Effect of eccentric-based resistance exercise on bone (re)modelling markers across the menstrual cycle and oral contraceptive cycle.

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Abstract

Purpose: To investigate the acute effects of eccentric-based resistance exercise and sex hormone fluctuations on P1NP and β-CTX-1 concentrations in premenopausal females.

Methods: Nine eumenorrheic females and ten oral contraceptive (OC) users performed eccentric-based resistance exercise, consisted of 10x10 repetitions of parallel back squats with a 4-second eccentric phase, in the early-follicular (EFP), late-follicular (LFP) and mid-luteal (MLP) phases of the menstrual cycle (MC) or in the withdrawal (WP) and active pill-taking (APP) phases of the OC cycle.

Results: 17β-oestradiol (pg·ml-1) was lower in EFP (36.63±29.93) compared to LFP (224.81±233.81;p=<0.001) and MLP (161.45±110.08;p<0.001) and higher in WP (24.857±29.428) compared to APP (12.72±13.36;p=0.004). Progesterone (ng·ml-1) was higher in MLP (8.30±5.23) compared to EFP (0.33±0.33;p<0.001) and LFP (0.21±0.18;p<0.001), no significant differences were observed between the WP and APP. In eumenorrheic females, β-CTX-1 (ng·ml-1) was lower in MLP (0.395±0.126) compared to LFP (0.472±0.137;p=0.044). Comparing MC vs OC phases, eumenorrheic females had higher P1NP levels (ng·ml-1) compared to OC users: EFP (62.54±13.13) vs APP (50.69±8,91;p=0.034), LFP (67.32±18.96) vs WP (52.16±10.72; p=0.047), LFP vs APP (p=0.025), MLP (67.51±19.34;p=0.049) vs WP, MLPvsAPP (p=0.027). Exercise time effect showed lower β-CTX-1 concentrations 2h post-exercise (MC: 0.376±0.114,p<0.001; OC: 0.340±0.156,p=0.030) compared to pre-exercise (MC: 0.485±0.137; OC: 0.428±0.188) in all participants.

Conclusions: β-CTX-1 concentrations were lower in the mid-luteal phase, emphasizing the importance of standardizing bone marker measurements to a specific MC phase. OC users exhibited reduced P1NP levels, underscoring the need to investigate synthetic and endogenous hormones' impact on long-term bone structure and strength.

Trial registration

The study was registered at Clinicaltrials.gov NCT04458662 on 2 July 2020.

Keywords: oestradiol; progesterone; sex hormones; P1NP; β-CTX; training

58 **Abbreviations**

P1NP	Procollagen type I N-propeptide
β-CTX-1	Cross-linking telopeptide of type I collagen
DXA	Dual-energy X-ray absorptiometry
OC	Oral contraceptive
MC	Menstrual cycle
WP	Withdrawal phase
APP	Active pill-taking phase
EFP	Early follicular phase
LFP	Late follicular phase
MLP	Mid luteal phase
1RM	1-repetition maximum
SHBG	Sex hormone binding globulin
SD	Standard deviation

59

Introduction

Exercise guidelines to improve bone strength generally recommend exercises that transmit both ground and joint reaction forces (e.g., impact and resistance-based modalities) (Beck et al. 2017). Therefore, exercise provides a stimulus for bone tissue, hence the analysis of procollagen type I N-propeptide (P1NP), as a biomarker of bone formation, and cross-linking telopeptide of type I collagen (β-CTX-1), reflecting bone resorption, may elucidate how exercise can affect bone metabolism given that traditional techniques [dual-energy X-ray absorptiometry (DXA), computed tomography or magnetic resonance imaging] are slow to respond to stimuli, and measurable changes take months or even years to occur (Hart et al. 2020; Eriksen 2010). Bone biomarker measurements are frequently used to provide insight into the bone response to acute exercise interventions (Dolan et al. 2022); specifically, they provide information about physiological alterations in bone metabolism, such as the prevalence of formative or resorptive activity (Hart et al. 2020). A recent meta-analysis (Dolan et al. 2022) shows that the typical bone response to an acute bout of exercise is an increase bone resorption and formation markers. Nevertheless, the circulating biomarkers response depends on the exercise type and impact loading, leading to highly variable outcomes (Dolan et al. 2022). Insufficient data were available to evaluate response to resistance exercise while bone resorption biomarkers showed no response (Dolan et al. 2022). However, sample timing is important as CTX-1 peaked within 2 h post-exercise (Dolan et al. 2022).

