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Osteocalcin and its forms respond similarly to exercise in males and females

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Abstract

Introduction: Acute exercise increases osteocalcin (OC), a marker of bone turnover, and in particular the undercarboxylated form (ucOC). Males and females differ in baseline levels of total OC and it is thought the hormonal milieu may be driving these differences. Males and females adapt differently to the same exercise intervention, however it is unclear whether the exercise effects on OC are also sex-specific. We tested whether the responses of OC and its forms to acute High Intensity Interval Exercise (HIIE) and High Intensity Interval Training (HIIT) differed between males and females. Secondly, we examined whether sex hormones vary with OC forms within sexes to understand if these are driving factor in any potential sex differences.

Methods: Total OC (tOC), undercarboxylated OC (ucOC), and carboxylated OC (cOC) were measured in serum of 96 healthy participants from the Gene SMART cohort (74 males and 22 females) at rest, immediately after, and 3 h after a single bout of HIIE, and at rest, 48h after completing a four week HIIT intervention. Baseline testosterone and estradiol were also measured for a subset of the cohort (Males = 38, Females = 20). Linear mixed models were used to a) uncover the sex-specific effects of acute exercise and short-term training on OC forms and b) to examine whether the sex hormones were associated with OC levels.

Results: At baseline, males had higher levels of tOC, cOC, and ucOC than females ($q < 0.01$). In both sexes tOC, and ucOC increased to the same extent after acute HIIE. At baseline, in males only, higher testosterone was associated with higher ucOC ($\beta = 3.37$; $q < 0.046$). Finally, tOC and ucOC did not change following 4 weeks of HIIT.

Conclusion/Discussion: While there were no *long-term* changes in OC and its forms. tOC and ucOC were *transiently enhanced* after a bout of HIIE similarly in both sexes. This may be important in metabolic signalling in skeletal muscle and bone suggesting that regular exercise is needed to maintain these benefits. Overall, these data suggest that the sex differences in exercise adaptations do not extend to the bone turnover marker, OC.

Introduction

Bone remodelling is the cellular mechanism in maintaining bone health across the lifespan and is tightly regulated by both biochemical and mechanical factors. It is a complex process that involves the balance between the breakdown of bone tissue by osteoclasts (bone resorption) and the formation of new bone by osteoblasts (bone formation)[1]. Physical activity and diet are important in maintaining bone health, reducing the risks of metabolic and cardiovascular disease, and are the key to healthy ageing [2, 3]. The positive effect of exercise on bone and metabolic health may be partly due to changes in markers of bone turnover and their interaction with other organs, such as skeletal muscle [4, 5]. One such marker is total osteocalcin (tOC), which is used in clinical settings as a marker of bone turnover [6]. The undercarboxylated form of osteocalcin (ucOC) can act as a hormone and is shown to be involved in glucose regulation in some [4, 7-13] but not all studies [14, 15]. The carboxylated form of osteocalcin (cOC) is involved in bone mineralisation [5, 16, 17], and elevated cOC is positively correlated with bone formation and osteoblast number [5, 18].

Ageing is associated with a "U" shaped pattern of circulating levels of OC and its forms, with circulating levels of OC higher in young adults (aged from 18-30 years) and older individuals (aged > ~50yrs)[19, 20]. Dependent on the age of an individual this can either be beneficial or detrimental to bone health. When young, higher levels of OC are related to beneficial increases in bone formation while, in older individuals, it is related to bone loss due to an imbalance or uncoupling of bone resorption and formation [21]. Sex also influences circulating levels of tOC, in males the circulating level of tOC is higher compared to pre-menopausal females [22-24]. However, this changes once a female reaches menopause and the relationship reverses, with tOC higher in females than males in this age group [23-25]. In addition to age and sex, several studies have shown that an acute bout of exercise can elicit an increase total tOC and ucOC [26-28] and following an exercise training program[29-32]. However, it is unknown whether OC and its forms respond differently to exercise between sexes. This is important as literature has shown that males and females adapt differently to the same exercise stimulus [33-39].

