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

3
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14
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37 **Abstract**

38 There is a need to further understand the impact of the menstrual cycle and phase of combined oral
39 contraceptive (COC) use on the pre-analytical variability of markers of bone metabolism in order to
40 improve standardisation procedures for clinical practice and research. The aim of this study was to
41 assess bone metabolism marker concentrations across the menstrual cycle and phases of COC use.
42 Carboxy-terminal cross-linking telopeptide of type I collagen (β -CTX), procollagen type 1 N propeptide
43 (P1NP) and Bone alkaline phosphatase (Bone ALP) concentrations were assessed in eumenorrheic
44 women (n = 14) during the early follicular, ovulatory and mid-luteal phases of the menstrual cycle and
45 in COC (Microgynon®) (n = 14) users on day 2-3 of pill consumption (PC1), day 15-16 pill
46 consumption (PC2) and day 3-4 of the pill free interval (PFI). β -CTX was significantly (-16%) lower at
47 PC2 compared to PC1 (P = 0.015) in COC users and was not affected by menstrual cycle phase (P >
48 0.05). P1NP and Bone ALP were not significantly different across either menstrual cycle phase or phase
49 of COC use (all P > 0.05). There was no difference in pooled bone marker concentrations between
50 eumenorrheic women and COC users (P > 0.05). In contrast to some previous studies, this study showed
51 that bone marker concentrations do not significantly fluctuate across the menstrual cycle. Furthermore,
52 bone resorption markers are significantly affected by phase of COC use, although bone formation
53 markers do not significantly vary by COC phase. Therefore, the phase of COC use should be considered
54 in clinical practice and research when assessing markers of bone metabolism as this can impact
55 circulating concentrations of bone metabolic markers yet is not currently considered in existing
56 guidelines for best practice.

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

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65 **Highlights**

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

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96 **1. Introduction**

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

111

112 Monophasic COCs are the most common form of hormonal contraceptive and typically consist of 21
113 pill consumption days, followed by a 7-day pill free interval (PFI), repeated in a continuous manner [8].
114 On pill consumption days, 17- α -ethinyl oestradiol (EO) provides negative feedback to the anterior
115 pituitary, inhibiting the production of endogenous 17- β -oestradiol [9]. During the 7-day PFI, the
116 withdrawal of this negative feedback results in a 3-4 fold increase in 17- β -oestradiol concentrations [9–
117 11]. Furthermore, although a consistent dose of synthetic oestrogen and progestin is supplied on pill
118 consumption days, concentrations of exogenous synthetic hormones accumulate over the course of an
119 COC cycle, with peak EO (~52%) and levonorgestrel (LNG; 123-153%), and area under the curve for
120 both EO (75-87%) and LNG (261-273%) higher on the 21st day of pill consumption compared to the 1st
121 day of consumption [12]. Mean trough concentrations also increase throughout pill consumption days
122 for LNG [13] and EO [14,15] and reach a steady state around day 14 of pill consumption [13]. These

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

126

127 In COC users, PINP has only been assessed across a pill cycle in women that had been using an COC
128 for 2 months, which may result in poor cycle control [17], and that had chronic posterior pelvic pain
129 [18], which may present with altered collagen metabolism [19]. β -CTX has only been studied on one
130 occasion where 24 h urinary β -CTX was 26% and 27% lower during early (day 3-5) and late (day 17-
131 19) pill consumption compared to the PFI. The use of creatinine-corrected β -CTX measurements,
132 however, should be interpreted with caution, since COC use increases creatinine clearance [20], which
133 is affected by reproductive hormone concentrations [21,22]. Therefore, any differences between pill
134 consumption and omission days may not be solely reflective of changes in bone resorption. Further
135 research is required across phases of COC use using IOF recommended measurement practices to assess
136 the impact on bone metabolism.