This relationship between exercise and bone remodelling becomes even more complex when considering the role of sex hormones. Beyond its reproductive function, 17β-oestradiol is also a key regulator of bone metabolism (Khosla and Monroe 2018). In fact, *in vitro* studies have shown that the oestrogen receptor is involved in the osteogenic response to mechanical stress, thus low concentrations of 17β-oestradiol could reduce the mechanosensitivity of osteocytes and the responsiveness of bone cells to mechanical load (Riggs et al. 2002; Windahl et al. 2013; Klein-Nulend et al. 2015). In addition, progesterone has been shown to stimulate osteoblast differentiation *in vitro* (Seifert-Klauss and Prior 2010). The female menstrual cycle (MC) is characterized by fluctuating 17β-oestradiol and progesterone (Oosthuyse et al. 2022), although not all females follow these patterns of hormonal fluctuations, given that there is a large

proportion of the athletic population that uses oral contraceptives (OC) (Martin et al. 2018). During the hormonal active pill-taking phase (APP) 17α-ethinyl oestradiol inhibits endogenous 17-β-oestradiol production, while in the placebo or withdrawal phase (WP), endogenous 17β-oestradiol increases again (Willis et al. 2006). Based on this theoretical justification, there is *in vivo* evidence on the effect of sex hormones on bone remodelling markers. Some investigations show that CTX-1 is lower during the luteal phase of ovulating females compared to the follicular phase (Mozzanega et al. 2013; Gass et al. 2008; Guisado-Cuadrado et al. 2024), and P1NP higher during luteal phase (Gass et al. 2008), although results remain inconsistent, as some studies show no differences between phases (Guzman et al. 2022). In turn, CTX-1 has been found to be lower in the APP compared to the WP (Martin et al. 2021). And when comparing ovarian hormonal profiles, OC users during the APP have lower P1NP and CTX-1 concentrations compared to eumenorrheic/non-OC users females (He et al. 2022; Guisado-Cuadrado et al. 2024; Glover et al. 2009). Considering this evidence, it is important to investigate whether different endogenous sex hormone concentrations could affect circulating concentrations of bone (re)modelling markers, both at rest and in response to resistance training.

Therefore, attending the close relationship between bone metabolism and ovarian sex hormones, this study aimed to examine the bone (re)modelling marker concentrations in eumenorrheic females and OC users at rest and in response to resistance training across the different phases of the MC and OC cycle.

Methods

Participants

Participants included in this study were a subsample selected from the participants enrolled in the IronFEMME project (Peinado et al. 2021). The purpose of IronFEMME was to determine the influence of sex hormones on iron metabolism and muscle damage, hence, the present study is a secondary analysis that was carried out after the trial was completed. This trial was registered at clinicaltrials.gov (NCT04458662). To be included in the IronFEMME study, participants were required to meet the following criteria: (i) healthy adult females between 18 and 40 years; (ii) regular MCs (defined as normally occurring MCs from 21 to 35 days in length) (Elliott-

Sale et al. 2021) at least 6 months prior to the study ; (iii) or using monophasic combined OC pills for at least 6 months prior to the study; (iv) no regular consumption of medication or nutritional supplements; (v) non-smokers; (vi) non-pregnant; and (vii) experienced in resistance training performing at least 30 min session two times per week during a minimum of a year. The present trial was performed as a secondary analysis using serum samples collected and frozen from the IronFEMME project. Participants from the IronFEMME project were further selected for inclusion in the current analysis according to: (i) aged between 20 and 30 years; (ii) bone injury free for the at least 12 months or muscle injury free for at least 6 months. Following this further selection, the data from nine eumenorrheic females and ten monophasic OC users (see Table 1 for participants' characteristics and training volume) were included in the current analysis. Participants received ethical clearance from the Research Ethics Committee of the Universidad Politécnica de Madrid and were informed of the study procedures (*i.e.*, for the present study on bone (re)modelling) and risks prior to participation and written informed consent was obtained from each subject prior to inclusion. Participants also agreed to the use of their data for other scientific purposes *a posteriori*.

