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

by

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Abstract

The aim of this study was to investigate potential fluctuations in bone metabolic markers across the menstrual cycle both at rest and after a 30-minute bout of vigorous-intensity running at 80% of $\dot{V}O_2max$. Resting and post-exercise (0, 30, 90 min) sclerostin (inhibitor of bone formation), parathyroid hormone (PTH, regulator of calcium homeostasis), carboxy-terminal cross-linking telopeptide of type I collagen (β-CTX, marker of bone resorption), and procollagen type 1 N propeptide (P1NP, marker of bone formation) were assessed in 10 young, eumenorrheic women (21.7 ± 3.2 years, 23.2 ± 3 $kg \cdot m^2$) 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 phase was subsequently confirmed by measurement of plasma estradiol and progesterone taken on study days, confirming an increase in both hormones during the mid-luteal phase. At rest, there were no significant differences in sclerostin (FP: 266.5 ± 48.6 $pg \cdot mL^{-1}$; LP: 296.0 ± 37.5 $pg \cdot mL^{-1}$; p=0.507), PTH (FP: 1.00 ± 0.22 $pmol \cdot L^{-1}$; LP: 0.71 ± 0.16 $pmol \cdot L^{-1}$; p=0.485), β-CTX (FP: 243.1± 52.7 $ng \cdot mL^{-1}$; LP: 202.4 ± 30.8 $ng \cdot mL^{-1}$; p=0.691), or P1NP (FP: 56.9 ± 11.30 $ng \cdot mL^{-1}$; LP: 64.30 ± 18.32 $ng \cdot mL^{-1}$; p=0.133) between menstrual cycle phases. As there were no main effects for menstrual phase and no significant interaction, post-exercise responses did not differ between menstrual phases for any of the markers. Significant main effects for time were found in sclerostin, PTH, β-CTX and P1NP. Specifically, sclerostin and PTH increased from pre- to immediately post-exercise (+46% and +43%, respectively; p<0.0001), then returned to resting concentrations at 30 min post-exercise. P1NP also increased immediately post-exercise (+29%; p<0.0001), returning to resting concentrations at 30 min post-exercise. β-CTX decreased from pre- to immediately post-exercise (-20%; p=0.004) and remained below its pre-exercise concentrations at 30 min post-exercise (-12%; p=0.039) and 90 min post-exercise (-17%; p=0.002). These results demonstrate that sclerostin, PTH, β-CTX and P1NP do not differ at rest or in response to exercise across the menstrual cycle.

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Chapter 1: Introduction

1.1 Rationale

It is well established that there are cyclical changes in female sex hormones during the menstrual cycles of healthy, young, eumenorrheic females[1]. What is less clear in respect to the menstrual cycle is whether different phases of the cycle are related to significant changes in markers and hormones related to bone metabolism and whether the bone response to exercise remains consistent throughout the different phases of the menstrual cycle.

The female hormones, including estradiol, follicle stimulating hormone (FSH), luteinizing hormone (LH) and progesterone have a predictable cyclical undulation throughout the normal menstrual cycle. Thus, at different points during the menstrual cycle, estrogen and progesterone concentrations fluctuate, in that they peak and dip in accordance with the different phases. A normal eumenorrheic cycle can last between 21 and 35 days[2,3,4] and variability is common even within the same woman's cycle. A eumenorrheic cycle is recognized by a mid cycle peak in estrogen and LH at the time of ovulation[3,4]. These fluctuations are known to affect other physiological functions, including hepatic glucose production during exercise executed above certain intensities[5], and regulatory markers and hormones such as the adipokine asprosin, which stimulates hepatic glucose release and is associated with obesity[6,7]. It is, therefore, important to also understand whether these hormonal fluctuations influence markers of bone metabolism in order to optimise data collection when performing research with a healthy young eumenorrheic population.

