The influence of α-actinin-3 deficiency on bone remodelling markers in young men

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Abstract

There is a large individual variation in the bone remodelling markers (BRMs) osteocalcin (OC), procollagen 1 N-terminal propeptide (P1NP) and β-isomerized C-terminal telopeptide (β-CTx), as well as undercarboxylated osteocalcin (ucOC), at rest and in response to exercise. *ACTN3*, sarcomeric protein, gene is expressed in skeletal muscle and osteoblasts, and may influence BRM levels and the cross-talk between muscle and bone. We tested the levels of serum BRMs in α-actinin-3 deficient humans (*ACTN3* XX) at baseline, and following a single bout of exercise. Forty three healthy Caucasian individuals were divided into three groups (*ACTN3* XX, n=13; *ACTN3* RX (haploinsufficient), n=16; *ACTN3* RR, n=14). Participants completed a single session of High Intensity Interval Exercise (HIIE) on a cycle ergometer (8×2-min intervals at 85% of maximal power). Blood samples were taken before, immediately after, and three hours post exercise to identify the peak changes in serum BRMs. There was a stepwise increase in serum BRMs across the *ACTN3* genotypes (XX>RX>RR) with significantly higher levels of tOC ~26%, P1NP ~34%, and β -CTX ~33%) in those with *ACTN3* XX compared to *ACTN3* RR. Following exercise BRMs and ucOC were higher in all three *ACTN3* genotypes, with no significant differences between groups.

Serum levels of tOC, P1NP and β-CTX are higher in men with *ACTN3* XX genotype (α-actinin-3 deficiency) compared to RR and RX. It appears that the response of BRMs and ucOC to exercise is not explained by the *ACTN3* genotype.

Key words: ACTN3; Bone remodelling markers; Exercise; Gene variant

Introduction

Skeletal health is maintained throughout life by the cellular machinery of bone remodelling, the final common pathway mediating the effects of all genetic and environmental factors on the material composition, microstructure and strength of bone [1]. Bone remodelling is challenging to assess *in vivo* and as consequences, surrogate measures in the serum, known collectively as bone remodelling markers (BRMs), are commonly used. Common serum BRMs include osteocalcin (OC), procollagen 1 N-terminal propeptide (P1NP), and β-isomerized C-terminal telopeptide (β-CTX) [2]. Higher levels of serum BMRs have been associated with higher bone turnover [3].

Exercise is an important, non-pharmacological intervention that improves bone health and reduces the risk of osteoporosis and also improves glucose regulation and insulin sensitivity [4,5]. The above mentioned effects of exercise are partly via the undercarboxylated form of osteocalcin (ucOC) [6]. Even a single bout of exercise can increase circulating levels of BRMs and ucOC, and can improve insulin sensitivity [6-8]. However, we, and others, have reported large individual variability for the changes in BRMs and ucOC in response to similar exercise [9,6,7,10,8,11]. Indeed, some individuals are 'low-responders' (do not increase some aerobic-related traits in response to exercise training), while others respond well or very well ('high-responders') to similar exercise, and this is, at least partly, likely due to genetic variability [12]. One gene that has been shown to have an association with the response to exercise training in humans [13] and mice [14] is *ACTN3*, which encodes for the sarcomeric protein α-actinin-3 in skeletal muscle fibres. Homozygosity for a common null single nucleotide polymorphism (*ACTN3* R577X, rs1815739) in the *ACTN3* gene results in complete deficiency of the α-actinin-3 protein (*ACTN3* XX genotype) in an estimated 18% of humans worldwide, whereas individuals with the *ACTN3* RR or RX (haploinsufficinet) genotypes express the α-actinin-3 protein[15]. We, and others, have established an association between the *ACTN3* R577X genotype and athletic performance. It appears that the *ACTN3* XX genotype is associated with increased aerobic performance ([16,17]whereas the RR genotype is more prevalence in

sprint/power athletes [13,18].Importantly, *ACTN3* is also expressed in osteoblasts, the bone forming cells - the same cells that produce and secrete OC and ucOC, and therefore may influence BRM levels and the cross-talk between muscle and bone [19]. The *ACTN3* XX genotype has been associated with reduced muscle mass in the elderly [20], and with an increased risk of falling in post-menopausal women [21]. Recently, it has been shown that α-actinin-3-deficient mice (*Actn3* knockout mice, mimicking the *ACTN3* XX genotype in humans) exhibit lower bone mineral density and bone formation rates per unit bone surface (BFR/BS) compared to wild type (WT) littermates, partly due to altered osteoblastic function [19]. Although not investigated to date, this suggests *ACTN3* genotype might influence basal and exercise-induced serum BRM levels.