MC and OC cycle monitoring

The protocols used for MC and OC cycle monitoring have been previously described (Peinado et al. 2021; Guisado-Cuadrado et al. 2024). In brief, for the MC group, menstruation, ovulation, and mid-luteal progesterone levels were established using gold-standard techniques (Elliott-Sale et al. 2021). Finally, MC phases were verified using blood samples taken on each of the eccentric testing days. The EFP was characterised by lower levels of 17β-oestradiol and progesterone. The LFP was characterised by higher 17β-oestradiol concentrations than in the EFP and MLP and higher progesterone concentrations than in the EFP, but lower than 6.36 nmol/L. The MLP was characterised by a progesterone concentration greater than 16 nmol/L and 17β-oestradiol higher than in the EFP but lower than in the LFP.

OC users took their active hormone pill daily for 21 days during the APP, followed by a 7-day WP (pill without hormonal content). Endogenous sex hormone concentrations were analyzed in serum in each phase. The mean duration of the OC use was 3.9±3 years (mean±SD). The brands and dosages of exogenous sex hormones in the

monophasic combined OC preparations used by these participants were as follows: Yasmin® (n=2): 0.03 mg ethinyl oestradiol and 3 mg drospirenone; Linelle® (n=1): 0.02 mg ethinyl oestradiol and 0.1 mg levonorgestrel; Sibilla® (n=1): 0.03 mg ethinyl oestradiol and 2 mg dienogest; Yasminelle® (n=1): 0.02 mg ethinyl oestradiol and 3 mg drospirenone; Levobel® (n=1): 0.02 mg ethinyl oestradiol and 0.10 mg levonorgestrel; YAZ® (n=1): 0.02 mg ethinyl oestradiol and 3 mg drospirenone; Diane 35® (n=1): 0,035 mg ethinyl oestradiol and 2 mg ciproterone; and Loette® (n=2): 0.02 mg ethinyl oestradiol and 0.1 mg levonorgestrel.

Experimental overview

Eumenorrheic participants came to the laboratory on four occasions, the first one to perform a 1-repetition maximum (1RM) test and the following three times (Figure 1) to perform an eccentric-based resistance exercise in each of the MC phases evaluated (EFP, LFP and MLP). Testing sessions took place on cycle days 4±1 for the EFP, 12±3 for the LFP and 23±2 for the MLP. The LFP testing session was arranged 2 days prior to estimated LH surge, which was based upon retrospective cycles' LH surge confirmation. If LH peak was not observed during the 2 subsequent days to LFP testing session, this trial was considered invalid. OC users came to the laboratory on 3 occasions, the first visit for the 1RM test and the following two occasions to carry out the eccentric-based resistance exercise on days 5±2 and 13±2 of WP and APP the OC cycle (Figure 1). This study analysed only the early pill-taking phase (first week after the first pill); however, it should be noted that exogenous sex hormone concentrations increase over the days of pill-taking (Willis et al. 2006). 24 h prior to all laboratory visits, all participants were instructed to refrain from alcohol, caffeine, and any intense physical activity or sport. Cycle phases order to perform the eccentric-based exercise protocol was randomized and counterbalanced for both eumenorrheic and OC participants.

1 RM estimation

On screening day, volunteers attended the laboratory between 8:00 a.m. and 10:00 a.m. in a resting and fasted state during the EFP in the eumenorrheic group and day 4-7 of the WP in the OC users. Baseline antecubital venous blood samples were collected for complete blood count, biochemical, and hormonal analysis. After collecting