Estrogen is a key regulator of bone metabolism[8,9,10]. In eumenorrheic women, typical estrogen concentrations, ranging from 15-350 pg·mL^{-1}, provide a protective effect on bone[11,12]. Evidence has shown, chronic low levels of estrogen due to menopause, calorie restriction, congenital conditions, Turners syndrome or conditions associated with ovarian function, can negatively impact bone health in women[13,8]. Decreased estrogen levels are associated with an increased rate of bone remodeling, the physiological process that preserves the skeleton through a process of balanced bone formation and resorption (bone coupling) within bone's basic multicellular unit (BMU), which includes osteoclasts, osteoblasts and osteocytes[14]. With decreased estrogen, the BMUs are formed at a faster rate, or activation frequency (AF), which increases both bone formation and resorption[15]. Menopause is marked by increased bone resorption, which cannot be overcome by bone formation within the BMU. Although bone remodeling is necessary for bone health, when bone formation and resorption become uncoupled, with the balance tipping chronically towards higher resorption than formation, a loss of bone mass ensues. Decreased estrogen levels have also been associated with increased oxidative stress, which has been also been associated with bone loss in both *in vivo* and *in vitro* studies[16.] Recent evidence has emerged about the role of estrogen on oxidative stress across the menstrual cycle[17]. Evidence, mainly *in vitro*, exists showing estrogen has a protective effect on bone through inhibition of osteoclast (resorption cells) maturation, as well as osteoblast (formation cells) apoptosis[18,19]. Through its binding to estrogen receptors on bone cells, estrogen also has anti resorptive effects through downregulation of the NF-κB ligand (RANKL) signaling pathway, which is achieved by increased production of osteoprotegerin (OPG) leading to suppression of the RANK/RANKL binding [14]. Taken together, these data suggest that a chronic

reduction of circulating estrogen could underpin the increased resorption seen under these circumstances, which eventually leads to bone mass loss[8,20,19].

Although there are many studies investigating estrogen and bone health, what is less well understood is how cyclical fluctuations in estrogen throughout the menstrual cycle could affect resting and post-exercise bone metabolism. Several studies have measured baseline markers of bone metabolism at different phases of the menstrual cycle, although these studies have reported contradictory outcomes[21,22,23,24,25,26,27,28,29,30,31,32]. Differences between menstrual cycle phases in parathyroid hormone (PTH), sclerostin (an osteokine antagonist of the anabolic Wnt/βcatenin signaling pathway), carboxyl-terminal crosslinking telopeptide of type I collagen (β-CTXI; a marker of bone resorption and procollagen type 1 N-terminal propeptide (P1NP; a marker of bone formation), have been measured. Specifically, studies investigating baseline PTH levels between the follicular and luteal phases have shown contradictory results. One study measuring PTH showed a mid-cyclic increase[30], while another study showed significantly higher levels of PTH in the follicular compared to luteal phase[33] and other studies showed no difference between follicular and luteal phases despite sharply fluctuating estrogen levels[34,35,36]. In addition, although research regarding the bone related responses to exercise in different menstrual phases is limited. One study including participants taking oral contraceptives, reported significant decreases in PTH levels in response to resistance exercise during both phases. This significant decrease was observed post-exercise in the follicular phase only, and 1 hour post-exercise in both the luteal and follicular phases[28]. Two studies have measured sclerostin at different phases of the menstrual cycle, reporting no significant differences between follicular and luteal phases[37,38]. Although some evidence suggests that sclerostin and PTH have an inverse relationship in relation to the osteogenic effects of mechanical loading, animal studies have shown that

sclerostin downregulation is not necessary for the anabolic effects of PTH[39]. To date we have not seen any literature measuring both PTH and sclerostin in the same study. Examining sclerostin and PTH together in each menstrual phase, at rest and post-exercise, will give us new insights whether their responses are of similar magnitude and direction in both phases.

Discrepancies also exist among studies measuring β-CTXI. One study showed serum β-CTX to decrease progressively through the follicular phase with a significant drop from menstruation toward the late luteal phase, returning quickly to starting values after this point[22]. Similar patterns have been shown in studies measuring serum β-CTX[21,37,22,40]. However, a recent study showed no significant differences in serum β-CTX levels between phases[32]. As β-CTXI measurements are impacted by fasting, circadian rhythms, poor research design may explain some of the different outcomes seen in the literature. The same is true for P1NP measurements. A recent study investigating resting P1NP concentrations showed no differences between phases in both eumenorrheic females and oral contraceptive users[,32], however two previous studies showed significantly lower resting levels of P1NP in follicular and mid follicular phase compared to the mid luteal phase[21,37]. It is possible that differences seen between these studies could be due to different statistical analysis used between studies, non-standardized nutrition, as neither study standardized nutrition and or individual bone marker variability within and between participants.

Determining whether the process of bone metabolism responds differently to exercise during different phases of the cycle will be important and might even be useful in scheduling more osteogenic types of exercise including high intensity and high impact exercises, should the menstrual cycle exert bone specific effects. Furthermore, learning whether certain processes are more, or less dominant during a particular menstrual phase could direct to which phase of the

cycle studies should be performed at or provide a greater degree of interpretation over study results in women.

1.2 Objective and Hypotheses

The purpose of this study was to investigate potential fluctuations in bone metabolic markers and regulatory hormones between the follicular and luteal phases of the menstrual cycle, both at rest and in response to vigorous running. The aim of this study was to determine whether there are differences in either resting or post-exercise markers of bone metabolism between the follicular and luteal stages of the menstrual cycle in healthy, young, eumenorrheic women.

