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 ($\beta = 0.193$ng/mL; p=0.037; 95% CI $= 0.012, 0.361$) and cOC ($\beta = 0.188$ng/mL; p=0.04; 95% CI $= 0.004, 0.433$). Following exercise, GRS was associated with ucOC levels, ($\beta = 3.864$ng/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.

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

63

64 **Introduction**

65 The primary function of the skeletal system is to provide mechanical support of the body,

66 respond to outside mechanical forces, and is a reservoir for normal mineral metabolism [1, 2].

67 Throughout the lifespan the skeleton undergoes continuous bone remodelling, it is tightly

68 controlled by osteoclasts and osteoblasts which balance bone removal and bone formation [3].

69 In-vivo bone remodelling is difficult to assess, therefore circulating bone turnover markers

70 (BTMs) are commonly used in a clinical setting as a surrogate measure for bone metabolism

71 and give an indication of bone turnover [4, 5].

72 Osteocalcin (OC), a BTM, is secreted by osteoblasts into the extracellular matrix and is

73 important in bone matrix formation, mineralization and maintenance [6, 7]. OC can be post-

74 translationally modified by gamma (ɣ)-carboxylation at one or more of the three (17, 21 and

75 24) glutamic acid residues and found in two different forms, carboxylated (cOC) and

76 undercarboxylated OC (ucOC). While they share a similar tertiary structure they are thought

77 to have different biological functions. The undercarboxylated form (ucOC) was implicated in

78 energy metabolism and cardiovascular health [4, 8-10]. UcOC may also play a role in bone

79 health as higher levels of ucOC have been found to be associated with a higher risk of hip

80 fracture [11-13]. While cOC, where all three glutamic acid residues are carboxylated, is

81 considered predominantly a protein of the bone matrix [6, 9]. After carboxylation

82 conformational changes occur in OC, increasing the affinity for calcium ions [9]. Calcium helps

83 to stabilize OC and facilitates the binding of OC to the surface of hydroxyapatite, the bone

84 mineral component of the bone and is closely aligned with the bone mineral density (BMD) [4,

85 6].

86 BMD is a parameter used in the identification of people at risk for osteopenia and osteoporosis

87 [14, 15]. BMD has a high genetic heritability with contributions from environmental factors

88 such as exercise and nutrition [16-18]. A recent Genome-Wide Association (GWAS) study

89 identified nine single nucleotide polymorphisms (SNPs) that are associated with BMD (p< 5

90 X 10^{-6}) [16]. Environmental factors such as acute exercise can also affect BTMs by

91 mechanically loading the skeleton [19-21]. We have also previously shown that the *ACTN3*

92 R577X common SNP is associated with serum levels of tOC in men with serum levels higher

93 in the *ACTN3* XX genotype (α-actinin-3 deficiency) compared to RR and RX at baseline [22].

94 This illustrates both genetic and environmental factors can influence bone turnover and the

95 associated markers [23, 24]. Any imbalances to this process can cause bone loss and a reduction

96 in bone strength leading to common bone disorders such as osteopenia and osteoporosis [25].

97 However, it is currently unclear whether bone related genetic variants are associated with levels

98 of bone turnover, nor if genetic variants can alter the response of BTMs following acute

99 exercise. This could be clinically important in identifying people at a younger age at risk of

100 bone related disorders. Therefore, we tested the hypothesis that the GWAS SNPs that were

101 previously identified to be associated with bone-related phenotypes [16], can predict

102 circulating tOC, ucOC and cOC at baseline, and following an acute bout of High-Intensity

103 Interval Exercise (HIIE).

104 **Materials and methods**

105 *Participants*

106 This study is a part of the Genes and Skeletal Muscle Adaptive Response to Training (Gene

107 SMART) study. The detailed methodology has previously been published [26]. Briefly,

108 seventy-three apparently healthy, Caucasian men (age = 31.4 years ± 8.2; BMI = 25.2 kg/m^2 ±

109 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

132 Participants completed a single session of High Intensity Interval Exercise (HIIE) on a cycle

133 ergometer (Velotron®, Racer Mate Inc.). The acute exercise session consisted of 8 X 2min

134 intervals at 40% of [(Wpeak-LT) + LT] with 1 minute active recovery intervals at a power of

135 60W.