As such, the aims of the current study were: A) to test the hypothesis that α-actinin-3 deficient men (*ACTN3* XX genotype) have higher levels of serum BRMs compared to men with *ACTN3* RR or RX; and B) to test the hypothesis that BRMs and ucOC, which has been implicated in glucose regulation and insulin sensitivity [6,22], will increase to a greater degree following exercise in individuals with the *ACTN3* XX genotype, compared to those with *ACTN3* RR and RX.

Materials and Methods

Participants: Forty-three young, healthy, Caucasian men were given written and verbal explanations about the study before signing an informed consent form. The study protocol was approved by the Human Research Ethics Committee, Victoria University. The study is part of the Genes and Skeletal Muscle Adaptive Response to Training (Gene SMART) study (www.vu.edu.au/speed-gene), which aims to discover genetic variants that influence health-related phenotypes. A detailed medical history was assessed by questionnaire. Participants with bone disease, taking hypoglycaemic medications, or medications known to affect bone metabolism, insulin secretion or insulin sensitivity, musculoskeletal or other conditions that prevent daily activity, or receiving warfarin or vitamin K supplementation, were excluded from

the study. Flow diagram of recruitment is illustrated in Figure 1.

Peak aerobic capacity (VO_{2peak}): VO_{2peak} was assessed using a graded exercise test (GXT). This test was performed on an electronically-braked cycle-ergometer (Lode-excalibur sport, Groningen, the Netherlands), and consisted of 4-min stages separated by 30-s rest periods until voluntary exhaustion. VO_{2peak} was measured using a calibrated Quark CPET metabolic system (COSMED, Rome, Italy). The high intensity interval exercise (HIIE) phase commenced 48 - 72 h after the GXT. Capillary blood samples were collected prior to the GXT test.

Genotyping: Genomic DNA was extracted from residual capillary blood samples from the GXT tests using the GeneJET Genomic Whole Blood DNA Purification Kit (#K0781 Thermo Scientific, MA, USA). Candidate gene variants were determined using the TaqMan SNP assay (rs1815739 Cat. # 4351379, Life Technologies Australia), by Mastercycler® ep realplex2 (Eppendorf, Hamburg, Germany). Genotyping was replicated in another institute, as previously described [23] to validate the results. Participants were then divided into three groups according to their *ACTN3* genotype (*ACTN3* XX, N=13; *ACTN3* RX, N=16; *ACTN3* RR, N=14, Table 1).

Nutrition consultation: Prior to the HIIE each participant was provided with individualised, pre-packaged meals for the 48 h prior to the resting blood sample. The content of the diets are constructed based on the current National Health and Medical Research Council (NHMRC) guidelines. Participants were asked not to eat any food and avoid caffeine and alcohol 12 h prior to the HIIE phase and the blood collection.

HIIE phase: Participants completed a single session of High Intensity Interval Exercise (HIIE) on a cycle ergometer (8×2-min intervals at 85% of peak aerobic power, with 1 min of recovery between intervals). Peak aerobic power was set as the highest watts (W_{peak}) the participants paddled against in the final stage of the GXT. Venous blood samples were collected, by venepuncture or cannulation, before, immediately after, and three hours post exercise, to identify the peak change in BRMs. Five millilitres of venous blood were stored in BD Vacutainer SST ® II ™ tubes (Becton, Dickinson and Company, USA), inverted 6-10 times,

centrifuged at 3,500 rpm for 10 minutes at 4 ˚C, and subsequently analyses for BMRs was performed.

Bone remodelling marker (BRMs) assays

Serum osteocalcin and undercarboxylated osteocalcin: Total serum OC was measured using an automated immunoassay (Elecsys 170; Roche Diagnostics). This assay has a sensitivity of 0.5μg·L^{-1}, with an intra-assay precision of 1.3%. Serum ucOC was measured by the same immunoassay after adsorption of carboxylated OC on 5 mg/mL hydroxyl-apatite slurry, following the method described by Gundberg et al.[24]

β-CTX and P1NP: β-isomerized C-terminal telopeptide (β-CTX, bone resorption marker) and procollagen I N-terminal propeptide (P1NP, a bone formation marker) were analysed at Austin pathology, Melbourne using a Roche Hitachi Cobas e602 immunoassay analyser, according to the manufacturer's guidelines.

