# Influence of menstrual cycle and oral contraceptive phases on bone (re)modelling markers in response to interval running

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**ABSTRACT**

Introduction: To explore how sex hormone fluctuations may affect bone metabolism, this study aimed to examine P1NP and β-CTX-1 concentrations across the menstrual and oral contraceptive (OC) cycle phases in response to running.

Methods: 17β-oestradiol, progesterone, P1NP and β-CTX-1 were analysed pre- and post-exercise in eight eumenorrheic females in the early-follicular, late-follicular, and mid-luteal phases, while 8 OC users were evaluated during the withdrawal and active pill-taking phases. The running protocol consisted of 8x3min treadmill runs at 85% of maximal aerobic speed.

Results: 17β-oestradiol concentrations ($pg \cdot ml^{-1}$) were lower in early-follicular (47.22±39.75) compared to late-follicular (304.95±235.85;p=<0.001) and mid-luteal phase (165.56±80.6;p=0.003) and higher in withdrawal (46.51±44.09) compared to active pill-taking phase (10.88±11.24;p<0.001). Progesterone ($ng \cdot ml^{-1}$) was higher in mid-luteal (13.214±4.926) compared to early-follicular (0.521±0.365; p<0.001) and late-follicular phase (1.677±2.586;p<0.001). In eumenorrheic females, P1NP concentrations ($ng \cdot ml^{-1}$) were higher in late-follicular (69.97±17.84) compared to early-follicular (60.96±16.64;p=0.006;) and mid-luteal phase (59.122±11.77;p=0.002). β-CTX-1 concentrations ($ng \cdot ml^{-1}$) were lower in mid-luteal (0.376±0.098) compared to late-follicular (0.496±0.166; p=0.001) and early-follicular phase (0.452±0.148; p=0.039). OC users showed higher post-exercise P1NP concentrations in withdrawal phase (61.75±8.32) compared to post-exercise in active pill-taking phase (45.45±6;p<0.001). Comparing hormonal profiles, post-exercise P1NP concentrations were higher in early-follicular (66.91±16.26;p<0.001), late-follicular (80.66±16.35;p<0.001) and mid-luteal phases (64.57±9.68;p=0.002) to active pill-taking phase.

Conclusion: These findings underscore the importance of studying exercising females with different ovarian hormone profiles, as changes in sex hormone concentrations affect bone metabolism in response to running, showing a higher post-exercise P1NP concentrations in all menstrual cycle phases compared with active pill-taking phase of the OC cycle.

Keywords: endurance; oestradiol; eumenorrheic; bone health; P1NP; βCTX-1

## Introduction

Exercise is commonly considered beneficial for bone health and a preventive strategy for age-related bone loss [1] or to achieve a higher peak bone mass during the growth stage [2]. Exercise characteristics are, however, important to produce an osteogenic stimulus, including multidirectional movements, high impact intensity and load changes during training [3, 4]. On the contrary, participation in endurance sports that involve repetitive or lower impact loads (e.g., running) or non-weight bearing sports (e.g., cycling or swimming) generally do not produce skeletal benefits [5], although some controversy remains about the osteogenic effect of running [6]. Furthermore, bone stress injuries are a concern that directly affects female athletes, as their incidence is higher than in men (for a review, please see [7]). These injuries occur when excessive repetitive loads are introduced into bone tissue and an imbalance in bone metabolism favours the accumulation of microdamage over its removal and replacement by new bone tissue during the remodelling process (for a review, please see [7]).

Beyond its reproductive function, 17β-oestradiol has an important effect on bone cells, producing a longer osteoblast lifespan and an increase in their metabolic activity, thus participating in the bone formation/resorption balance [8]. Consequently, menstrual status is directly linked to bone health; menstrual disturbances have been identified as a risk factor for stress-related bone injuries and lower bone mineral density (BMD) [9, 10]. Regarding this issue, there are contradictory results in the literature, while general recommendations [11] for the analysis of bone (re)modelling markers analysis suggest that hormonal fluctuations across the menstrual cycle (MC) should be taken into account when measuring these parameters, other authors such as Guzman et al. [12] and Martin et al. [13] have found no differences in concentrations at different phases of the MC.

Given the high prevalence of oral contraceptive (OC) use among female athletes [14], different hormonal profiles should be considered when assessing bone health. During the hormonal active pill-taking phase (APP) 17α- ethinyl oestradiol inhibits endogenous 17β-oestradiol production, while in the placebo or withdrawal phase (WP), endogenous 17β-oestradiol increases again [15]. Nevertheless, there is limited evidence relating to the effect of taking OCs and the associated implications (exogenous hormone

supply and reduction of endogenous sex hormones) on bone (re)modelling markers, as well as their influence on the post-exercise bone remodelling markers concentrations.

A major challenge in assessing the bone metabolic response to a specific exercise intervention has been the methodology, since both BMD assessment, by dual-energy X-ray absorptiometry (DXA), and microarchitectural assessment, by computed tomography, require long-term interventions before changes become apparent [16], which increases the potential for many confounding factors to exert an affect. Thus, bone (re)modelling marker measurements have been used as an alternative to assess acute responses to a stimulus, such as exercise, although some questions remain over how they relate to longer-term outcomes (BMD, material and structural properties, etc.) [17, 18]. Specifically, the International Osteoporosis Foundation and the International Federation of Clinical Chemistry and Laboratory Medicine recommend the use of procollagen type I N-propeptide (P1NP) and carboxy-terminal cross-linking telopeptide of type I collagen (β-CTX-1) concentrations, as reference markers of bone formation and bone resorption [11]. Therefore, the general aim of this study was to examine the P1NP and β-CTX-1 responses to high-intensity running in eumenorrheic females and OC users. The specific objectives of this study were: 1) To compare P1NP and β-CTX-1 concentrations between the early follicular (EFP), late follicular (LFP) and mid-luteal (MLP) phases of the MC; 2) To compare P1NP and β-CTX-1 concentrations between the WP and APP of the OC cycle; 3) To compare P1NP and β-CTX-1 concentrations between the phases of the MC and OC cycle; 4) To compare pre- and post-exercise P1NP and β-CTX-1 concentrations in eumenorrheic females and OC users.