It has been suggested that differences in the hormonal milieu are, in part, driving sex differences in bone turnover and adaptation to exercise [40, 41]. Estrogens and androgens are critical for bone maintenance in both sexes [40, 42, 43] as they slow the bone remodelling rate

and maintain the balance between bone resorption and formation [44, 45]. Estrogens include estrone (E1), 17-β estradiol (E2) and estriol (E3), the levels of which change throughout the lifespan, particularly in females. Testosterone is the primary circulating androgen and can act unmodified or be converted to the more potent dihydrotestosterone (DHT) or alternatively converted to E2 through aromatase actions [42]. There is discrepancies in the literature regarding whether sex hormones associate with OC forms. Some studies, but not all[46], reported that higher tOC levels are correlated with higher testosterone levels in healthy males, and males who suffer from chronic disease including; type 2 diabetes mellitus[47], hyperthyroidism[48], testosterone deficiency syndrome[49] and common bone disorders (i.e. Osteopenia and Osteoporosis)[50]. The discrepancies in the literature indicate the need for more clinical research to elucidate the relationship between OC and sex hormones.

A recent meta-analysis reported that acute exercise can induce changes in many serum bone turnover markers (BTMs) and the response of these BTMs was dependent on exercise modality, intensity, age and sex [51]. One such marker is ucOC which has been shown in the literature to consistently increase in response to an acute bout of exercise in different cohorts and has the strongest links to other aspects outside bone metrics[8, 27, 28, 52-54]. Therefore, we examined whether OC and its forms respond differently between sexes following both acute bout of High-Intensity Interval Exercise (HIIE), and four weeks of High-Intensity Interval Training (HIIT). We also examined whether testosterone and E2 vary with OC forms within sexes to understand if these are driving factors in any potential sex differences.

Methods

Participants: The tissue used in this study was from the Gene and Skeletal Muscle Adaptive Response to Training (Gene SMART) cohort, which is a part of on-going biobank[55]. We have previously investigated the effect of age[19] and genes[26] on the OC forms in the *males at* baseline, and after an acute exercise bout. The detailed methodology has been previously published [26, 56, 57]. Briefly, 74 healthy males (age = 31.4 ± 8.3 years-old; BMI = 25.0 ± 3.1 kg/m^2), and 22 healthy pre-menopausal females (age = 34.3 ± 7.2 years-old; BMI = 24.3 ± 4.7 kg/m2) participated in the study. Volunteers were excluded if they had a bone disease, were taking hypoglycaemic medications, warfarin or vitamin K supplementation, were using hormonal contraceptives, or any other 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 and all participants provided written informed consent.

Aerobic Capacity (Graded exercise test): Aerobic capacity was assessed by a graded exercise test (GXT) performed on an electronically-braked cycle-ergometer (Lode-Excalibur sport, Groningen, the Netherlands) to measure maximal oxygen uptake ($\dot{V}O_{2peak}$) and peak power output (W_{peak}). The $\dot{V}O_{2peak}$ was determined using a calibrated Quark CPET metabolic system (COSMED, Rome, Italy). The GXT consisted of four minute stages separated by 30 second rest periods until voluntary exhaustion with incremental increases in resistance at each stage. Capillary blood samples were collected at the end of each four minute stage and immediately after exhaustion and were analysed by the YSI 2300 STAT Plus system (Ohio, USA) to establish lactate concentration. Lactate Threshold (LT) was calculated by the modified DMAX method as previously described [56]. The GXT was performed in duplicate at both baseline and after the intervention and the average was calculated for all parameters between the two tests. In addition at baseline, participants performed a familiarisation test of the GXT.

Diet control (48h prior to testing): To standardise diet across the participants and minimise the effects of this confounding factor, each participant was provided with an individualised pre-packaged diet 48 hours prior to providing the blood samples [58, 59]. The energy content of the provided meals was calculated using the Mifflin St-Jeor equation using the participant's body mass, height and age [60]. 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 caffeine and alcohol throughout the 48 hour diet as well as food consumption 12 hours prior to blood collection.

Blood collection: Blood samples were collected at rest, immediately after and, three hours after the acute bout of HIIE, as well as at rest, 48h after completing a four week HIIT intervention. Venous blood samples were collected via venepuncture or cannulation in BD SST Vacutainers (Becton and Dickson Company, USA). 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.