The hypothesis was that there would be no significant differences between resting, fasting levels of sclerostin, PTH, β-CTXI and P1NP concentrations between the two phases of the menstrual cycle. Similarly, it is hypothesized that there will be no differences in the exercise response of these markers between the two phases of the menstrual cycle.

Chapter 2. Literature Review

2.1 Markers of Bone Metabolism

There are a variety of methods to measure the bone's response to acute mechanical loading. Since changes in bone mineral density (BMD) are not immediate, and only occur over longer time periods, this measurement is not appropriate to use to investigate changes in bone metabolism after one acute bout of exercise. After an acute bout of exercise, it is common to measure circulating bone turnover markers, osteokines and related hormones. These markers are products of bone formation or resorption. There are a variety of bone turnover markers, however some of the more commonly used are procollagen I intact N-terminal (PINP) and C-terminal crosslinking telopeptides of type I collagen (CTX), which are recognized by the International Osteoporosis Foundation (IOF) and are products of osteoblastic or osteoclastic cell activation, respectively[41]. Additionally, key osteokines are known to regulate bone metabolism, including sclerostin, osteoprotegerin (OPG) and receptor activator of NF-κB (RANK) and RANK [2]ligang (RANKL). Hormones also play a critical role in bone metabolism, including but not limited to insulin growth factor, estrogen, and parathyroid hormone (PTH).

2.2 Bone Anatomy and Turnover

The human skeleton gives our body shape and creates a physical support for all systems within. In addition, the skeleton is part of our musculoskeletal system which enables our body to move and partake in physical activity. Consequently, our bone structure is such that it allows bone to be both strong and light. There are several components to bone including the hard-outer layer of cortical bone and the inner spongy trabecular bone. The interior of bone is called bone marrow. Different bones will have different compositions of each of these components[42,43].

Cortical or compact bone makes up 80% of our skeletal mass. Cortical bone is very strong and resistant to compression. For this reason, most cortical bone is in the long bones of the body, where it provides robust structure to the human frame. It is found mostly in the diaphysis of bone, or long tube like areas [44]. Cortical bone is composed of basic structural units referred to as osteons. Osteons are cylindrical structures made up of concentric bone layers called lamellae, where osteocytes are located. Osteocytes, mature osteoblasts embedded within the bone matrix, play an important role skeletal homeostasis. The osteocyte containing lamellae surround a Haversian canal, which is a long hollow pathway at the center of the osteon. Small blood vessels and nerves are found within the Haversian canal. The blood vessels in the Haversian canal facilitate the exchanges between osteocytes and the blood. The cylindrical osteon units are connected to one another by canaliculi, which transport blood, parallel to the long axis of the bone. Together these elements create a strong layer in the bone that is resistant to bending[42,43,45,44,46].

Trabecular bone, also referred to as spongy or cancellous bone, can be found in the inner layer of bones. It has one tenth the compression strength of cortical bone and is unlike cortical bone in that it has high porosity and a reduced matrix per mass[44]. The increased porosity and space provided by trabecular bone allows it to be the main area in bone where blood vessels, connective tissue and red marrow can be in contact with bone. This makes trabecular bone ideal for hematopoiesis. Although trabecular bone does not have the same compressive strength it does add valuable mechanical support by helping bone evenly distribute load. It is often found in the epiphysis of bone, such as the head of the femur, and when healthy and strong can take multidirectional stresses. Like cortical bone, osteocytes are found within trabecular bone and act as sensors to local changes in strength. Unique to trabeculae is that they are covered in flattened

lining cells involved in the process of bone remodelling[42,43]. Different bones will have different compositions of cortical and trabecular bone. Bone remodelling occurs in both cortical and trabecular bone.

Bone marrow is found in the inner most portion of bones. The cells found within this portion of bone are protected by the trabeculae and vascular network. The cellular components of blood are formed within the bone marrow, through a process called hematopoiesis[42]. Bone matrix is part of the bone tissue which makes up a significant proportion of the bone's mass. It is composed from both organic and inorganic materials. The organic matrix is predominantly composed of collagen protein, in particular type I collagen, synthesized within osteoblasts, while the remainder is composed from noncollagenous proteins and proteoglycans. The inorganic mineral matrix is composed ions calcium, phosphorous, magnesium and sodium[43,42,44]. The combination of these ions form bone crystals in the form of calcium hydroxyapatite, which are found along and within the collagen fibers. The combination of collagen and minerals in bone provides bone with the properties of flexibility or toughness and stiffness, respectively. During the aging process or due to disease, decreased levels of collagen in bone can lead to increased brittleness and increased fracture risk. Excess or low mineral levels can result in excess or reduced stiffness. The appropriate balance of organic and inorganic materials is paramount for optimal bone health[44,43].

