

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 ( $\beta$ -CTX), procollagen type 1 N propeptide  
43 (P1NP) and Bone alkaline phosphatase (Bone ALP) concentrations were assessed in eumenorrhic  
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).  $\beta$ -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 eumenorrhic 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 •  $\beta$ -CTX concentrations were affected by COC phase but not menstrual cycle phase.
- 67 • Lowest  $\beta$ -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 ( $\beta$ -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  $\beta$ -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.

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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- $\alpha$ -ethinyl oestradiol (EO) provides negative feedback to the anterior  
115 pituitary, inhibiting the production of endogenous 17- $\beta$ -oestradiol [9]. During the 7-day PFI, the  
116 withdrawal of this negative feedback results in a 3-4 fold increase in 17- $\beta$ -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 21<sup>st</sup> day of pill consumption compared to the 1<sup>st</sup>  
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.

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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].  $\beta$ -CTX has only been studied on one  
130 occasion where 24 h urinary  $\beta$ -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  $\beta$ -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.

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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  $\beta$ -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  $\beta$ -CTX concentrations across the menstrual cycle  
147 using standardised procedures recommended by the IOF to reduce pre-analytical variability.

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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  $\beta$ -CTX across the menstrual cycle or during the COC cycle.

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## 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  $28 \pm 2$  days) over the 6 months prior to recruitment. COC users  
168 were required to use a low dose, COC preparation (Microgynon<sup>®</sup>), 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.

|                                      | <b>Eumenorrheic</b><br><b>n = 14</b> | <b>Oral contraceptive</b><br><b>n = 14</b> |
|--------------------------------------|--------------------------------------|--|
| 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 <sup>2</sup> ) | 23.8 ± 3.5                           | 22.1 ± 1.6                                 |

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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- $\beta$ -Oestradiol,  $\beta$ -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- $\beta$ -oestradiol was < 4.3% between 150-3000 pmol·L<sup>-1</sup> with a detection limit of 18.4-1581 pmol·L<sup>-1</sup>.  
215 Inter-assay CV for Bone ALP was 5.8%, with a detection limit of 0.7 U·L<sup>-1</sup>. Inter-assay CV for  $\beta$ -CTX  
216 was < 3% between 200 and 150 ng·L<sup>-1</sup>, with a sensitivity of 10 ng·L<sup>-1</sup>. Inter-assay CV for P1NP was <  
217 3% between 20-600  $\mu$ g·L<sup>-1</sup> with a sensitivity of 8  $\mu$ g·L<sup>-1</sup>.

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- $\beta$ -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- $\beta$ -oestradiol  
229 concentrations and bone metabolic markers for eumenorrhic participants and COC users



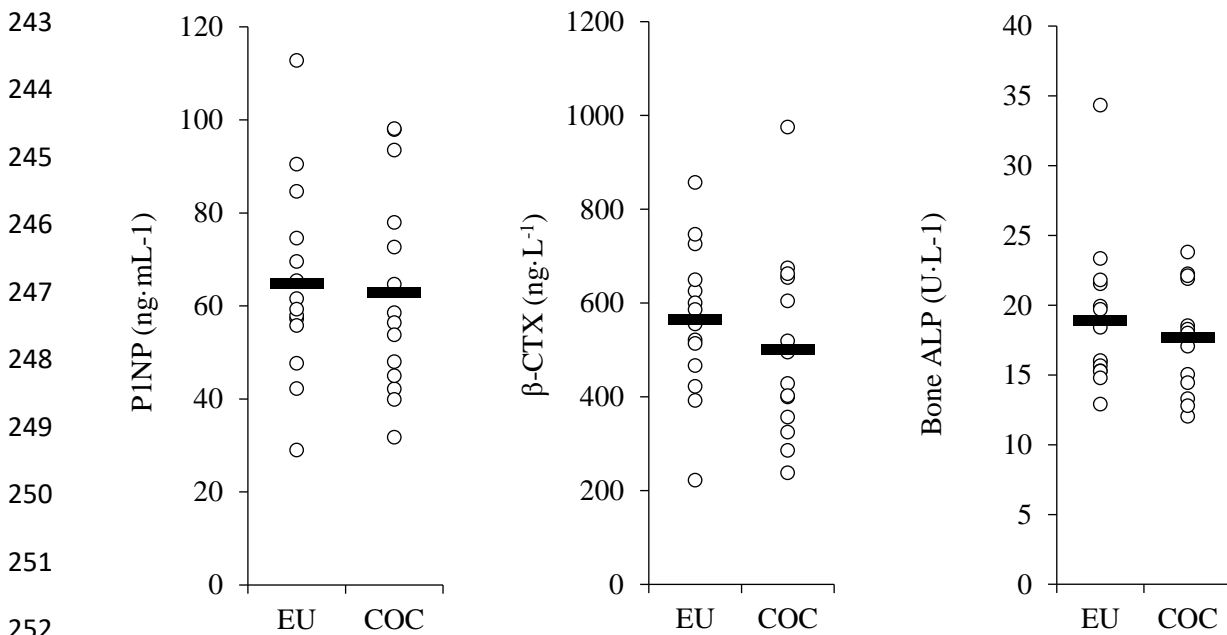
230 independently. For bone metabolism markers, mean % change between different phases of the  
231 menstrual cycle or COC cycle were calculated and individual % change responses were characterised  
232 by presenting the range of responses in addition to the relative number of participants whose bone  
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- $\beta$ -oestradiol concentrations were significantly ( $P < 0.001$ ;  $d = 3.05$ ) higher in eumenorrheic  
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 eumenorrheic and COC groups for  $\beta$ -CTX (EU =  $560 \pm 180$ , COC =  $500 \pm 200$   
241  $\text{ng}\cdot\text{L}^{-1}$ ;  $P = 0.37$ ;  $d = 0.32$ ), P1NP (EU =  $64.9 \pm 21.9$ , COC =  $62.9 \pm 22.1 \text{ ng}\cdot\text{mL}^{-1}$ ;  $P = 0.81$ ;  $d = 0.03$ )  
242 and Bone ALP (EU =  $18.9 \pm 5.4$ , 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 eumenorrheic (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 ( $\beta$ -CTX), Procollagen type I N propeptide  
256 (P1NP) and Bone alkaline phosphatase (Bone ALP) concentrations.

