The association between bone mineral density gene variants and osteocalcin at baseline, and in response to exercise: the gene SMART study

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Abstract

Introduction: Osteocalcin (OC) is used as a surrogate marker for bone turnover in clinical settings. As bone mineral density (BMD) is largely heritable, we tested the hypothesis that a) bone-associated genetic variants previously identified in Genome-Wide Association Studies (GWAS) and combined into a genetic risk score (GRS) are associated with a) circulating levels of OC and b) the changes in OC following acute exercise.

Methods: Total OC (tOC), undercarboxylated OC (ucOC), and carboxylated OC (cOC) were measured in serum of 73 healthy Caucasian males at baseline and after a single bout of high-intensity interval exercise. In addition, genotyping was conducted targeting GWAS variants previously reported to be associated with BMD and then combined into a GRS. Potential associations between the GRS and tOC, ucOC and cOC were tested with linear regressions adjusted for age.

Results: At baseline none of the individual SNPs associated with tOC, ucOC and cOC. However, when combined, a higher GRS was associated with higher tOC (β = 0.193ng/mL; p=0.037; 95% CI = 0.012, 0.361) and cOC (β = 0.188ng/mL; p=0.04; 95% CI = 0.004, 0.433). Following exercise, GRS was associated with ucOC levels, (β = 3.864ng/mL; p-value = 0.008; 95% CI = 1.063, 6.664) but not with tOC or cOC.

Conclusion: Screening for genetic variations may assist in identifying people at risk for abnormal circulating levels of OC at baseline/rest. Genetic variations in BMD predicted the ucOC response to acute exercise indicating that physiological functional response to exercise may be influenced by bone-related gene variants.

Key words: Osteocalcin, SNP, exercise, bone turnover, biomarkers, bone mineral density.

Introduction

The primary function of the skeletal system is to provide mechanical support of the body, respond to outside mechanical forces, and is a reservoir for normal mineral metabolism [1, 2]. Throughout the lifespan the skeleton undergoes continuous bone remodelling, it is tightly controlled by osteoclasts and osteoblasts which balance bone removal and bone formation [3]. In-vivo bone remodelling is difficult to assess, therefore circulating bone turnover markers (BTMs) are commonly used in a clinical setting as a surrogate measure for bone metabolism and give an indication of bone turnover [4, 5].

Osteocalcin (OC), a BTM, is secreted by osteoblasts into the extracellular matrix and is important in bone matrix formation, mineralization and maintenance [6, 7]. OC can be post-translationally modified by gamma (γ)-carboxylation at one or more of the three (17, 21 and 24) glutamic acid residues and found in two different forms, carboxylated (cOC) and undercarboxylated OC (ucOC). While they share a similar tertiary structure they are thought to have different biological functions. The undercarboxylated form (ucOC) was implicated in energy metabolism and cardiovascular health [4, 8-10]. UcOC may also play a role in bone health as higher levels of ucOC have been found to be associated with a higher risk of hip fracture [11-13]. While cOC, where all three glutamic acid residues are carboxylated, is considered predominantly a protein of the bone matrix [6, 9]. After carboxylation conformational changes occur in OC, increasing the affinity for calcium ions [9]. Calcium helps to stabilize OC and facilitates the binding of OC to the surface of hydroxyapatite, the bone mineral component of the bone and is closely aligned with the bone mineral density (BMD) [4, 6].

BMD is a parameter used in the identification of people at risk for osteopenia and osteoporosis [14, 15]. BMD has a high genetic heritability with contributions from environmental factors such as exercise and nutrition [16-18]. A recent Genome-Wide Association (GWAS) study identified nine single nucleotide polymorphisms (SNPs) that are associated with BMD ($p< 5 X 10^{-6}$) [16]. Environmental factors such as acute exercise can also affect BTMs by mechanically loading the skeleton [19-21]. We have also previously shown that the *ACTN3* R577X common SNP is associated with serum levels of tOC in men with serum levels higher in the *ACTN3* XX genotype (α-actinin-3 deficiency) compared to RR and RX at baseline [22]. This illustrates both genetic and environmental factors can influence bone turnover and the associated markers [23, 24]. Any imbalances to this process can cause bone loss and a reduction in bone strength leading to common bone disorders such as osteopenia and osteoporosis [25].