Bone formation and resorption are the processes that underpin bone remodelling, a process necessary for bone growth and maintenance. Four cells within the organic matrix, osteoblasts, osteoclasts, osteocytes and osteoprogenitor cells mediate the remodelling process and are derived from hematopoietic and mesenchymal stem cells. During bone remodelling, old bone is resorbed by osteoclasts, derived from haematopoietic progenitors (Figure 1). These

hematopoietic stem cells give rise to mononuclear cells and subsequently preosteoclasts which enter the bloodstream after the expression of transcription and colony stimulating factors[44]. Once at the site where bone resorption will occur there is a fusing of the osteoclastic precursors with one another, resulting in a multinucleated immature osteoclast[47].

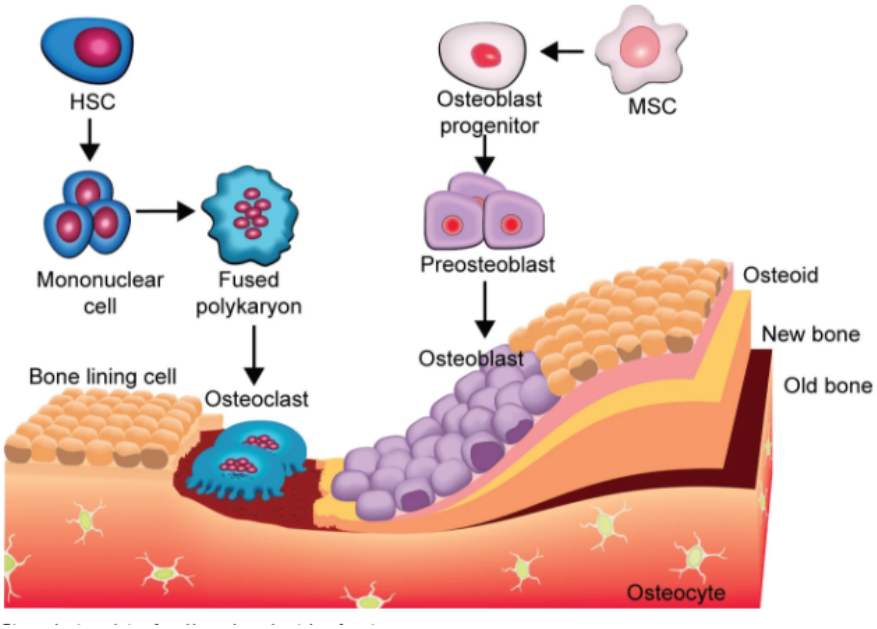

Figure 1 – Bone Remodeling: Osteoclasts derive from hematopoietic stem cells and transform into mononuclear cells and eventually fused polykaryon or immature osteoclasts. Mature osteoclasts resorb old bone and make room for new osteoid to be laid down from osteoblasts. Osteoblasts are derived from mesenchymal cells. Osteoblast progenitor cells mature into preostoeblasts and eventually mature osteoblasts which lay down new bone. Once embedded in the bone matrix osteoblasts become osteocytes[48].

Immature osteoclast or fused polykaron production requires the presence of both macrophage colony stimulating hormone and (M-CSF) and receptor for activation of nuclear factor kappa κB ligand (RANKL). Following successful production of immature osteoclasts, tartrate-resistant acid phosphatase (Trap) expression is activated. The enzyme Trap takes part in bone resorption in differentiated osteoclasts. Osteoclast differentiation requires the consistent

presence of RANKL along with other genes and transcription factors. Interestingly, RANKL is derived from osteoblastic lineage including osteocytes. Increased mobilization of RANKL from osteocytes, which binds to the receptor activator of NF-κB (RANK) on osteoclasts, contributes to increased osteoclastogenesis or bone resorption[49,50]. Contrary to this, a soluble factor of mesenchymal origin called osteoprotegerin (OPG) acts as a decoy receptor for RANKL (Figure 2). OPG binding to RANKL reduces osteoclastogenesis, meaning cells of osteoblastic lineage can either increase or decrease osteoclastogenesis.