137

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

148

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

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

155

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

158

159 **2. Methods and methods**

160 *2.1. Participants*

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

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

	Eumenorrhic 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·m ²)	23.8 ± 3.5	22.1 ± 1.6

179

180

181 *2.2. Experimental design*

182 Eumenorrhic 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

202 24 h and caffeine for a minimum of 4 h. Blood was drawn from an antecubital forearm vein and
203 separated into ethylenediaminetetraacetic acid (EDTA) and serum tubes. EDTA tubes were
204 immediately centrifuged (accuSpin, 1R centrifuge, Fisher Scientific, Germany) for 10 min at 3000 g
205 and 4°C, with plasma transferred into Eppendorf tubes and frozen at -80°C. Serum tubes were left to
206 clot at room temperature for 30 minutes, before being centrifuged at 3000 g for 10 minutes at 4°C, and
207 serum was transferred into Eppendorf tubes and frozen at -80°C.

208

209 Plasma 17- β -Oestradiol, β -CTX and P1NP (where referring to our specific methods and data, P1NP
210 will be used rather than PINP as is the terminology used by our Roche commercial assay) were analysed
211 using an electro-chemiluminescence immunoassay (ECLIA) on a COBAS e601 analyser (Roche
212 Diagnostics, Mannheim, Germany). Serum Bone ALP was determined by MicroVue™ enzyme-linked
213 immunosorbent assay ELISA kit (Quidel Corporation, US) Inter-assay coefficient of variation (CV) for
214 17- β -oestradiol was < 4.3% between 150-3000 pmol·L⁻¹ with a detection limit of 18.4-1581 pmol·L⁻¹.
215 Inter-assay CV for Bone ALP was 5.8%, with a detection limit of 0.7 U·L⁻¹. Inter-assay CV for β -CTX
216 was < 3% between 200 and 150 ng·L⁻¹, with a sensitivity of 10 ng·L⁻¹. Inter-assay CV for P1NP was <
217 3% between 20-600 μ g·L⁻¹ with a sensitivity of 8 μ g·L⁻¹.

218

219 *2.4. Statistical analysis*

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

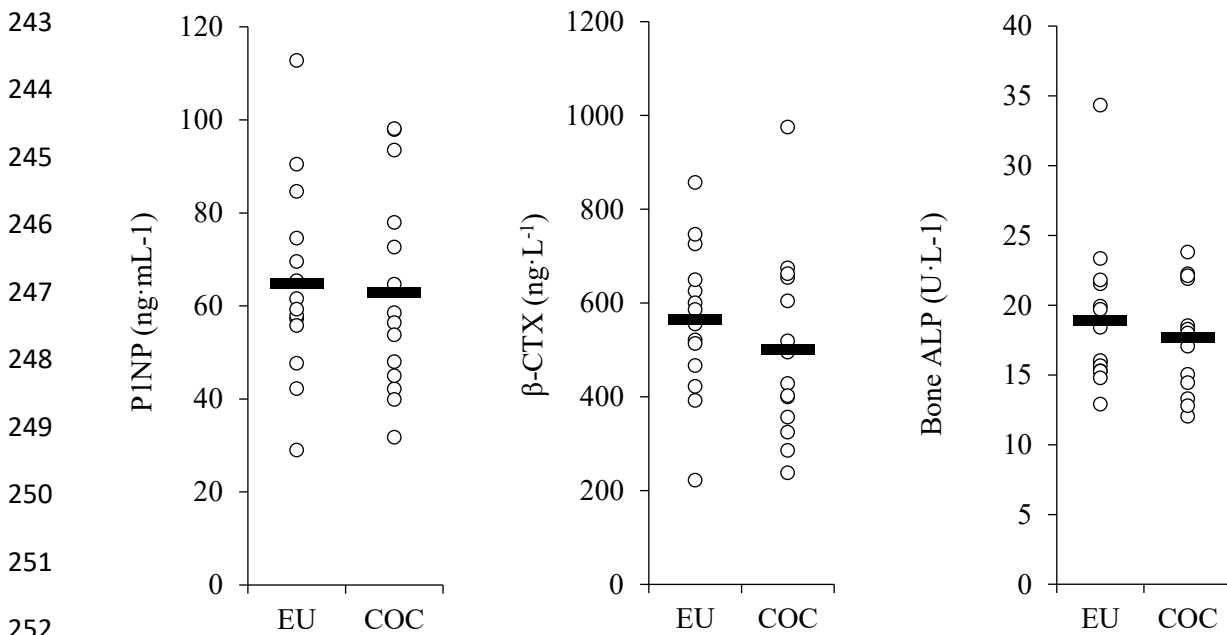
230 independently. For bone metabolism markers, mean % change between different phases of the
231 menstrual cycle or COC cycle were calculated and individual % change responses were characterised
232 by presenting the range of responses in addition to the relative number of participants whose bone
233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

