Menstrual Cycle Related Fluctuations in Circulating Markers of Bone Metabolism at Rest and in Response to Running in Eumenorrheic Females

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Abstract

This study examined potential fluctuations in bone metabolic markers across the menstrual cycle both at rest and after a 30-min bout of continuous running at 80% of $\dot{V}O_{2max}$. Resting and post-exercise (0, 30, 90 min) sclerostin, parathyroid hormone (PTH), carboxy-terminal cross-linking telopeptide of type I collagen (β-CTXI), and procollagen type 1 N propeptide (PINP) were assessed in 10 eumenorrheic women (age: 21±3 y, BMI: 23.2±3.0 $kg \cdot m^2$) during the mid- to late-follicular (FP: day 8.0±1.4) and mid-luteal (LP: day 22.0±2.5) phases of the menstrual cycle. Ovulation was determined using ovulation kits and daily measurement of oral body temperature upon awakening. Menstrual cycle phase was subsequently confirmed by measurement of plasma estradiol and progesterone. On average, resting estradiol concentrations increased from 46.3±8.9 $pg \cdot mL^{-1}$ in the FP to 67.3±23.4 $pg \cdot mL^{-1}$ in the LP (p=0.015), and resting progesterone increased from 4.12±2.36 $ng \cdot mL^{-1}$ in the FP to 11.86±4.49 $ng \cdot mL^{-1}$ in the LP (p<0.001). At rest, there were no differences between menstrual cycle phases in sclerostin (FP: 260.1±135.0 $pg \cdot mL^{-1}$; LP: 303.5±99.9 $pg \cdot mL^{-1}$; p=0.765), PTH (FP: 0.96±0.64 $pmol \cdot L^{-1}$; LP: 0.79±0.44 $pmol \cdot L^{-1}$; p=0.568), β-CTXI (FP: 243.1±158.0 $ng \cdot L^{-1}$; LP: 202.4 ± 92.3 $ng \cdot L^{-1}$; p=0.198), and PINP (FP: 53.6±8.9 $\mu g \cdot L^{-1}$; LP: 66.2±20.2 $\mu g \cdot L^{-1}$; p=0.093). Main effects for time (p<0.05) were shown in sclerostin, PTH, β-CTXI and PINP, without phase or interaction effects. Sclerostin increased from pre- to immediately post-exercise (45%; p=0.007), and so did PTH (43%; p=0.011), both returning to resting concentrations 30 min post-exercise. β-CTXI decreased from pre- to post-exercise (20%; p=0.027) and was below still its pre-exercise concentrations at 90 min post-exercise (17%; p=0.013). PINP increased immediately post-exercise (29%; p<0.001), returning to resting concentrations at 30 min post-exercise. These results demonstrate no effect of menstrual cycle phase on resting bone marker concentrations or on the bone metabolic marker response to intense exercise.

Introduction

It is well established that female sex hormones, including estrogen, follicle stimulating hormone, luteinizing hormone and progesterone have a predictable cyclical undulation throughout the normal menstrual cycle [1]. Although there is individual variation within each women's menstrual cycle, typically a eumenorrheic cycle lasts on average 28 days and is recognized by a mid-cycle peak in estrogen and LH during the ovulatory phase [2, 3]. Estrogen and progesterone concentrations fluctuate in accordance with the different menstrual phases; both having lower concentrations pre-ovulation, *i.e.*, during the follicular phase (FP), and higher concentrations post-ovulation, *i.e.*, during the luteal phase (LP) [2, 3]. Since these fluctuations have been shown to affect other physiological functions, regulatory markers and hormones [4, 5, 6], it is important to investigate whether they could potentially also affect the circulating concentrations of bone biomarkers, osteokines and related hormones, either at rest or in response to vigorous running.

Estrogens are an important group of steroid hormones that play a key role in reproductive and sexual development. Estradiol is the estrogen that regulates the female menstrual cycle and is also a key regulator of bone metabolism [7, 8, 9]. In eumenorrheic women, typical estradiol concentrations, ranging from 30 to 350 $pg \cdot mL^{-1}$, provide a protective effect on bone [11]. *In vitro* studies have shown that this protective effect occurs through inhibition of osteoclast maturation, as well as osteoblast apoptosis [12, 13]. In humans, chronic low levels of estradiol due to menopause, calorie restriction, congenital conditions, Turners syndrome or conditions associated with ovarian function, can negatively impact bone health in women [7, 14]. Decreased estradiol levels are associated with an increased rate of bone remodeling and activation frequency within bone's basic multicellular unit [15], which increases bone formation and resorption [16]. Furthermore, decreased estradiol levels have been associated with increased oxidative stress,

which has been associated with bone loss in *in vivo* and *in vitro* [17]. Taken together, these data suggest that a cyclical reduction of circulating estradiol could underpin the increased bone resorption seen under these circumstances, potentially leading to a subsequent loss of bone mass [7, 13, 18].