Serum osteocalcin measurements: Circulating tOC, ucOC and cOC were measured using an automated immunoassay (Elecys 170; Roche Diagnostics). This assay has a sensitivity of 0.5 µg/L with an inter-assay imprecision of 5.4% at 24.1 ug/L. We measured ucOC with the same immuno-assay after absorption of carboxylated OC on 5mg/ml hydroxyl-apatite slurry as described by Gundberg et al. [61]. Inter-assay imprecision was 5.6% at 12 ug/L ucOC. cOC

was calculated by the subtracting the ucOC from the tOC. The peak tOC, ucOC and cOC were considered the maximal concentration immediately after, or three hours post exercise, which we abbreviated as PEAK. We used six samples as batch controls which had inter-assay variability of 2.8% for tOC and three samples for ucOC which had 3.9% CV. All participants fasted overnight (from 12am) and attend our laboratory between 8-9.30am in a fasted state, which minimises the diurnal effect of OC.

Hormone analysis: As the age of our cohort was 18-45 yrs, we measured the two major circulating sex hormones for this life stage, testosterone, and E2 [62, 63]. The assays were completed in the accredited pathology laboratory at Monash Health, Australia. Testosterone was measured using high performance liquid chromatography–mass spectrometry (HPLCMS/MS) method using a liquid sample extraction (AB Sciex Triple Quad 5500 LC/MS/MS system). Estradiol (E2) was measured using a competitive binding immunoenzymatic assay performed on a Beckman Coulter Unicel DXI 800 analyser.

Acute HIIE bout: Male and female participants completed HIIE on an electronically braked cycle ergometer (Velotron, Racer Mate Inc, Seattle, USA). Participants completed approximately five minutes of warm up at an intensity of their own choosing [range 25-60W] and then cycled for six x two minute intervals and this was interspersed with 1-min recovery periods at a power of 60 W (work to-rest ratio of 2:1). Intensity was individually-determined based on baseline GXT results and calculated as power at lactate threshold (LT) + 40% of the difference between peak aerobic power (Wpeak) and power at LT.

HIIT Intervention: Male and female participants trained three times/week under supervision. All training sessions were completed on an electronically braked cycle ergometer (Velotron, Racer Mate Inc, Seattle, USA) and were preceded by a five minute warm up at 50W. Each session consisted of six to fourteen 2-min intervals and was interspersed with 1-min recovery periods at a power of 60 W (work to-rest ratio of 2:1). Intensity was once again individually-determined based on baseline GXT results and calculated as power at lactate threshold (LT) + 40-70% of the difference Wpeak and power at LT. The intensity and/or the number of intervals were altered each session in order to maintain progression [56].

Statistical Analysis

All data were analysed using R studio version 4.0.2. Mann-Whitney tests were used to compare age, weight, BMI and fitness parameters between females and males. Robust linear models were performed to examine differences in sex hormones and OC forms at baseline between

sexes and was adjusted for age. A likelihood ratio test was used to compare the full model containing both age and sex, with a null model containing only age. We then fitted linear mixed-effects models, to see if an acute bout of HIIE or 4 weeks of HIIT training could alter the OC forms and if this was specific to sex. The model was of the form [outcome ~ age + timepoint*sex + BMI + baseline fitness + random intercept (participant ID)], where outcome was OC and its forms (tOC, ucOC, cOC, ucOC ratio and cOC ratio); the fixed effects were age, baseline BMI, baseline fitness (z-score), timepoint (PRE or PEAK for the acute response; PRE or 4WKP for the chronic response), sex, and the interaction between sex and timepoint. Baseline fitness was a z-score that combines W_{peak}, LT, and $\dot{V}O_{2peak}$ into a single value. First, we calculated the z-score for each fitness measure relative to body weight, and then we averaged those z-scores to obtain the final z-score. The random effect was the participants unique ID, accounting for repeated measures. Finally, to examine whether the sex hormones (testosterone and E2) were associated with OC levels *within* each sex, we ran the following model [OC ~ Age + Testosterone + E2 + random intercept (participant ID)], with baseline testosterone, and E2 as fixed effects. Only the sex hormones (Testosterone and Estradiol) required log-transformation. We assessed each model for normality by plotting the residuals against predicted values, the plots were equally spread and without any distinct patterns and therefore were deemed normal. P-values from the statistical analyses were adjusted for multiple testing using the false discovery rate (FDR) [64], and q-values < 0.05 were deemed significant. The following packages were used in our analysis *lme4* [65], *lmerTest* [66], *tidyverse* [67] *MASS* [68] and *lmtest* [69].

Results:

There were no differences in age between males and females (**Table 1**). BMI was slightly higher and fitness slightly lower in females compared with males. Males had higher circulating levels of tOC, ucOC and cOC than females ($p<0.001$). There were no differences in the ucOC ratio or cOC ratio between sexes.

