1 **Bone metabolic marker concentrations across the menstrual cycle and**

2 **phases of combined oral contraceptive use**

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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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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

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.

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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 eumenorrheic 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.

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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 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·m^2)	23.8 ± 3.5	22.1 ± 1.6

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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

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 µg·L^{-1} with a sensitivity of 8 µg·L^{-1}.

218

219   *2.4. Statistical analysis*

220   Data were checked for normality using the Shapiro-Wilk test. Eumenorrheic 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 eumenorrheic 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 eumenorrheic 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 ± 1SD and

234 the level of significance was set at $P \leq 0.05$.

235

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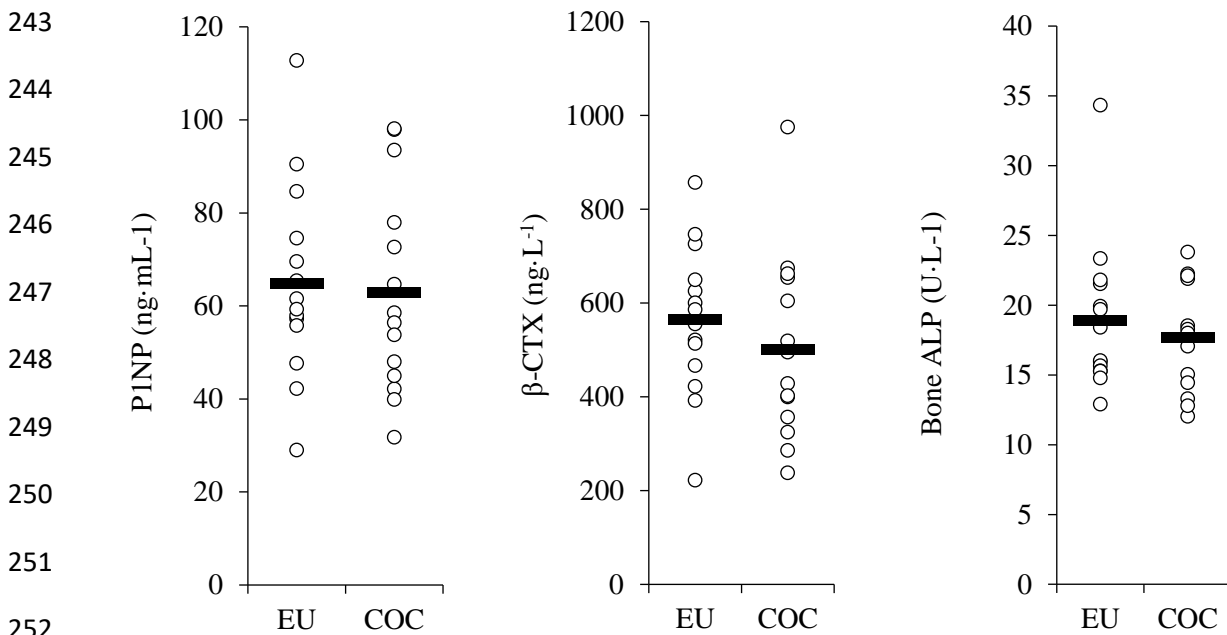
Figure 1. Univariate scatter plots with individual data points and mean values for eumenorrheic (EU)
participants and combined oral contraceptive (COC) users mean values across all phases measured for
Carboxy-terminal cross-linking telopeptide of type I collagen (β-CTX), Procollagen type I N propeptide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