Several studies have examined resting concentrations of markers of bone metabolism, including parathyroid hormone (PTH), sclerostin, C-terminal cross-linking telopeptide of type I collagen (β-CTXI, and procollagen type 1 N propeptide (PINP), to determine whether there are differences between phases. These studies have shown contradictory outcomes [19, 20, 21, 22, 23, 24, 25, 26, 27]. Studies investigating baseline PTH levels between the FP (from the onset of menses until ovulation, approximately days 1-14) and the LP (from ovulation until the start of menses, approximately days 14-28), have shown significant increases in the late FP [26][28] and no difference between phases [29, 30, 31], while no phase-induced differences have been reported in sclerostin [32, 33]. Several studies have shown higher concentrations of β-CTXI in FP compared to LP [19, 20, 32, 34], but more recent data show no significant differences in serum β-CTXI between phases [27]. As β-CTXI measurements are impacted by fasting and circadian rhythms, among other things, poor research design may explain some of these differing outcomes. The same is true for PINP measurements, as recent data show no significant differences between phases in eumenorrheic females [27], although two previous studies showed significantly lower resting levels of PINP in FP compared to LP [19, 32].

These contradictory results are only relevant during rest, with no data existing on the effects of menstrual cycle phase on circulating levels of bone markers following exercise. Confirming whether menstrual fluctuations influence markers of bone metabolism both at rest and post-exercise is important for three reasons; firstly, to optimise data collection during

research by making sure that data are collected during a specific phase, should there be an influence of menstrual fluctuations. Secondly, to remove participation barriers when recruiting eumenorrheic females into research related to these bone metabolic markers in case that there is no influence of menstrual fluctuations, in which case, investigators would not have to account for menstrual cycle phase during data collection, thus simplifying the process. Third, to understand whether one menstrual phase could provide an environment that leads to a greater osteogenic response to exercise, which would be important to capitalize on when prescribing exercise with the purpose to increase/maintain bone mass or recover from a bone stress injury. The purpose of this secondary analysis study was to investigate potential fluctuations in bone metabolic markers and regulatory hormones, including sclerostin, PTH, β-CTXI and PINP, between the FP (day 1-14) and LP (day 14-28), both at rest and in response to high intensity (80% of $\dot{V}O_{2max}$) running in healthy, eumenorrheic women.

Methods

Participants

Data presented in this study represent a secondary analysis of blood samples taken from a convenient university population of 10 healthy, eumenorrheic females between the ages of 18 and 30 years (average age 22 ± 3 y), who volunteered for an ongoing larger study originally designed to examine the potential involvement of sex hormones in appetite regulation. As per the CSEP Get Active Questionnaire, all participants were considered 'healthy' (62.6 ± 10.4 kg, 1.64 ± 0.34 m; 23.2 ± 3.4 $kg \cdot m^{-2}$), non-smokers and recreationally active, performing no more than 2 weekly exercise sessions and were not involved in any structured exercise training programs in the four months prior to participation [35]. Participants were eumenorrheic, minimum 3 y post-

menarche, and were not taking hormonal contraceptives. Eumenorrhea was defined as regularly occurring menstrual cycles lasting 26-35 days for a minimum of 1 year, with cycle length variability [3]. Participants not actively tracking their menstrual cycles were asked to do so for three months to insure they met the inclusion criterion of being eumenorrheic. Participants were excluded if they were pregnant or had been pregnant for over 3 months within the past 3 years or had plans to become pregnant within the study period. Participants were also excluded if they had been diagnosed with any eating disorder or metabolic diseases. All study procedures were approved by the Research Ethics Boards of Brock (REB# 20-218) and Wilfrid Laurier (REB# 5856) Universities.

Menstrual Phase Determination

Day 1 of the menstrual cycle was defined by the onset of menses. Menstrual cycle phase was monitored in each participant, and an LH surge was measured using ovulation kits (Easy@Home, Easy Healthcare Corporation, IL, USA), which were provided by investigators along with specific instructions on when to commence testing and how to use the kit. Participants were provided with the user manual in PDF format as an additional reference. In addition to the ovulation kits, participants were provided with a thermometer to take oral body temperature daily upon waking. Both ovulation kit outcomes and temperature were reported daily to researchers via text or email. Ovulation was assumed when a participant had sustained oral body temperature increase of ~0.3°C [36]. Menstrual cycle phase was subsequently confirmed with analysis of plasma estradiol (E2) and progesterone (P4) taken on study days, confirming a peak in P4 during the mid luteal phase.

