [46] and increased permeability to K^+ and ethidium$^+$ in comparison to *P2X7R* wild-type mice [45]. Increased cortical thickness and CSA at the 14% site were also shown when comparing homozygotes of the common allele with heterozygotes. These differences are interesting, since the rare allele would have been expected to confer a gain of function based

on its known mechanistic function [45]. It can be speculated that gene-gene and/or gene-environment interactions may have occurred in which those homozygous for the common allele compensated for the loss of function via another SNP. It is impossible to substantiate this hypothesis in the present study, but data in mice have demonstrated gene-gene and gene-environment modulation related to exercise [47].

The variant C allele of SNP rs3751143 was associated with less bone strength (SSI), periosteal circumference, total and cortical area. The known cellular function of rs3751143 makes the present findings unsurprising. Homozygosity for the C allele has been shown to cause a complete loss of receptor function, whereas heterozygotes have half of the receptor functionality [48]. Our data are in line with studies conducted in *vitro,* showing the C allele of rs3751143 to be associated with osteoclast apoptosis [49], reduced pore formation [48] and a reduction in pro-inflammatory cytokine secretion [50]. *In vivo,* the C allele has been associated with lower hip BMD [43] and a greater risk of fracture [42;49] in elderly participants, and stress fracture prevalence in military personal and elite athletes [9]. Taking these findings as a whole, it might be suggested that *P2X7R* SNP rs3751143 has an influence on the bone remodelling cycle across a range of populations over the lifespan.

The genotype dependent difference in bone phenotypes in the present study may have implications for bone health, and injury risk. Cortical bone size and density are important factors in the determination of bone strength [41;51]. Genotype dependent bone phenotypes could highlight an area of weakness in the bone remodelling response to loading. This would be symptomatic of the early stages of bone injury or reflect subtle bone weaknesses, which could have the same mechanisms as bone disease in later life. 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.

Despite being the largest known study to undertake such an investigation, it is not without limitation. Despite the participants being largely homogenous; 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 their club), ethnicity and specific training stimulus (academy players were from 5 different clubs, and played in numerous positions) were not controlled. The impossibility of recruiting a large number of participants in the present study, due to the uniqueness of the population, meant that population stratification methods, such as family-based design, genomic control and principal components analysis were not conducted. Whilst heterogeneity in ethnicity is acknowledged as variable factors in the present study, in order to gain a representative sample group from the population studied, all ethnicities were included in the analysis. Epiphyseal growth plate fusion and maturation status was not assessed. Artifacts as a result of unfused growth plates could have produced artifacts in these indiviudals at the distal measurement site, but maturation is unlikely to have influenced the findings due to the relatively short follow-up period (12 weeks) [52]. Although studies have shown changes in bone phenotypes [19;20] in a ~12 week period, the short follow-up time of 12 weeks in the present study may be the reason for no genotype by time differences being shown. A study with a longer follow-up time is advised, but this type of study is difficult to administer in an elite athlete population. No significant differences occurred in tibial length, ensuring the same tibial site was being scanned during both visits. 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.

Conclusions

Although, no genotype dependent change in bone phenotypes related to pQCT measures were shown following 12 weeks of increased training, six SNPs were associated with bone characteristics and an increased training volume in elite male, adolescent footballers. These data highlight the importance of SNPs in the vicinity of the *RANK/RANKL/OPG, Wnt, P2X7R* metabolic regulatory pathways with bone phenotypes in adolescents. The associations of SNPs with distinct bone phenotypes at different tibial sites highlight the complexity of the genetic contribution to bone morphology.

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