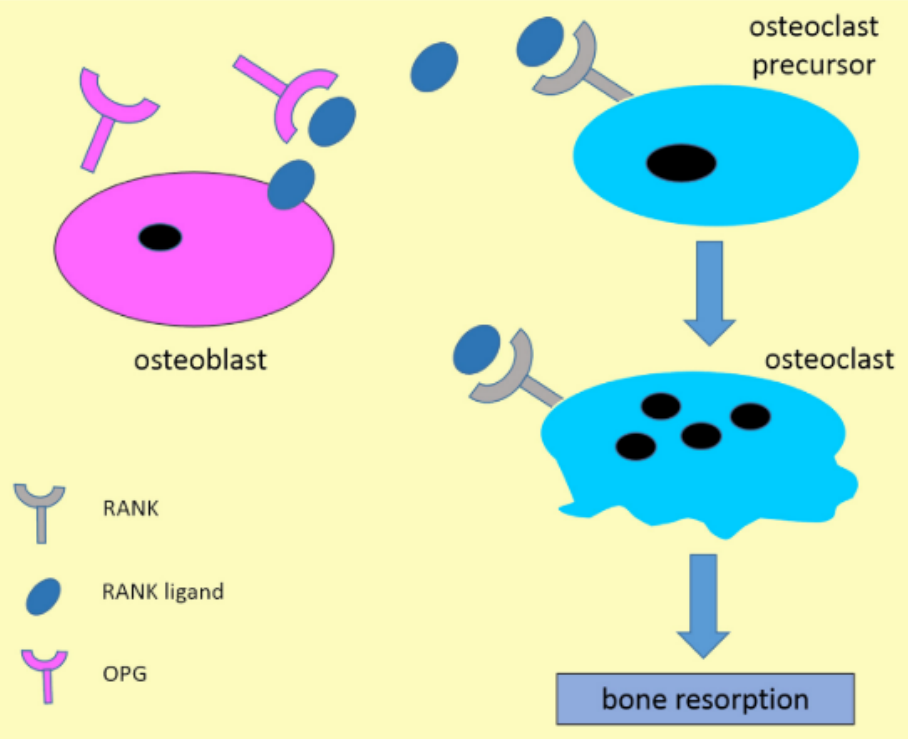

Figure 2 – RANK-L Decoy Receptor: Cells from osteoblastic lineage, osteoprotegerin (OPG) act as a decoy receptor for RANK-ligand reducing the amount of RANK-L binding to osteoclast receptor RANK, reducing osteoclastogenesis.

Osteoblasts are derived from mesenchymal stem cells in the bone marrow stroma and periosteum. Mesenchymal stem cells can lead to different lineages, so in order to lead to the osteoblastic lineage certain transcription factors and homeoblox homologues are required. An osteoblast precursor cell or immature osteoblast expresses collagen and bone sialoprotein (BSP), a non collagenous protein. The process of differentiation into mature osteoblasts includes several processes involving in the Wnt signalling pathway as well as the expression of Runx2, osterix (Osx). Mature osteoblasts will be found within the lining of the bone they will be forming and have the role of synthesizing and mineralizing the osteoid which makes up the organic matrix. Once mineralization is complete the osteoblasts become osteocytes within the bone through processes that are still not well understood[44,42,45,48].

2.3 Sclerostin and the Wnt/β-catenin Signalling Pathway

More recently, bone metabolism has been investigated through measures of the glycoprotein sclerostin, a product of the SOST gene, which encodes sclerostin, within osteocytes. Sclerostin is found within osteocytic lucanae and along osteocytic canaliculi. The presence of sclerostin indicates mature osteocytes within the bone matrix[39]. Sclerosteosis and van Buchem's disease are both rare diseases that lead to increases in bone mass and strength through different mechanisms, both which are due to defects in the SOST gene chromosome that encodes sclerostin[51]. This impaired sclerostin synthesis negates sclerostin's inhibitory effects within the Wnt signalling pathway. Sclerostin has been identified as an inhibitor of the Wnt pathway, which leads to decreased bone formation[51].

Sclerostin binds to the LRP5/6 receptor, in place of the Wnt protein, interfering with the Wnt LRP 5/6 frizzled interaction (Figure 3). In response, β-catenin is phosphorylated from the activity of the glycogen synthase kinase 3 (GSK3) with the protein complex Axin, adenomatous

polyposis coli (APC) Axin and Disheveled (Dsh) which are released into the cytosol (Figure 3). Degradation of the β-catenin occurs which interrupts β-catenin accumulation in the cytoplasm, a necessary step leading to the proliferation and differentiation of bone cells[52]. Although the exact mechanisms are still unclear, there is an inhibition of bone formation with increased levels of circulating sclerostin[53]. The Wnt/β-catenin signalling process also has influence on the mobilization of OPG and RANKL, the osteokines that regulate osteoclast formation. Upregulation of OPG from osteoblasts occurs when the Wnt/β-catenin signalling pathway is initiated by the Wnt molecule. This same process initiated by the Wnt molecule in the Wnt/B-catenin pathway downregulates the expression of RANKL from osteoblasts, indirectly leading to decreased bone resorption with less RANKL available to bind to receptor RANK on osteoclasts. Therefore, the two pathways, Wnt/β-catenin and OPG/RANKL have a relationship that can help us to better understand the processes of bone resorption and formation[54,39,52]. Ultimately, sclerostin is capable of uncoupling the remodelling unit of bone formation and resorption through inhibition of bone forming osteoblasts and stimulation of osteoclasts[39].