Study Design

The study used a randomized, repeated measures design. A random computer-based number generator was used to determine which phase the participants started with. The study protocol included a 1-h familiarization session, including running on the treadmill to determine $\dot{V}O_{2max}$, followed by 2 identical 30-min continuous running trials performed at 80% of each participant's predetermined $\dot{V}O_{2max}$. During the familiarization session, $\dot{V}O_{2max}$ was determined and verified with a graded running test to exhaustion. The running trials were performed in the FP and the LP phases of the menstrual cycle. For the FP, testing occurred on day 8 ± 1, which corresponds to mid to late FP. The LP sessions were scheduled 1 week after a positive test from a LH detection kit. With LH spiking ~36 h before ovulation, the LP session was timed near the mid LP (day 22 ± 3).

Familiarization Session

Familiarization and information collection occurred approximately 1 week before the first experimental session. Upon arrival to the laboratory, participants provided informed consent and were screened using the Get Active Questionnaire (GAQ) [35], to assess health status, as well as the Godin Leisure Time Exercise questionnaire [37], to get a baseline of activity levels. They were asked in person about their menstrual history at this time, including when their first menses was and if they had any history of menstrual irregularities. During this time, height (to the nearest 0.01 m) and body mass (to the nearest 0.1 kg) were recorded using a mechanical scale (Health-o-meter Professional, Sunbeam Products Inc., Ill., USA).

Next, participants were familiarized with the motorized treadmill (4Front, Woodway, WI, USA) used for the graded running test and the exercise sessions, with instruction from the investigators. Participants were then fitted with a silicon facemask (7400 series Vmask, Hans

Rudolph Inc. KS, USA), to prevent leaking and to ensure comfort. The graded running test to exhaustion started with a walking warmup at 3.5 $mi \cdot h^{-1}$ for 5 min, after which, participants began running at their own selected pace between 5-7 $mi \cdot h^{-1}$ until test completion. Incremental increases of 2% were applied to the treadmill every two minutes, until volitional fatigue. To verify $\dot{V}O_{2max}$, a follow-up test was performed after a 20-min rest period. During this test, participants ran at 110% of their maximal work rate, until volitional fatigue. Oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) were measured using an online breath by breath gas collection analysis system (MAX II, AEI technologies, Pittsburgh, PA, USA). Heart rate was monitored continuously using an integrated HR monitor (FT1, Polar Electro, QC, Canada). $\dot{V}O_{2max}$ was defined as the highest 30-second average where $\dot{V}O_2$ values plateaued, irrespective of increases in workload. In addition, one of the three following criteria were also met: 1) respiratory exchange ratio (RER) > 1.10, 2) maximal HR within ± 10 $b \cdot min^{-1}$ of age predicted maximum defined as 220 $b \cdot min^{-1}$ - age in years, or 3) voluntary exhaustion. From the data collected during the test to exhaustion, 80% of $\dot{V}O_{2max}$ was determined and became the target intensity for the high intensity, continuous running trials. Familiarity with the equipment and study exercise session protocols was complete at this point, minimizing any learning effects during the experimental exercise trials.

Participants were given a food log and were instructed how to record their food intake, including the quantity of food and beverage intake. Food intake was recorded over a 3-d period including the day prior, day of and day following each trial. This same food intake was repeated by participants the day before each subsequent trial to replicate the exact diet for all sessions. To maximize accuracy, participants were provided with a sample log with specific instructions to get the most accurate measurements and recordings. The Nutribase software (Nutribase Pro

Edition, Cybersoft Inc., AZ, USA) was used to analyze all dietary logs for daily total energy intake (kcal) and macronutrient content.

Vigorous Intensity Continuous Running Trials

All participants arrived at the laboratory at 0800 h after a 12-h overnight fast. Participants also refrained from caffeine, alcohol, and vigorous exercise for 12 h prior to all visits. A standardized breakfast was provided to each participant, which consisted of a Chocolate Chip Clif bar, 29 $kJ \cdot kg^{-1}$ of the participant's body weight. Each bar contained 250 cal, 44 g of carbohydrate, 10 g of protein, 5 g of fat and 294 mg of calcium. Fifteen minutes was allotted for consuming the bar and 45 min to digest it, while water was consumed *at libitum* during this time. Participants sat quietly eating and digesting from 0800 to 0845 h and then had their resting gas exchange measured between 0845 and 0900 h.