235

236 3. Results

237 3.1. Between group comparisons

238 Mean 17- β -oestradiol concentrations were significantly ($P < 0.001$; $d = 3.05$) higher in eumenorrhic
239 participants ($367.4 \pm 182.3 \text{ pmol}\cdot\text{L}^{-1}$) compared to COC users ($47.3 \pm 27.4 \text{ pmol}\cdot\text{L}^{-1}$). There were no
240 differences between eumenorrhic and COC groups for β -CTX (EU = 560 ± 180 , COC = 500 ± 200
241 $\text{ng}\cdot\text{L}^{-1}$; $P = 0.37$; $d = 0.32$), P1NP (EU = 64.9 ± 21.9 , COC = $62.9 \pm 22.1 \text{ ng}\cdot\text{mL}^{-1}$; $P = 0.81$; $d = 0.03$)
242 and Bone ALP (EU = 18.9 ± 5.4 , COC = $17.6 \pm 3.8 \text{ U}\cdot\text{L}^{-1}$; $P = 0.47$; $d = 0.27$; Figure 1).



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

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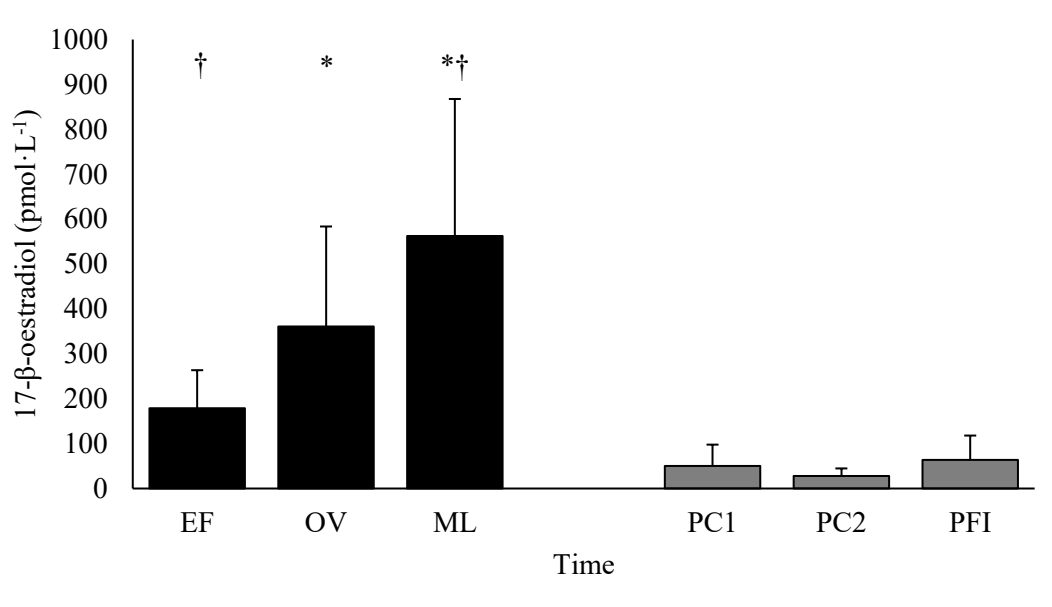
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259 3.2. Within group comparisons

260 3.2.1. 17- β -oestradiol

261 For eumenorrhic participants, EF phase (178.8 ± 84.7 pmol·L⁻¹) 17- β -oestradiol concentrations were
262 significantly lower than OV (360.9 ± 222.7 pmol·L⁻¹, $P = 0.02$; $d = 1.18$) and ML phases (562.4 ± 305.2
263 pmol·L⁻¹, $P < 0.001$; $d = 1.97$) and ML phase 17- β -oestradiol concentrations were significantly higher
264 than the OV phase ($P = 0.03$; $d = 0.76$; Figure 2). For COC users, there was no significant effect of
265 COC phase on 17- β -oestradiol concentrations ($P = 0.076$), but there was a medium effect size when
266 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
267 effect size when comparing PC2 to the PFI (63.7 ± 54.2 pmol·L⁻¹, $d = 1.01$, $P = 0.075$).