Statistical analysis

A multivariate analysis with a post-hoc, LSD, correction was used to identify differences between the three groups at baseline and to compare the percentage change following acute exercise between groups. A paired t test was used to identify changes within group relative to baseline in response to a single bout of exercise. Multi-linear regression Backward model with age, BMI and aerobic fitness were used to determine association with BRMs. All data are reported as mean ± standard error of mean (SEM) and all statistical analyses were conducted at the 95% level of significance ($p \leq 0.05$).

Results

Genotype groups (XX, RX and RR) were matched for age, BMI and aerobic capacity ($p > 0.05$, Table 1). At baseline, there was a stepwise increase in the circulating levels of tOC (Figure 2A), P1NP (Figure 2C) and β-CTX (Figure 2 D) between *ACTN3* genotypes (XX, RX and RR). Individuals with *ACTN3* XX had a higher tOC (~26%, $p < 0.05$), P1NP (~34%, $p < 0.05$,) and

β-CTX (~33%, p <0.05), compared to those with *ACTN3* RR (p<0.05). ucOC was similar across genotypes (all p > 0.37, Figure 1B).

Multiple regression analysis: in all three *ACTN3* genotypes (RR, RX and XX), age, but not BMI or aerobic power, was the strongest predictor for tOC (β= -0.72, p=0.004; β= -0.77, p=0.001 and β= -0.70, p=0.008, respectively), ucOC (β= -0.48, p=0.055; β= -0.73, p=0.001 and (β= -0.39, p=0.19, respectively), P1NP (β= -0.77, p=0.001; β= -0.69, p=0.003 and β= -0.79, p=0.001, respectively) and β-CTX (β= -0.62, p=0.019; β= -0.84, p<0.001 and β= -0.67, p=0.013, respectively).

Effect of exercise: Overall, participants in all three *ACTN3* genotypes responded similarly to a single session of HIIE. There were no differences between groups in response to HIIE with the exception of tOC between *ACTN3* RX and *ACTN3* XX (p=0.02, Figure 3). Compared to baseline, individuals with *ACTN3* RX exhibited an increase in tOC, ucOC, P1NP and β-CTX (9-15%, p<0.05) following a single bout of HIIE (Figure 3). Individuals with *ACTN3* XX exhibited a significant increase in ucOC and P1NP (p<0.05). However, no significant difference was noted for β-CTX (~7%, p=0.104, Figure 3) following HIIE in these individuals. Individuals with *ACTN3* RR, exhibited an increase in P1NP (p<0.001). However, no significant differences were noted for ucOC and β-CTX (~7% and ~5% p<0.12, respectively, Figure 3) following HIIE.

Multiple regression analysis following exercise: In participants with the *ACTN3* RR and the *ACTN3* RX, age, BMI or VO_{2peak} did not predict the change in tOC, ucOC, P1NP or β-CTX following exercise. In participants with the *ACTN3* XX, age, BMI or VO_{2peak} did not predict the change in tOC. However, VO_{2peak}, but not age or BMI, predicted exercise-induced change in ucOC (β= 0.57, p=0.04) and tended to predict changes in β-CTX (β= -0.51, p=0.073) following exercise.

Discussion

We reported that there was a stepwise increase in baseline tOC, P1NP and β-CTX across the *ACTN3* genotypes (XX>RX>RR). This may indicate that α-actinin-3 -deficient individuals (*ACTN3* XX genotype) have higher bone turnover at rest compared to *ACTN3* haploinsufficient RX and normally expressing RR individuals. However, resting ucOC levels and the response of serum BRMs and ucOC to exercise is not explained by the *ACTN3* R577X gene variant; the magnitude of exercise-induced increase in circulating BRMs and ucOC was similar regardless of *ACTN3* genotype.

