Bone metabolic marker concentrations across the menstrual cycle and phases of combined oral contraceptive use

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Abstract

There is a need to further understand the impact of the menstrual cycle and phase of combined oral contraceptive (COC) use on the pre-analytical variability of markers of bone metabolism in order to improve standardisation procedures for clinical practice and research. The aim of this study was to assess bone metabolism marker concentrations across the menstrual cycle and phases of COC use. Carboxy-terminal cross-linking telopeptide of type I collagen (β-CTX), procollagen type 1 N propeptide (P1NP) and Bone alkaline phosphatase (Bone ALP) concentrations were assessed in eumenorrheic women (n = 14) during the early follicular, ovulatory and mid-luteal phases of the menstrual cycle and in COC (Microgynon®) (n = 14) users on day 2-3 of pill consumption (PC1), day 15-16 pill consumption (PC2) and day 3-4 of the pill free interval (PFI). β-CTX was significantly (-16%) lower at PC2 compared to PC1 ($P = 0.015$) in COC users and was not affected by menstrual cycle phase ($P > 0.05$). P1NP and Bone ALP were not significantly different across either menstrual cycle phase or phase of COC use (all $P > 0.05$). There was no difference in pooled bone marker concentrations between eumenorrheic women and COC users ($P > 0.05$). In contrast to some previous studies, this study showed that bone marker concentrations do not significantly fluctuate across the menstrual cycle. Furthermore, bone resorption markers are significantly affected by phase of COC use, although bone formation markers do not significantly vary by COC phase. Therefore, the phase of COC use should be considered in clinical practice and research when assessing markers of bone metabolism as this can impact circulating concentrations of bone metabolic markers yet is not currently considered in existing guidelines for best practice.

Keywords: Bone, Marker, Metabolism, Oestrogen, Oral contraceptive, Menstrual cycle

Highlights

- β-CTX concentrations were affected by COC phase but not menstrual cycle phase.
- Lowest β-CTX concentrations occurred after two weeks COC use.
- P1NP and Bone ALP were not affected by menstrual cycle or COC phase.
- The phase of COC use should be considered in clinical practice and research.

1. Introduction

Biochemical markers of bone (re)modelling can be used to evaluate responses to therapeutic agents [1] , examine responses to dietary or exercise manipulations [2,3] and have been suggested to be useful in the prediction of fracture risk [4,5]. The International Osteoporosis Foundation (IOF) and International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) suggest the use of Carboxy-terminal cross-linking telopeptide of type I collagen (β-CTX) and Procollagen type I N Propeptide (PINP) as the preferred markers of bone resorption and formation, emphasising the need to control pre-analytical variability by standardising factors such as fasting status, exercise and circadian rhythm [6,7]. The menstrual cycle is currently considered a 'moderately important' variable to account for when assessing bone marker concentrations, with Szulc et al. [7] advising that samples should be collected in the early follicular phase where possible as PINP and β-CTX may fluctuate across the menstrual cycle. Currently, the impact of varying exogenous and endogenous reproductive hormone concentrations across phases of combined oral contraceptive (COC) use on bone markers have not been considered. There is a need to identify how the phase of COC use affects biochemical markers of bone metabolism, in addition to further research exploring the role of the menstrual cycle on biochemical markers of bone metabolism.

Monophasic COCs are the most common form of hormonal contraceptive and typically consist of 21 pill consumption days, followed by a 7-day pill free interval (PFI), repeated in a continuous manner [8]. On pill consumption days, 17-α-ethinyl oestradiol (EO) provides negative feedback to the anterior pituitary, inhibiting the production of endogenous 17-β-oestradiol [9]. During the 7-day PFI, the withdrawal of this negative feedback results in a 3-4 fold increase in 17-β-oestradiol concentrations [9–11]. Furthermore, although a consistent dose of synthetic oestrogen and progestin is supplied on pill consumption days, concentrations of exogenous synthetic hormones accumulate over the course of an COC cycle, with peak EO (~52%) and levonorgestrel (LNG; 123-153%), and area under the curve for both EO (75-87%) and LNG (261-273%) higher on the 21st day of pill consumption compared to the 1st day of consumption [12]. Mean trough concentrations also increase throughout pill consumption days for LNG [13] and EO [14,15] and reach a steady state around day 14 of pill consumption [13]. These

variations in exogenous reproductive hormone concentrations may affect markers of bone (re)modelling as EO activates oestrogen receptors in a similar manner to endogenous oestrogen [16], although limited research has explored this.

In COC users, PINP has only been assessed across a pill cycle in women that had been using an COC for 2 months, which may result in poor cycle control [17], and that had chronic posterior pelvic pain [18], which may present with altered collagen metabolism [19]. β-CTX has only been studied on one occasion where 24 h urinary β-CTX was 26% and 27% lower during early (day 3-5) and late (day 17-19) pill consumption compared to the PFI. The use of creatinine-corrected β-CTX measurements, however, should be interpreted with caution, since COC use increases creatinine clearance [20], which is affected by reproductive hormone concentrations [21,22]. Therefore, any differences between pill consumption and omission days may not be solely reflective of changes in bone resorption. Further research is required across phases of COC use using IOF recommended measurement practices to assess the impact on bone metabolism.