Each running trial began at 0910 h on a motorized treadmill (4Front, Woodway, WI, USA), and consisted of the same standardized 5-min warm up at 3.5 $mi \cdot h^{-1}$ followed by 30 min of high intensity, continuous running at 80% of $\dot{V}O_{2max}$, and a 5-min cool down. Heart rate and gas exchange ($\dot{V}O_2$ and $\dot{V}CO_2$) were continuously measured throughout the trial using an online breath by breath gas collection analysis system (MAX II, AEI technologies, Pittsburgh, PA, USA), and an integrated HR monitor (FT1, Polar Electro, QC, Canada). By calculating the $\dot{V}O_2$ reserve, a pre-determined work rate was established [38]. Target $\dot{V}O_2$ was determined using the following formula: target $\dot{V}O_2$ = (intensity fraction) ($\dot{V}O_{2max}$ - $\dot{V}O_{2rest}$) + $\dot{V}O_{2rest}$. Then, a mode-specific standardized equation (the ACSM running equation) was used to determine the percent grade necessary to elicit the target $\dot{V}O_2$ at each participant's chosen speed (Swain 2000): $\dot{V}O_2$ = 0.2 (speed) + 0.9 (speed)(grade) + 3.5. In order to maintain the target intensity, speed and grade were adjusted throughout the trial.

Blood Samples and Analysis

Blood samples were obtained from an antecubital vein at four time points: pre-exercise (0900 h), immediately post-exercise (0950 h), 30 min post-exercise (1020 h) and 90 min post-exercise (1120 h). Blood samples were collected from the antecubital vein while participants were in a supine position. Two 3 mL whole blood samples were collected into separate pre chilled vacutainer tubes coated with K2 ethylenediaminetetraacetic acid (EDTA; 5.4mg) at each time point. All tubes were centrifuged at 3000 g for 10 min at -4°C, then the plasma supernatant was aliquoted into microcentrifuge tubes, which were stored at -80°C until further analysis.

Estradiol and progesterone were measured in duplicate using standard enzyme-linked immunosorbent (ELISA) assay kits (DiaMetra, Spello, Italy) at both rest and post-exercise. The in-house, intra-assay coefficients of variation were 9.1% for estradiol, and 10.5% for progesterone. Serum sclerostin was measured in duplicate using two panels of an ELISA assay kit (SCL, cat.# DSST00, R&D Systems, Inc., Minneanapolis, MI, USA), with the average in-house intra- and inter-assay coefficients of variation being 9.2%, and 7.2%. Total PTH, β-CTXI, and PINP were measured at the Mount Sinai Hospital Core Laboratory (Toronto, Ontario) using a Roche Cobas e602 automated analyzer for β-CTXI and PTH, and a Roche e411 Elecsys automated analyzer (Roche Diagnostics, Rotkreuz, Switzerland) for PINP. Lower and upper detection limits were 0.010–6.00 $ng \cdot mL^{-1}$ (quality control standard CV: 4.8%), 0.127-530 $pmol \cdot L^{-1}$ (quality control standard CV: 6.2%), and 5-1200 $\mu g \cdot L^{-1}$ (quality control standard CV: 5.2%), for β-CTXI, PTH, and PINP.

Statistical Analysis

Upon inspection of estradiol and progesterone individual data, one participant was found with atypical estrogen and progesterone pattern and was excluded from the analysis of sclerostin,

PTH, β-CTXI and PINP. In addition, although all participants did complete both exercise trials there were a few missing values due to either a missed blood draw or an insufficient sample volume to run all assays. Participants with more than two missing values of a specific marker were excluded from the analysis of that marker (1 from the β-CTXI analysis and 1 from the PTH analysis). If a participant had one or two missing values, each of the missing values (5 of 64 for sclerostin, 2 of 56 for PTH, 6 of 56 for β-CTXI and 4 of 64 for PINP) was replaced with the group mean value at the corresponding timepoint for the specific menstrual phase. The replacement of missing values with the group mean value is often used in a repeated measures design, as it does not affect the group mean of a particular time point while preserving the rest of these participants' data in the analysis [39]. Subsequently, all variables were screened for normality using the Shapiro-Wilk test, z-scores for skewness and kurtosis of ±3 and visual screening of histograms for symmetry. The screening showed that PTH and PINP were not normally distributed and, as such, data were log-transformed for the analysis. Sphericity was tested using Mauchly's Test of Sphericity. For the analytes that did not pass the Mauchly's Test (estradiol and progesterone) the Greenhouse-Geisser correction was used.

Exercise data between the two phases of the menstrual cycle were analysed using paired *t*-tests. Differences between phases in bone biomarker concentrations were analysed using a two-factor repeated measures ANOVA with the factors being phase (FP and LP) and time (pre-exercise, 0 min, 30 min and 90 min post-exercise). In the case of a significant main effect of time, post hoc comparisons using paired *t*-tests with Bonferroni adjustment were performed. Significance was assumed at $\alpha<0.05$ and was adjusted for 3 comparisons ($\alpha<0.017$) in the *post hoc* analysis. All statistical analyses were done using the SPSS IBM version 26 (SPSS Inc., Chicago, IL, USA). Effect sizes are reported as partial eta squared (η_p^2) for ANOVA and

Cohen's d for significant *post hoc* comparisons and were interpreted based on the Cohen's criteria: for partial η^2: 0.01=small, 0.06=moderate, 0.14=large effect; for Cohen's d: 0.2=small, 0.5=medium, 0.8=large effect [40, 41]. Data are reported as means ± 1SD.