257

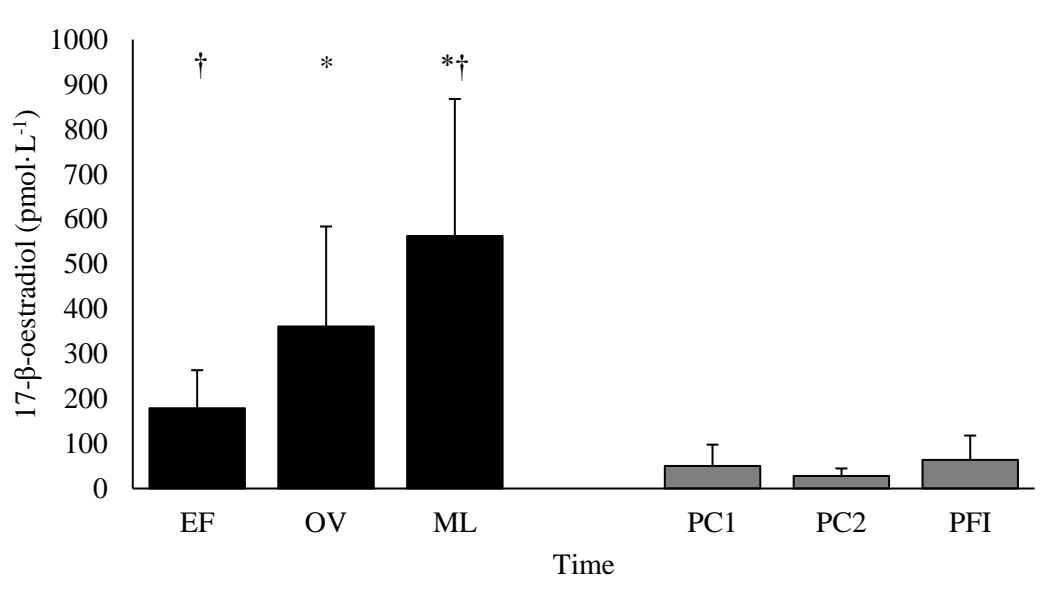
258

259 3.2. Within group comparisons

260 3.2.1. 17- $\beta$ -oestradiol

261 For eumenorrhic participants, EF phase ( $178.8 \pm 84.7$  pmol·L<sup>-1</sup>) 17- $\beta$ -oestradiol concentrations were  
262 significantly lower than OV ( $360.9 \pm 222.7$  pmol·L<sup>-1</sup>,  $P = 0.02$ ;  $d = 1.18$ ) and ML phases ( $562.4 \pm 305.2$   
263 pmol·L<sup>-1</sup>,  $P < 0.001$ ;  $d = 1.97$ ) and ML phase 17- $\beta$ -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- $\beta$ -oestradiol concentrations ( $P = 0.076$ ), but there was a medium effect size when  
266 comparing PC1 ( $50.2 \pm 47.5$  pmol·L<sup>-1</sup>) to PC2 ( $27.9 \pm 16.8$  pmol·L<sup>-1</sup>,  $d = 0.69$ ,  $P = 0.25$ ) and a large  
267 effect size when comparing PC2 to the PFI ( $63.7 \pm 54.2$  pmol·L<sup>-1</sup>,  $d = 1.01$ ,  $P = 0.075$ ).