259   *3.2. Within group comparisons*

260   *3.2.1. 17-β-oestradiol*

261   For eumenorrheic 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).

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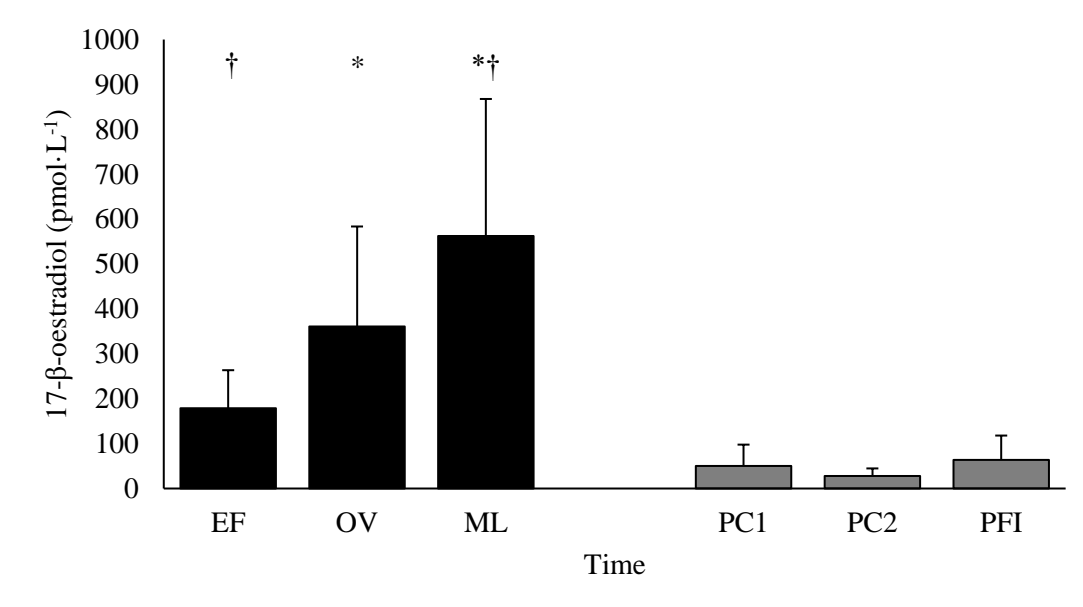
269

270   Figure 2. Mean ± 1SD 17-β-oestradiol concentrations in eumenorrheic 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%.

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

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%.

313
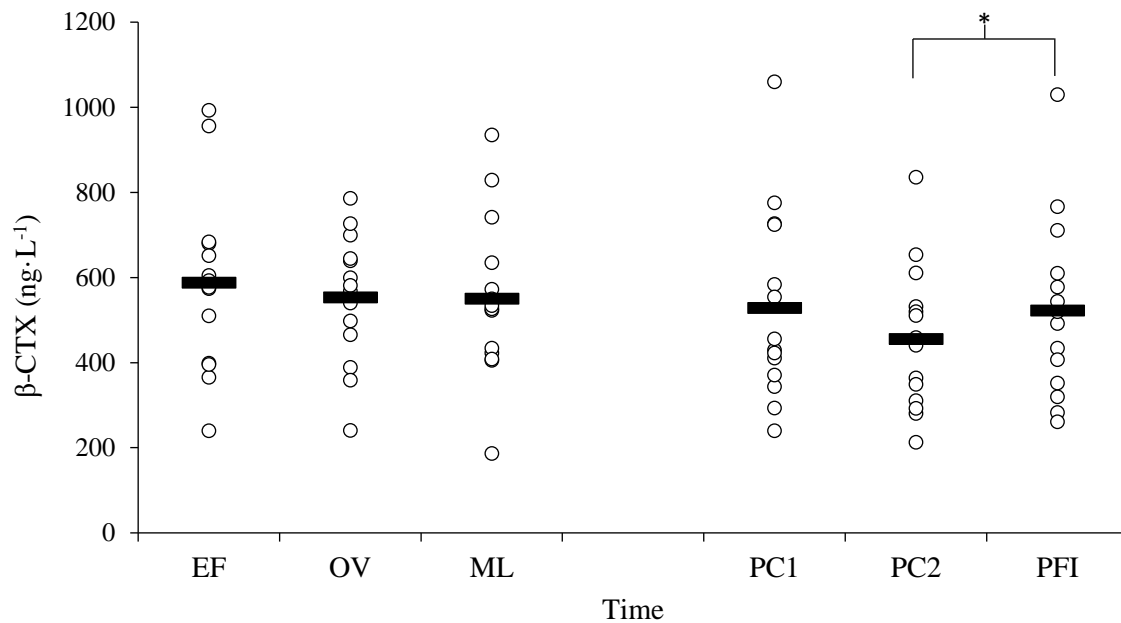
314

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).