In eumenorrheic women, PINP concentrations have been reported to be 6.4% [23] and 11.4% [24] higher in the luteal phase compared to the follicular phase, while β-CTX concentrations were ~9-13% higher in the luteal phase [23–26]. The ability to interpret these studies, however, is limited as standardisation procedures recommended by the IOF [7] were not followed; including not restricting exercise in the 24 h before measurements [23–26] and not using fasted measurements or controlling for the time of day appropriately [25,26]. Furthermore, two studies [23,26] did not provide details of the assays used to measure bone markers and Niethammer et al., [26] did not clearly define the menstrual cycle phases in which measurements were taken. All of these factors limit the ability to interpret these data. Further research is required to assess PINP and β-CTX concentrations across the menstrual cycle using standardised procedures recommended by the IOF to reduce pre-analytical variability.

Although the bone formation marker Bone alkaline phosphatase (Bone ALP) is not an IOF specified marker, it provides a more complete picture of bone metabolism across the menstrual cycle as, unlike

PINP, it is specific to bone [7] and represents mineralisation rather than collagen turnover [27]. Previous research relating to Bone ALP has shown contrasting results across the menstrual cycle [23,26,28,29] (Chiu et al., 1999; Gass et al., 2008; Nielsen et al., 1990; Niethammer et al., 2015) and this has not been studied across phases of COC use.

Therefore, the aim of this study was to examine if there are changes in circulating concentrations of PINP, Bone ALP and β-CTX across the menstrual cycle or during the COC cycle.

2. Methods and methods

2.1. Participants

Thirty-seven recreationally active participants were recruited to take part in the study (eumenorrheic, n=21; COC users, n=16). Seven eumenorrheic participants were unable to complete the study due to anovulatory cycles (n=4), menstrual cycle length > 35 days (n=1), relocation (n=1) and personal issues (n=1). Two COC users were unable to complete the study due to cessation of COC use (n=1) and blood sampling issues (n=1). These withdrawals resulted in a total of 14 eumenorrheic and 14 COC participants (Table 1). Eumenorrheic participants were required to have had a regular menstrual cycle with a duration of 21-35 days (mean 28 ± 2 days) over the 6 months prior to recruitment. COC users were required to use a low dose, COC preparation (Microgynon®), with a regimen of 21 pill consumption days and a 7-day PFI for a minimum of 6 months prior to recruitment to limit the occurrence of improper cycle regulation [17]. A homogenous COC group using the same preparation was employed to reduce inter-participant variability [30]. Exclusion criteria were amenorrhea, oligomenorrhea, known history of reproductive disorders, pregnancy or trying to become pregnant, use of medications known to affect bone metabolism and aged < 18 or > 35 years. The study was approved by the Nottingham Trent University Research (Humans) Ethics Committee (Reference number 280). Participants were provided with a participant information sheet, completed a health screen and gave their written informed consent prior to commencing the study. Participants could withdraw from the study at any time.

178 Table 1. Demographic information for eumenorrheic participants and oral contraceptive users.

	Eumenorrheic n = 14	**Oral contraceptive n = 14**
Age (y)	21 ± 2	22 ± 4
Height (m)	1.65 ± 0.07	1.66 ± 0.06
Body mass (kg)	64.8 ± 10.1	61.1 ± 6.7
Body mass index ($kg \cdot m^2$)	23.8 ± 3.5	22.1 ± 1.6

179

180

181 *2.2. Experimental design*

182 Eumenorrheic participants were tested during the early follicular phase (EF; day 2-3), ovulatory phase
183 (OV; day immediately following a surge in luteinising hormone as confirmed by ovulation detection
184 kit [Clearblue®]) and mid luteal phase (ML; 7-8 days following LH surge). These phases were used to
185 represent three distinct profiles of 17-β-oestradiol. Oral contraceptive users were tested in the first week
186 of pill consumption (pill consumption day 2-3; PC1), after two weeks of pill consumption (day 15-16;
187 PC2) and during the PFI (day 3-4 PFI). Early (PC1) and late (PC2) pill consumption phases were used
188 as circulating exogenous steroid hormone concentrations increase across pill-taking days [12,13,15].
189 The PFI was used to represent a time when no exogenous hormones were supplied. The order of testing
190 for both groups was determined by the participant's cycle (*e.g.,* the first testing session corresponded
191 with the next testing time point following recruitment) and availability for testing (*e.g.,* a testing time-
192 point could be completed the following cycle if the participant was unavailable).