268



269

270 Figure 2. Mean  $\pm$  1SD 17- $\beta$ -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  $\beta$ -  
281 CTX concentrations. For COC users,  $\beta$ -CTX concentrations were significantly different between  
282 different pill consumption phases ( $P = 0.006$ ; Figure 3). Compared to PC2,  $\beta$ -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  $\beta$ -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  $\beta$ -CTX concentrations were reduced from PC1 to  
291 PC2, ranging from -30.7% to +12.1%, and 11 out of 14 COC participant's  $\beta$ -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

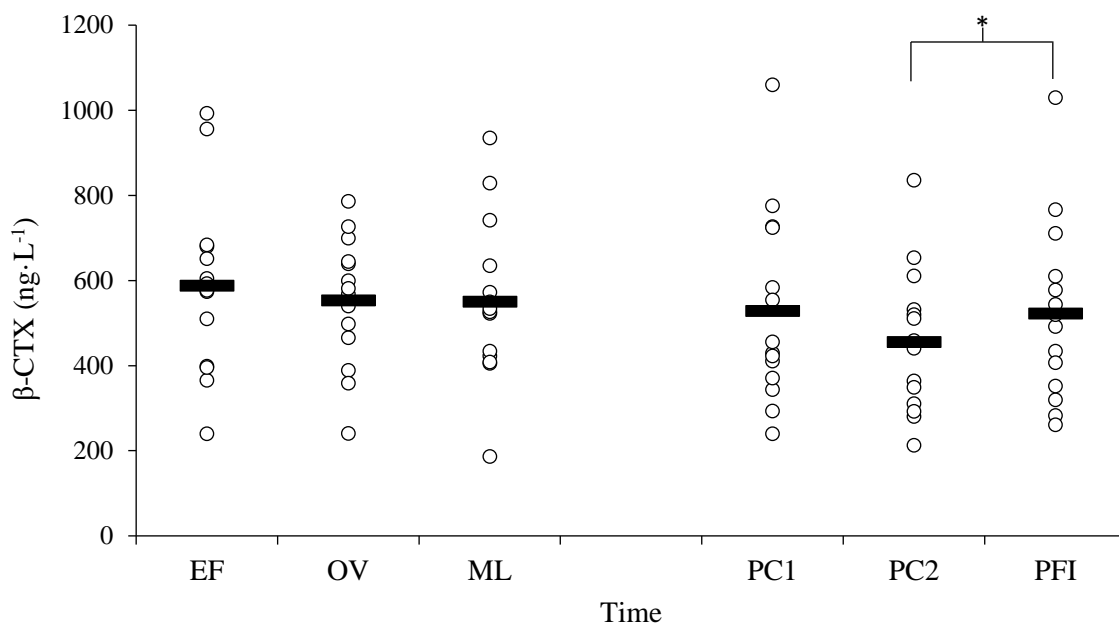
303 3.2.4. *Bone ALP*

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%.