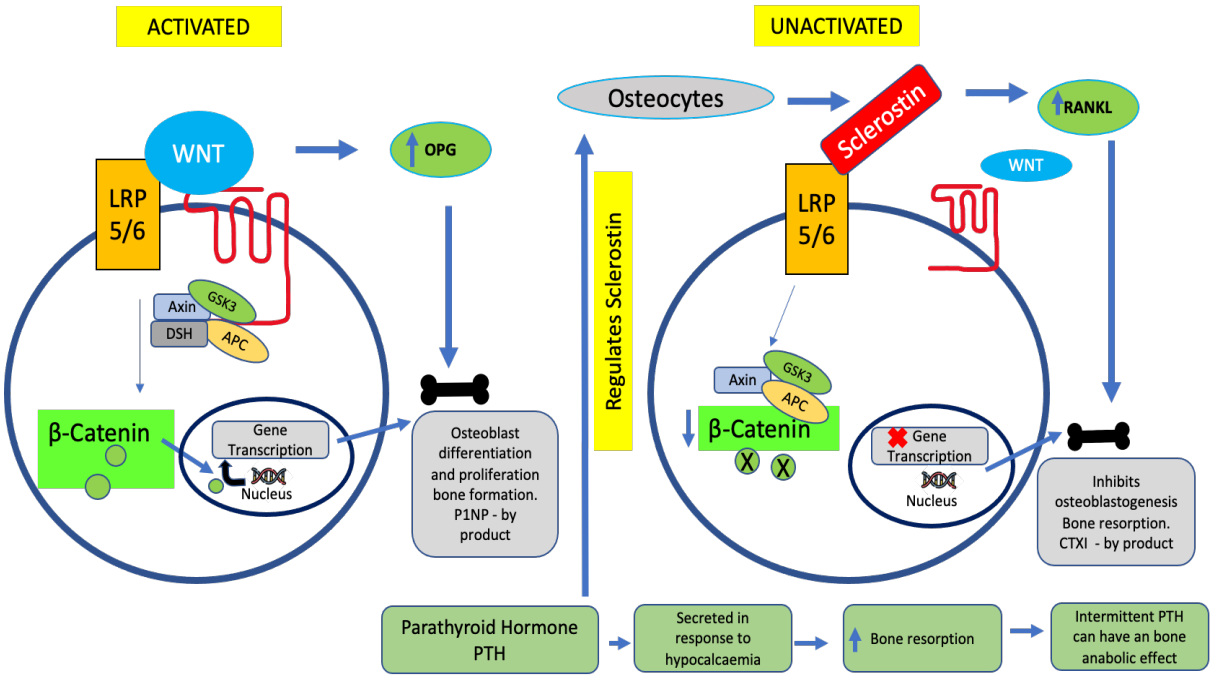

Figure 3 – Wnt/ β-catenin signaling pathway and sclerostin: Sclerostin an antagonist of the Wnt/β-catenin signalling process, binds to receptor LRP5/6 and blocks the frizzled interaction with the receptor. In response β-catenin is phosphorylated from the activity of the glycogen synthase kinase 3 (GSK3) with the protein complex Axin and adenomatous polyposis coli (APC). Axin and Disheveled (Dsh) are released into the cytosol. Degradation of the β-catenin occurs as a result, which negatively impacts osteoblast differentiation and proliferation from mesenchymal stem cells[54].

2.4 Parathyroid Hormone

2.4.1 PTH Synthesis and Secretion

Parathyroid hormone (PTH), a peptide hormone produced and secreted by the chief cells of parathyroid gland, plays an important role in the regulation of bone metabolism. A reduction in serum blood calcium levels result in an increased expression of the PTH gene which leads to production of PreproPTH. PreproPTH then converts to ProPTH and finally PTH which can be secreted from the parathyroid glands (Figure 4). PTH (1-84) is the bioactive circulating form of PTH, consisting of 84 amino acid peptides, with the majority of the bioactive activity being within it's amino (NH_2) terminal domain. PTH, a major regulator of extracellular fluid calcium

(Ca++) and phosphate (Pi) homeostasis, has a goal if maintaining constant levels of ionized calcium in blood and is essential for bone remodelling[55,56]. PTH secretion from the parathyroid gland is inhibited as a response to the binding of calcium to the calcium binding receptor (CaSR). The concentration of calcium in the circulation will determine the magnitude of inhibition as will the rate at which the calcium levels fall. Rapid decreases in calcium concentration result in a more pronounced response. Whether PTH is administered intermittently as treatment for osteoporosis or is chronically elevated, for example due to hyperparathyroidism, also appears to impact its effect on bone metabolism[57]. Chronic elevated levels of PTH are associated with increased bone turnover and elevated resorption in tandem with increased release of Ca+ and Pi from bone[57]. On the contrary, intermittent treatments regimens with PTH analogues have been shown to result in increased BMD in postmenopausal women and are a current treatment option for osteoporosis[58,55]. Less is understood about intermittent PTH responses to exercise and bone metabolism[56,57].