187 the blood sample, a total body DXA was performed. The 1RM of the parallel back-squat
188 exercise was estimated by using the Powerlift App (Carlos Balsalobre-Fernández,
189 Madrid, Spain) (Balsalobre-Fernández et al. 2017), based on the force load-velocity
190 relationship (González-Badillo and Sánchez-Medina 2010). This app has been proved to
191 be highly valid, reliable, and accurate for the measurement of barbell velocity in the
192 squat exercise (Balsalobre-Fernández et al. 2017). Participants performed an
193 standardized warm-up (Peinado et al. 2021). After that, the test consisted of 4 sets of 1
194 repetition with submaximal loads proportionally increased between 70% and 90% of
195 participants' maximum self-reported. To record the videos, a researcher (always the
196 same) held an iPhone 6S (Apple Inc., Cupertino, CA, USA) in portrait position and
197 recorded each lift with a high-speed camera (240 Hz) (see the detailed methodology in
198 Peinado et al. (Peinado et al. 2021)).
199
200 *Eccentric-based resistance exercise*
201 After 1RM estimation, the eccentric-based resistance exercise sessions were
202 performed based upon the obtained values. The exercise protocol consisted of 10 sets
203 of 10 reps of plate-loaded barbell parallel back squats, at 60% of their 1RM, with 2 min
204 of rest between sets. Squats were performed at a tempo of 4-seconds eccentric
205 movement, 1-s pause at the bottom, 1-s concentric movement, and a 1-second pause
206 at the top of the lift. This protocol was designed for the IronFEMME project with the aim
207 of triggering muscle damage (MacDonald et al. 2014). Although, this work may extend
208 existing evidence, as the characteristics of the exercise protocol in this study differ from
209 others (Sherk et al. 2013; Rogers et al. 2011), as it focuses on the eccentric phase of
210 exercise.
211
212 *Blood collection*
213 Blood samples were taken between 8 and 11 a.m. to avoid diurnal variability of
214 biochemical parameters (Szulc et al. 2017) and within a participant the timeframe was
215 minimised to 1 hour within the 3 h total window in the different phases of the MC and
216 OC to reduce the intra-participant variability of the results. Two samples (pre- and 2h
217 post- eccentric-based resistance exercise) were drawn from each participant at each MC
218 or OC phase, from an antecubital vein while they were seated to determine the bone

(re)modelling marker [procollagen type I N-propeptide (P1NP) and carboxy-terminal cross-linking telopeptide of type I collagen (β-CTX-1)] and sex hormone (17β-oestradiol and progesterone) concentrations. Sex hormone binding globulin (SHBG) was measured only at rest. All venous blood samples were obtained using a 21-gauge (0.8 mm × 19 mm, Terumo®) needle. Blood samples for serum variables were collected in a 9 mL Z serum separator clot activator tubes (Vacuette®) and allowed to clot at room temperature for 60 minutes. They were then centrifuged for 10 minutes at 1610 g to obtain the serum (supernatant), divided into 600 µL aliquots, and stored at −80°C.

Blood analysis

17β-oestradiol, progesterone, SHBG, P1NP and β-CTX-1 were analysed in serum by electrochemiluminescent immunoassay using Roche Diagnostics reagents in a Cobas e411 Elecsys automated analyser (Roche Diagnostics GmbH, Mannheim, Germany) in the Spanish National Centre of Sport Medicine (Madrid, Spain). Inter-assay and intra-assay CV were: 1.8 and 2.4% at 57.2 $ng \cdot ml^{-1}$ level for P1NP; were 2.1 and 2.8% at 0.403 $ng \cdot ml^{-1}$ level for β-CTX; 11.9% and 8.5% at 93.3 $pg \cdot ml^{-1}$ and 6.8% and 4.7% at 166 $pg \cdot ml^{-1}$ for 17β-oestradiol; 23.1% and 11.8% at 0.7 $ng \cdot ml^{-1}$ and 5.2% and 2.5% at 9.48 $ng \cdot ml^{-1}$ for progesterone; and 2.4 and 2.8% at 44.2 $nmol \cdot l^{-1}$ level and 2.7 and 5.6% at 204 $nmol \cdot l^{-1}$ level for SHBG.

Nutritional recommendations

A nutritionist prescribed the breakfast meal, and participants replicated the same breakfast at least 2h prior to the eccentric-based resistance protocol in all the MC and OC phases. Nutritional recommendations were standardised 48 h prior the eccentric-based resistance protocol (for diet composition see Supplementary Material 1).