## Material and methods

*Participants*

Participants included in this study were part of the IronFEMME project, which received ethical clearance from the Research Ethics Committee of the University. 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. 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) [19] 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 or oophorectomized; and (vii) participating in endurance training between 3 and 12 h per week. By using blood samples collected as part of the IronFEMME study, the present trial was designed as a secondary analysis, for which the inclusion criteria were further narrowed beyond those determined for the IronFEMME project. These additional criteria were (i) age between 20 and 32 years; (ii) not taking collagen supplements, calcium, or any substance that interferes/participates in bone metabolism; (iii) not having suffered any bone fracture for at least one year prior to the start of the study; and (iv) participating in endurance training involving running (i.e., long distance running, trail running, triathlon) between 3 and 12 h per week (see Table 1 for training volume). Therefore, the study sample was limited to eight eumenorrheic females and eight monophasic OC users (see Table 1 for participants' characteristics and training volume). All participants 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*, which applies to the present study.

*MC and OC cycle monitoring*

MC monitoring was based on the three-step methodology (see Peinado et al. [20] for detailed protocol). The theoretical MC phases were predicted by a gynecologist using the calendar-based counting method, based on records of the length of each participant's last six MCs. Secondly, a urine-based predictor kit (Ellatest, Alicante, Spain) was used to identify the LH surge and subsequent ovulation. Participants collected their mid-morning urine (always at the same time of day) starting three to five days before the estimated LFP testing day until the test result was positive. A participant was excluded from the trial if a positive LH test result was not obtained in three MCs, was as they were considered to have anovulatory MCs, and if the progesterone concentration in the MLP was lower than 16 nmol/L. Finally, all phases were confirmed by serum sex hormone analysis taken on study days prior to the exercise bout. 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.

OC users took their active hormone pill daily for 21 days during the active pill-taking phase, followed by a 7-day withdrawal phase (pill without hormonal content). The mean duration of the OC use was 4.09±2.93 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=2): 0.02 mg ethinyl oestradiol and 0.1 mg levonorgestrel; Sibilla® (n=2): 0.03 mg ethinyl oestradiol and 2 mg dienogest (n=2); Edelsin® (n=1): 0.035 mg ethinyl oestradiol and 25 mg norgestimate; and Yasminelle® (n=1): 0.02 mg ethinyl oestradiol and 3 mg drospirenone.

*Experimental overview*

Eumenorrheic participants came to the laboratory on four occasions (Figure 1), the first for a maximal incremental treadmill test and the following three times to perform the intervallic running test in each phase of the MC phases (EFP, LFP and MLP). The EFP testing session took place on day 4±1 of the MC. The LPF testing session took place 2 days prior to predicted ovulation, on the day 12±2 of the MC; predicted ovulation was based on previous cycles in which ovulation was confirmed. If ovulation did not occur within 2 days of the predicted LPF testing session, the testing session was deemed invalid. The MLP took place on the day 21±3 of the MC. OC users came to the laboratory on 3 occasions, the first for the maximal incremental test and the following 2 times to carry out the intervallic test in the WP (day 6±1) and APP (day 22±5) of the OC cycle. The first session consisted of participants screening, while on the following sessions participants performed the interval running test in each of the MC and OC cycle phases in a randomized and counterbalanced manner. In the eumenorrheic group, the order of performance of the intervallic tests was randomized according to the phases of the MC as follows: EFP-LFP-MLP; LFP-MLP-EFP; MLP-EFP-LFP; LFP-EFP-MLP and EFP-MLP-LFP. For the group of OC users, the randomization was: WP-APP and APP-WP.

On day of screening, volunteers attended the laboratory between 8:00 a.m. and 10:00 a.m. in a resting and fasting 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 the blood sample, a total body DXA was performed. This screening session was completed with an incremental running exercise to exhaustion on a computerised treadmill (H/P/COSMOS 3PW 4.0, H/P/Cosmos Sports & Medical, Nussdorf-Traunstein, Germany) to determine their maximal oxygen uptake. Expired gases were measured breath-by-breath with a Jaeger Oxycon Pro gas analyser (Erich Jaeger, Viasys Healthcare, Friedberg, Germany). This incremental maximal protocol began with a 3 min warm-up at 6 km/h followed by the incremental test in which the initial speed was set at 8 km/h and then increased by 0.2 km/h every 12 s until exhaustion. Prior to the maximal aerobic test and all the intervallic running tests all participants were instructed to refrain from alcohol, caffeine, and any intense physical activity or sport 24 hours before to visit the laboratory.

*Intervallic running protocol*

After the screening day in which the maximal incremental treadmill test was performed with the objective of determining maximal aerobic speed (vVO2peak), interval running tests were performed based upon the obtained values. This intervallic protocol consisted of a 5-min warm-up at 60% of the vVO2peak followed by eight bouts of 3 min at 85% of the vVO2peak with 90-s recovery at 30% of the vVO2peak between bouts. Finally, a 5-min cooling down was performed at 30% of vVO2peak. The intervallic tests were performed in a maximum of two consecutive MCs or two consecutive OC cycles. This protocol was designed for the IronFEMME project with the aim of stimulating IL-6 production, resulting in the subsequent elevation of hepcidin 3 hours after exercise. [21] However, this protocol differs in characteristics with respect to those that have been used to study the bone (re)modelling markers response to exercise, which are typically continuous protocols (60–120 min) and intensity between at 65–75% $VO_{2max}$ [22].

*Blood collection*

Blood samples were taken between 8 and 10 am to avoid diurnal variability of biochemical parameters [11]. Intervallic tests were always performed between 8 a.m. and 10 a.m. as well, and the time window was reduced to 1 hour between tests in the different phases of the MC and OC to reduce the intra-participant variability of the results. Two samples (at rest and immediately post-exercise) were drawn from each participant at each MC and OC phase, from an antecubital vein while they were seated to determine the bone (re)modelling marker and sex hormone concentrations. 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*

17-β-oestradiol, progesterone, 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·ml$^{-1}$ level for P1NP; were 2.1 and 2.8% at 0.403 ng·ml$^{-1}$ level for β-CTX; 11.9% and 8.5% at 93.3 pg·ml$^{-1}$ and 6.8% and 4.7% at 166 pg·ml$^{-1}$ for 17β-oestradiol; and 23.1% and11.8% at 0.7 ng·ml$^{-1}$ and 5.2% and 2.5% at 9.48 ng·ml$^{-1}$ for progesterone.