Results

The continuous running trials were performed in both the FP and LP at a work rate corresponding to 29.31 ± 3.30 mL·kg^{-1}·min^{-1} (79.3 ± 0.03% $\dot{V}O_{2max}$) and 29.42 ± 3.10 mL·kg^{-1}·min^{-1} (79.6 ± 0.02% $\dot{V}O_{2max}$), with no significant difference between phases (p = 0.753; d = 0.10). Likewise, there were no significant differences in the average heart rate between the trials performed in the FP and LP (171 ± 15 b·min^{-1} and 171 ± 12 b·min^{-1}; p = 0.902; d = 0.01), or in RER achieved in the running trials between the FP and the LP (1.01 ± 0.05 and 1.00 ± 0.04; p = 0.058; d = 0.64). $\dot{V}O_2$-derived estimates of energy expenditure (assuming a relationship of 5 kcal·L^{-1} O_2) were 270.9 ± 36.0 kcal and 272.5 ± 45.5 kcal in the FP and LP and were not significantly different between phases (p = 0.523; d = 0.20).

All participants had resting estradiol levels within adult premenopausal reference values in both phases, with the reference range derived from ELISA assay kits (DiaMetra, Spello, Italy) being 30-100 pg·mL^{-1} for FP and 50-180 pg·mL^{-1} for LP. In line with previous research [27], estradiol concentrations were on average lower in the FP than in the LP (46.3 ± 8.9 pg·mL^{-1} versus 67.3 ± 23.4 pg·mL^{-1}; p = 0.015; d = 1.2), and this difference was observed in 8 of the 10 participants while 2 participants did not show an increase in estradiol (Figure 1A). Similarly, resting progesterone was lower in the FP than in the LP, on average (4.12 ± 2.36 ng·mL^{-1} versus 11.86 ± 4.49 ng·mL^{-1}; p < 0.001, d = 2.1), as well as individually (Figure 1B). In addition, resting progesterone fell within the reference range of <1.0 ng·mL^{-1} in the FP for all participants. For the LP, all participants levels fell within the reference range of 4-25 ng·mL^{-1} (DiaMetra,

Spello, Italy), with only two participants near the lower limit of this range; one of whom did not show an increase in estradiol either, so she was considered atypical (i.e., anovulatory) and was excluded from further analysis. Moreover, estradiol showed significant main effects of time ($F = 3.39$, $p = 0.027$; $_p\eta^2 = 0.19$) and menstrual phase ($F = 11.68$; $p = 0.004$; $_p\eta^2 = 0.45$), but no interaction ($F = 0.41$; $p=0.747$; $_p\eta^2 = 0.03$), reflecting an increase from pre- to immediately post-exercise in both phases (15 ± 5%; $p=0.006$; d = 0.81) returning to pre-exercise levels 30 min post-exercise. Significant main effects of time ($F = 4.42$; $p = 0.009$; $_p\eta^2 = 0.24$) and menstrual phase ($F = 47.30$; $p < 0.001$; $_p\eta^2 = 0.77$), with no interaction ($F = 2.53$; $p = 0.07$; $_p\eta^2 = 0.15$) were also shown in progesterone, which also increased from pre- to immediately post-exercise in both phases (20 ± 4%; $p = 0.006$; d = 0.80) and remained elevated up to 30 min post-exercise (19 ± 4%; $p = 0.002$; d = 0.93).

[INSERT FIGURE 1]

Sclerostin showed no effect for menstrual phase ($F = 0.25$; $p = 0.627$; $_p\eta^2 = 0.015$) and no interaction ($F = 1.06$; $p = 0.375$; $_p\eta^2=0.06$), but there was a significant main effect of time ($F = 4.19$; $p = 0.010$; $_p\eta^2 = 0.21$). At rest, sclerostin was not different between FP and LP (260.1 ± 135.0 and 303.5 ± 99.9 pg·mL^{-1}, respectively; $p=0.450$). Following running, sclerostin increased immediately post-exercise in FP and LP (45%; $p = 0.007$; d = 0.91), then returned to near its pre-exercise levels at 30 min and 90 min post-exercise (Figure 2). Likewise, PTH showed no main effects of menstrual cycle phase ($F = 1.31$; $p = 0.271$; $_p\eta^2 = 0.09$) and no interaction ($F = 0.83$; $p = 0.483$; $_p\eta^2 = 0.06$), so resting PTH was not different between FP and LP (0.96 ± 0.64 and 0.79 ± 0.44 pmol·L^{-1}, respectively; $p=0.256$). However, we found a significant main for time ($F = 4.57$; $p = 0.007$; $_p\eta^2 = 0.25$), reflecting an overall increase (43%; $p = 0.011$; d = 0.79) in PTH irrespective of menstrual phase (Figure 3).