Statistical analysis

Normality tests were performed using the Shapiro-Wilk test. 17β-oestradiol, progesterone and SHBG were non-normally distributed, thus, they were log-transformed for analysis (Hackney and Viru 2008).

Participant characteristics were analysed using independent samples t-tests. SHBG was compared between MC phases (EFP vs LFP vs MLP) using a one-way ANOVA and OC cycle phases (WP vs APP) using a paired t-test. To compare SHBG between MC vs OC phases an independent t-test was conducted for each comparison. Mean concentrations of 17β-oestradiol, progesterone, P1NP and β-CTX-1 were compared between MC phases and OC cycle phases using the mixed linear model to analyse repeated measures. The phases and time were set as fixed effects (both intra-subject), and participants were set as random effects. Comparing hormonal profiles, the mixed linear model analysis was also performed, conducting a separate analysis for each of the following comparisons: EFP vs WP, EFP vs APP, LFP vs WP, LFP vs APP, MLP vs WP, and MLP vs APP. Ovarian hormonal profile (inter-subject) and time (intra-subject) were set as fixed effects, and participants were set as random effects. Bonferroni's post hoc test was applied to pairwise comparisons when the main effect was significant ($p<0.05$). The ANOVAs effects sizes are reported as partial eta squared (η^2p) whose interpretation is 0.01 = small, 0.06 = moderate, 0.14 = large effect. For pairwise comparisons Cohen's d was used and interpreted based upon the following criteria: 0.2 = small, 0.5 = medium, 0.8 = large effect (Cohen 1992). Data are presented as mean±1SD.

Results

Sex hormones

Significant main effect of phase was observed for 17β-oestradiol in eumenorrheic females, showing lower 17β-oestradiol levels in the EFP compared to the LFP ($p<0.001$; d=-2.144) and MLP ($p<0.001$; d=-2.036) (Table 2). No main effect of time or interaction was observed. While a significant main effect of phase was observed for progesterone, where concentrations were significantly higher in the MLP compared to the EFP ($p<0.001$; d=-2.840) and LFP ($p<0.001$; d=-3.194). No main effect of time or interaction was observed (Table 2). SHBG concentrations at rest were not significantly different between MC phases (see Table 2).

In OC users, significant main effect of phase was observed, where 17β-oestradiol concentrations were lower in the APP than in the WP. No main effect of time or interaction was observed (Table 2). Progesterone showed no main effect of phase, time,

or interaction (see on Table 2). SHBG concentrations at rest were not significantly different between OC phases (see Table 2).

Comparing different ovarian hormone profiles, a significant main effect of hormonal profile (MC vs OC) was observed for endogenous 17β-oestradiol in the following comparisons: EFPvsAPP ($F=8.288$; $p=0.010$; $\eta^2p=0.328$), LFPvsWP ($F=25.322$; $p<0.001$; $\eta^2p=0.598$), LFPvsAPP ($F=51.870$; $p<0.001$; $\eta^2p=0.753$), MLPvsWP ($F=30.173$; $p<0.001$; $\eta^2p=0.639$), and MLPvsAPP ($F=73.763$; $p<0.001$; $\eta^2p=0.813$), where 17β-oestradiol was higher in these MC phases compared to the OC phases indicated above (see 17β-oestradiol concentrations in Tables 2). Progesterone concentrations showed a significant main effect of hormonal profile for the following analyses: MLPvsWP ($F=34.120$; $p<0.001$; $\eta^2p=0.667$) and MLPvsAPP ($F=32.288$; $p<0.001$; $\eta^2p=0.655$), where progesterone concentrations were higher in the MLP compared to both OC phases (see concentrations in Tables 2). SHBG concentrations were higher in OC phases compared to MC phases: EFPvsWP ($p<0.001$; $d=-2.078$), EFPvsAPP ($p<0.001$; $d=-2.561$), LFPvsWP ($p=0.009$; $d=-1.355$), LFPvsAPP ($p=0.002$; $d=-1.732$), MLPvsWP ($p=0.022$; $d=-1.157$) and MLPvsAPP ($p=0.004$; $d=-1.508$) (see Table 2).

P1NP

No main effect of phase, time, or interaction (phase*time) within group was shown (see Figure 2).