193

194 *2.3. Sampling*

195 Participants arrived at the laboratory at 08.00 (± 30 min), at the same time for each participant, having
196 fasted from 22.00 the previous night and having consumed 600 ml of water upon awakening. Oral
197 contraceptive users were asked to consume their pill 1 h prior to arriving at the laboratory and were
198 asked to consume it at this time for the duration of the study. Dietary intake and physical activity were
199 recorded in the 24 h prior to the initial laboratory visit and participants were asked to replicate this in
200 the day preceding each testing session, which was verbally confirmed by the experimenter. Participants
201 were asked to arrive at the laboratory in a rested state, having abstained from alcohol for a minimum of

24 h and caffeine for a minimum of 4 h. Blood was drawn from an antecubital forearm vein and separated into ethylenediaminetetraacetic acid (EDTA) and serum tubes. EDTA tubes were immediately centrifuged (accuSpin, 1R centrifuge, Fisher Scientific, Germany) for 10 min at 3000 g and 4°C, with plasma transferred into Eppendorf tubes and frozen at -80°C. Serum tubes were left to clot at room temperature for 30 minutes, before being centrifuged at 3000 g for 10 minutes at 4°C, and serum was transferred into Eppendorf tubes and frozen at -80°C.

Plasma 17-β-Oestradiol, β-CTX and P1NP (where referring to our specific methods and data, P1NP will be used rather than PINP as is the terminology used by our Roche commercial assay) were analysed using an electro-chemiluminescence immunoassay (ECLIA) on a COBAS e601 analyser (Roche Diagnostics, Mannheim, Germany). Serum Bone ALP was determined by MicroVue™ enzyme-linked immunosorbent assay ELISA kit (Quidel Corporation, US) Inter-assay coefficient of variation (CV) for 17-β-oestradiol was < 4.3% between 150-3000 $pmol \cdot L^{-1}$ with a detection limit of 18.4-1581 $pmol \cdot L^{-1}$. Inter-assay CV for Bone ALP was 5.8%, with a detection limit of 0.7 $U \cdot L^{-1}$. Inter-assay CV for β-CTX was < 3% between 200 and 150 $ng \cdot L^{-1}$, with a sensitivity of 10 $ng \cdot L^{-1}$. Inter-assay CV for P1NP was < 3% between 20-600 $\mu g \cdot L^{-1}$ with a sensitivity of 8 $\mu g \cdot L^{-1}$.

2.4. Statistical analysis

Data were checked for normality using the Shapiro-Wilk test. Eumenorrheic and COC participant characteristics were compared using independent samples t-tests. 17-β-oestradiol concentrations and bone metabolic markers were analysed independently for eumenorrheic and COC participants using one-way repeated measures ANOVAs (SPSS v 23.0), with significant effects explored using Bonferroni adjusted t-tests. Where sphericity of data were violated, Greenhouse-Geisser adjustments were used. Between-group comparisons were made using independent samples t-tests on the mean values for each participant calculated across the three phases. Effect sizes were calculated using Cohen's d (Cohen & Jacob, 1992) and were described as trivial (0.0 – 0.19), small (0.20 – 0.49), medium (0.50 – 0.79) and large (> 0.80). Pearson's correlation coefficients were used to cross-correlate 17-β-oestradiol concentrations and bone metabolic markers for eumenorrheic participants and COC users

independently. For bone metabolism markers, mean % change between different phases of the menstrual cycle or COC cycle were calculated and individual % change responses were characterised by presenting the range of responses in addition to the relative number of participants whose bone marker concentrations increased or decreased between phases. Data are presented as mean ± 1SD and the level of significance was set at $P \leq 0.05$.

3. Results

3.1. Between group comparisons

Mean 17-β-oestradiol concentrations were significantly ($P < 0.001$; $d = 3.05$) higher in eumenorrheic participants (367.4 ± 182.3 pmol·L^{-1}) compared to COC users (47.3 ± 27.4 pmol·L^{-1}). There were no differences between eumenorrheic and COC groups for β-CTX (EU = 560 ± 180, COC = 500 ± 200 ng·L^{-1}; $P = 0.37$; $d = 0.32$), P1NP (EU = 64.9 ± 21.9, COC = 62.9 ± 22.1 ng·mL^{-1}; $P = 0.81$; $d = 0.03$) and Bone ALP (EU = 18.9 ± 5.4, COC = 17.6 ± 3.8 U·L^{-1}; $P = 0.47$; $d = 0.27$; Figure 1).