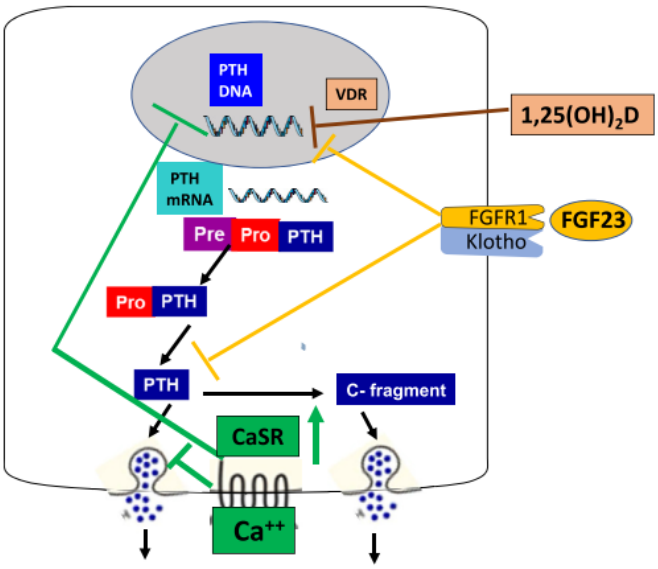

Figure 4 – PTH Production and Signaling: Decreased extracellular Ca++ increases the expression of the PTH gene which leads to production of PreproPTH. PreproPTH then converts to ProPTH and finally PTH which can be secreted[56].

2.4.2 PTH – Wnt /β-catenin and OPG/RANKL Pathways

As a result of PTH binding to PTH receptor PTH1R on cells of osteoblastic lineage including osteoprogenitor cells, lining cells, mature and immature osteoblasts, several growth factors and osteokines are stimulated, leading to increased proliferation of mesenchymal cells which eventually differentiate into osteoblasts[56]. It is the lineage specific transcription factors that will determine a progenitor's fate, as osteoblasts and chondrocytes have the same progenitor. In vivo studies have also shown that intermittent PTH can inhibit osteoblast apoptosis resulting, in the survival of more osteoblasts and increased bone formation[59]. PTH can also act on mature osteoblasts stimulating new bone matrix and can inhibit sclerostin by acting on osteocytes within the bone matrix. Since sclerostin deactivates the Wnt /β-catenin pathway, PTH inhibition culminates in an osteogenic effect. Ultimately this cascade of events leads to bone formation through osteoblast production.

The interplay between PTH and osteocyte derived bone inhibitor sclerostin, is mediated by cyclic adenosine monophosphate (cAMP). cAMP inhibits the transcription factors MEF2, which act as an enhancer of the SOST gene encoding sclerostin. By inhibiting this process, Wnt then binds to the LRP5/6 receptor protein, leading to a cascade of events including the accumulation of β-catenin in the cytoplasm, which results in an proliferation and differentiation of bone cells[56]. However, mature osteoblasts can also produce catabolic osteokines, i.e., receptor activator of nuclear factor *k*-B (RANK) and ligand (RANKL), which stimulate osteoclast production and action, while inhibiting the anabolic osteokine OPG, which acts as decoy receptor for RANK, all together resulting in increased osteocatabolic bone resorption[56]. Therefore, PTH can have either anabolic or catabolic effects on bone, depending on which pathways are being impacted more.