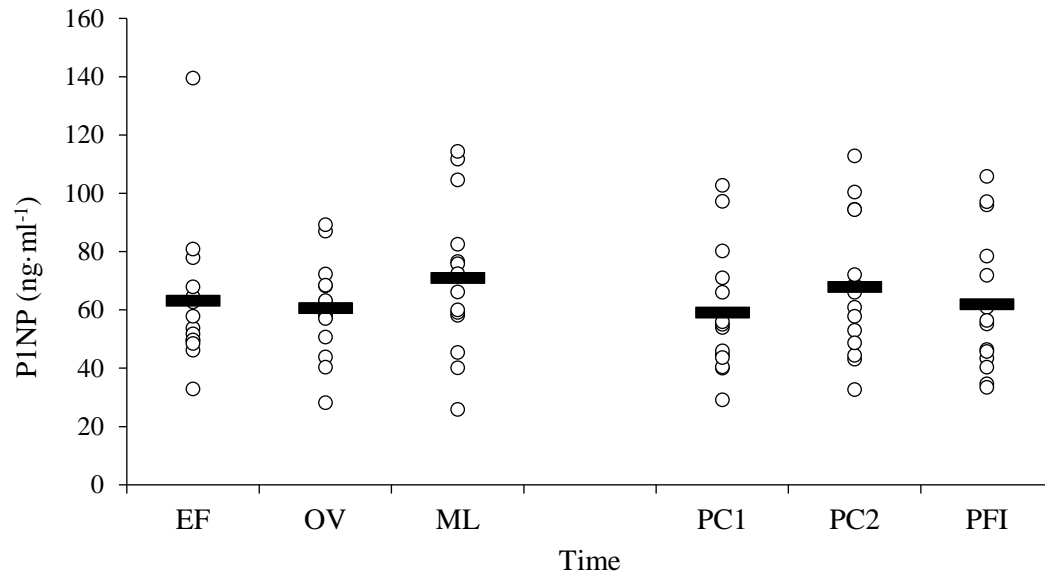
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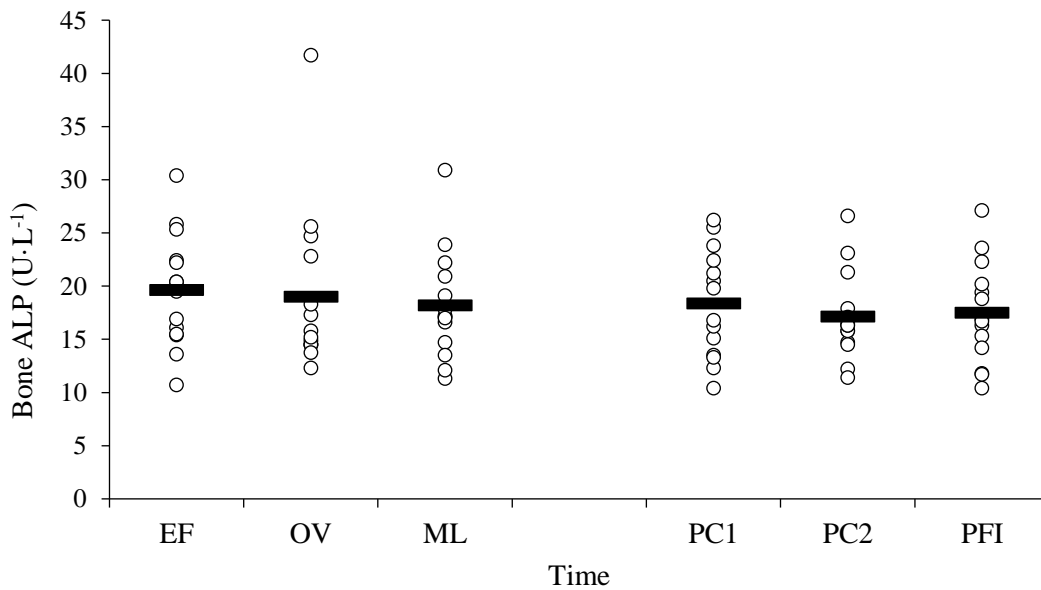
315 **Figure 3.** Univariate scatter plots with individual data points and mean values for Carboxy-terminal  
316 cross-linking telopeptide of type I collagen ( $\beta$ -CTX) in the early follicular (EF), ovulatory (OV) and  
317 mid-luteal (ML) phase and oral contraceptive users at first (PC1) and second (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  
 324 oral 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.

|                           | <b>β-CTX</b> | <b>P1NP</b> | <b>Bone ALP</b> |
|---------------------------|--------------|-------------|-----------------|
| <i>Eumenorrhic</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 eumenorrhic 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,  $\beta$ -CTX was significantly (-16%) lower during late pill consumption compared to early  
361 pill consumption. 17- $\beta$ -oestradiol was only correlated to Bone ALP in eumenorrheic participants and  
362  $\beta$ -CTX in COC users, although these correlations occurred with 17- $\beta$ -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  $\beta$ -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'  $\beta$ -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  $\beta$ -CTX concentrations were significantly (~9-14%) lower in the follicular phase  
371 compared to the luteal phase [23–26]. Individual variations in  $\beta$ -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  $\beta$ -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  $\alpha$  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  $\beta$ -  
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,  $\beta$ -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  $\beta$ -  
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  $\beta$ -CTX, which may be influenced by changes in creatinine excretion  
399 across the COC cycle [20], while the current study measured  $\beta$ -CTX in serum which avoids this  
400 potential measurement error. Typically, low 17- $\beta$ -oestradiol concentrations are associated with an  
401 increased rate of bone resorption [33], although the lowest  $\beta$ -CTX concentrations occurred on D15-16  
402 of pill consumption, at a time where endogenous 17- $\beta$ -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- $\beta$ -oestradiol as  $\beta$ -CTX  
407 concentrations during the PFI were negatively correlated with 17- $\beta$ -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- $\beta$ -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- $\beta$ -oestradiol concentrations compared to COC users, there were no  
433 differences in  $\beta$ -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  $\beta$ -CTX concentrations were not different between phases of the menstrual cycle.  $\beta$ -  
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  $\beta$ -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**