A significant main effect of hormonal profile was shown comparing MC vs OC phases, where EFP ($F=5.329$; $p=0.034$; $\eta^2p=0.239$), LFP ($F=5.999$; $p=0.025$; $\eta^2p=0.261$) and MLP ($F=5.588$; $p=0.027$; $\eta^2p=0.257$) reflected higher P1NP concentrations compared to APP, while LFP ($F=4.580$; $p=0.047$; $\eta^2p=0.212$) and MLP ($F=4.516$; $p=0.049$; $\eta^2p=0.210$) showed a higher concentration in comparison with WP (see Figure 2).

β-CTX-1

Significant main effect of phase was observed in eumenorrheic females ($F=3.390$; $p=0.044$; $\eta^2p=0.257$), where serum concentrations were lower in the MLP compared to LFP ($p=0.044$; $d=0.617$); and main effect of time ($F=19.861$; $p<0.001$; $\eta^2p=0.871$), showing lower concentrations post-exercise (0.376 ± 0.114 ng·ml^{-1}) than pre-exercise (0.485 ± 0.137 ng·ml^{-1}). No interaction (phase*time) was observed (see Figure 2).

Significant main effect of time in OC users was observed (F=5.224; p=0.030; η^2p=0.445), showing lower values post-exercise (0.340±0.156 ng·ml^{-1}) compared to pre-exercise (0.428±0.188 ng·ml^{-1}). No main effect of phase or interaction was observed.

It was observed a main effect of hormonal profile in LFP vs APP analysis, reflecting higher β-CTX-1 concentrations in the LFP of the MC (F=14.181; p=0.040; η^2p=0.225) (see Figure 2).

Discussion

This study is the first to investigate the effect of eccentric-based resistance exercise on bone formation (P1NP) and resorption (β-CTX-1) markers in young eumenorrheic females and OC users, within and between both ovarian hormonal profiles. β-CTX-1 concentrations were significantly lower 2h after resistance exercise without any change in P1NP, regardless of ovarian hormonal profile or phase. This acute reduction in bone resorption may be succeeded by a reduction in bone formation, since, based on the bone remodelling traditional theory, bone resorption and bone formation processes are typically coupled (Heaney 1994). Nevertheless, it is unlikely that an acute decrease in bone resorption represents the beginning of the cycle of bone remodelling in response to exercise and is subsequently accompanied by a decrease in bone formation, as combined reductions in bone resorption and bone formation are more likely to occur under conditions of disuse (Hughes et al. 2020). What has been described in the literature is an increase in serum markers of bone resorption after the onset of mechanical loading, which is understandable considering the cellular response to this new stimulus (Hughes et al. 2020). Although the relationship between acute change in bone remodelling marker concentrations and long-term structural bone changes is not yet well understood and future research should investigate the long-term potential for a protective effect of eccentric resistance exercise on bone health, this study highlights the need to understand the characteristics of resistance training for optimal bone health in female athletes with different ovarian hormonal profiles.

The effect of resistance training on β-CTX-1 concentrations can only be directly compared with the study by Rogers et al. (2011), as it measured β-CTX-1 concentrations 2 hours post-exercise. The significantly lower β-CTX-1 concentrations observed 2 hours

after the eccentric-based resistance exercise in the present study do not align with the findings of Rogers et al. (2011), where no significant change in β-CTX-1 was reported 2 hours post-resistance exercise. Additionally, the present findings contradict the results of Dolan et al. (2022) in their meta-analysis, where no biomarker response was observed following a single session of resistance training. Nevertheless, it should be highlighted the different effects of this eccentric-based exercise modality performed in the present study, achieved by extending the eccentric phase over time (4 s eccentric movement), in comparison with previous studies. Eccentric contractions have unique characteristics, approximately 20-60% more force can be generated during eccentric contractions compared to concentric contractions, this fact is highly relevant in explaining the acute responses after exercise (Douglas et al. 2017). Given that muscle contraction is the main source of mechanical loading that causes bone adaptations, because of the mechanical, biochemical and molecular muscle-bone interplay (Brotto and Bonewald 2015), there is a need to better understand the characteristics of resistance exercise training that may further benefit bone health. In addition, it should be noted that none of these investigations (Sherk et al. 2013; Rogers et al. 2011) included eumenorrheic females, so it is unknown whether the influence of sex hormones may have affected the response to resistance exercise in these studies. Given the timing of the exercise session and the sample collection, it is possible that the lower β-CTX-1 concentrations observed 2 hours post-exercise were influenced by the biomarker's typical circadian pattern. β-CTX-1 follows a diurnal rhythm, peaking around 5:00 a.m. and reaching its lowest levels by approximately 2:00 p.m. (Szulc et al. 2017).