Table 1: Baseline participant characteristics

	Females	**Males**	**p**
Age (years)	34.8 (7.0)	31.2 (8.2)	0.06
Weight (kg)	69.3 (13.7)	81.3 (12.3)	<0.001
BMI (kg/m2)	24.3 (4.7)	25.2 (3.3)	0.04

$\dot{V}O_{2peak}$ (mL/min/kg)	43.1 (10.1)	47.9 (8.1)	0.04
W_{peak} (W/kg)	3.2 (0.9)	3.7 (0.8)	0.03
LT (W/kg)	2.3 (0.8)	2.5 (0.6)	0.04
Testosterone (nmol/L)	0.81 (0.3)	20.2 (6.8)	<0.001
E2 (pmol/L)	218.4 (215)	102.9 (24.5)	0.01
tOC (ug/L)	20.1 (5.5)	30.5 (10.9)	<0.001
ucOC (ug/L)	8.4 (2.9)	11.8 (4.3)	<0.001
cOC (ug/L)	12.0 (3.9)	18.67 (8.2)	<0.001
ucOC/tOC	0.42 (0.09)	0.40 (0.9)	0.47
cOC/tOC	0.58 (0.09)	0.60 (0.9)	0.47

BMI, body mass index; W_{peak}, peak power output; LT, Lactate Threshold; OC, Osteocalcin; tOC, total OC; ucOC, Undercarboxylated OC; cOC, carboxylated OC; E2, estradiol.
Data is shown as mean ± SD.

tOC and ucOC increase after acute exercise to a similar degree in males and females

Overall, in the majority (68%) of participants, the OC level peaked immediately post exercise, with the remaining 32% of participants peaking at 3HP (see supplementary figure 1). Males had higher circulating levels of tOC, ucOC and cOC, compared with females ($q<0.001$) (**Figure 1**). tOC increased by 1.23 ug/L ($q < 0.001$) and ucOC by 0.97 ug/L ($q < 0.001$) after acute HIIE, and this increase was similar between males and females ($q > 0.05$) (**Figure 1, Supplementary table 1**). cOC did not change after acute HIIE ($q > 0.05$). Four weeks of HIIT increased $\dot{V}O_{2peak}$ ($q <0.05$), W_{peak} ($q < 0.001$) and LT ($q < 0.001$) to a similar degree in males and females ($q > 0.05$) (**Table 2**). Body mass and BMI did not significantly change after 4 weeks of HIIT (**Supplementary Table 4)**. OC and its forms did not change following 4 weeks of HIIT **(Figure 3, Supplementary table 2).**

Effect of sex hormones

In a subset of the original cohort (N=53), we examined whether sex hormone levels *within* each sex may contribute to sex differences in OC levels. Testosterone was significantly higher in males than females ($p<0.0001$), while E2 was significantly higher in females than males ($p=0.01$) (**Table 1**). In males, but not females those with higher baseline testosterone had higher levels of ucOC ($\beta = 3.37$; 95% CI= 0.62, 6.1; $q < 0.046$) **(Figure 2).** Testosterone was not

239 associated with tOC or cOC in either sex. E2 did not explain any variability in OC forms in
240 either males or females **(Figure 2).**
241
242

243 **Table 2: Participant characteristic before and after 4-week HIIT intervention.**

	Overall comparison				Female		Male		Sex-specific differences		Sex*Timepoint (Interaction)	
	PRE	**4WKP**	**p-value**	**q**	**Baseline**	**4WKP**	**PRE**	**4WKP**	**p-value**	**q**	**p-value**	**q**
$\dot{V}O_{2peak}$ (ml.min.kg)	46.9 (8.7)	47.8 (8.7)	0.03	**0.04**	43.1 (10.1)	44.1 (9.4)	47.9 (8.1)	48.9 (8.2)	0.02	**0.05**	0.48	0.64
Wpeak (W.kg.-1)	3.6 (0.8)	3.8 (0.8)	p<0.0001	**p<0.001**	3.2 (0.9)	3.4 (0.9)	3.7 (0.8)	3.9 (0.8)	0.01	**0.05**	0.65	0.65
LT (W.kg.-1)	2.5 (0.7)	2.7 (0.7)	p<0.0001	**p<0.001**	2.3 (0.8)	2.4 (0.8)	2.5 (0.6)	2.75 (0.7)	0.07	0.08	0.41	0.64