268



269

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

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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%.

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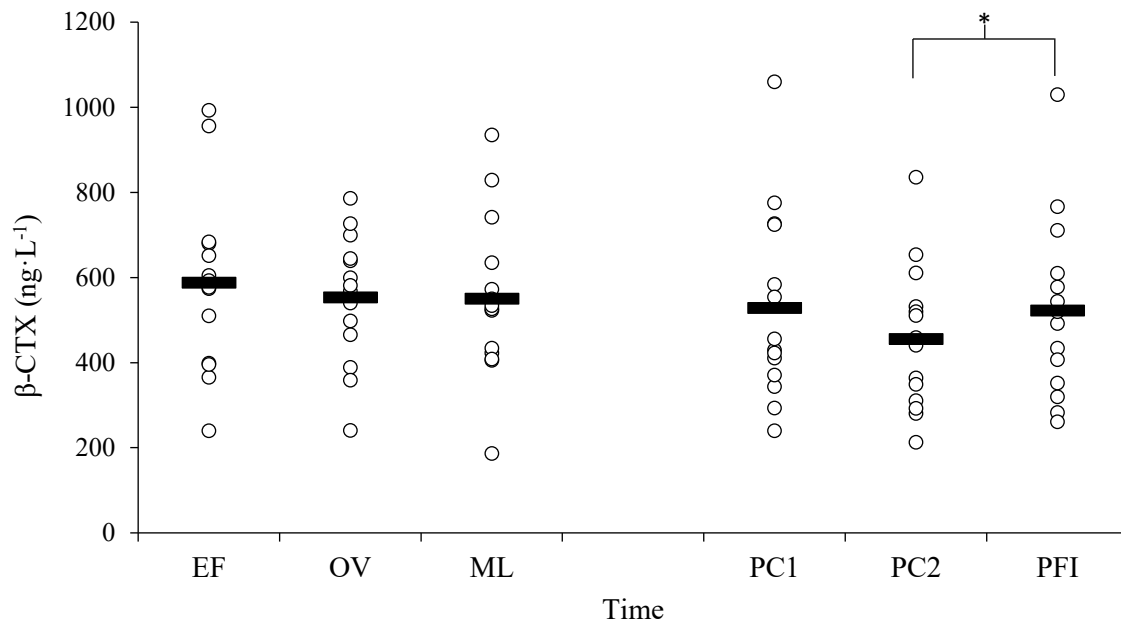
303 3.2.4. *Bone ALP*