*Corrections for plasma volume changes*

Plasma volume changes (ΔPV) can affect the interpretation of biochemical measurements in blood. In the current study the Dill and Costill equation was used for calculation of the % ΔPV using changes in serum total albumin levels post-exercise in each subject, given their correlation with % ΔPV [23]. The following equations [23] for P1NP and β-CTX-1 corrections were used:

a) $\%\,\Delta\mathrm{PV} = 100 * \left(\frac{(Albumin\ post) - (Albumin\ pre)}{(Albumin\ pre)}\right)$

b) $\mathrm{Bone\ marker}_{\mathrm{corrected}} = (\mathrm{Bone\ marker}_{\mathrm{uncorrected}}) * \left(1 - \frac{\%\,\Delta PV}{100}\right)$

Sex hormone concentrations were not corrected because, although part of the increase in post-exercise circulating hormone concentrations was a result of a decrease in plasma volume, the biological action of these hormones is of greater interest and the concentration of a hormone determines its effect [24].

*Nutritional recommendations*

In order to ensure that nutrient intake was not a confounding factor in our results, a nutritionist prescribed the breakfast meal, and participants replicated the same breakfast at least 2h prior to the intervallic tests in all the MC and OC phases before the different blood draws. Nutritional recommendations were standardised 48 h prior and 24 h following the running protocol (for diet composition see Supplementary Material 1).

*Statistical analysis*

Normality tests were performed using the Shapiro-Wilk test. Data for non-normally distributed variables were log-transformed for analysis [25].

Participant characteristics were analysed using independent samples t-tests. To explore our objectives, mean concentrations of bone (re)modelling markers and sex hormones were compared between MC phases (EFP vs LFP vs MLP) and OC cycle phases (WP vs APP) using the mixed linear model to analyse repeated measures. The phases and time were set as fixed effects (both intra-subject), and subjects were set as random effect. 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. In this case, the ovarian hormonal profile (inter-subject) and time (intra-subject) were set as fixed effects, and subjects 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 effects sizes are reported as partial eta squared ($\eta^2p$) whose interpretation is 0.01 = small, 0.06 = moderate, 0.14 = large effect. For significant post hoc comparisons Cohen's d was used and interpreted based upon the following criteria: 0.2 = small, 0.5 = medium, 0.8 = large effect [26]. Data are presented as mean±1SD.

## Results

*17 β-oestradiol*

17β-oestradiol showed significant main effects of phase in eumenorrheic females, showing lower 17β-oestradiol levels in the EFP compared to the LFP (p=<0.001; d=-2.099) and MLP (p=0.003; d=-1.731); and time, reflecting an increase from pre- to post exercise. There was, however, no interaction effect (see 17β-oestradiol levels on Table 2).

On the other hand, in OC users 17β-oestradiol showed a significant main effect of phase, reflecting lower endogenous 17β-oestradiol concentrations in the APP than in the WP (see 17β-oestradiol levels on Table 3); but no main effect of time and no interaction were observed.

Comparing sex hormone concentrations between ovarian hormonal profiles, endogenous 17β-oestradiol showed significant main effect of hormonal profile for EFPvsAPP, LFPvsWP, LFPvsAPP, MLPvsWP, and MLPvsAPP analyses, where 17β-oestradiol was higher in these MC phases compared to the APP phases. Moreover, a significant interaction (hormonal profile*time) was observed in LFPvs WP and LFPvsAPP, where pre- and post-exercise 17-β-oestradiol was higher in the LFP compared to WP (pre-: p=0.008, d=1.984; post-: p=0.018, d=1.786) and APP (pre-: p<0.001, d=3.452; post-: p<0.001, d=3.687) (see values in Tables 2 and 3 and statistics in Table 4).

*Progesterone*

Progesterone showed a significant main effect of phase, where concentrations were significantly higher in the MLP compared to the EFP (p<0.001; d=-4.047) and LFP (p<0.001; d=-3.381); and time, showing an increase from pre- to post-exercise; but no interaction (see progesterone levels on Table 2).

In OC users, endogenous progesterone showed a significant main effect of time, where post-exercise concentrations increased from pre-exercise; but no main effect of phase and no interaction were shown (see progesterone levels on Table 3).

Comparing sex hormone concentrations between ovarian hormonal profiles, progesterone showed a significant main effect of hormonal profile for MLPvsWP and MLPvsAPP, where progesterone concentrations were higher in the MLP compared to

298 WP and APP. Furthermore, a significant interaction (hormonal profile*time) was shown
299 for MLPvsWP and MLPvsAPP, where pre- and post-exercise progesterone was higher in
300 the MLP compared to WP (pre-: p<0.001, d=8.144; post-: p<0.001, d=6.467;
301 respectively) and APP (pre-: p=0.001, d=9.888; post-: p<0.001, d=7.871; respectively)
302 (see values in Tables 2 and 3 and statistics in Table 4).

303 *P1NP*

304 In eumenorrheic females, significant main effects of phase and time were
305 observed, but no interaction was shown. Higher P1NP concentrations were shown in the
306 LFP compared to the EFP (p=0.006; d=-0.659) and the MLP (p=0.002; d=0.734).
307 Moreover, post-exercise concentrations were higher than pre-exercise (see P1NP levels
308 on Table 2).

309 In OC users, significant main effects of phase, showing higher P1NP
310 concentrations in the WP compared to the APP; and time were observed, where post-
311 exercise concentrations were higher than pre-exercise. Moreover, a significant time
312 *phase interaction was shown, highlighting greater post-exercise concentrations in the
313 WP compared to the APP (p<0.001, d=2.419) (see P1NP concentrations on Table 3).

314 When P1NP levels were compared between participants with different ovarian
315 hormonal profiles (eumenorrheic vs OC users), P1NP main effect of hormonal profile in
316 EFPvsAPP, LFPvsWP, LFPvsAPP, MLPvsAPP was shown; where EFP, LFP and MLP
317 reflected a higher level of P1NP compared to APP, while LFP showed a higher
318 concentration in comparison with WP. In addition, a significant hormonal profile*time
319 interaction was shown in EFPvsAPP, LFPvsAPP and MLPvsAPP; showing lower post-
320 exercise P1NP concentrations in APP compared with EFP (p<0.001; d=1.879), LFP
321 (p<0.001; d=3.371) and MLP (p=0.002; d=2.311), without significant differences
322 between pre-exercise P1NP concentrations between MC phases and APP (see
323 concentrations in Tables 2 and 3 and statistics in Table 4).

324 *β-CTX-1*

325 There was a significant main effect of phase for β-CTX-1 in eumenorrheic
326 females, where pairwise comparisons reflected lower concentrations in the MLP
327 compared to the LFP (p=0.001; d=0.804) and the EFP (p=0.039; d=0.540). No other main

effects or interactions were shown (see values in Tables 2 and 3 and statistics in Table 4).