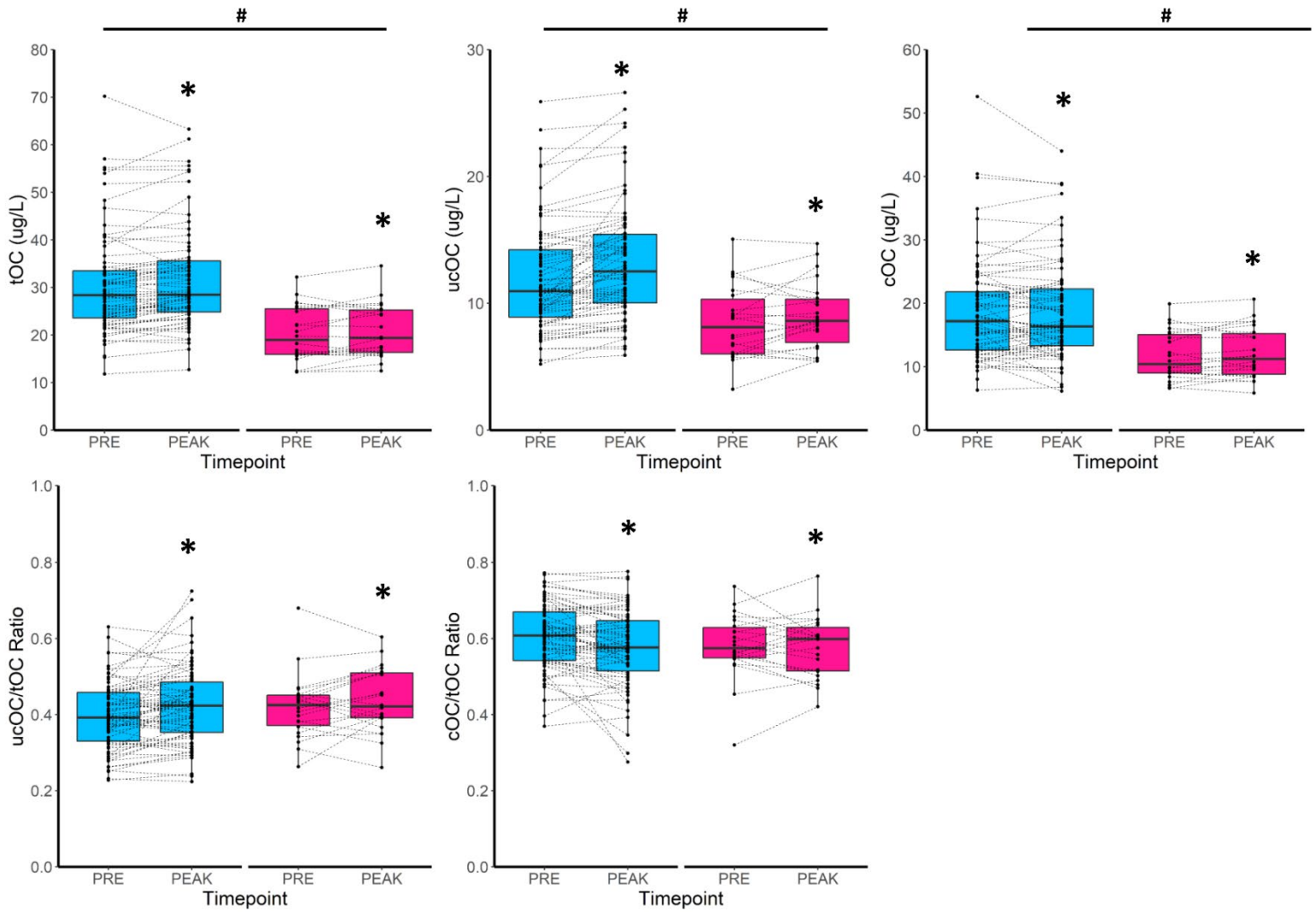


Figure 1: Circulating OC forms at baseline (PRE) and post exercise (PEAK) faceted by sex.

*Significant difference to PRE, #Significant difference between sex, adjusted for age, BMI and baseline fitness. FDR q<0.05 was deemed significant. Blue represents males and pink represents females.