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

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

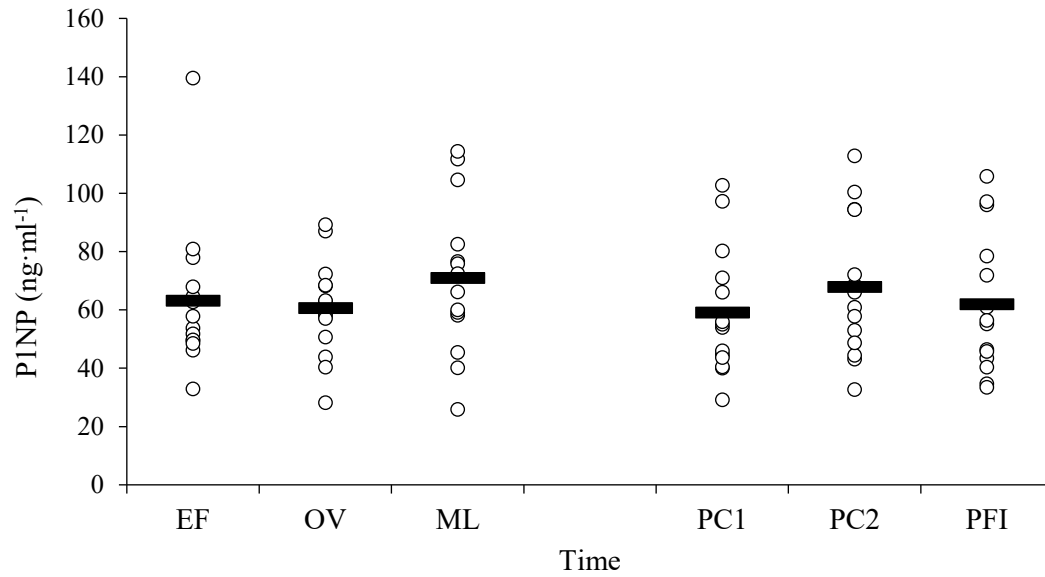
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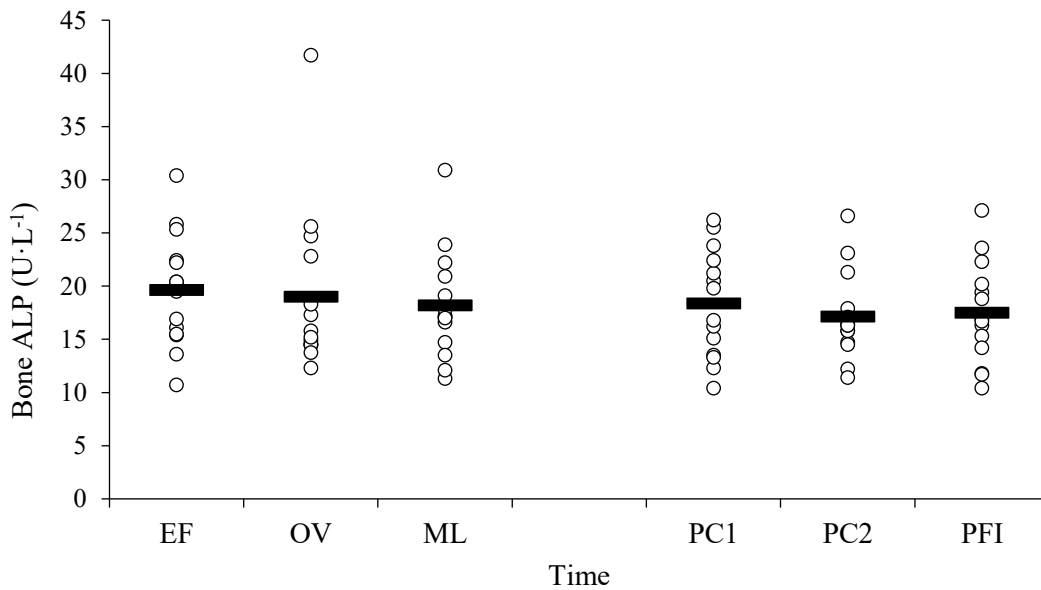
315 Figure 3. Univariate scatter plots with individual data points and mean values for Carboxy-terminal
316 cross-linking telopeptide of type I collagen (β -CTX) in the early follicular (EF), ovulatory (OV) and
317 mid-luteal (ML) phase and oral contraceptive users at first (PC1) and second (PC2) pill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

320



321

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



326

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

331

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

	β-CTX	P1NP	Bone ALP
<i>Eumenorrheic</i>			
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%
<i>Oral contraceptive</i>			
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%

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

339
 340

341 3.3. Bone marker correlations

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

348
 349 For COC users, PC2 17-β-oestradiol concentrations were significantly negatively correlated to PFI β-
 350 CTX concentrations ($P = 0.041$, $r = -0.550$), with no other significant correlations to 17-β-oestradiol.
 351 Bone ALP concentrations at PC2 were significantly positively correlated to P1NP concentrations at
 352 PC1 ($P = 0.001$, $r = 0.764$) and PFI ($P = 0.005$, $r = 0.700$). β-CTX and P1NP concentrations were
 353 positively correlated at all time points ($P > 0.05$; $r = 0.638-0.841$).