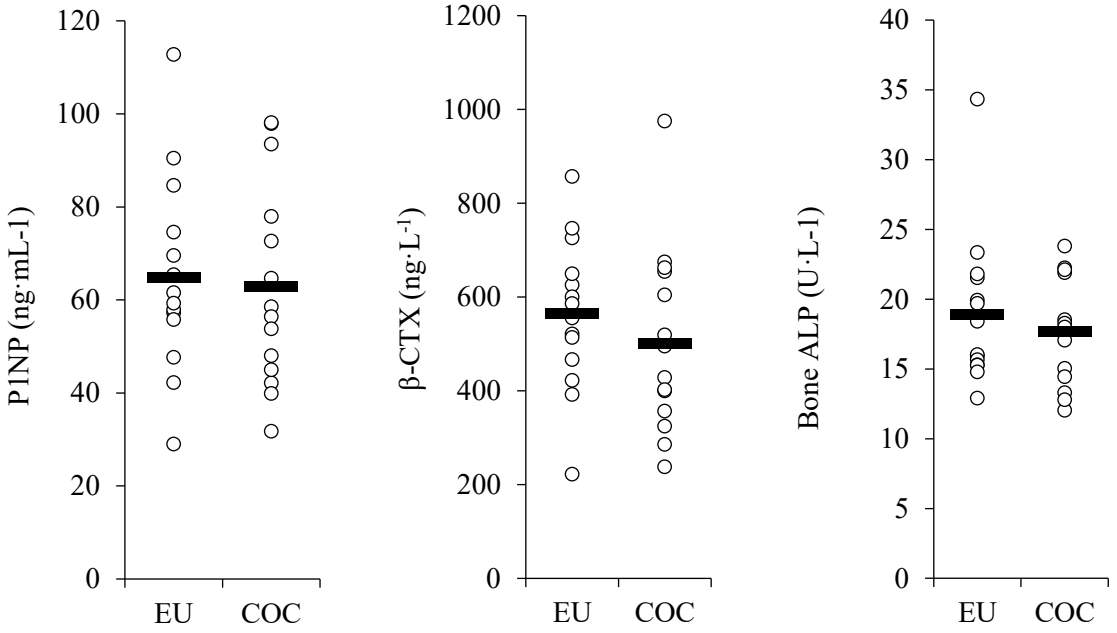

Figure 1. Univariate scatter plots with individual data points and mean values for eumenorrheic (EU) participants and combined oral contraceptive (COC) users mean values across all phases measured for Carboxy-terminal cross-linking telopeptide of type I collagen (β-CTX), Procollagen type I N propeptide (P1NP) and Bone alkaline phosphatase (Bone ALP) concentrations.

3.2. Within group comparisons

3.2.1. 17-β-oestradiol

For eumenorrheic participants, EF phase (178.8 ± 84.7 pmol·L^{-1}) 17-β-oestradiol concentrations were significantly lower than OV (360.9 ± 222.7 pmol·L^{-1}, P = 0.02; d = 1.18) and ML phases (562.4 ± 305.2 pmol·L^{-1}, P < 0.001; d = 1.97) and ML phase 17-β-oestradiol concentrations were significantly higher than the OV phase (P = 0.03; d = 0.76; Figure 2). For COC users, there was no significant effect of COC phase on 17-β-oestradiol concentrations (*P* = 0.076), but there was a medium effect size when comparing PC1 (50.2 ± 47.5 pmol·L^{-1}) to PC2 (27.9 ± 16.8 pmol·L^{-1}, d = 0.69, P = 0.25) and a large effect size when comparing PC2 to the PFI (63.7 ± 54.2 pmol·L^{-1}, d = 1.01, P = 0.075).

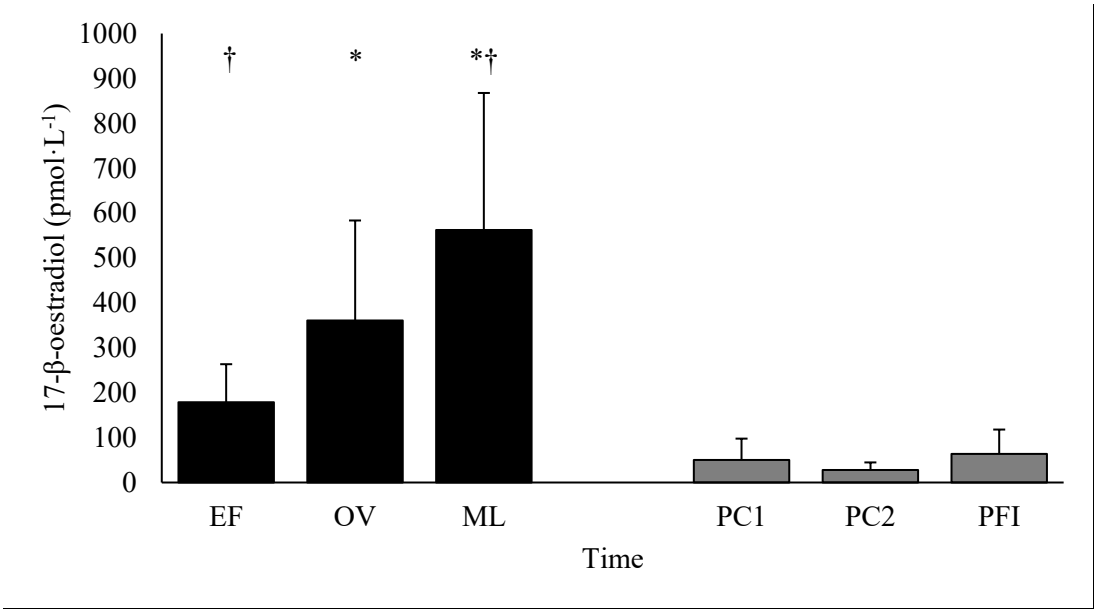

Figure 2. Mean ± 1SD 17-β-oestradiol concentrations in eumenorrheic participants (black bars) in the early follicular (EF), ovulatory (OV) and mid-luteal (ML) phases and oral contraceptive users (grey bars) at first (PC1) and second (PC2) pill consumption time points and during the pill-free interval (PFI). * Indicates a significant difference to EF and † indicates a significant difference to OV (P < 0.05).