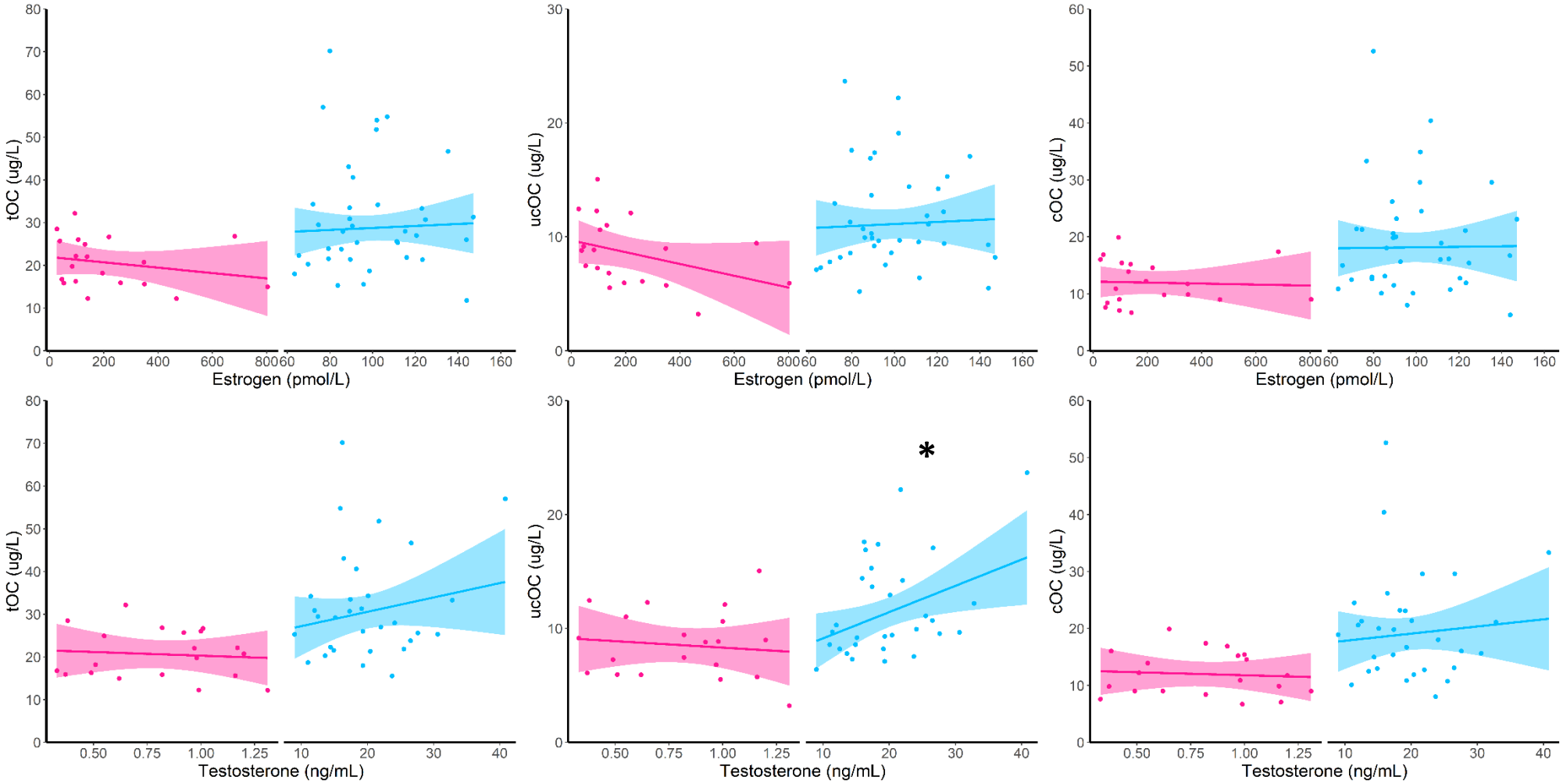