354
 355

356 4. Discussion

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

364

365 In eumenorrheic participants, mean β -CTX concentrations were 6.3% and 6.7% lower in the ovulatory
366 and mid-luteal phases compared to the early follicular phase, although this was not statistically
367 significant. For both the ovulatory and mid-luteal phases, 8 out of 14 participants' β -CTX
368 concentrations were reduced compared to the early follicular phase, with a wide range of individual
369 responses (+35.0% to -60.2%), showing that this was a non-uniform effect. This contrasts with previous
370 studies where β -CTX concentrations were significantly (~9-14%) lower in the follicular phase
371 compared to the luteal phase [23–26]. Individual variations in β -CTX concentrations have either been
372 unreported in previous menstrual cycle research [23] or were relatively high; with standard deviations
373 being 36-55% [25] and 59-60% [26] of total β -CTX concentrations, similar to the current study (31-
374 36%). Furthermore, the variability in responses between phases was large, with standard deviation of
375 the total change ~30% of total values [26] and standard deviations of the percentage change greater than
376 the actual percentage change [23]. Large standard deviations and inter-individual responses reduce the
377 likelihood of significant differences occurring as these are integral to the calculation of the t statistic.
378 One reason why significant differences may have been observed in previous research is due to less
379 stringent statistical procedures being employed, such as non-corrected multiple comparisons [23] or
380 more flexible α corrections for repeated comparisons (e.g., Tippets step-down procedure; [25]), which
381 significantly increase the likelihood of type 1 errors in these studies. This discrepancy in statistical
382 approaches may also be responsible for the differences in PINP results between the current study and

383 previous research. PINP concentrations were not significantly different across the menstrual cycle
384 despite mean values being 14.6% higher in the mid luteal phase compared to the ovulatory phase. The
385 absolute difference was greater than the 6.4% significant difference previously shown by Gass et al.
386 [23]. The current study highlights that the changes between menstrual cycle phases for PINP and β -
387 CTX concentrations are not as clear as previous research suggests, and that large individual variations
388 in bone marker concentrations, coupled with individuality of responses between different phases, affects
389 the interpretation of results.

390

391 In COC users, β -CTX concentrations on day 15-16 of COC consumption were significantly lower than
392 days 2-3 of COC use (16.0%) and the PFI (14.6%), although this was not significant. The reduced β -
393 CTX concentrations after approximately two weeks of pill consumption is similar to previous research
394 [32], although Zitterman et al. [32] also showed reduced concentrations in the first week (day 3-5) of
395 pill consumption, which was not shown in the current study. This disparity may be due to an earlier
396 sampling date during pill consumption in the current study (day 2-3), where the effects of synthetic
397 hormones may not yet have manifested. Alternatively, it may be due to analytical differences whereby
398 Zitterman et al. [32] used urinary β -CTX, which may be influenced by changes in creatinine excretion
399 across the COC cycle [20], while the current study measured β -CTX in serum which avoids this
400 potential measurement error. Typically, low 17- β -oestradiol concentrations are associated with an
401 increased rate of bone resorption [33], although the lowest β -CTX concentrations occurred on D15-16
402 of pill consumption, at a time where endogenous 17- β -oestradiol concentrations were lowest. As
403 circulating EO concentrations are elevated by > 50% during late pill consumption and activate oestrogen
404 receptors in a similar manner to endogenous oestrogen [16], this may suggest that differences shown
405 across the pill cycle were due to an inhibitory effect of synthetic oestrogens on bone resorption.
406 Alternatively, this may be due to delayed effects of endogenous 17- β -oestradiol as β -CTX
407 concentrations during the PFI were negatively correlated with 17- β -oestradiol measured 8-9 days earlier
408 on D15-16 pill consumption. This is in line with other studies showing that the effect of 17- β -oestradiol
409 may occur with a time-lag, as these processes are based upon protein transcription activities that can
410 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. P1NP has only been studied across a COC
419 cycle on one other occasion, where there was a 21% reduction in P1NP 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

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

440

441 **5. Conclusions**

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

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466 **6. References**

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