279 *3.2.2. β-CTX*

280 For eumenorrheic participants, there was no main effect of menstrual cycle phase (P = 0.632) for β-
281 CTX concentrations. For COC users, β-CTX concentrations were significantly different between
282 different pill consumption phases (P = 0.006; Figure 3). Compared to PC2, β-CTX concentrations were
283 significantly higher at PC1 (16.0%; P = 0.015; d = 0.37) and were 14.7% higher at PFI, however this
284 was not significantly different (P = 0.065; d = 0.35). Mean percentage differences between menstrual
285 cycle and COC phases are shown in Table 2.

286

287 In the eumenorrheic group, 8 out of 14 participant's β-CTX concentrations were higher in the EF phase
288 compared to the OV phase, with differences between phases ranging from +42.3% to -62.4%, and 8 out
289 of 14 were higher in the EF phase compared to the ML phase, ranging from +33.6% to -21.2%. In the
290 COC group, 12 out of 14 COC-using participant's β-CTX concentrations were reduced from PC1 to
291 PC2, ranging from -30.7% to +12.1%, and 11 out of 14 COC participant's β-CTX concentrations were
292 lower in PC2 compared to PFI, ranging from -40.4% to + 7.2%.

293

294 *3.2.3. P1NP*

295 There was no effect of phase for eumenorrheic (P = 0.074) and COC participants (P = 0.096; Figure 4)
296 for P1NP and mean percentage differences between phases are shown in Table 2.

297

298 In the eumenorrheic group, 10 out of 14 participant's P1NP concentrations were increased from the OV
299 phase to the ML phase, with the differences between phases ranging from -8.4% to +52.7% and with 6
300 participant's P1NP concentrations increasing by > 25%. In the COC group, 12 out of 14 participant's
301 P1NP concentrations increased from PC1 to PC2, with the differences ranging from -8.1% to +70.8%.

302

303 *3.2.4. Bone ALP*

304 There was no significant effect of phase for eumenorrheic (P = 0.588) and COC participants (P = 0.602;
305 Figure 5) for Bone ALP and mean percentage differences between phases are shown in Table 2.

306

In the eumenorrheic group, 7 out of 14 eumenorrheic participant's Bone ALP concentrations were reduced from EF to OV, ranging from -42% to + 37.2%, and 8 out of 14 EU participant's Bone ALP concentrations were reduced from EF phase to ML phase, ranging from -42.1% to +26.2%. In the COC group, 7 out of 14 participant's Bone ALP concentrations were reduced from PC1 to PC2, with differences ranging from -49.1% to -56.7%, and 9 out of 14 participant's Bone ALP concentrations were reduced from PC1 to PFI, ranging from -31.5% to +27.8%.

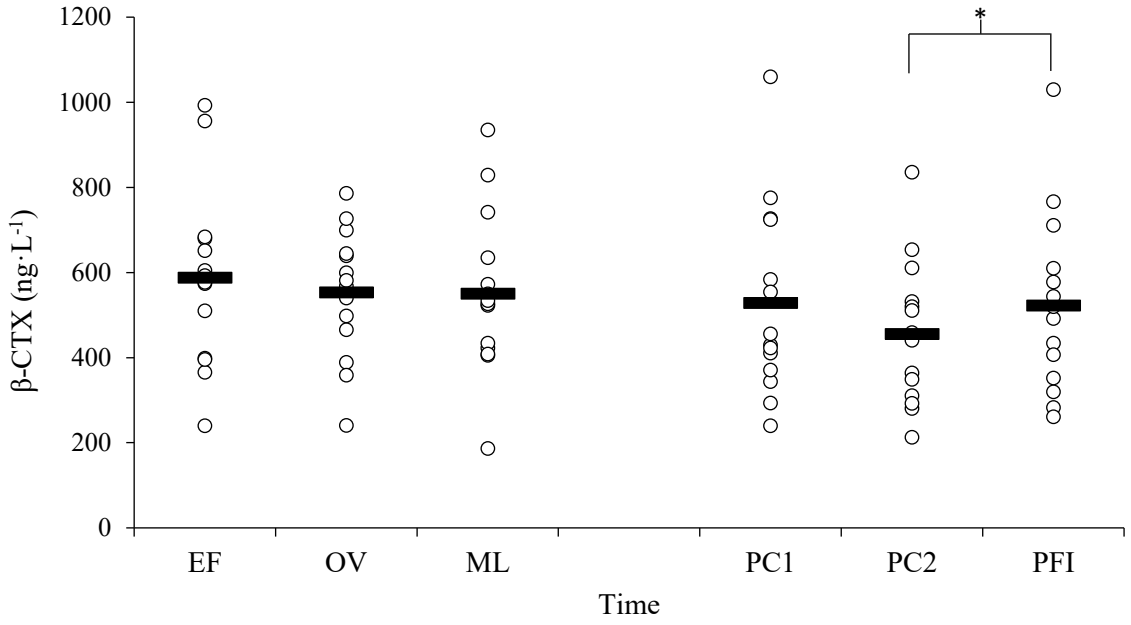

Figure 3. Univariate scatter plots with individual data points and mean values for Carboxy-terminal cross-linking telopeptide of type I collagen (β-CTX) in the early follicular (EF), ovulatory (OV) and mid-luteal (ML) phase and oral contraceptive users at first (PC1) and second (PC2) pill consumption time points and during the pill free interval (PFI). *Indicates a significant post-hoc difference between phases ($P < 0.05$).