## Discussion

This study investigated β-CTX-1 and P1NP responses to intervallic running throughout the MC and OC phases, while comparing ovarian hormonal profiles. Bone formation, measured by P1NP concentrations, and bone resorption, determined by β-CTX-1, were affected by MC-related fluctuations following exercise, showing greater bone formation in the LFP and reduced bone resorption during the MLP. These results are not in line with those shown by Guzman et al. [12] where no differences were shown between MC phases [mid-late follicular phase (day 8±1) and luteal phase (day 22±3)] in a group of seven eumenorrheic females. Nevertheless, it should be noted that the Guzman et al. [12] study did not measure the EFP (in this study day 4±1) nor the LFP (as defined in the present study as 1-3 days before the ovulation day, day 12±2), but rather measured the mid or late follicular phase, depends on the individual characteristics of each female's MC. Moreover, luteal phase testing was scheduled 1 week after a positive LH detection kit test (day 22±3) in the Guzman et al. [12] trial, coinciding with our timing of MLP measurement (day 21±3). These differences in the timing of measurement during the MC between the present study and the Guzman et al. [12] trial could explain the discordant results, since the participants included in the Guzman et al. [12] study were not evaluated when progesterone concentrations were as low as our participants in the EFP (follicular phase [12]: 4.12±2.36 $ng \cdot ml^{-1}$) and did not reach as high 17β-oestradiol values in the follicular phase [12] (46.3±8.9 $pg \cdot ml^{-1}$) or the luteal phase [12] (67.3±23.4 $pg \cdot ml^{-1}$) as our participants in the LFP (see Table 2). Therefore, the fact that the present study showed a significant main effect of MC phase in P1NP in contrast to Guzman et al. may be supported by the known beneficial effect of 17β-oestradiol on bone metabolism in addition to the recognized role of progesterone as an 17β-oestradiol partner in bone metabolism, which has been shown to promote bone formation by increasing the number, maturation, and differentiation of osteoblasts *in vitro* [27].

Regarding results from OC users, no differences in P1NP and β-CTX-1 levels were observed at rest between OC phases (see days on Figure 1), in contrast to the results shown by He et al. [28] in which β-CTX-1 concentrations were lower in the mid APP (day 22 to 28) and P1NP concentrations were lower in the mid and late APP (day 10 to 26) at rest. Moreover, the results presented herein disagree with the Martin et al. [13] study, in which β-CTX-1 were lower in the APP (days 15-16) compared to the WP (days 3-4) at rest. It is worth noting that the participants included in the He et al. [28] and Martin et al. [13] studies used a specific formulation of OCs containing 30 µg ethinyl oestradiol and 150 µg levonorgestrel, as opposed to the participants included in the present study who did not standardize the composition and dosage of OC they used, which may explain the difference in resting results.

Additionally, this study is the first to show bone (re)modelling marker concentrations after exercise in OC users. The main finding is the greater increase in post-exercise P1NP in the WP compared to the APP following the same protocol, which may suggest the same exercise protocol may involve a different stimulus between phases of the OC cycle. This non-variation of post-exercise P1NP in APP, when 17β-oestradiol concentration is lower, could be linked to the existence of an 17β-oestradiol concentration threshold, which other studies hypothesize to be approximately 26-31 $pg \cdot ml^{-1}$, representing the threshold level below which oestrogen receptor α (ERα) in bone cells are not occupied by oestrogens, leading to a skeletal functional oestrogen deficiency [29]. Thus, the findings from the present study appear to support other studies conducted in *in vitro* models showing that the ERα 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 [30]. Nonetheless, exogenous sex hormones must be taken into consideration. Although ethinyl oestradiol shows a similar affinity to 17β-oestradiol to ERα [31], other factors could intervene in its effect on bone metabolism such as the low dose of ethinyl oestradiol contained in these OCs, the possible binding of progestins to the ERα [32] and the increase that other studies reported in levels of sex hormone-binding globulin [33], decreasing the bioavailability of ethinyl oestradiol to bind to the

ERα. Therefore, other factors associated with these synthetic hormones could mediate bone metabolism, decreasing the P1NP response to this running protocol.

Comparing bone (re)modelling markers between participants with different ovarian hormonal profiles, lower post-exercise P1NP concentrations were shown in the APP of the OC cycle compared to the EFP, LFP and MLP of the MC without significant differences in pre-exercise P1NP levels. Furthermore, significant differences between pre- and post-exercise in P1NP concentrations in all MC phases versus no difference between pre- and post-exercise in the APP of the OC cycle can be observed. Thus, considering the existence of significant differences between pre- and post-exercise values of 17β-oestradiol in the MC phases cycle versus the absence of differences in pre- and post-exercise values in OC users, it could be suggested that the increase in post-exercise P1NP concentrations could be associated with this increase in 17β -oestradiol, apart from the stimulus derived from exercise. This fact reinforces the role of ovarian sex hormones, especially 17-β-oestradiol, in the osteogenic response to an exercise stimulus [29, 30, 34]. In addition a significant main effect has also been shown, where higher P1NP levels were observed in the LFP of the MC compared to the WP of the OC cycle. Thus, the higher P1NP levels observed in the LFP could be positively influenced by the higher 17β-oestradiol concentrations, which may contribute to higher bone formation [29, 30, 34]. This higher bone formation might not be achieved by OC users due to its lower levels of 17β-oestradiol. These findings may provide some evidence of differences in bone metabolism in females with different ovarian hormonal profiles. Nonetheless, these results need to be supported by long-term studies conducted with healthy OC users using additional methods of bone health assessment (*i.e.* DXA or Quantitative Computed Tomography) to examine the possible effects that this difference in acute exercise response may have on bone health in the long-term, since an association has been observed between exposure to the use of hormonal contraceptives and BMD, which should be taken into account when assessing the OC effect on bone health [35, 36].