However, it is currently unclear whether bone related genetic variants are associated with levels of bone turnover, nor if genetic variants can alter the response of BTMs following acute exercise. This could be clinically important in identifying people at a younger age at risk of bone related disorders. Therefore, we tested the hypothesis that the GWAS SNPs that were previously identified to be associated with bone-related phenotypes [16], can predict circulating tOC, ucOC and cOC at baseline, and following an acute bout of High-Intensity Interval Exercise (HIIE).

Materials and methods

Participants

This study is a part of the Genes and Skeletal Muscle Adaptive Response to Training (Gene SMART) study. The detailed methodology has previously been published [26]. Briefly, seventy-three apparently healthy, Caucasian men (age = 31.4 years ± 8.2; BMI = 25.2 kg/m^2 ± 3.2) participated in the study following a written informed consent. Participants attended the

VU laboratory on 2 separate occasions, to perform two graded exercise tests, and on one occasion for the blood sampling and the acute exercise intervention. Volunteers were excluded if they had a bone disease, were taking hypoglycaemic medications, warfarin or vitamin K supplementation, or medications that affect bone metabolism, insulin secretion, or sensitivity. Further, participants with known musculoskeletal or other conditions that prevent daily activity were excluded from the study. This study was approved by the Human Ethics Research Committee at Victoria University (HRE13-223) and all participants provided written informed consent.

Aerobic Capacity (Graded exercise test)

Aerobic capacity was assessed by a graded exercise test (GXT) to establish peak power output (W_{peak}) and lactate threshold (LT). Briefly the test consisted of 4 minute exercise stages, separated by 30 second rest periods until voluntary exhaustion. Capillary blood samples were collected and analysed by the YSI 2300 STAT Plus system (Ohio, USA) at the end of each 4 minute stage and immediately after exhaustion to establish lactate (LT) concentration. LT was calculated by the modified DMAX method as previously described [26].

Nutrition consultation

Each participant was provided with an individualised pre-packaged diet 48 hours prior to providing the blood samples to standardise diet across the participants and minimise the effects of this confounding factor [27, 28]. The content of the diets were based on the current Australian National Health and Medical Research Council (NHMRC) guidelines. Participants were asked to abstain from food, caffeine and alcohol 12 hours prior to blood collection.

Acute exercise session

Participants completed a single session of High Intensity Interval Exercise (HIIE) on a cycle ergometer (Velotron®, Racer Mate Inc.). The acute exercise session consisted of 8 X 2min intervals at 40% of [(Wpeak-LT) + LT] with 1 minute active recovery intervals at a power of 60W.

Serum osteocalcin measurements

Before acute exercise (baseline), immediately after the acute exercise, and 3 hours post exercise, venous blood samples were collected via venepuncture or cannulation in BD SST Vacutainers (Becton and Dickson Company, USA). All participants abstained from food, caffeine and alcohol 12 hours prior to blood collection in the morning to control for variation in serum levels of OC. They were left at room temperature (10 mins) before being centrifuged at 3500 rpm for 10mins at 4°C. Serum was collected and stored at -80°C. tOC was measured using an automated immunoassay (Elecys 170; Roche Diagnostics). This assay has a sensitivity of 0.5 $\mu g.L^{-1}$ with an intra-assay precision of 1.3%. We measured ucOC by the same immuno-assay after absorption of carboxylated OC on 5mg/ml hydroxyl-apatite slurry as described by Gundberg et al. [29]. cOC was calculated by the subtracting the ucOC from the tOC. Circulating tOC, ucOC and cOC were measured at baseline (before acute exercise), immediately after, and 3 hours post exercise. The peak OC, ucOC and cOC was considered the maximal concentration immediately after or 3 hours post exercise

Genotyping

Genomic DNA was extracted from residual blood samples from BD Vacutainer EDTA tubes using the MagSep Blood gDNA kit (0030 451.00, Eppendorf, Hamburg, Germany) [26]. The genotype of each SNP was assessed by the Australian Genome Research Facility (AGRF). We chose the SNPs based on previous literature from a GWAS study assessing BMD [16]. The SNPs, locus, type, effect allele and closest gene are described in supplementary table 1. Based

on the Moayyeri et al GWAS, nine genetic variants known to be associated with broadband ultrasound attenuation (BUA), that estimates BMD, were used to calculate genetic risk scores (GRS).The SNPs were measured by MassARRAY® combined with iPLEX® chemistry (Agena Bioscience).