Comparing bone resorption and formation markers between phases of the MC, β-CTX-1 was lower in the MLP compared to LFP, regardless of whether pre or post exercise. As the 17β-oestradiol/progesterone ratio may be important in interpreting the effect of the menstrual cycle, LFP was included in this study because 17β-oestradiol concentrations are very high, and progesterone is very low compared to MLP. Thus, the LFP may represent an ideal time to assess the effect of 17β-oestradiol with relatively low progesterone concentrations. Whereas high 17β-oestradiol concentrations seem to increase bone formation *in vitro* (Klein-Nulend et al. 2015; Windahl et al. 2013) and *in vivo* models (Guisado-Cuadrado et al. 2024; Gass et al. 2008), progesterone seems to reduce β-CTX-1 concentrations. These findings agree with previous studies (Guisado-

375 Cuadrado et al. 2024; Mozzanega et al. 2013; Gass et al. 2008), suggesting that a lower
376 17β-oestradiol/progesterone ratio may decrease β-CTX-1 concentrations. However,
377 these findings differ from those reported by Guzman et al. (2022), where no differences
378 were found between MC phases (mid-late follicular and luteal phases) following a
379 running protocol. Nonetheless, it should be noted that the Guzman et al. (2022) study
380 did not measure the LFP (1–3 days before ovulation, day 12 ± 3), as defined in the
381 present study, but instead measured the mid-late follicular phase. The differences in
382 timing between the present study and Guzman et al. (2022) may explain the conflicting
383 results, as the participants in Guzman et al. (2022) had lower concentrations of 17β-
384 oestradiol in the follicular phase than in the luteal phase, whereas in the present study,
385 17β-oestradiol levels were higher in the LFP compared to the MLP. Therefore, as
386 previously suggested in the literature (Hackney et al. 2022), the relationship between
387 17β-oestradiol and progesterone must be taken into account when interpreting the
388 effect of the MC.
389 Regarding the results from OC users, no differences in P1NP and β-CTX-1 levels
390 were observed between OC phases (see days in Fig. 1). This contrasts with the findings
391 of He et al. (2022), where β-CTX-1 concentrations were lower in the mid APP (days 22 to
392 28) and P1NP concentrations were lower in the mid and late APP (days 10 to 26) at rest.
393 Additionally, our results disagree with those of Martin et al. (2021), who found lower β-
394 CTX-1 levels in the APP (days 15–16) compared to the WP (days 3–4) at rest. It is
395 important to highlight that the participants in the studies by He et al. (2022) and Martin
396 et al. (2021) used a specific OC formulation (30 µg ethinyl oestradiol and 150 µg
397 levonorgestrel), in contrast to the participants in our study, which may explain the
398 differences in results.
399 When MC and OC phases were compared, β-CTX-1 was lower in the APP
400 compared to the LFP. This finding is in line with other studies in which OC users were
401 shown to have significantly lower bone resorption marker levels (Glover et al. 2009; He
402 et al. 2022), suggesting an inhibition of bone metabolism. On the other hand, P1NP was
403 lower in the WP compared to the LFP and MLP of the MC, and in the APP compared to
404 all the MC phases. As mentioned above, 17β-oestradiol plays an important role in bone
405 metabolism by promoting bone formation (Klein-Nulend et al. 2015; Windahl et al.
406 2013). Over the course of the OC cycle, the concentration of endogenous 17β-oestradiol