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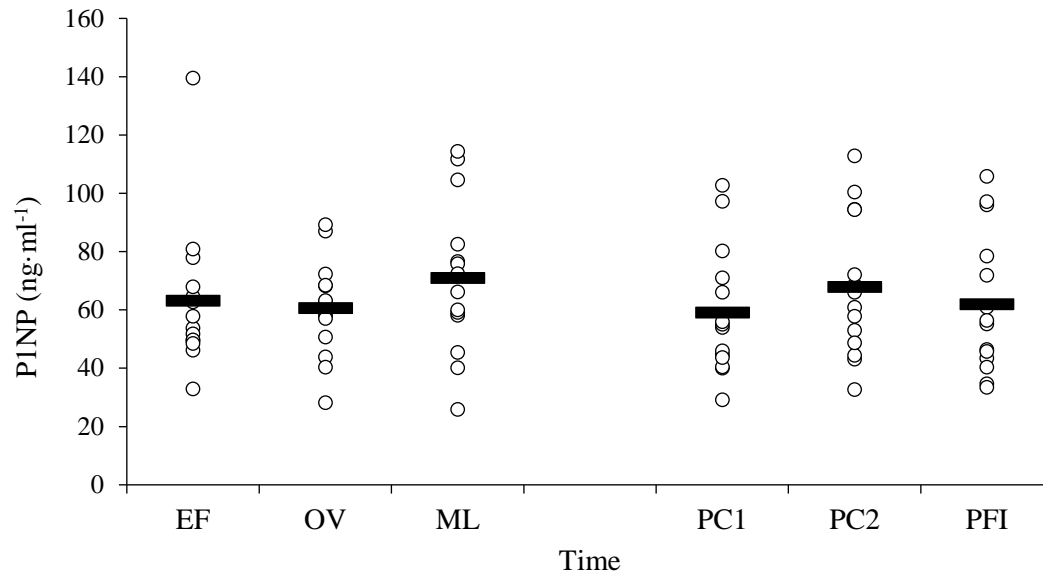
Figure 4. Univariate scatter plots with individual data points and mean values for Procollagen type I N propeptide (P1NP) in the early follicular (EF), ovulatory (OV) and mid-luteal (ML) phase and oral contraceptive users at first (PC1) and second (PC2) pill consumption time points and during the pill free interval (PFI).
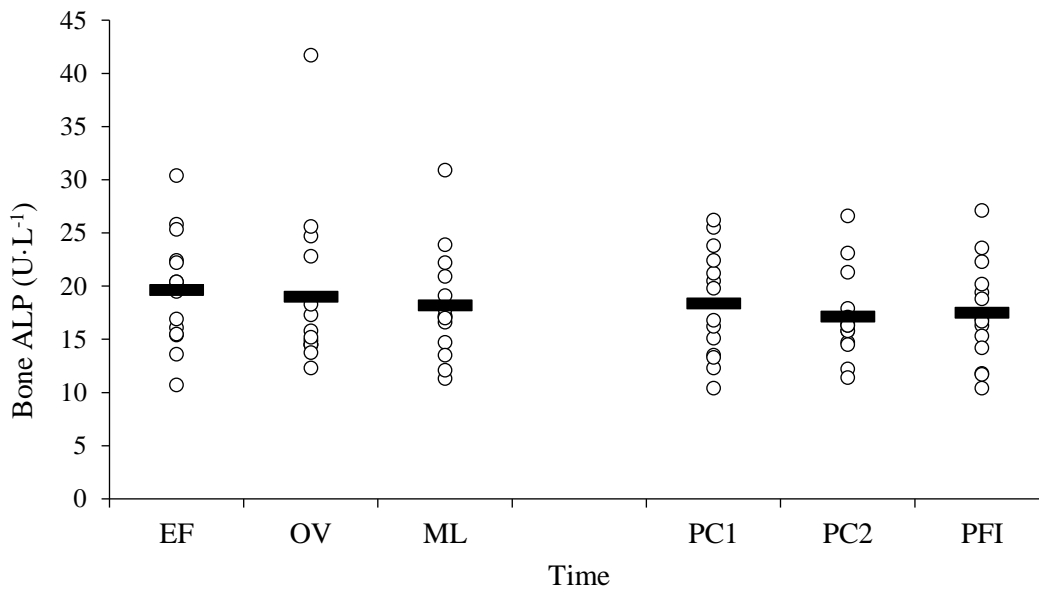
Figure 5. Univariate scatter p·ots with individual data points and mean values for Bone alkaline phosphatase (Bone ALP) in the early follicular (EF), ovulatory (OV) and mid-luteal (ML) phase and oral contraceptive users at first (PC1) and second (PC2) pill consumption time points and during the pill 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

383   previous research. P1NP 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. 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

438 representative of bone (re)modelling marker concentrations compared to previous research, which used

439 measurements from one time point only.

440

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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