Statistical analysis

We conducted linear regressions to test for potential associations between individual SNPs and tOC or cOC levels, using an additive genetic model (i.e. homozygotes for non-effect allele coded as 0; heterozygotes coded as 1; homozygotes for effect allele coded as 2). The GRS was calculated by coding each SNP with the number of effect alleles (0, 1 or 2), multiplying this with the published regression coefficient (beta) [16] then summed up to obtain a weighted GRS. Linear regressions were conducted to test whether the GRS was associated with tOC, ucOC or cOC. The distribution of tOC, ucOC and cOC were checked for normality visually using histograms, and tOC, ucOC and cOC were log-transformed to meet normality assumptions. In the linear regressions, we used log-transformed tOC, ucOC or cOC as the dependent variable; and GRS and age as the independent variables. We also tested BMI, and fitness parameters (VO_2peak, lactate threshold and peak power) as additional covariates, but as they did not significantly associate with levels of tOC, ucOC or cOC they were removed from the model. To investigate the response of tOC, ucOC and cOC to exercise and potential associations with the GRS, we used the delta change of baseline to peak before and after the acute exercise session. Where required p-values from the statistical analyses were adjusted for multiple testing using the false discovery rate (FDR) [30], and q-values<0.05 were deemed significant. Post-hoc power analysis was conducted in R using the pwr package using effect sizes and notations from Cohen J [31].

Results

Participants' characteristics and the tOC, ucOC and cOC responses to exercise are described in Table 1. A small, but significant (4.6%, $p<0.05$) increase was observed for tOC, and ucOC (10.1%, $p<0.01$) but not cOC, following exercise (Table 1).

Table 1- Participant characteristics (n=73)

	Baseline		
Age (years)	31.4 ± 8.2		
BMI ($kg.m^{-2}$)	25.2 ± 3.2		
VO_2peak ($mL.kg^{-1}.min^{-1}$)	47.2 ± 8.0		
Lactate threshold (W)	206.7 ± 55.3		
W_{peak} (W)	294.9 ± 64.7		
Circulating Osteocalcin		**Peak**	**p-value**
tOC (ng/ml)	30.5 ± 10.9	31.9 ± 10.65	p=0.004
cOC (ng/ml)	18.7 ± 8.2	19.1 ± 7.8	p=0.372
ucOC (ng/ml)	11.9 ± 4.3	13.1 ± 4.6	P<0.01

BMI, Body Mass Index; VO2peak, Peak oxygen uptake during graded exercise test; Peak, peak level of tOC, ucOC and cOC at either immediately post or 3 hours post exercise; tOC, total osteocalcin; cOC, Carboxylated OC; ucOC, under-carboxylated osteocalcin; W, watts. Values are mean ± SD.

Individual SNPs were not associated with tOC, ucOC and cOC

We first assessed the contribution of each individual SNP to the variance in tOC, ucOC and cOC, but no significant associations were found (Table 2).

Table 2- Individual SNP regression analysis with tOC, ucOC and cOC.

	tOC				cOC				ucOC			
SNP ID	**B (ng/ml)**	**p-value**	**FDR q-value**	**Post-Hoc Power (%)**	**B (ng/ml)**	**p-value**	**FDR**	**Post-Hoc Power (%)**	**B (ng/ml)**	**p-value**	**FDR q-value**	**Post-Hoc Power (%)**
rs7741021	0.08	0.82	0.92	5.3	0.144	0.71	0.71	5.6	0.124	0.889	0.889	0.05
rs4869739	-0.19	0.54	0.69	7.9	-0.218	0.57	0.64	7.5	-0.385	0.655	0.75	0.066
rs3020331	0.29	0.38	0.68	11.6	0.348	0.39	0.64	11.6	0.449	0.616	0.75	0.072
rs2982552	0.80	0.02	0.19	55	0.82	0.059	0.3	39.3	1.75	0.07	0.63	0.075
rs2908007	0.22	0.13	0.39	27	0.232	0.18	0.54	21.3	0.309	0.423	0.75	0.054
rs597319	0.01	0.98	0.98	0.5	0.217	0.57	0.64	7.6	-0.873	0.299	0.672	0
rs10416265	-0.25	0.49	0.69	8.8	-0.32	0.47	0.64	9.1	-0.429	0.667	0.75	0.14
rs6974574	0.62	0.21	0.47	19.1	0.544	0.37	0.64	11.6	1.588	0.243	0.67	0.17
rs38664	0.90	0.06	0.28	37.8	1.091	0.068	0.31	37	1.672	0.212	0.67	0.9

The p-values were adjusted for multiple testing using the false discovery rate (FDR). Additive genetic models were used. Effect size correspond to the regression coefficient in the linear models, and is interpreted as the change in tOC, ucOC or cOC (log-transformed) per effect allele at the SNP.