- 467 [1] C.J. Rosen, C.H. Chesnut, N.J.S. Mallinak, The Predictive Value of Biochemical Markers of  
468 Bone Turnover for Bone Mineral Density in Early Postmenopausal Women Treated with  
469 Hormone Replacement or Calcium Supplementation 1 , *J. Clin. Endocrinol. Metab.* 82 (1997)  
470 1904–1910. <https://doi.org/10.1210/jcem.82.6.4004>.
- 471 [2] M. Heer, C. Mika, I. Grzella, C. Drummer, B. Herpertz-Dahlmann, Changes in bone turnover  
472 in patients with anorexia nervosa during eleven weeks of inpatient dietary treatment, *Clin.*  
473 *Chem.* 48 (2002) 754–760. <https://doi.org/10.1093/clinchem/48.5.754>.
- 474 [3] M. Papageorgiou, D. Martin, H. Colgan, S. Cooper, J.P. Greeves, J.C.Y. Tang, W.D. Fraser,  
475 K.J. Elliott-Sale, C. Sale, Bone metabolic responses to low energy availability achieved by diet  
476 or exercise in active eumenorrheic women, *Bone*. 114 (2018) 181–188.  
477 <https://doi.org/10.1016/j.bone.2018.06.016>.
- 478 [4] Z. Dai, R. Wang, L.W. Ang, J.M. Yuan, W.P. Koh, Bone turnover biomarkers and risk of  
479 osteoporotic hip fracture in an Asian population, *Bone*. 83 (2016) 171–177.  
480 <https://doi.org/10.1016/j.bone.2015.11.005>.
- 481 [5] D. Massera, S. Xu, M.D. Walker, R.J. Valderrábano, K.J. Mukamal, J.H. Ix, D.S. Siscovick,  
482 R.P. Tracy, J.A. Robbins, M.L. Biggs, X. Xue, J.R. Kizer, Biochemical markers of bone  
483 turnover and risk of incident hip fracture in older women: the Cardiovascular Health Study,  
484 *Osteoporos. Int.* 30 (2019) 1755–1765. <https://doi.org/10.1007/s00198-019-05043-1>.
- 485 [6] S. Vasikaran, R. Eastell, O. Bruyère, A.J. Foldes, P. Garnero, A. Griesmacher, M. McClung,  
486 H.A. Morris, S. Silverman, T. Trenti, D.A. Wahl, C. Cooper, J.A. Kanis, Markers of bone  
487 turnover for the prediction of fracture risk and monitoring of osteoporosis treatment: A need  
488 for international reference standards, *Osteoporos. Int.* 22 (2011) 391–420.  
489 <https://doi.org/10.1007/s00198-010-1501-1>.
- 490 [7] P. Szulc, K. Naylor, N.R. Hoyle, R. Eastell, E.T. Leary, National Bone Health Alliance Bone  
491 Turnover Marker Project, Use of CTX-I and PINP as bone turnover markers: National Bone  
492 Health Alliance recommendations to standardize sample handling and patient preparation to  
493 reduce pre-analytical variability, *Osteoporos. Int.* 28 (2017) 2541–2556.