P1NP concentrations increased post-exercise, whereas β-CTX-1 values did not vary significantly in response to exercise in both groups. Although the running protocol

417 performed in this study (intervallic at 85% $VO_{2max}$) differs from others reported in the
418 literature (continuous protocols between 60-120 min and intensity at 65-75% $VO_{2max}$)
419 [22], the increase in P1NP appears to be in line with other studies in which participants
420 have performed running protocols [12, 37]. While some investigations have reported
421 post-exercise β-CTX-1 data in which the concentration did not vary [38], agreeing with
422 the present results, others have shown a decrease in this bone resorption marker [12].
423 Despite the fact that P1NP is considered an indicator of bone formation, some authors
424 have suggested that transient increases in P1NP may be related to exercise-induced
425 damage resulting in a small release of connective tissue content into the blood [22].
426 Nevertheless, controversy still surrounds the microdamage repair mechanism
427 suggesting that there are alternative mechanisms of direct repair of the bone matrix
428 that don´t need to involve removal and replacement of bone by remodelling [39]. In fact
429 the results of Seref-Ferlengez et al.,[39] suggest that alternative repair mechanisms exist
430 in bone to address matrix micro-cracks *in vitro*, given that previously damaged bone
431 tissue recovered control values 14 days after damage occurred. This alternative
432 mechanism may also explain this increase in post-exercise P1NP without any variation
433 in β-CTX-1. Although the results of the present study show an increase in bone formation
434 after exercise, this fact may not imply a long-term osteogenic effect, as there is still
435 limited evidence to interpret these bone (re)modelling marker data and some studies
436 suggest that endurance running training may decrease spine BMD [35, 40]. Therefore, a
437 long-term follow-up should be performed to really draw conclusions about the stimulus
438 of running training on bone health and to establish a relationship with the increase in
439 P1NP after high-intensity exercise.

440 The main strength of this study is the consideration of the hormonal
441 environments throughout the MC and OC cycle, by measuring serum 17β-oestradiol and
442 progesterone, and using ovulation tests to measure LH surge according to guidelines
443 [19]. In addition, exercise trials were performed in the morning, with a maximum
444 interval of one hour between trials, using standardized protocols and indications [11]
445 for the preservation and measurement of serum sex hormones and bone (re)modelling
446 markers to avoid within- and between-subject variability. Furthermore, this original
447 research could expand knowledge on this topic, as a recent systematic review with

meta-analysis only included studies on P1NP and/or β-CTX-1 responses to running exercise in healthy young adult males, evidencing the lack of similar studies in female populations, especially in premenopausal females [22].

There were some limitations, such as the fact that a specific type of OCs 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 [32]. Moreover, it should be mentioned that although endogenous sex hormones have been measured in serum and in the case of OC users the OC dosages have been reported, a good practice could be to measure the synthetic sex hormone concentrations in serum. Finally, it was not possible to provide the meals to the participants, which may be a limitation, as it was not possible to check whether the participants followed strictly the recommendations.

**Conclusions**

MC phase affected bone (re)modelling markers by showing higher bone formation, measured by P1NP concentrations, in the LFP and lower bone resorption, as measured by β-CTX-1, during the MLP. OC users showed decreased P1NP levels post-exercise in the APP without differences in pre-exercise levels, when endogenous 17β-oestradiol was lower and exogenous ethinyl oestradiol and progestins were higher. Moreover, a different behaviour of P1NP in post-exercise was seen between eumenorrheic females in all MC phases, where a significant increase in P1NP was shown, and OC users in the APP, where no post-exercise increase was observed. These findings underscore the importance of studying exercising females with different ovarian hormone profiles, as these changes in sex hormone concentrations affect bone metabolism in response to high intensity running exercise and could have long-term implications for bone health that should be studied. Therefore, since exercise is one of the stimuli that can influence bone health in female athletes, and as observed in this study, different sex hormone concentrations influence the acute response to a running stimulus, these two groups of female athletes should be studied independently if the objective is to assess bone health.

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## References


1. Allison, S.J., et al., *The Influence of High-Impact Exercise on Cortical and Trabecular Bone Mineral Content and 3D Distribution Across the Proximal Femur in Older Men: A Randomized Controlled Unilateral Intervention.* J Bone Miner Res, 2015. **30**(9): p. 1709-16.
2. Dowthwaite, J.N., et al., *A 2-yr, School-Based Resistance Exercise Pilot Program Increases Bone Accrual in Adolescent Girls.* Transl J Am Coll Sports Med, 2019. **4**(11): p. 74-83.
3. Ikedo, A., et al., *Comparison of Site-Specific Bone Mineral Densities between Endurance Runners and Sprinters in Adolescent Women.* Nutrients, 2016. **8**(12): p. 781.
4. Bellver, M., et al., *Bone mineral density and bone mineral content among female elite athletes.* Bone, 2019. **127**: p. 393-400.
5. Scofield, K.L. and S. Hecht, *Bone health in endurance athletes: runners, cyclists, and swimmers.* Curr Sports Med Rep, 2012. **11**(6): p. 328-34.
6. Chen, X., et al., *Nrf2 epigenetic derepression induced by running exercise protects against osteoporosis.* Bone Research, 2021. **9**(1).
7. Hoenig, T., et al., *Bone stress injuries.* Nat Rev Dis Primers, 2022. **8**(1): p. 26.
8. Khosla, S., M.J. Oursler, and D.G. Monroe, *Estrogen and the skeleton.* Trends Endocrinol Metab, 2012. **23**(11): p. 576-81.
9. Hutson, M.J., et al., *Incidence of bone stress injury is greater in competitive female distance runners with menstrual disturbances independent of participation in plyometric training.* Journal of Sports Sciences, 2021. **39**(22): p. 2558-2566.
10. Southmayd, E.A., et al., *Energy Deficiency Suppresses Bone Turnover in Exercising Women With Menstrual Disturbances.* J Clin Endocrinol Metab, 2019. **104**(8): p. 3131-3145.
11. Szulc, P., et al., *Use of CTX-I and PINP as bone turnover markers: National Bone Health Alliance recommendations to standardize sample handling and patient preparation to reduce pre-analytical variability.* Osteoporosis International, 2017. **28**(9): p. 2541-2556.
12. Guzman, A., et al., *Menstrual Cycle Related Fluctuations in Circulating Markers of Bone Metabolism at Rest and in Response to Running in Eumenorrheic Females.* Calcified Tissue International, 2022.
13. Martin, D., et al., *Bone metabolic marker concentrations across the menstrual cycle and phases of combined oral contraceptive use.* Bone, 2021. **145**: p. 115864.
14. Martin, D., et al., *Period Prevalence and Perceived Side Effects of Hormonal Contraceptive Use and the Menstrual Cycle in Elite Athletes.* Int J Sports Physiol Perform, 2018. **13**(7): p. 926-932.