136 *Serum osteocalcin measurements*

137 Before acute exercise (baseline), immediately after the acute exercise, and 3 hours post

138 exercise, venous blood samples were collected via venepuncture or cannulation in BD SST

139 Vacutainers (Becton and Dickson Company, USA). All participants abstained from food,

140 caffeine and alcohol 12 hours prior to blood collection in the morning to control for variation

141 in serum levels of OC. They were left at room temperature (10 mins) before being centrifuged

142 at 3500 rpm for 10mins at 4°C. Serum was collected and stored at -80°C. tOC was measured

143 using an automated immunoassay (Elecys 170; Roche Diagnostics). This assay has a sensitivity

144 of 0.5 μg.L^{-1} with an intra-assay precision of 1.3%. We measured ucOC by the same immuno-

145 assay after absorption of carboxylated OC on 5mg/ml hydroxyl-apatite slurry as described by

146 Gundberg et al. [29]. cOC was calculated by the subtracting the ucOC from the tOC.

147 Circulating tOC, ucOC and cOC were measured at baseline (before acute exercise),

148 immediately after, and 3 hours post exercise. The peak OC, ucOC and cOC was considered the

149 maximal concentration immediately after or 3 hours post exercise

150 *Genotyping*

151 Genomic DNA was extracted from residual blood samples from BD Vacutainer EDTA tubes

152 using the MagSep Blood gDNA kit (0030 451.00, Eppendorf, Hamburg, Germany) [26]. The

153 genotype of each SNP was assessed by the Australian Genome Research Facility (AGRF). We

154 chose the SNPs based on previous literature from a GWAS study assessing BMD [16]. The

155 SNPs, locus, type, effect allele and closest gene are described in supplementary table 1. Based

156   on the Moayyeri et al GWAS, nine genetic variants known to be associated with broadband

157   ultrasound attenuation (BUA), that estimates BMD, were used to calculate genetic risk scores

158   (GRS).The SNPs were measured by MassARRAY® combined with iPLEX® chemistry

159   (Agena Bioscience).

160   *Statistical analysis*

161   We conducted linear regressions to test for potential associations between individual SNPs and

162   tOC or cOC levels, using an additive genetic model (i.e. homozygotes for non-effect allele

163   coded as 0; heterozygotes coded as 1; homozygotes for effect allele coded as 2). The GRS was

164   calculated by coding each SNP with the number of effect alleles (0, 1 or 2), multiplying this

165   with the published regression coefficient (beta) [16] then summed up to obtain a weighted GRS.

166   Linear regressions were conducted to test whether the GRS was associated with tOC, ucOC or

167   cOC. The distribution of tOC, ucOC and cOC were checked for normality visually using

168   histograms, and tOC, ucOC and cOC were log-transformed to meet normality assumptions. In

169   the linear regressions, we used log-transformed tOC, ucOC or cOC as the dependent variable;

170   and GRS and age as the independent variables. We also tested BMI, and fitness parameters

171   (VO$_2$peak, lactate threshold and peak power) as additional covariates, but as they did not

172   significantly associate with levels of tOC, ucOC or cOC they were removed from the model.

173   To investigate the response of tOC, ucOC and cOC to exercise and potential associations with

174   the GRS, we used the delta change of baseline to peak before and after the acute exercise

175   session. Where required p-values from the statistical analyses were adjusted for multiple testing

176   using the false discovery rate (FDR) [30], and q-values<0.05 were deemed significant. Post-

177   hoc power analysis was conducted in R using the pwr package using effect sizes and notations

178   from Cohen J [31].

179

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$_2$peak (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).