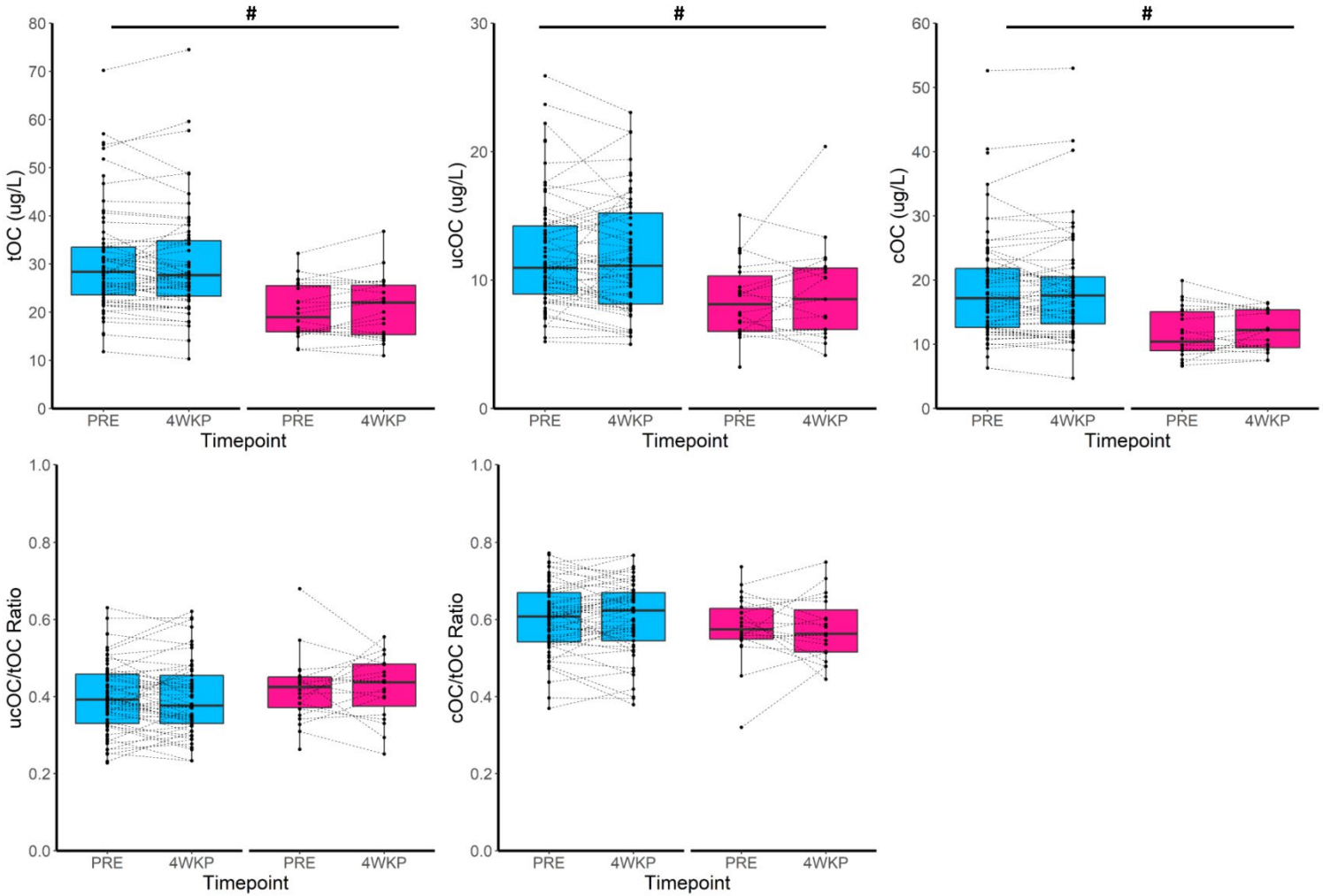