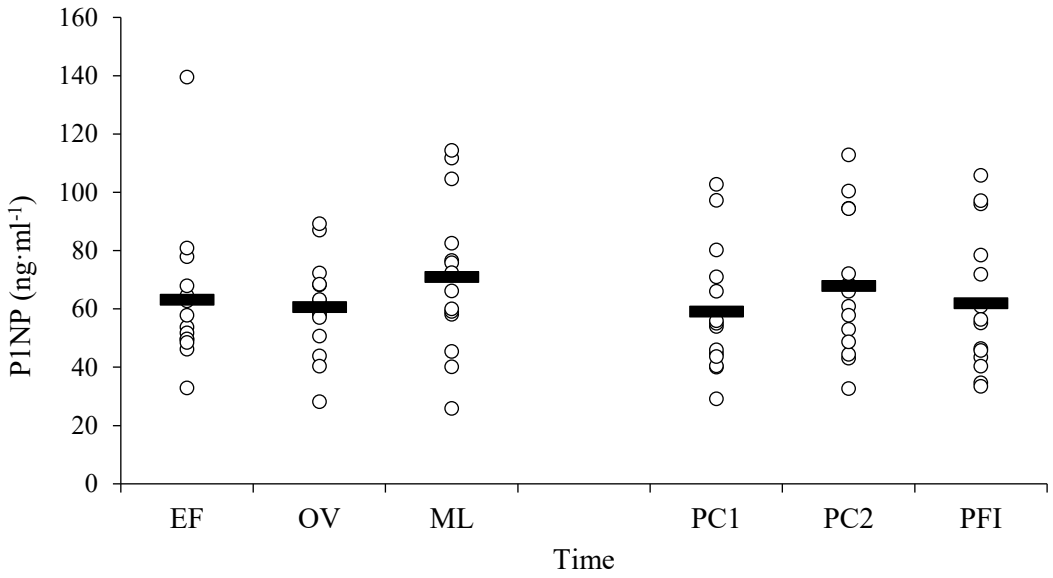

Figure 4. Univariate scatter plots with individual data points and mean values for Procollagen type I N propeptide (P1NP) in the early follicular (EF), ovulatory (OV) and mid-luteal (ML) phase and oral contraceptive users at first (PC1) and second (PC2) pill consumption time points and during the pill free interval (PFI).

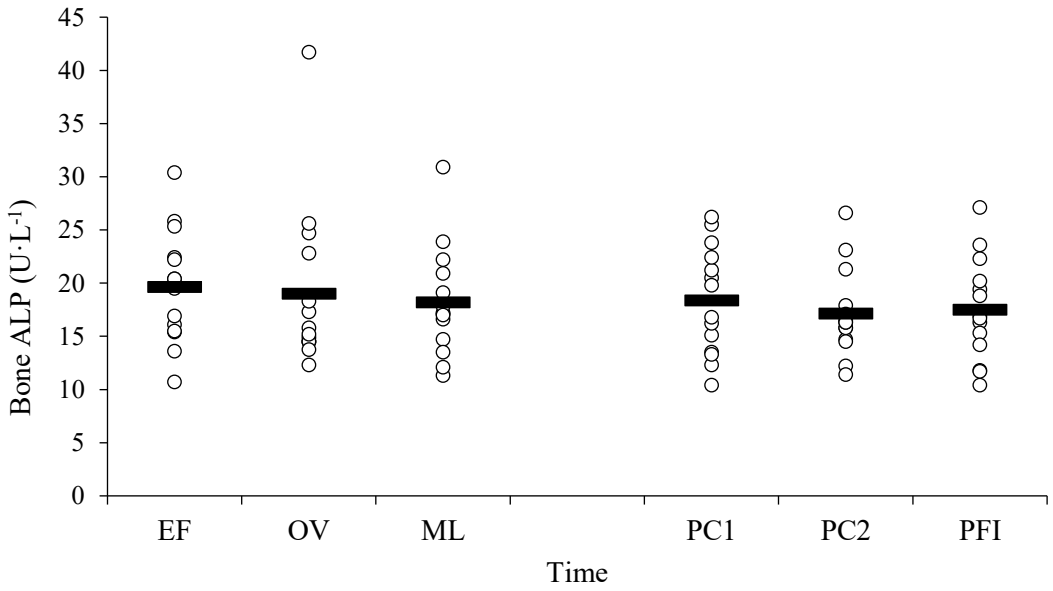

Figure 5. Univariate scatter p·ots with individual data points and mean values for Bone alkaline phosphatase (Bone ALP) in the early follicular (EF), ovulatory (OV) and mid-luteal (ML) phase and oral contraceptive users at first (PC1) and second (PC2) pill consumption time points and during the pill free interval (PFI).