- 494 <https://doi.org/10.1007/s00198-017-4082-4>.
- 495 [8] G. Benagiano, F.M. Primiero, M. Farris, Clinical profile of contraceptive progestins, *Eur. J.*  
496 *Contracept. Reprod. Heal. Care.* 9 (2004) 182–193.  
497 <https://doi.org/10.1080/13625180400007736>.
- 498 [9] Z.M. Van Der Spuy, U. Sohnius, C.A. Pienaar, R. Schall, Gonadotropin and estradiol secretion  
499 during the week of placebo therapy in oral contraceptive pill users, *Contraception.* 42 (1990)  
500 597–609. [https://doi.org/10.1016/0010-7824\(90\)90001-C](https://doi.org/10.1016/0010-7824(90)90001-C).
- 501 [10] A.M. Van Heusden, B.C.J.M. Fauser, Activity of the pituitary-ovarian axis in the pill-free  
502 interval during use of low-dose combined oral contraceptives, *Contraception.* 59 (1999) 237–  
503 243. [https://doi.org/10.1016/S0010-7824\(99\)00025-6](https://doi.org/10.1016/S0010-7824(99)00025-6).
- 504 [11] S.A. Willis, T.J. Kuehl, A.M. Spiekerman, P.J. Sulak, Greater inhibition of the pituitary-  
505 ovarian axis in oral contraceptive regimens with a shortened hormone-free interval,  
506 *Contraception.* 74 (2006) 100–103. <https://doi.org/10.1016/j.contraception.2006.02.006>.
- 507 [12] W. Carol, G. Klinger, R. Jäger, R. Kasch, A. Brandstädt, Pharmacokinetics of Ethinylestradiol  
508 and Levonorgestrel after Administration of Two Oral Contraceptive Preparations, *Exp. Clin.*  
509 *Endocrinol. & Diabetes.* 99 (1992) 12–17. <https://doi.org/10.1055/s-0029-1211124>.
- 510 [13] W. Kuhnz, G. Al-Yacoub, A. Fuhrmeister, Pharmacokinetics of levonorgestrel and  
511 ethinylestradiol in 9 women who received a low-dose oral contraceptive over a treatment  
512 period of 3 months and, after a wash-out phase, a single oral administration of the same  
513 contraceptive formulation, *Contraception.* 46 (1992) 455–469. [https://doi.org/10.1016/0010-7824\(92\)90149-N](https://doi.org/10.1016/0010-7824(92)90149-N).
- 514
- 515 [14] L. Dibbelt, R. Knuppen, G. Jütting, S. Heimann, C.O. Klipping, H. Parikka-Olexik, Group  
516 comparison of serum ethinyl estradiol, SHBG and CBG levels in 83 women using two low-  
517 dose combination oral contraceptives for three months, *Contraception.* 43 (1991) 1–21.  
518 [https://doi.org/10.1016/0010-7824\(91\)90122-V](https://doi.org/10.1016/0010-7824(91)90122-V).
- 519 [15] W. Kuhnz, D. Back, J. Power, B. Schütt, T. Louton, Concentration of ethinyl estradiol in the  
520 serum of 31 young women following a treatment period of 3 months with two low-dose oral  
521 contraceptives in an intraindividual cross-over design., *Horm. Res.* 36 (1991) 63–9.

- 522 [16] T. Rabe, M.K. Bohlmann, S. Rehberger-Schneider, S. Prifti, Induction of estrogen receptor-  
523 alpha and -beta activities by synthetic progestins., *Gynecol. Endocrinol.* 14 (2000) 118–26.
- 524 [17] J.M. Foidart, W. Wuttke, G.M. Bouw, C. Gerlinger, R. Heithecker, A comparative  
525 investigation of contraceptive reliability, cycle control and tolerance of two monophasic oral  
526 contraceptives containing either drospirenone or desogestrel., *Eur. J. Contracept. Reprod.*  
527 *Health Care.* 5 (2000) 124–34.
- 528 [18] U. Wreje, J. Brynhildsen, H. Aberg, B. Byström, M. Hammar, B. von Schoultz, Collagen  
529 metabolism markers as a reflection of bone and soft tissue turnover during the menstrual cycle  
530 and oral contraceptive use., *Contraception.* 61 (2000) 265–70.
- 531 [19] P. Kristiansson, K. Svärdsudd, B. von Schoultz, Serum relaxin, symphyseal pain, and back  
532 pain during pregnancy., *Am. J. Obstet. Gynecol.* 175 (1996) 1342–7.
- 533 [20] E. Brändle, E. Gottwald, H. Melzer, H.G. Sieberth, Influence of oral contraceptive agents on  
534 kidney function and protein metabolism., *Eur. J. Clin. Pharmacol.* 43 (1992) 643–6.
- 535 [21] J.M. Davison, M.C.B. Noble, Serial changes in 24 hour creatinine clearance during normal  
536 menstrual cycles and the first trimester of pregnancy, *BJOG An Int. J. Obstet. Gynaecol.* 88  
537 (1981) 10–17. <https://doi.org/10.1111/j.1471-0528.1981.tb00930.x>.
- 538 [22] W.R. Phipps, A.M. Duncan, B.E. Merz, M.S. Kurzer, Effect of the menstrual cycle on  
539 creatinine clearance in normally cycling women, *Obstet. Gynecol.* 92 (1998) 585–588.  
540 [https://doi.org/10.1016/s0029-7844\(98\)00241-5](https://doi.org/10.1016/s0029-7844(98)00241-5).
- 541 [23] M.L. Gass, R. Kagan, J.D. Kohles, M.G. Martens, Bone turnover marker profile in relation to  
542 the menstrual cycle of premenopausal healthy women, *Menopause.* 15 (2008) 667–675.  
543 <https://doi.org/10.1097/gme.0b013e31815f8917>.
- 544 [24] C.G. Liakou, G. Mastorakos, K. Makris, I.G. Fatouros, A. Avloniti, H. Marketos, J.D.  
545 Antoniou, A. Galanos, I. Dontas, D. Rizos, S. Tournis, Changes of serum sclerostin and  
546 Dickkopf-1 levels during the menstrual cycle. A pilot study, *Endocrine.* 54 (2016) 543–551.  
547 <https://doi.org/10.1007/s12020-016-1056-9>.
- 548 [25] B. Mozzanega, S. Gizzo, D. Bernardi, L. Salmaso, T.S. Patrelli, R. Mioni, L. Finos, G.B.  
549 Nardelli, Cyclic variations of bone resorption mediators and markers in the different phases of