15. Willis, S.A., et al., *Greater inhibition of the pituitary--ovarian axis in oral contraceptive regimens with a shortened hormone-free interval.* Contraception, 2006. **74**(2): p. 100-3.
16. Dolan, E., et al., *The Bone Metabolic Response to Exercise and Nutrition.* Exercise and Sport Sciences Reviews, 2020. **48**(2): p. 49-58.
17. Dolan, E., et al., *The Bone Biomarker Response to an Acute Bout of Exercise: A Systematic Review with Meta-Analysis.* Sports Medicine, 2022.
18. Hart, N.H., et al., *Biological basis of bone strength: anatomy, physiology and measurement.* J Musculoskelet Neuronal Interact, 2020. **20**(3): p. 347-371.
19. Elliott-Sale, K.J., et al., *Methodological Considerations for Studies in Sport and Exercise Science with Women as Participants: A Working Guide for Standards of Practice for Research on Women.* Sports Medicine, 2021. **51**(5): p. 843-861.
20. Peinado, A.B., et al., *Methodological Approach of the Iron and Muscular Damage: Female Metabolism and Menstrual Cycle during Exercise Project (IronFEMME Study).* International Journal of Environmental Research and Public Health, 2021. **18**(2): p. 735.
21. Sim, M., et al., *Effect of Exercise Modality and Intensity on Postexercise Interleukin-6 and Hepcidin Levels.* International Journal of Sport Nutrition and Exercise Metabolism, 2013. **23**(2): p. 178-186.
22. Civil, R., et al., *P1NP and β-CTX-1 Responses to a Prolonged, Continuous Running Bout in Young Healthy Adult Males: A Systematic Review with Individual Participant Data Meta-analysis.* Sports Medicine - Open, 2023. **9**(1).
23. Alis, R., et al., *Hemoconcentration induced by exercise: Revisiting the Dill and Costill equation.* Scand J Med Sci Sports, 2015. **25**(6): p. e630-7.
24. Borer, K.T., *Advanced exercise endocrinology*. 2013, Human Kinetics. p. 219-228.
25. Hackney, A.C. and A. Viru, *Research methodology: endocrinologic measurements in exercise science and sports medicine.* J Athl Train, 2008. **43**(6): p. 631-9.
26. Cohen, J., *Statistical Power Analysis.* Current Directions in Psychological Science, 1992. **1**(3): p. 98-101.
27. Li, D., et al., *Negative Spinal Bone Mineral Density Changes and Subclinical Ovulatory Disturbances—Prospective Data in Healthy Premenopausal Women With Regular Menstrual Cycles.* Epidemiologic Reviews, 2013. **36**(1): p. 137-147.
28. He, W., et al., *Fluctuations in Metabolites and Bone Markers Across the Menstrual Cycle in Eumenorrheic Women and Oral Contraceptive Users.* The Journal of Clinical Endocrinology & Metabolism, 2022. **107**(6): p. 1577-1588.
29. Riggs, B.L., S. Khosla, and L.J. Melton, *Sex Steroids and the Construction and Conservation of the Adult Skeleton.* Endocrine Reviews, 2002. **23**(3): p. 279-302.
30. Windahl, S.H., et al., *Estrogen receptor-α is required for the osteogenic response to mechanical loading in a ligand-independent manner involving its activation function 1 but not 2.* Journal of Bone and Mineral Research, 2013. **28**(2): p. 291-301.
31. Gutendorf, B. and J. Westendorf, *Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens.* Toxicology, 2001. **166**(1): p. 79-89.
32. Louw-Du Toit, R., et al., *Comparing the androgenic and estrogenic properties of progestins used in contraception and hormone therapy.* Biochemical and Biophysical Research Communications, 2017. **491**(1): p. 140-146.
33. Noé, G., M. Pavez, and H.B. Croxatto, *Changes in serum levels of SHBG, endogenous ligands and levonorgestrel induced by ethinylestradiol in NORPLANTR users.* Contraception, 1992. **45**(3): p. 187-201.
34. Klein-Nulend, J., et al., *Bone cell mechanosensitivity, estrogen deficiency, and osteoporosis.* J Biomech, 2015. **48**(5): p. 855-65.

35. Guisado-Cuadrado, I., et al., *Influence of sex hormones status and type of training on regional bone mineral density in exercising females.* European Journal of Sport Science, 2023: p. 1-19.
36. Rocca, M.L., et al., *Bone health and hormonal contraception.* Minerva Obstet Gynecol, 2021. **73**(6): p. 678-696.
37. Scott, J.P., et al., *The role of exercise intensity in the bone metabolic response to an acute bout of weight-bearing exercise.* J Appl Physiol (1985), 2011. **110**(2): p. 423-32.
38. Stunes, A.K., et al., *Acute Effects of Strength and Endurance Training on Bone Turnover Markers in Young Adults and Elderly Men.* Front Endocrinol (Lausanne), 2022. **13**: p. 915241.
39. Seref-Ferlengez, Z., et al., *Structural and Mechanical Repair of Diffuse Damage in Cortical Bone In Vivo.* Journal of Bone and Mineral Research, 2014. **29**(12): p. 2537-2544.
40. Hind, K., J.G. Truscott, and J.A. Evans, *Low lumbar spine bone mineral density in both male and female endurance runners.* Bone, 2006. **39**(4): p. 880-885.

588 TABLES
589

| | *Eumenorrheic females* | *Oral contraceptives users* | *p* |
|---|---|---|---|
| Age (years) | 30.45±5.28 | 25.52±3.99 | 0.016* |
| % Fat | 23.11±8.78 | 25.75±7.26 | 0.523 |
| Total BMD (gr/cm$^2$) | 1.21±0.1 | 1.19±0.0.06 | 0.699 |
| $VO_2$max (ml·kg$^{-1}$·min$^{-1}$) | 49.55±4.416 | 47.31±5.91 | 0.406 |
| Running training volume (min·week$^{-1}$) [&] | 127.81±126.82 | 204.0±119.28 | 0.236 |
| Menstrual cycle length | 30.4±3.2 | | |

590 ***Table 1.*** *Participant characteristics show as mean±SD. [&]Training volume during the 6 months prior to*
591 *recruitment. Eumenorrheic participants = 8, Oral contraceptive participants = 8. VO2max = maximal*
592 *oxygen uptake; BMD = total bone mineral density. * Significant differences between groups (p<0.05).*