Table 3. Percentage differences in bone marker concentrations between phases of the menstrual cycle and oral contraceptive cycle.

	β-CTX	**P1NP**	**Bone ALP**
Eumenorrheic			
EF vs. OV	+5.9%	+4.2%	+3.3%
EF vs. ML	+6.7%	-11.0%	+8.0%
OV vs. ML	-0.4%	-14.6%	+4.5%
Oral contraceptive			
PC1 vs. PC2	+16.0%*	-12.9%	+7.3%
PC1 vs. PFI	+1.2%	-4.6%	+5.0%
PC2 vs. PFI	+12.8%	+9.3%	-2.1%

Bone alkaline phosphatase, Bone ALP; Carboxy-terminal cross-linking telopeptide of type I collagen, β-CTX; Early follicular, EF; Mid-luteal, ML; Ovulatory, OV; Pill consumption, PC; Procollagen type I N propeptide, P1NP. *Indicates a significant post-hoc difference between phases ($P < 0.05$). N.B. the reference phase for the percentage difference calculation is the second-mentioned phase e.g., where 'EF vs. OV' is 5.9%, this states that mean EF values are 5.9% higher than those in OV.

3.3. Bone marker correlations

For eumenorrheic participants, ML phase 17-β-oestradiol concentrations were significantly negatively correlated with EF phase Bone ALP concentrations ($P = 0.007$, $r = -0.681$), with no other significant correlations being shown with 17-β-oestradiol. EF phase β-CTX concentrations were positively correlated to OV phase and ML phase P1NP concentrations ($P < 0.05$; $r = 0.798$-0.838). β-CTX and P1NP were correlated during the OV phase ($P = 0.017$; $r = 0.626$), and ML phase β-CTX concentrations were correlated to P1NP at all time points ($P < 0.05$; $r = 0.662$-0.926).

For COC users, PC2 17-β-oestradiol concentrations were significantly negatively correlated to PFI β-CTX concentrations ($P = 0.041$, $r = -0.550$), with no other significant correlations to 17-β-oestradiol. Bone ALP concentrations at PC2 were significantly positively correlated to P1NP concentrations at PC1 ($P = 0.001$, $r = 0.764$) and PFI ($P = 0.005$, $r = 0.700$). β-CTX and P1NP concentrations were positively correlated at all time points ($P > 0.05$; $r = 0.638$-0.841).

4. Discussion

There were no significant differences in bone metabolism between eumenorrheic participants and COC users. Bone (re)modelling marker concentrations were also not significantly different between menstrual cycle phases. Although concentrations of P1NP and Bone ALP were not different between COC phases, β-CTX was significantly (-16%) lower during late pill consumption compared to early pill consumption. 17-β-oestradiol was only correlated to Bone ALP in eumenorrheic participants and β-CTX in COC users, although these correlations occurred with 17-β-oestradiol concentrations from the preceding phase, suggesting a possible time lag of approximately 8 days in both instances.

In eumenorrheic participants, mean *β*-CTX concentrations were 6.3% and 6.7% lower in the ovulatory and mid-luteal phases compared to the early follicular phase, although this was not statistically significant. For both the ovulatory and mid-luteal phases, 8 out of 14 participants' β-CTX concentrations were reduced compared to the early follicular phase, with a wide range of individual responses (+35.0% to -60.2%), showing that this was a non-uniform effect. This contrasts with previous studies where β-CTX concentrations were significantly (~9-14%) lower in the follicular phase compared to the luteal phase [23–26]. Individual variations in β-CTX concentrations have either been unreported in previous menstrual cycle research [23] or were relatively high; with standard deviations being 36-55% [25] and 59-60% [26] of total β-CTX concentrations, similar to the current study (31-36%). Furthermore, the variability in responses between phases was large, with standard deviation of the total change ~30% of total values [26] and standard deviations of the percentage change greater than the actual percentage change [23]. Large standard deviations and inter-individual responses reduce the likelihood of significant differences occurring as these are integral to the calculation of the *t* statistic. One reason why significant differences may have been observed in previous research is due to less stringent statistical procedures being employed, such as non-corrected multiple comparisons [23] or more flexible α corrections for repeated comparisons (e.g., Tippets step-down procedure; [25]), which significantly increase the likelihood of type 1 errors in these studies. This discrepancy in statistical approaches may also be responsible for the differences in PINP results between the current study and

previous research. P1NP concentrations were not significantly different across the menstrual cycle despite mean values being 14.6% higher in the mid luteal phase compared to the ovulatory phase. The absolute difference was greater than the 6.4% significant difference previously shown by Gass et al. [23]. The current study highlights that the changes between menstrual cycle phases for PINP and β-CTX concentrations are not as clear as previous research suggests, and that large individual variations in bone marker concentrations, coupled with individuality of responses between different phases, affects the interpretation of results.