Figure 2: Associations between circulating OC forms with sex hormones (Testosterone and E2) stratified by sex and adjusted for age.

*Significantly associated. FDR q<0.05 was deemed significant. Blue represents males and pink represents females.



272 **Figure 3: Circulating OC forms at baseline (PRE) and post HIIT (4WKP) faceted by sex.**

273 #significant difference between sex, adjusted for age, BMI and baseline fitness. FDR q<0.05 was deemed significant. Blue represents males and

274 pink represents females.

Discussion

We report that males have higher tOC and ucOC than females but, both forms increase similarly between males and females after acute HIIE. The baseline differences in ucOC, may in part, be due to the differences in the hormonal milieu between sexes. We found that in males only, testosterone was positively correlated with ucOC. A 4-week HIIT intervention did not alter the circulating levels of OC and its forms in either sex.

Physical activity is recognised as one of the most effective lifestyle strategies to maintain bone health and metabolic function during ageing [5, 7, 18, 70, 71]. This effect of exercise has been shown, at least in part, to be mediated/associated with the exercise effect on bone metabolism, including its effect on OC [4, 72, 73]. Our results are in line with previous studies showing that an acute bout of exercise at both moderate and high intensities increase the level of ucOC in obese and healthy males, pre- and post-menopausal females and additionally, these studies have further shown that it is associated with a concomitant increase in insulin sensitivity [27-29, 74, 75]. We add to the growing literature that an acute bout of exercise can mediate transient changes in tOC and ucOC levels in young healthy males and females, before the levels return to baseline in both sexes after 3h-48h. Further, we showed that the response to an acute bout of exercise was similar in both sexes, suggesting that the sex differences in exercise adaptations do not extend to the bone turnover marker, OC.

We then examined whether the effect of high intensity exercise on OC forms can be accumulated following four weeks of exercise training. We report that the OC forms were not altered from baseline in the 48 hours after the last HIIT session. Bone formation is a slow process and we could hypothesise that to induce long-term changes in circulating levels of OC would perhaps require a longer intervention timeframe[76]. This has been shown in a recent meta-analysis where regardless of the exercise modality, a training intervention greater than 8 weeks induces a significant increase in ucOC [31]. Impact and the modality of exercise training is also an important factor as bone mechanical properties are modified depending on workload where mechanical stress must reach a minimum level to promote structural changes in the bone[77-79]. Our data suggest that HIIT (aerobic training) does not induce long-term changes in OC forms [31, 76]. This is in line with previous literature whereby, *long-term* changes in OC are not found following an aerobic only training intervention until it is combined with resistance training[29, 31, 76, 80]. As such, for long-term benefits, exercise training should include resistance/high impact exercises. While no *long-term* changes were detected in our

cohort, we cannot rule out that the transient ucOC enhancement after a bout of HIIE may be important in metabolic signalling in skeletal muscle, adipose tissue and bone[27, 71, 81-83].

Sex explained a large proportion of differences in levels of OC forms at baseline and following exercise, with males having higher levels of tOC, in line with previous literature [20, 23, 24]. We hypothesised that sex hormones may explain these differences, and we showed that testosterone was positively correlated with circulating levels of ucOC in young healthy males, consistent with some [84, 85], but not all literature [81]. In both sexes, E2 was not associated with any OC forms. The lack of association of E2 could be due to the younger age (18-45yrs) of the cohort [86]. As we age, particularly for females post menopause, there is a steep decline in E2 levels that is associated with elevated tOC and increased risk of osteoporosis [40, 43]. Taken together, this indicates that hormonal milieu, in particular testosterone, may mediate the differences in the level of ucOC, but not tOC or cOC, between males and females. This expands on previous literature [87] where we show that ucOC is associated with testosterone in males only, indicating a sex-specific mechanisms. The mechanistic pathways underlying the regulation of ucOC are controversial with some studies indicating a male-specific phenomenon in which circulating ucOC induces testosterone production in the Leydig cells of the testes, [88, 89], while others were unable to find a connection between testosterone production and ucOC [14, 15, 90]. Clearly more work is required to elucidate the causal mechanisms and the role of sex steroids in bone metabolism, in particular the bone turnover marker OC, to explain these sex differences.

We acknowledge there are limitations to consider in the interpretation of these findings. Several intrinsic and extrinsic factors may affect circulating OC levels, including vitamin K status. We did minimise these potential confounding effects of diet by supplying pre-packaged, personalised meals 48 hours prior to testing and blood sampling for each participant. We acknowledge that while we included a larger cohort of males, there was a smaller number of females included in the study. However, both sexes completed the same exercise intervention and testing. We analysed OC forms according to the methods proposed by Gundberg *et al*. [61], enabling the assessment of tOC and the OC forms from the *same* serum samples minimising batch effect. Furthermore, all samples were collected fasted and at the same time of day, which minimises the diurnal effect of OC.

While there were no *long-term* changes in OC and its forms. tOC and ucOC are *transiently enhanced* after a bout of HIIE similarly in both sexes. The transient increase in the bioactive form, ucOC, may play an important role in glucose regulation in skeletal muscle and suggest that regular exercise is needed to maintain these benefits. Clearly further work in humans is required to elucidate the mechanistic role of ucOC. Overall, these data suggest that the sex differences in exercise adaptations do not extend to OC.

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