593

| | | ***Eumenorrheic females*** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *EFP* | *LFP* | *MLP* | *Total* | | ***Main effects*** | | |
| | | | | | | | *Phase* | *Time* | *Phase*Time* |
| *P1NP (ng·ml$^{-1}$)* | *Pre* | 55.02±15.76 | 58.87±11.98 | 53.67±11.62 | 55.86±12.86 | **F** | 8.45 | 50.193 | 1.381 |
| | *Post* | 66.91±16.26 | 80.66±16.35 | 64.57±9.68 | 70.71±15.59$^{\$}$ | **p** | 0.001 | <0.001 | 0.265 |
| | *Total* | 60.96±16.64 | 69.97±17.84* | 59.122±11.77$^{\#}$ | | **η²p** | .414 | 0.939 | .261 |
| *ß-CTX-1 (ng·ml$^{-1}$)* | *Pre* | 0.478±0.168 | 0.483±0.197 | 0.388±0.126 | 0.45±0.165 | **F** | 7.929 | .044 | 1.126 |
| | *Post* | 0.426±0.132 | 0.509±0.140 | 0.364±0.068 | 0.433±0.128 | **p** | 0.001 | 0.835 | 0.336 |
| | *Total* | 0.452±0.148 | 0.496±0.166 | 0.376±0.098*$^{\#}$ | | **η²p** | .454 | .007 | .185 |
| 17-β-oestradiol (pg·ml$^{-1}$) | *Pre* | 40.73±33 | 239.94±182.36 | 127.96±45.47 | 136.21±134.23 | **F** | 39.956 | 4.397 | 0.088 |
| | *Post* | 53.7±46.93 | 369.96±276.18 | 203.159±92.85 | 208.94±209.57$^{\$}$ | **p** | <0.001 | 0.043 | 0.916 |
| | *Total* | 47.22±39.75 | 304.95±235.85* | 165.56±80.6* | | **η²p** | 0.702 | 0.924 | 0.209 |
| Progesterone (ng·ml$^{-1}$) | *Pre* | 0.292±0.151 | 1.471±2.845 | 12.463±4.96 | 4.74±6.42 | **F** | 91.145 | 0.783 | 1.404 |
| | *Post* | 0.749±0.378 | 1.884±2.477 | 13.966±5.109 | 5.533±6.869$^{\$}$ | **p** | <0.001 | 0.005 | 0.259 |
| | *Total* | 0.521±0.365 | 1.677±2.586 | 13.214±4.926*$^{\#}$ | | **η²p** | 0.875 | 0.619 | 0.445 |

***Table 2.*** *P1NP, ß-CTX-1, 17-ß-oestradiol, and progesterone (mean±SD) of eumenorrheic participants in the different menstrual cycle phases. EFP: early follicular phase; LFP: late follicular phase; MLP: mid-luteal phase. *Significantly different from total EFP. # Significantly different from total LFP. $ Significantly different from total pre-exercise. Significant differences p<0.05.*

| | | Oral contraceptive users | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *WP* | *APP* | *Total* | ***Main effects*** | | | |
| | | | | | | Phase | Time | Time*Phase |
| *P1NP (ng·ml$^{-1}$)* | *Pre* | 49.43±6.26 | 47.19±6.1 | 48.31±6.08 | **F** | 45.72 | 14.884 | 26.346 |
| | *Post* | 61.75±8.32 | 45.45±6$^{\#}$ | 53.6±10.95$^{\$}$ | **p** | <0.001 | <0.001 | <0.001 |
| | *Total* | 55.59±9.54 | 46.32±5.91* | | **η²p** | 0.876 | 0.674 | 0.782 |
| *β-CTX-1 (ng·ml$^{-1}$)* | *Pre* | 0.411±0.136 | 0.368±0.092 | 0.390±0.114 | **F** | 2.516 | 0.353 | 0.106 |
| | *Post* | 0.415±0.156 | 0.354±0.106 | 0.384±0.132 | **p** | 0.128 | 0.559 | 0.748 |
| | *Total* | 0.413±0.141 | 0.361±0.096 | | **η²p** | 0.149 | 0.061 | 0.138 |
| 17-β-oestradiol (pg·ml$^{-1}$) | *Pre* | 32.75±26.47 | 9.60±10.06 | 21.17±22.74 | **F** | 37.899 | 3.148 | 0.058 |
| | *Post* | 60.28±55.06 | 12.16±12.87 | 36.22±45.94$^{\$}$ | **p** | <0.001 | 0.091 | 0.380 |
| | *Total* | 46.51±44.09 | 10.88±11.24* | | **η²p** | 0.653 | 0.875 | 0.674 |
| Progesterone (ng·ml$^{-1}$) | *Pre* | 0.304±0.151 | 0.355±0.109 | 0.329±0.13 | **F** | 3.321 | 56.654 | 0.138 |
| | *Post* | 0.772±0.307 | 0.834±0.312 | 0.778±0.305$^{\$}$ | **p** | 0.083 | <0.001 | 0.812 |
| | *Total* | 0.513±0.319 | 0.595±0.335 | | **η²p** | 0.589 | 0.779 | 0.013 |

***Table 3**. P1NP, β-CTX-1, 17-β-oestradiol, and progesterone (mean±SD) of oral contraceptive users in the different oral contraceptive cycle phases. WP: withdrawal phase; APP: active pill-taking phase. *Significantly different from total WP. # Significantly different from post-exercise WP. $ Significantly different from total pre-exercise. Significant differences p<0.05.*