- 550 the menstrual cycle, *J. Bone Miner. Metab.* 31 (2013) 461–467.  
551 <https://doi.org/10.1007/s00774-013-0430-4>.
- 552 [26] B. Niethammer, C. Körner, M. Schmidmayr, P.B. Luppá, V.R. Seifert-Klauss, Non-  
553 reproductive Effects of Anovulation: Bone Metabolism in the Luteal Phase of Premenopausal  
554 Women Differs between Ovulatory and Anovulatory Cycles., *Geburtshilfe Frauenheilkd.* 75  
555 (2015) 1250–1257. <https://doi.org/10.1055/s-0035-1558298>.
- 556 [27] J.C. Crockett, M.J. Rogers, F.P. Coxon, L.J. Hocking, M.H. Helfrich, Bone remodelling at a  
557 glance, *J. Cell Sci.* 124 (2011) 991–998. <https://doi.org/10.1242/jcs.063032>.
- 558 [28] K.M. Chiu, J. Ju, D. Mayes, P. Bacchetti, S. Weitz, C.D. Arnaud, Changes in bone resorption  
559 during the menstrual cycle, *J. Bone Miner. Res.* 14 (1999) 609–615.  
560 <https://doi.org/10.1359/jbmr.1999.14.4.609>.
- 561 [29] H.K. Nielsen, K. Brixen, R. Bouillon, L. Mosekilde, Changes in Biochemical Markers of  
562 Osteoblastic Activity during the Menstrual Cycle\*, *J. Clin. Endocrinol. Metab.* 70 (1990)  
563 1431–1437. <https://doi.org/10.1210/jcem-70-5-1431>.
- 564 [30] K.J. Elliott-Sale, S. Smith, J. Bacon, D. Clayton, M. McPhilimey, G. Goutianos, J. Hampson,  
565 C. Sale, Examining the role of oral contraceptive users as an experimental and/or control group  
566 in athletic performance studies, *Contraception.* 88 (2013) 408–412.  
567 <https://doi.org/10.1016/j.contraception.2012.11.023>.
- 568 [31] J. Cohen, Jacob, A power primer., *Psychol. Bull.* 112 (1992) 155–159.  
569 <https://doi.org/10.1037/0033-2909.112.1.155>.
- 570 [32] A. Zittermann, J. Rühl, H.K. Berthold, T. Sudhop, H. Van der Ven, J. Reinsberg, P. Stehle,  
571 Oral contraceptives moderately effect bone resorption markers and serum-soluble interleukin-6  
572 receptor concentrations, *Calcif. Tissue Int.* 70 (2002) 16–21.  
573 <https://doi.org/10.1007/s002230020035>.
- 574 [33] G.E. Krassas, P. Papadopoulou, Oestrogen action on bone cells., *J. Musculoskelet. Neuronal*  
575 *Interact.* 2 (2001) 143–51.
- 576 [34] I. Gorai, Y. Taguchi, O. Chaki, R. Kikuchi, D. Obstetrics, I.G. Gynecology, Serum Soluble  
577 Interleukin-6 Receptor and Biochemical Markers of Bone Metabolism Show Significant, 83