[INSERT FIGURES 2 and 3]

For β-CTXI we found no main effect of menstrual cycle phase (F = 0.16; $p = 0.691$; ${}_p\eta^2 = 0.01$) and no interaction (F = 0.27; $p = 0.845$; ${}_p\eta^2 = 0.02$), but there was a significant main effect of time (F = 4.79; $p = 0.005$; ${}_p\eta^2 = 0.23$). At rest, β-CTXI was not different between FP and LP (243.1 ± 158.0 and 202.4 ± 92.3 $ng \cdot L^{-1}$, respectively; p=0.198). Following running, β-CTXI decreased significantly immediately post-exercise (20%; $p = 0.027$; d = 0.78) with no difference between phases (Figure 4). Interestingly, β-CTXI remained below its pre-exercise levels at 30 min post-exercise (12%; $p = 0.151$; d = 0.53), although this *post hoc* comparison was not significant upon Bonferroni adjustment, and then continued to remain below its pre-exercise concentration at 90 min post-exercise (17%; $p = 0.013$; d = 0.75). For PINP, there was no main effect of menstrual cycle phase (F = 2.47; $p = 0.133$; ${}_p\eta^2 = 0.12$) and no interaction (F = 1.16; $p = 0.333$; ${}_p\eta^2 = 0.06$), but there was a significant main effect of time (F = 26.50; $p < 0.001$; ${}_p\eta^2 = 0.59$). At rest, PINP was not different between FP and LP (56.9 ± 8.9 and 64.3 ± 20.2 $\mu g \cdot L^{-1}$, respectively; p=0.253). In response to exercise, PINP increased immediately post-exercise (29%; $p < 0.001$; d = 0.14), returning to resting concentrations at 30 min and 90 min post-exercise (Figure 5).

[INSERT FIGURES 4 and 5]

Discussion

This study investigated differences in circulating sclerostin, PTH, β-CTXI and PINP at rest and in response to exercise between the follicular and luteal phases of the menstrual cycle in eumenorrheic women. Despite a significantly lower concentration of estradiol in the FP compared to the LP, there were no effects of menstrual cycle phase on sclerostin, PTH, β-CTXI and PINP at rest or in response to exercise. PTH and PINP concentrations were significantly

higher immediately post-exercise in both phases of the menstrual cycle, while β-CTXI was significantly lower immediately post-exercise, as well as 90 min post-exercise compared to pre-exercise.

The lack of a menstrual cycle effect on resting sclerostin, despite significant estradiol fluctuations, is in line with previous research [32, 33]. Although the chronic estrogen deficiency seen during menopause increases serum sclerostin, leading to decreased bone formation and potential bone loss [43], based on the 'estrogen threshold' theory, it is possible that healthy eumenorrheic women do not reach low enough estrogen levels to significantly impact sclerostin concentrations. According to this theory, estradiol concentrations below 20 $pg \cdot mL^{-1}$ and between 10-20 $pg \cdot mL^{-1}$ are osteocatabolic. In our study, although we cannot confirm that we measured estradiol at its lowest point, the lowest average concentration during FP was 46 $pg \cdot mL^{-1}$ with the lowest individual concentration at 30 $pg \cdot mL^{-1}$, which was presumably not low enough to elicit significant changes in sclerostin levels. Indeed, serum levels of estradiol during the menstrual cycle remain higher than those seen post menopause [44]. Therefore, the cyclical decrease in estradiol levels seen during the follicular phase in our eumenorrheic females suggests there may be a enough estradiol present to avoid a catabolic effect on bone [11]. Sclerostin concentrations significantly increased immediately post-exercise, with no difference between phases. This was expected based upon previous research investigating sclerostin responses to running [45, 46] and other forms of exercise [47] in females. The exact mechanisms underlying the transient post-exercise increases in sclerostin concentrations are unknown, although it seems to occur in response to intense exercise of both high and low impact [45]. There were no differences in post-exercise sclerostin between phases.

There were no significant fluctuations in resting PTH across the phases of the menstrual cycle, which is in line with previous studies [22, 30, 31], although an increase in PTH during the follicular phase has also been reported in two studies [26, 28]. Study design and large individual biological variations in PTH, up to 20% in healthy people, could explain some of the differences in results between studies [48]. With increased age, and a decrease in estrogen, in particular in the first two decades post menopause, there is an increase in PTH [49]. Despite significant fluctuations in estradiol during our study, and three other studies [22, 30, 31], this inverse relationship does not appear to be present in the eumenorrheic population. Like sclerostin, PTH may not differ between phases because the normal estradiol fluctuations during a healthy eumenorrheic menstrual cycle do not result in low enough estrogen levels, which falls in line with the estrogen threshold theory. The transient nature of the fluctuations during a menstrual cycle may also not be long enough to elicit the PTH response compared to when estradiol is chronically lower during menopause or after an ovariectomy.