in the APP was low, while in the WP it increases but remains low, similar to the EFP of the MC (see Table 2). This low concentration of endogenous 17β-oestradiol may explain the lower bone formation shown by OC users compared to eumenorrheic females, in line with other investigations (Glover et al. 2009; He et al. 2022). Nevertheless, OC users show a low concentration of endogenous sex hormones resulting from the negative feedback effect of synthetic hormones on the anterior pituitary (Willis et al. 2006). During APP the dose of synthetic hormones in these participants is 0.02-0.035 mg ethinyl oestradiol. However, although this synthetic hormone shows a similar affinity for the oestrogen receptor α as 17β-oestradiol (Gutendorf and Westendorf 2001), other factors may mediate the bioavailability of this hormone such as the low dose of synthetic hormone contained in these OCs, the possible binding of progestins to the oestrogen receptor α (Louw-Du Toit et al. 2017) or the significantly higher concentration of SHBG in OC users compared to eumenorrheic females (see Table 2 and Results section). In fact, other studies have already observed that ethinyl oestradiol (in OCs) has a dose-dependent stimulatory effect on hepatic SHBG production, leading to a reduction in bioavailable 17β-oestradiol (Riggs et al. 2002). Notably, other studies have already observed a negative association between SHBG and P1NP concentrations (Ackerman et al. 2019). These results may provide some evidence for differences in bone metabolism in women with different ovarian hormonal profiles, especially in altered bone formation. However, as mentioned previously, these results need to be supported by long-term studies to understand the effect of these bone markers on bone health. Although there is already some evidence of an association between exposure to OCs and lower BMD (Rocca et al. 2021; Guisado-Cuadrado et al. 2023).

The main strength of this study was the consideration of the hormonal environments throughout the MC and OC cycle, measuring serum 17β-oestradiol and progesterone, and using ovulation tests to measure LH surge according the most recent guidelines (Elliott-Sale et al. 2021). Furthermore, exercise trials were performed in the morning at the same time, using standardized protocols and indications (Szulc et al. 2017) for the preservation and measurement of serum sex hormones and bone (re)modelling markers to avoid variability within and between subjects. The exercise sessions were supervised by sports science professionals, which may help to

homogenise the stimulus achieved in each session by the participants. In addition, this original research could expand the knowledge on bone metabolism and exercise, since up to now most of the evidence refers to endurance (running or cycling) exercise in males (Dolan et al. 2022), while the data to evaluate response to resistance exercise were limited.

Methodological considerations

A specific type of OC with standardized composition and doses of synthetic hormones was not used. Given the different properties of different synthetic progestins in terms of binding affinities and transcriptional activities when binding to androgen or oestrogen receptors, there could be a different magnitude of effect and biological consequence (Louw-Du Toit et al. 2017). It should be mentioned that although endogenous sex hormones have been measured in serum and OC doses have been reported, in order to know the synthetic hormones bioavailability, ethinyl oestradiol and progestin serum concentrations should have been measured. Another limitation that may affect the interpretation of this study's results is that the β-CTX-1 marker exhibits diurnal variability (Szulc et al. 2017). This limitation could have been addressed by including a control group without exercise. Finally, the fact that samples were not taken immediately after exercise may have meant that some transient changes were missed.

Conclusion

In conclusion, after 2h post eccentric-based resistance β-CTX-1 concentrations were lower, regardless of ovarian hormonal status. This lower concentrations in β-CTX-1 do not seem to correspond to known physiological mechanisms triggered by exercise, suggesting the need for further exploration to understand the mechanism that triggers resistance training on bone metabolism. Analysing MC phases and OC use, our study revealed an influence of hormonal fluctuations on bone (re)modelling markers. Lower β-CTX-1 concentrations in the mid luteal phase suggesting that hormonal fluctuations impact bone resorption throughout the MC. In addition, when ovarian hormonal profiles were compared, OC users exhibited lower P1NP concentrations, emphasizing the importance of investigating the role of synthetic hormones and endogenous sex hormones in the potential long-term effects that these OCs may have on bone structure and strength.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethics approval and consent to participate

This study complied with the amended Declaration of Helsinki and was approved by Universidad Politécnica de Madrid Ethics Committee (December 21, 2015). All participants provided written informed consent to participate in the study and before the analyses, all data was depersonalized and anonymized.

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