The *ACTN3* gene is expressed in bone and fast glycolytic (Type 2X) skeletal muscle fibres, and *Actn3* KO mice have lower bone mineral density, lower bone mass, lower trabecular bone volume and reduced BFR/BS compared to WT littermates [19]. Similarly, older women with deficiency in α-actinin-3 (*ACTN3* XX genotype) have lower bone mineral density [19]. In the current study we report a stepwise increase in tOC, P1NP and β-CTX across the *ACTN3* genotypes, and individuals with the *ACTN3* XX genotype were characterised with the highest levels of BRMs and *ACTN3* RR the lowest. These findings in healthy young men are in agreement with those reported previously in older women [19], and may have practical and clinical implications. For instance, elite endurance athletes (i.e., long-distance runners) have a higher frequency of the *ACTN3* XX genotype compared to the general population [16,17]. However, long distance runners, both males and females, also have lower bone mineral density and a higher bone turnover that increases the risk of stress fracture [25-27]. Deficiency of α-actinin-3 has also been associated with reduced muscle mass in the elderly [28,20], and with an increased risk of falling in post-menopausal women [21]. As such, individuals with the *ACTN3* XX genotype may also be at a higher risk of future osteopenia or osteoporosis. Importantly, in the current study, individuals with *ACTN3* XX had higher tOC, P1NP and β-CTX, compared to their *ACTN3* RR counterparts despite the fact that they were recreationally active, not involved in elite sport, and the genotypes (XX, RX and RR) were matched for age, BMI and aerobic capacity. These data suggest that individuals with the *ACTN3* XX genotype

may be prone to higher BRMs, and greater future bone loss, compared to RR, independent of other confounding factors. We note that bone density and bone micro-architecture were not assessed in the current study, and future studies should examine the effect of *ACTN3* XX on bone structure over time.

In humans, ucOC, and tOC, are correlated with insulin sensitivity, fasting glucose and muscle strength [29-32]. As such, it is possible that higher circulating levels of ucOC enhance glucose metabolism in skeletal muscle. However, it appears that the *ACTN3* genotype plays little or no role in the levels of circulating serum ucOC, and, as such, *ACTN3* genotypes may not have a direct effect on glucose metabolism. It has been reported that the prevalence of the *ACTN3* XX genotype is higher, and not lower, as expected, in patients with type 2 diabetes; however, the genotype distribution was unrelated to metabolic control or obesity [33]. Although *ACTN3* genotype does not appear to influence metabolic control in type 2 diabetic individuals, Riedl et al. proposed that *ACTN3* genotype may influence glucose metabolism indirectly via effects on sarcomeric proteins and muscle function [33] .

Acute exercise increases ucOC, and this increase in ucOC is associated with a reduction in serum glucose levels in obese men [10]. In contrast to our hypothesis, participants BRMs responded similarly to HIIE regardless of their *ACTN3* genotype. However, the mean change in tOC was greatest in the *ACTN3* RX group. The overall findings highlight that, in men, serum BRMs increase following exercise independent of *ACTN3* genotype, and individual variation in response to exercise is not solely related to the *ACTN3* R577X polymorphisms. As such, future studies should examine other genetic variations or other factors that may affect individual responses to exercise.

A potential limitation of the study is the relatively small sample size in each genotype. Yet, significant differences were observed between individuals with different *ACTN*3 genotypes at rest and within groups in response to exercise. Furthermore, the trial was tightly-controlled in terms of aerobic capacity, the exercise stimulus and nutrition; thus, we are confident that our findings reflect the influence of the *ACTN3* genotype on the described phenotypes.

In conclusion, there was a stepwise increase in tOC, P1NP and β-CTX across the *ACTN3* genotypes (XX>RX>RR) in young men; *ACTN3* XX genotype is associated with higher basal levels of serum BRMs. It appears that the response of BRMs and ucOC to exercise is not explained by the *ACTN3* genotype. Future studies should explore the mechanism/s behind the influence of this variant on BRMs, and should also explore other genes or factors that may explain different individual responses following exercise.

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Figure legends:

Figure 1. Flow diagram of recruitment.

Figure 2. Comparison of serum bone remodelling markers (BRMs) and ucOC in men across the *ACTN3* genotypes. Total osteocalcin (tOC, A), uncarboxylated osteocalcin (ucOC, B), procollagen 1 N-terminal propeptide (P1NP, C) and β-isomerized C-terminal telopeptides (β-CTx, D). * $p<0.05$, ^ $p= 0.09$.

Figure 3. The influence of *ACTN3* genotype on the acute response of serum BRMs to High Intensity Interval Exercise (HIIE). * $p< 0.05$, ** $p<0.01$ compared to baseline levels. & indicates $p<0.05$ between genotypes.