PTH increased 43% from pre- to immediately post-exercise. There is only one study to our knowledge that has investigated pre and post-exercise PTH between phases of the menstrual cycle, using a resistance training protocol in women. [25]. They showed a time difference between phases, with an increase immediately post-exercise in the follicular phase, and 1 hour post-exercise in both the LP and the FP [25], although their estradiol levels also remained above 30 $pg \cdot mL^{-1}$ in both phases. It is possible that the 30-min running exercise protocol used in our study led to a different PTH response than the resistance training protocol used by Suzuki et al. [25]. Previous exercise studies have also shown conflicting post-exercise PTH responses, with some showing increases immediately post-exercise [50, 51], followed by a return to or below baseline at 1 h post-exercise [47, 52], and others showing no post-exercise PTH response when

exercise intensity was under 65% of $\dot{V}O_{2max}$ [50]. Although the exact mechanisms for the post-exercise PTH increases are not completely understood, studies investigating the role of dermal calcium and serum ionized calcium (iCa) pre, during and post-exercise have given important insights [53, 54]. In addition, PO_4 may contribute to PTH secretion during and following exercise [55]. Calcium ingestion pre and during exercise has been shown to attenuate increases in PTH [56, 57, 58, 59, 60]. Although we did not measure calcium in our study, participants consumed a bar containing 294 mg of calcium per serving before exercising. The average pre-exercise intake of calcium was 516 mg. Despite this calcium intake we still saw a significant exercise-induced transient increase in PTH immediately post-exercise in our study.

Bone resorption, as determined by β-CTXI concentrations, was not affected by menstrual cycle phase. This is in line with one recent study comparing β-CTXI between phases in eumenorrheic individuals and oral contraceptive users [27]. However, others have reported significant increases in β-CTXI concentrations in the FP as compared to the LP [19, 20, 32, 34]. Several factors could explain these conflicting results. Firstly, the average age in our study was 22 years, whereas previous studies examined women of 40 [34] and 45 [32] years of age. It is possible that, since vitamin D synthesis and intestinal absorption of calcium can be lower in middle-aged women compared to our younger group of women, this could have indirectly affected bone marker concentrations [61]. Secondly, our participants refrained from vigorous activity 12 hours prior to lab visits, which was not the case for the previous studies [19, 20, 34]. Controlling for pre-visit exercise potentially allowed us to show a more accurate representation of β-CTXI concentrations at rest. Thirdly, since previous studies have shown that nutrition can modulate β-CTXI levels [62, 63], it is possible that our pre-exercise meal reduced β-CTX1 levels; however, our nutrition protocol and energy intake was matched for the FP and LP

experimental sessions and no differences in β-CTXI were measured between phases. Additionally, our blood samples were drawn within a short window in the morning (0900 to 0910), in accordance with the range recommended by the National Bone Health Alliance [64]. Only one of the studies with contradicting results drew blood within the recommended 0800 to 1000 hours timeframe [64], although even this might be considered a broad timeframe from a research perspective given that β-CTXI levels decrease throughout the day to a nadir in the early afternoon [62, 65, 66]. As such, controlling the sampling time, as in our study, is essential in producing reliable data between menstrual cycle phases. Another factor to consider are the assays used between studies. Significant discrepancies between assays have been reported in the literature [67]. This limited commutability between assays makes comparing our results to previous studies problematic, and is an area that should be addressed in order to allow for legitimate comparisons between assays [68].

Post-exercise β-CTXI concentrations were significantly lower than pre-exercise concentrations, with this being the first study to examine the β-CTXI responses to exercise in different phases of the menstrual cycle. Previous studies examining β-CTXI concentrations following exercise have produced conflicting results, with many showing increases [52, 56, 69, 70, 71] and some showing decreases [47] or no change [50, 56]. An increase or decrease in β-CTXI could be osteogenic, with increases representing an upregulation of bone remodelling and decreases indicating lower osteoclastic activity. Studies involving running at similar intensities to our study, have shown increases in β-CTXI one hour post-exercise [52, 72], albeit in male participants. Indeed, research comparing men and women cycling at different intensities, has shown β-CTXI to increase post-exercise only in men [73]. There are also other possible reasons as to why β-CTXI decreased in response to running in our study. Evidence has shown that