| | | *EFPvsWP* | | | *EFPvsAPP* | | | *LFPvsWP* | | | *LFPvsAPP* | | | *MLPvsWP* | | | *MLPvsAPP* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ***F*** | ***p*** | **$\eta^2p$** | ***F*** | ***p*** | **$\eta^2p$** | ***F*** | ***p*** | **$\eta^2p$** | ***F*** | ***p*** | **$\eta^2p$** | ***F*** | ***p*** | **$\eta^2p$** | ***F*** | ***p*** | **$\eta^2p$** |
| ***P1NP*** | **Hormonal profile** | 0.623 | 0.443 | 0.042 | 7.141 | 0.018* | 0.329 | 6.909 | 0.020* | 0.878 | 22.933 | <0.001* | 0.621 | 0.635 | 0.439 | 0.043 | 10.318 | 0.006* | 0.424 |
| | **Time*H.Profile** | 0.629 | 0.784 | 0.005 | 14.711 | 0.002* | 0.513 | 3.113 | 0.099 | 0.184 | 65.414 | <0.001* | 0.824 | 0.276 | 0.608 | 0.018 | 30.183 | <0.001* | 0.684 |
| ***β-CTX-1*** | **Hormonal profile** | 0.373 | 0.551 | 0.026 | 2.164 | 0.163 | 0.134 | 1.115 | 0.309 | 0.074 | 3.925 | 0.068 | 0.068 | 0.136 | 0.718 | 0.01 | 0.085 | 0.774 | 0.006 |
| | **Time*H.Profile** | 1.338 | 0.267 | 0.088 | 0.333 | 0.537 | 0.024 | 1.147 | 0.302 | 0.076 | 2.068 | 0.172 | 0.173 | 0.000 | 0.985 | 0.001 | 0.113 | 0.742 | 0.008 |
| ***17-βoestradiol*** | **Hormonal profile** | 0.006 | 0.940 | 0.001 | 11.203 | 0.005* | 0.445 | 14.311 | 0.002* | 0.505 | 51.719 | <0.001* | 0.787 | 18.521 | <0.001* | 0570 | 93.680 | <0.001* | 0.870 |
| | **Time*H.Profile** | 11.485 | 0.004* | 0.451 | 0.203 | 0.602 | 0.020 | 5.963 | 0.028* | 0.299 | 3.818 | 0.010* | 0.214 | 4.024 | 0.065 | 0.223 | 4.162 | 0.060 | 0.230 |
| ***Progesterone*** | **Hormonal profile** | 0.010 | 0.996 | 0.001 | 1.649 | 0.220 | 0.105 | 1.552 | 0.233 | 0.100 | 0.621 | 0.444 | 0.043 | 310.473 | <0.001* | 0.957 | 369.378 | <0.001* | 0.963 |
| | **Time*H.Profile** | 0.010 | 0.920 | 0.002 | 1.132 | 0.721 | 0.009 | 0.005 | 0.946 | 0.001 | 0.005 | 0.945 | 0.001 | 9.002 | 0.010* | 0.391 | 27.838 | <0.001* | 0.665 |

***Table 4.*** *Statistics (F, p and $\eta^2p$) of each analysis performed to compare the phases of the different ovarian hormonal profiles. EFP: early follicular phase; LFP: late follicular phase; MLP: mid-luteal phase; WP: withdrawal phase; APP: active pill-taking phase. *Significant main effect.*

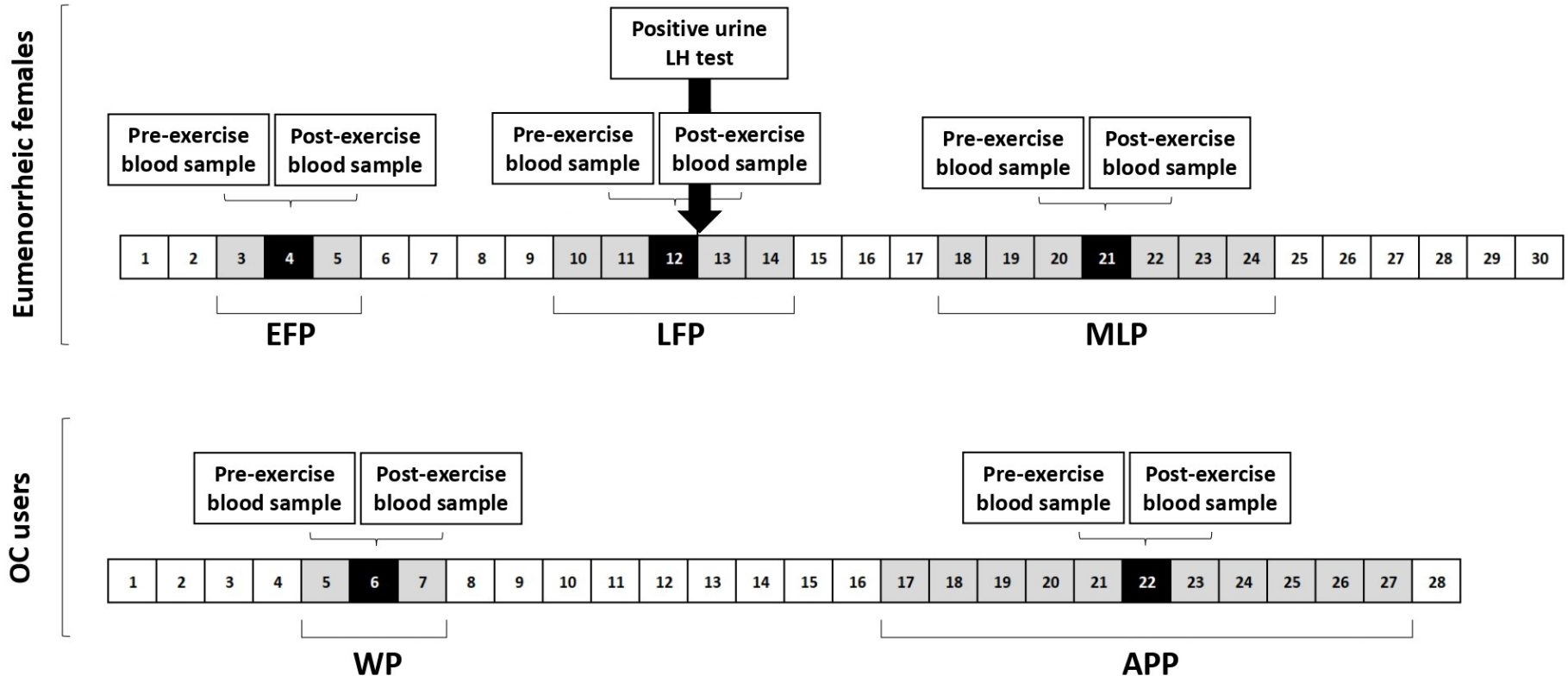


**Figure1.** *Diagram of the study design considering the mean length of the participants' menstrual cycles (30±3 days) and the mean day of LH positive (day 12±2) for eumenorrheic females and participants' oral contraceptive (OC) cycles. Intervallic trials days expressed as mean (black boxes) ± standard deviation (grey boxes) for the early follicular phase (EFP; 4±1 days), late follicular phase (LFP; 12±2 days) and mid luteal phase (21±3 days) for eumenorrheic females and for withdrawal phase (WP; 6±1 days) and active pill-taking phase (APP; 22±5 days) for OC users. The variables measured pre- and post-exercise in blood samples were 17-β oestradiol, progesterone, P1NP and β-CTX1.*