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

SNP ID	tOC				cOC				ucOC			
	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

193 The p-values were adjusted for multiple testing using the false discovery rate (FDR). Additive genetic models were used. Effect size correspond

194 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

195 SNP.

The GRS was positively associated with tOC, ucOC and cOC

197   As the contribution of each individual SNP may be too small to be detected in only n = 73

198   individuals, we calculated a GRS to increase statistical power (see Material & Methods). Age

199   was negatively associated with tOC (β = -0.608ng/ul; p<0.001; 95% CI = -0.017, -0.009), ucOC

200   (β = -0.018ng/ml; p<0.001; 95% CI= -0.027, -0.009) and cOC (β = -0.607ng/ml; p<0.001; 95%

201   CI = -0.017, -0.009). After adjusting for age, higher GRS was associated with higher levels of

202   tOC (β = 0.193ng/ml; p-value = 0.037; 95% CI = 0.012, 0.361) and with higher levels of cOC

203   (β = 0.188ng/ml p-value = 0.046; 95% CI = 0.004, 0.433). The GRS explained 6.1% of the

204   variance in tOC and 5.6% of the variance in cOC (Figure 1). ucOC was not associated with

205   GRS (β=0.261ng/ml; p-value = 0.289; 95% CI= -0.226, 0.747) after adjusting for age (Figure
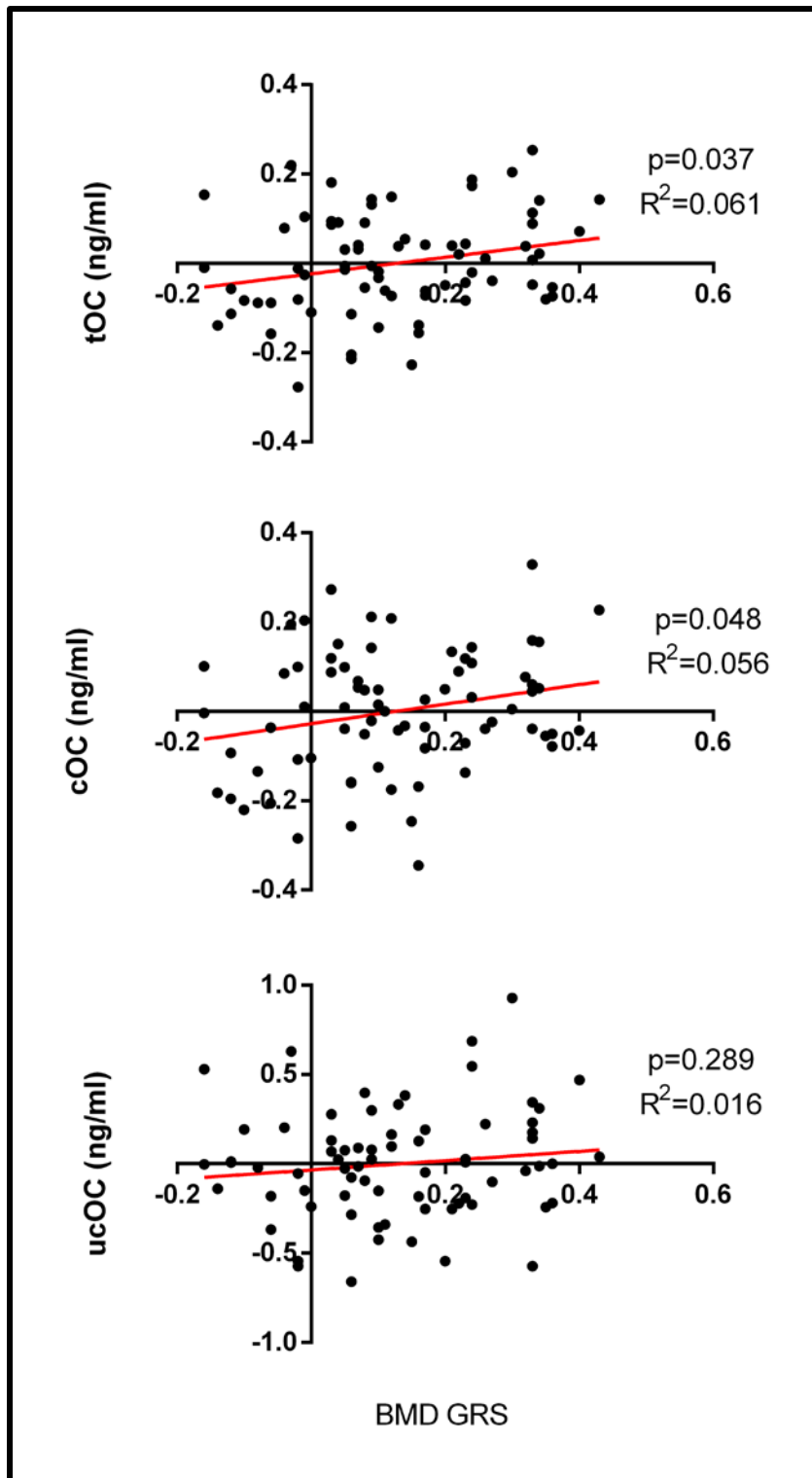
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Figure 1- Regression analysis of genetic risk score with tOC,ucOC and cOC adjusted for age. BMD GRS = Bone Mineral Density Genetic Risk Score. Significance $p<0.05$ after adjusting for age.