consumption of carbohydrates results in decreased markers of bone resorption, namely β-CTXI, as does consumption of protein and fat [62, 74]. These reductions in β-CTXI appear to be acute, lasting one up to as long as 6 hours post-exercise, indicating that food consumption acutely attenuates exercise induced increases in β-CTXI. It is possible, therefore, that the mixed meal our participants consumed pre-exercise contributed to the reduction in the β-CTXI levels we saw post-exercise. Exercise modality, duration and intensity may also have contributed to differences in the β-CTXI response between ours and other studies [47]. There is also the potential effect of circadian rhythm on the post-exercise β-CTXI response, which has β-CTXI decreasing throughout the morning with its nadir in the afternoon. Although it is possible that the lower β-CTXI concentration at 90 min post-exercise does not reflect a prolonged exercise effect, the 20% decrease immediately following the exercise was too early to be attributed to the natural circadian late morning drop in β-CTXI. For our study's purpose, participants performed the same exercise protocol and followed a controlled nutrition protocol, including the exact same meal and meal timing before the exercise trial in both menstrual phases.

There were no significant differences in resting PINP between menstrual cycle phases. Results from previous studies have been inconsistent, with two studies showing higher PINP in the LP versus the FP [19, 32], and one showing similar results to the present study with no differences between phases [75]. Reasons for the differences between studies may include individual variability of bone marker responses, and pre-visit exercise protocols. Our participants refrained from any vigorous activity for at least 12 hours before each trial. Within the basic multicellular unit, bone is remodelled first by the initiation of bone resorption followed by bone formation and mineralization [16]. Therefore, the potential lag of PINP response to exercise could affect resting PINP if standardization of exercise prior to lab visits is not the same

during both phases of testing. Although we did control for pre-visit factors, several previous studies did not mention the same controls [19, 32]. Furthermore, the PINP response to running was similar between menstrual cycle phases, marked by an increase in PINP immediately post-exercise in both phases. There are no previous studies that have compared exercise-induced changes in PINP between menstrual cycle phases. Studies investigating the PINP response to exercise, independent of menstrual cycle phase, also report inconclusive results. Although PINP has been shown to be mostly nonresponsive to acute exercise, including resistance training and exhaustive running [52, 76, 77], a couple of studies using aerobic exercise and/or jumping, have shown significant post-exercise increases in PINP, similar to ours [70, 78]. It is unclear which factors are contributing to differences in response, although it is possible that longer duration and higher intensity activities may elicit a greater post-exercise PINP response.

The main strength of this study is the detailed criteria used to determine phase, by measuring estradiol and progesterone, using ovulation tests in the morning to measure LH surge, insuring participants were not on any hormonal contraceptives for at least 3 years prior to participation, and making sure all participants had tracked regular cycles for at least 3 months to confirm inclusion criteria. Another strength of this study is that the exercise trials were performed in the morning using strict standardized protocols. The study also had limitations, mainly its small sample size, which makes it possible that the study was underpowered, thus increasing the likelihood of type 2 error. It is also possible that a well-trained population could have a different bone response compared to our recreationally active population since the strain from the running protocol would be less novel for the bone. However, it would be unlikely that this increased fitness would contribute to a different bone metabolic response between menstrual phases. Lastly, it is possible that we may have missed differences in exercise-induced changes

outside our blood sampling times, so extending sampling time points up to 24- and 48-hours post-exercise could help better characterize post-exercise changes in bone metabolism during each menstrual phase.

Conclusion

Menstrual cycle phase did not affect bone metabolism as determined by resting and post-exercise concentrations of sclerostin, PTH, β-CTXI and PINP. Although additional research is needed, our data suggest that future studies investigating bone metabolism in healthy eumenorrheic may not necessarily need to consider the impact of menstrual phase when interpretating results. For women, this finding can help reduce participation barriers in research related to bone metabolism at rest, and in response to vigorous running, since controlling for phase may not be necessary.

Author Contributions

AG contributed to the analysis of blood samples, data analysis and interpretation, and prepared the first draft. NK contributed to the analysis of the blood samples. SM, SM and TH designed the original study and completed all data collection. CS, HL-S and KE-S contributed to data interpretation and final draft. PK was the supervisor of the research and contributed to all aspects of data analysis and interpretation and prepared the final draft. All authors revised the paper critically for intellectual content and approved the final version. All authors agree to be accountable for the work and to ensure that any questions relating to the accuracy and integrity of the paper are investigated and properly resolved.

Conflicts of interest

The authors have no conflict of interest to declare.

Compliance with Ethical Standards

The study was conducted in accordance with the Declaration of Helsinki and received ethics approval from the Research Ethics Boards of Wilfrid Laurier and Brock Universities.

Informed consent

All participants agreed to participate in this study by signing a consent form.

Data Availability

Data are available only upon request from the corresponding author for researchers who are eligible for accessing confidential data, as all data of this study are restricted due to the Brock University Research Ethics Board privacy policy.

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