The GRS was positively associated with tOC, ucOC and cOC

As the contribution of each individual SNP may be too small to be detected in only n = 73 individuals, we calculated a GRS to increase statistical power (see Material & Methods). Age was negatively associated with tOC (β = -0.608ng/ul; p<0.001; 95% CI = -0.017, -0.009), ucOC (β = -0.018ng/ml; p<0.001; 95% CI= -0.027, -0.009) and cOC (β = -0.607ng/ml; p<0.001; 95% CI = -0.017, -0.009). After adjusting for age, higher GRS was associated with higher levels of tOC (β = 0.193ng/ml; p-value = 0.037; 95% CI = 0.012, 0.361) and with higher levels of cOC (β = 0.188ng/ml p-value = 0.046; 95% CI = 0.004, 0.433). The GRS explained 6.1% of the variance in tOC and 5.6% of the variance in cOC (Figure 1). ucOC was not associated with GRS (β=0.261ng/ml; p-value = 0.289; 95% CI= -0.226, 0.747) after adjusting for age (Figure 1).

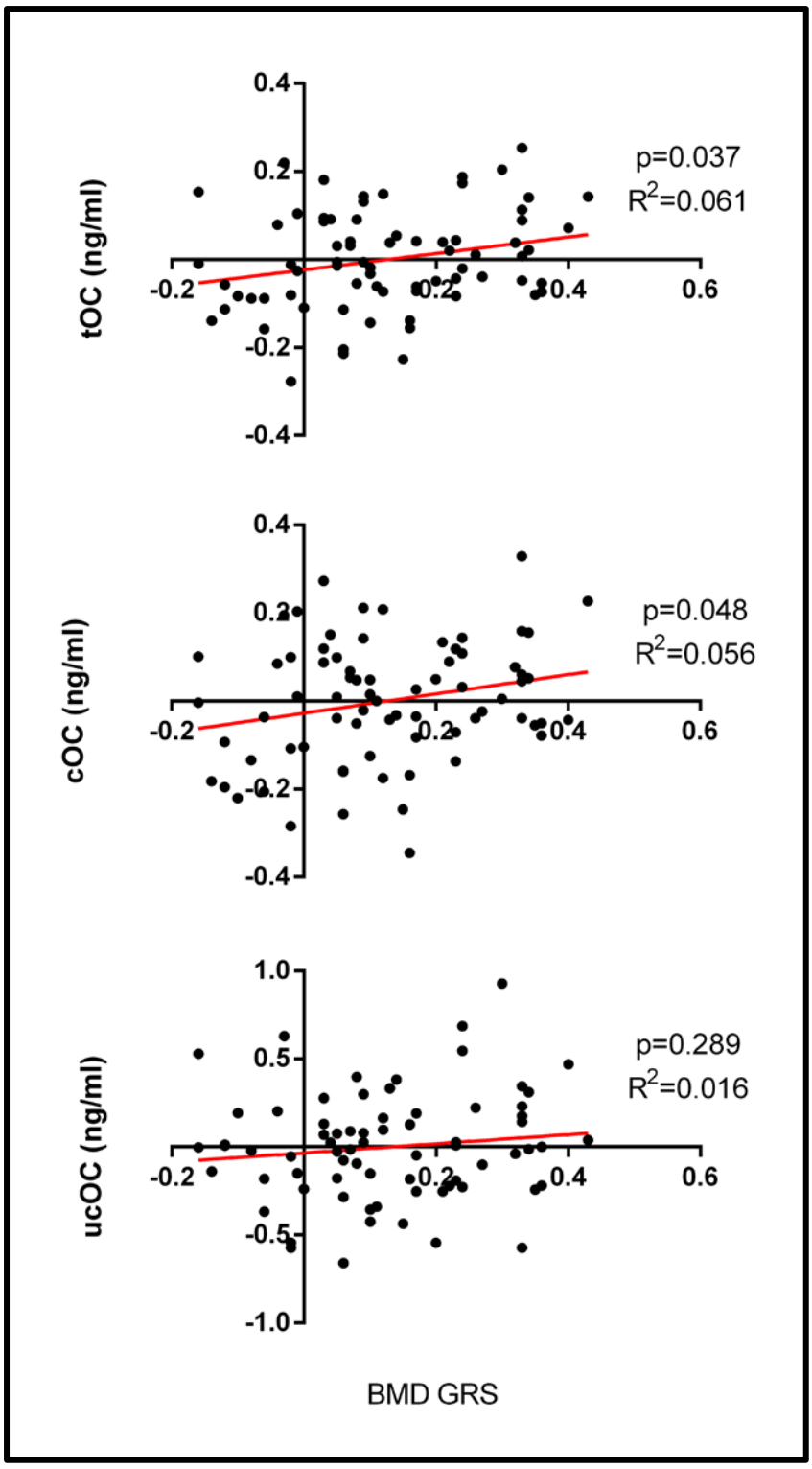

Figure 1- Regression analysis of genetic risk score with tOC,ucOC and cOC adjusted for age. BMD GRS = Bone Mineral Density Genetic Risk Score. Significance $p<0.05$ after adjusting for age.

The GRS was associated with exercise-induced peak in ucOC but not tOC or cOC

A positive association was identified between ucOC response to exercise and the GRS (β = 3.864ng/ml; p-value = 0.008; 95% CI = 1.063, 6.664). The GRS explained 9.8% of the variance in ucOC response to exercise (Figure 2). We were unable to identify an association between the changes in tOC or cOC and the GRS (p=0.617 and p=0.17, respectively).

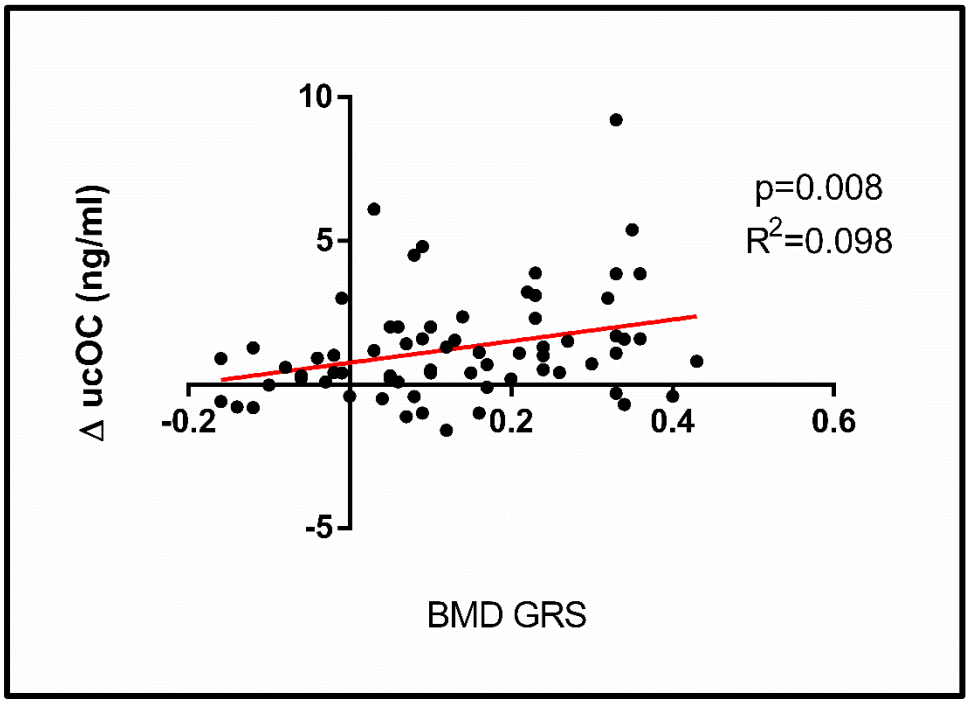

Figure 2- Regression analysis of genetic risk score with change in ucOC (Δ) after acute exercise. BMD GRS = Bone Mineral Density Genetic Risk Score. Significance p<0.05 after adjusting for age.