In COC users, β-CTX concentrations on day 15-16 of COC consumption were significantly lower than days 2-3 of COC use (16.0%) and the PFI (14.6%), although this was not significant. The reduced β-CTX concentrations after approximately two weeks of pill consumption is similar to previous research [32], although Zitterman et al. [32] also showed reduced concentrations in the first week (day 3-5) of pill consumption, which was not shown in the current study. This disparity may be due to an earlier sampling date during pill consumption in the current study (day 2-3), where the effects of synthetic hormones may not yet have manifested. Alternatively, it may be due to analytical differences whereby Zitterman et al. [32] used urinary β-CTX, which may be influenced by changes in creatinine excretion across the COC cycle [20], while the current study measured β-CTX in serum which avoids this potential measurement error. Typically, low 17-β-oestradiol concentrations are associated with an increased rate of bone resorption [33], although the lowest β-CTX concentrations occurred on D15-16 of pill consumption, at a time where endogenous 17-β-oestradiol concentrations were lowest. As circulating EO concentrations are elevated by > 50% during late pill consumption and activate oestrogen receptors in a similar manner to endogenous oestrogen [16], this may suggest that differences shown across the pill cycle were due to an inhibitory effect of synthetic oestrogens on bone resorption. Alternatively, this may be due to delayed effects of endogenous 17-β-oestradiol as β-CTX concentrations during the PFI were negatively correlated with 17-β-oestradiol measured 8-9 days earlier on D15-16 pill consumption. This is in line with other studies showing that the effect of 17-β-oestradiol may occur with a time-lag, as these processes are based upon protein transcription activities that can take approximately one week to occur [28,34]. Whilst this study shows that bone resorption

411 significantly varies across an COC cycle, further research is required to assess whether this is
412 attributable to variations in endogenous or exogenous hormones, or a combination of these.
413
414 Oral contraceptive phase did not significantly affect P1NP concentrations, although mean P1NP
415 concentrations were 12.9% higher on D15-16 of pill consumption compared to D2-3, with 11 out of 14
416 participant's P1NP concentrations increasing and changes ranging from -8.1% to +70.8%. As with other
417 metabolic markers, the lack of significant difference may be due to high inter-individual variation (36-
418 39%) and the large variation in the response between phases. PINP has only been studied across a COC
419 cycle on one other occasion, where there was a 21% reduction in PINP concentrations between the PFI
420 and day 18-21 pill consumption [18]. Data from the previous study, however, may not be applicable to
421 the general population as the participants had chronic posterior pain and had only used COCs for two
422 months, both of which may have affected responses [19,35]. This is the first study to assess P1NP
423 across an COC cycle in a healthy population and has shown that there was no significant difference in
424 bone formation concentrations between phases.
425
426 Bone ALP concentrations did not vary across the menstrual cycle or between pill consumption phases.
427 The lack of change in Bone ALP between menstrual cycle phases is similar to the majority of previous
428 research [23,26,36]. This is the first study to examine Bone ALP across an COC cycle and has shown
429 that COC phase does not need to be considered during sample collection.
430
431 Despite significantly different reproductive hormone profiles, with eumenorrheic participants
432 displaying significantly higher 17-β-oestradiol concentrations compared to COC users, there were no
433 differences in β-CTX, P1NP or Bone ALP concentrations between groups. This is in contrast to some
434 studies where COC use was shown to reduce bone marker concentrations [18,37–42], , although it does
435 agree with other studies that have shown no differences between eumenorrheic women and COC users
436 [35,43–45]. The between-group comparisons in the current study were conducted using mean values
437 from three different phases of the menstrual cycle and COC cycle, and, therefore, may be more

representative of bone (re)modelling marker concentrations compared to previous research, which used measurements from one time point only.

5. Conclusions

P1NP and Bone ALP concentrations were not changed between different phases of the menstrual or COC cycles and β-CTX concentrations were not different between phases of the menstrual cycle. β-CTX concentrations significantly varied across a COC cycle, with the lowest concentrations occurring after two weeks of pill consumption when endogenous oestrogen is lowest and exogenous oestrogen is highest, suggesting that synthetic hormones might play a role in regulating bone metabolism across an COC cycle. Contraceptive use is currently only considered as an uncontrollable source of pre-analytical variability in the long term (*e.g.*, use or non-use; Vasikaran et al. [6]), although this study has shown that the phase within the COC cycle affects bone resorption, as indicated by β-CTX concentrations. Therefore, the timing of sample collection within an COC cycle should be considered in the clinical use of bone (re)modelling markers and in research using these markers to assess changes in bone metabolism during interventions. This study has improved upon previous research by controlling for exercise, fasting status and time of day, and used a homogenous COC group using the same brand in order to reduce within-participant variability [30], although further research is required to assess if bone formation is similarly variable across COC phases in other COC preparations containing different doses and types of oestrogen and progestins.

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