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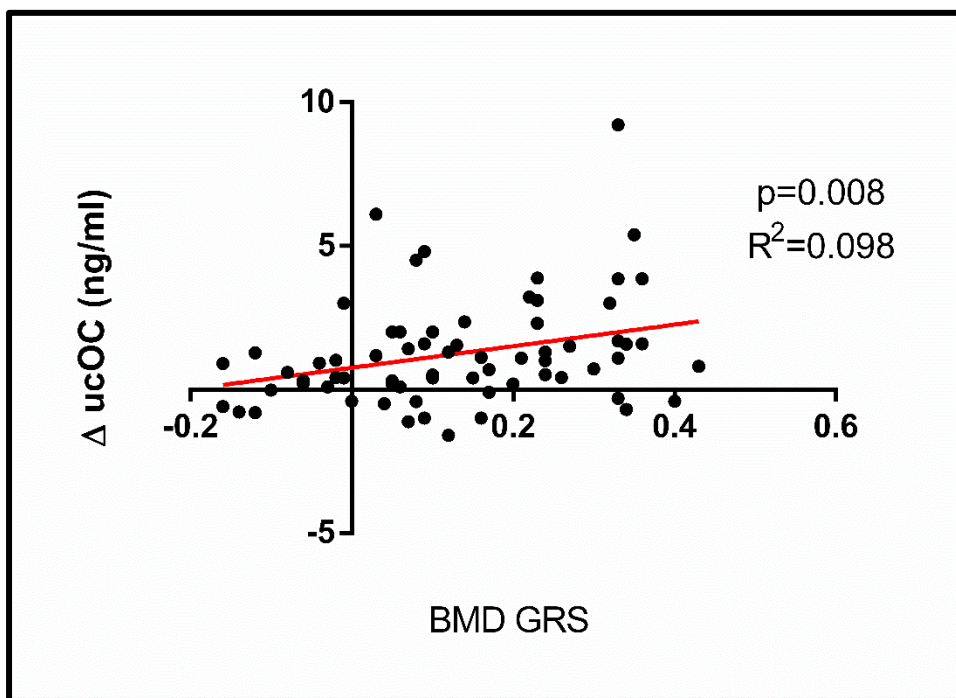
Figure 2- Regression analysis of genetic risk score with change in ucOC (Δ) after acute exercise. BMD GRS = Bone Mineral Density Genetic Risk Score. Significance p<0.05 after adjusting for age.

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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

235   following exercise. However, a higher genetic risk for lower BMD was associated with the

236   increased concentration of ucOC following exercise, providing a novel insight that BMD

237   genetic variants may play a role in this response.