Discussion

We report that a higher GRS is associated with higher tOC and cOC at baseline in healthy young men but not with ucOC. GRS did not appear to predict the change in tOC or cOC

following exercise. However, a higher genetic risk for lower BMD was associated with the increased concentration of ucOC following exercise, providing a novel insight that BMD genetic variants may play a role in this response.

BMD is a complex trait that, in part, relates to heritability and is used as a predictor for future risk to develop osteoporosis. Yet, each individual SNP may only contribute a small amount to the hereditary component of BMD, making this type of analysis not useful in predictive studies [32]. This is shown in table 2 where each individual SNP did not predict change in tOC, ucOC or cOC. GRS combines SNPs identified by GWAS into a score that can evaluate and provide further insights into the genetic contribution to BMD by increasing statistical power to detect these small contributions [32-34]. We used nine SNPs that were associated with BMD from an unbiased GWAS approach in large consortium [16] and found that these SNPs are also associated with the BTM, OC. The genetic variants used in the GRS determined 6.1% and 5.6% of the variability of circulating levels of tOC and cOC, respectively, at rest and 9.8% of the variability of circulating levels of ucOC in response to acute exercise.

We report that healthy men who have a higher genetic risk for lower BMD displayed increased circulating levels of tOC. Previous studies have shown that higher levels of serum BTMs, including tOC are associated with higher bone turnover [22, 35, 36]. While OC is conventionally used as a marker of bone formation, literature suggests that it may be a better indicator of overall bone turnover [37, 38]. Studies have shown that osteoporosis, vertebral fractures, and bone loss are associated with increased levels of tOC [39, 40]. Therefore, we provide evidence indicating that genetics may be playing a role in influencing bone turnover, previously shown to be associated with ongoing bone loss or higher risk of fracture [37, 39, 40].

Exercise mechanically loads the skeleton, improves insulin sensitivity and may partly mediate the interaction between muscle, bone and glucose metabolism [9, 20]. Therefore, we explored the hypothesis that BMD gene variants may play a role in tOC, ucOC or cOC response to acute exercise. We found that the genetic risk score was not associated with tOC or cOC in response to acute exercise, suggesting that the genetic variables examined cannot determine the response of tOC or cOC to acute exercise, at least in healthy-young men [22]. We could speculate that as bone formation is a slow process [41], to observe any influence of BMD gene variants on circulating levels of tOC or cOC would require a longer exercise intervention. Interestingly, an increased genetics risk for a lower BMD was associated with increased levels of ucOC in response to exercise. It is not clear why those with increased genetic risks exhibited increased levels of ucOC following exercise, however, previous studies suggest that increased ucOC levels are associated with increased fracture risk [11-13]. In addition, the increase in ucOC following exercise may be due to the increase metabolic demands by skeletal muscle. Indeed, we have previously shown that ucOC has a direct effect on muscle glucose uptake in insulin signalling proteins [9, 42]. We confirmed that ucOC is upregulated after exercise in humans and provide a novel insight that BMD genetic variants may play a role in this response.

The current study has some potential limitations. First, it includes a relatively small sample size in the context of genetic studies. Yet, even with a small sample size we were able to detect a significant association of GRS with tOC and cOC. We are confident in our findings, as they support previous findings of a GWAS that identified these SNPs to be associated with BMD [16]. We were underpowered for the individuals SNP analysis (on average tOC- 19.2%, ucOC- 0.2% and cOC- 16.7%). However, our power improved greatly with the use of the genetic risk score (tOC- 48%, ucOC- 14.9% and cOC- 44%) illustrating the strength of calculating a GRS for data analysis. We acknowledge that while greater sample sizes are required, our data provides hypothesis generating pilot data that offer interesting novel concepts for future

analysis. Secondly, we did not assess BMD, therefore a direct assessment of the SNPs with BMD cannot be performed. Finally, we only tested young-healthy males. Future studies should focus on young healthy females as well as older adults who have an increased risk for osteopenia and osteoporosis.

In conclusion, screening for genetic variations may assist in identifying people at risk for abnormal circulating levels of OC. Genetic variations in BMD predicted the response of ucOC to acute exercise, but not tOC or cOC, indicating that physiological functional response to exercise may be influenced by bone-related gene variants.

Acknowledgements

A/Prof Levinger was supported by a Future Leader Fellowship (ID: 100040) from the National Heart Foundation of Australia. This research was supported the National Health & Medical Research Council (NHMRC CDF # APP1140644) to Nir Eynon.

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