- 578 (2014) 326–332.
- 579 [35] J. Endrikat, E. Mih, B. Düsterberg, K. Land, C. Gerlinger, W. Schmidt, D. Felsenberg, A 3-  
580 year double-blind, randomized, controlled study on the influence of two oral contraceptives  
581 containing either 20 µg or 30 µg ethinylestradiol in combination with levonorgestrel on bone  
582 mineral density, *Contraception*. 69 (2004) 179–187.  
583 <https://doi.org/10.1016/j.contraception.2003.10.002>.
- 584 [36] M. Shimizu, Y. Onoe, M. Mikumo, Y. Miyabara, T. Kuroda, R. Yoshikata, K. Ishitani, H.  
585 Okano, H. Ohta, Variations in circulating osteoprotegerin and soluble RANKL during diurnal  
586 and menstrual cycles in young women, *Horm. Res.* 71 (2009) 285–289.  
587 <https://doi.org/10.1159/000208802>.
- 588 [37] P. Garnero, E. Sornay-Rendu, P.D. Delmas, Decreased bone turnover in oral contraceptive  
589 users, *Bone*. 16 (1995) 499–503. [https://doi.org/10.1016/8756-3282\(95\)00075-O](https://doi.org/10.1016/8756-3282(95)00075-O).
- 590 [38] S.J. Glover, M. Gall, O. Schoenborn-Kellenberger, M. Wagener, P. Garnero, S. Boonen, J.A.  
591 Cauley, D.M. Black, P.D. Delmas, R. Eastell, Establishing a Reference Interval for Bone  
592 Turnover Markers in 637 Healthy, Young, Premenopausal Women From the United Kingdom,  
593 France, Belgium, and the United States, *J. Bone Miner. Res.* 24 (2009) 389–397.  
594 <https://doi.org/10.1359/jbmr.080703>.
- 595 [39] R. Karlsson, S. Eden, B. von Schoultz, Oral contraception affects osteocalcin serum profiles in  
596 young women, *Osteoporos. Int.* 2 (1992) 118–121. <https://doi.org/10.1007/BF01623817>.
- 597 [40] S.M. Ott, D. Scholes, A.Z. LaCroix, L.E. Ichikawa, C.K. Yoshida, W.E. Barlow, Effects of  
598 Contraceptive Use on Bone Biochemical Markers in Young Women <sup>1</sup>, *J. Clin. Endocrinol.*  
599 *Metab.* 86 (2001) 179–185. <https://doi.org/10.1210/jcem.86.1.7118>.
- 600 [41] A.M. Paoletti, M. Orrù, S. Lello, S. Floris, F. Ranuzzi, R. Etzi, P. Zedda, S. Guerriero, S.  
601 Fratta, R. Sorge, G. Mallarini, G.B. Melis, Short-term variations in bone remodeling markers  
602 of an oral contraception formulation containing 3 mg of drospirenone plus 30 µg of ethinyl  
603 estradiol: Observational study in young postadolescent women, *Contraception*. 70 (2004) 293–  
604 298. <https://doi.org/10.1016/j.contraception.2004.04.004>.
- 605 [42] E. Rome, J. Ziegler, M. Secic, A. Bonny, M. Stager, R. Lazebnik, B.A. Cromer, Bone

- 606 biochemical markers in adolescent girls using either depot medroxyprogesterone acetate or an  
607 oral contraceptive, *J. Pediatr. Adolesc. Gynecol.* 17 (2004) 373–377.  
608 <https://doi.org/10.1016/j.jpag.2004.09.013>.
- 609 [43] V. Gargano, M. Massaro, I. Morra, C. Formisano, C. Di Carlo, C. Nappi, Effects of two low-  
610 dose combined oral contraceptives containing drospirenone on bone turnover and bone mineral  
611 density in young fertile women: a prospective controlled randomized study, *Contraception.* 78  
612 (2008) 10–15. <https://doi.org/10.1016/j.contraception.2008.01.016>.
- 613 [44] C. Nappi, A. Di Spiezio Sardo, G. Acunzo, G. Bifulco, G.A. Tommaselli, M. Guida, C. Di  
614 Carlo, Effects of a low-dose and ultra-low-dose combined oral contraceptive use on bone  
615 turnover and bone mineral density in young fertile women: a prospective controlled  
616 randomized study, *Contraception.* 67 (2003) 355–359. [https://doi.org/10.1016/S0010-](https://doi.org/10.1016/S0010-7824(03)00025-8)  
617 [7824\(03\)00025-8](https://doi.org/10.1016/S0010-7824(03)00025-8).
- 618 [45] C. Nappi, A. Di Spiezio Sardo, E. Greco, G. a Tommaselli, E. Giordano, M. Guida, Effects of  
619 an oral contraceptive containing drospirenone on bone turnover and bone mineral density.,  
620 *Obstet. Gynecol.* 105 (2005) 53–60. <https://doi.org/10.1097/01.AOG.0000148344.26475.fc>.

621

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623

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