238   BMD is a complex trait that, in part, relates to heritability and is used as a predictor for future

239   risk to develop osteoporosis. Yet, each individual SNP may only contribute a small amount to

240   the hereditary component of BMD, making this type of analysis not useful in predictive studies

241   [32]. This is shown in table 2 where each individual SNP did not predict change in tOC, ucOC

242   or cOC. GRS combines SNPs identified by GWAS into a score that can evaluate and provide

243   further insights into the genetic contribution to BMD by increasing statistical power to detect

244   these small contributions [32-34]. We used nine SNPs that were associated with BMD from an

245   unbiased GWAS approach in large consortium [16] and found that these SNPs are also

246   associated with the BTM, OC. The genetic variants used in the GRS determined 6.1% and 5.6%

247   of the variability of circulating levels of tOC and cOC, respectively, at rest and 9.8% of the

248   variability of circulating levels of ucOC in response to acute exercise.

249   We report that healthy men who have a higher genetic risk for lower BMD displayed increased

250   circulating levels of tOC. Previous studies have shown that higher levels of serum BTMs,

251   including tOC are associated with higher bone turnover [22, 35, 36]. While OC is

252   conventionally used as a marker of bone formation, literature suggests that it may be a better

253   indicator of overall bone turnover [37, 38]. Studies have shown that osteoporosis, vertebral

254   fractures, and bone loss are associated with increased levels of tOC [39, 40]. Therefore, we

255   provide evidence indicating that genetics may be playing a role in influencing bone turnover,

256   previously shown to be associated with ongoing bone loss or higher risk of fracture [37, 39,

257   40].

258 Exercise mechanically loads the skeleton, improves insulin sensitivity and may partly mediate

259 the interaction between muscle, bone and glucose metabolism [9, 20]. Therefore, we explored

260 the hypothesis that BMD gene variants may play a role in tOC, ucOC or cOC response to acute

261 exercise. We found that the genetic risk score was not associated with tOC or cOC in response

262 to acute exercise, suggesting that the genetic variables examined cannot determine the response

263 of tOC or cOC to acute exercise, at least in healthy-young men [22]. We could speculate that

264 as bone formation is a slow process [41], to observe any influence of BMD gene variants on

265 circulating levels of tOC or cOC would require a longer exercise intervention. Interestingly, an

266 increased genetics risk for a lower BMD was associated with increased levels of ucOC in

267 response to exercise. It is not clear why those with increased genetic risks exhibited increased

268 levels of ucOC following exercise, however, previous studies suggest that increased ucOC

269 levels are associated with increased fracture risk [11-13]. In addition, the increase in ucOC

270 following exercise may be due to the increase metabolic demands by skeletal muscle. Indeed,

271 we have previously shown that ucOC has a direct effect on muscle glucose uptake in insulin

272 signalling proteins [9, 42]. We confirmed that ucOC is upregulated after exercise in humans

273 and provide a novel insight that BMD genetic variants may play a role in this response.

274 The current study has some potential limitations. First, it includes a relatively small sample

275 size in the context of genetic studies. Yet, even with a small sample size we were able to detect

276 a significant association of GRS with tOC and cOC. We are confident in our findings, as they

277 support previous findings of a GWAS that identified these SNPs to be associated with BMD

278 [16]. We were underpowered for the individuals SNP analysis (on average tOC- 19.2%, ucOC-

279 0.2% and cOC- 16.7%). However, our power improved greatly with the use of the genetic risk

280 score (tOC- 48%, ucOC- 14.9% and cOC- 44%) illustrating the strength of calculating a GRS

281 for data analysis. We acknowledge that while greater sample sizes are required, our data

282 provides hypothesis generating pilot data that offer interesting novel concepts for future

283 analysis. Secondly, we did not assess BMD, therefore a direct assessment of the SNPs with

284 BMD cannot be performed. Finally, we only tested young-healthy males. Future studies should

285 focus on young healthy females as well as older adults who have an increased risk for

286 osteopenia and osteoporosis.

287 In conclusion, screening for genetic variations may assist in identifying people at risk for

288 abnormal circulating levels of OC. Genetic variations in BMD predicted the response of ucOC

289 to acute exercise, but not tOC or cOC, indicating that physiological functional response to

290 exercise may be influenced by bone-related gene variants.

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