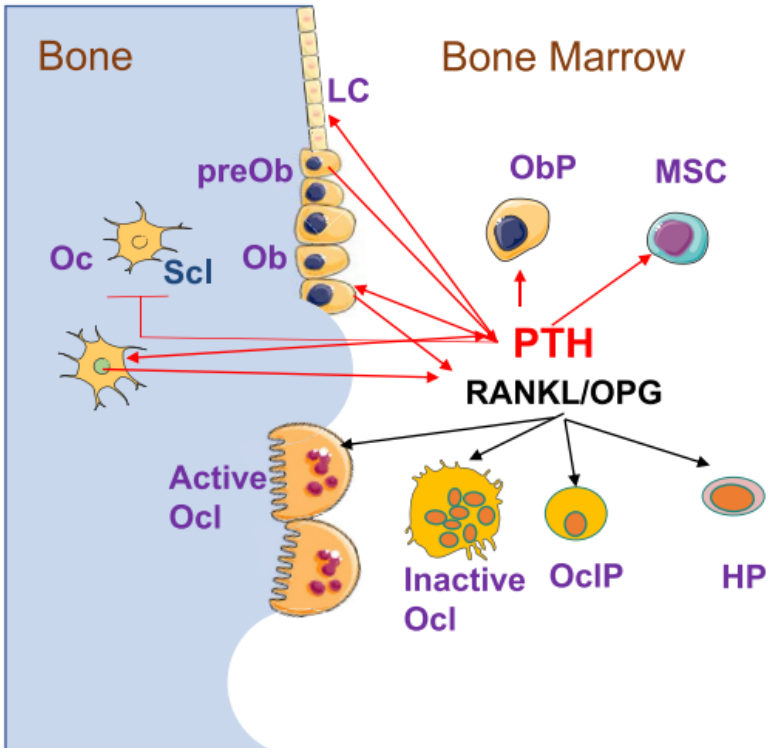

Figure 5 – Role of PTH in Bone Metabolism: PTH stimulates mesenchymal stem cells (MSc) enabling them to commit to the lineage of osteoblast progenitors (ObP). PTH binds to PTH receptor PTH1R on cells of osteoblastic lineage including osteoprogenitor cells, lining cells (LC), mature and immature osteoblasts, leading to increased proliferation of mesenchymal cells, lining cells and marrow, ObP form preosteoblasts and eventually differentiate into osteoblasts[56]. PTH can also inhibit sclerostin's effect on the Wnt/B Catenin pathway by acting on osteocytes (Oc) embedded within the bone matrix opening the pathway for bone formation. On the contrary, PTH can stimulate mature Oc and osteoblasts to secrete osteokine RANKL, leading to increased osteoclast precursors (OclP). RANKL can transform hematopoietic precursors to osteoclast precursors and osteoclast precursors to inactive osteoclasts and eventually active osteoclasts leading to increased bone resorption. PTH also inhibits osteoprotegerin, the decoy receptor for RANKL, therefor also promoting osteoclastogenesis. PTH can have either an osteoanabolic or osteocatabolic effect, which speaks to the importance of its balance in the body[56].

2.5 Markers of Bone Formation and Resorption

2.5.1 Cross Linked Telopeptide (β-CTXI)

Carboxyl-terminal crosslinking telopeptide of type I collagen (β-CTXI) is a bone marker formed during the bone resorption or degradation phase of bone remodelling. The International Osteoporosis Foundation (IOF) recommends β-CTXI as a reference bone resorption marker that should be measured in clinical studies[41,43]. β-CTXI are products of type I collagen breakdown in bone. Several factors that can interfere with β-CTXI measurements including circadian variations, calcium intake, exercise and food intake. It is recommended that β-CTXI be measured in a fasting state to reduce variability since β-CTXI measures after food consumption can lower as much as twenty percent[60]. In addition, measures from serum reduce variability as much as fifty percent in comparison to measures taken from urine[61].

2.5.2 Procollagen type 1 N-terminal Propeptide (P1NP)

Procollagen type 1 N-terminal Propeptide (P1NP) is the reference marker of bone formation recommended for clinical studies by the IOF and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC)[62]. P1NP is the precursor molecule for type 1 collagen, referred to as a procollagen. Type 1 collagen, which is synthesized and expressed from osteoblasts, is the most abundant bone protein constituting 90% of bone proteins. During synthesis of type 1 collagen, terminal and carboxyl extensions are cleaved from the molecule, leaving two peptides, P1NP and procollagen type 1 C-terminal propeptide (P1CP)[41,62]. Most P1NP is derived from bone collagen type 1 and P1NP is the more specific marker for bone collagen synthesis as well as the most sensitive marker of bone formation[61]. Although P1NP is also synthesized in skin and other connective tissues, bone synthesizes it quickly, and therefore,

most serum detected P1NP is derived from bone[60]. During times of rapid growth or if there are other pathologies such as osteomalacia, tissues besides bone can contribute more to increased serum P1NP levels[63,64]. In addition, P1NP is upregulated during exercise in both skeletal and tendinous tissue, however predominantly originates from osteoblasts[65]. In addition and of importance in our study, P1NP synthesized in tendinous and muscular tissue does not appear to be impacted by estrogen fluctuations throughout the menstrual cycle[66,67].

The analytical variability of P1NP has been well documented in the literature. Unlike bone resorption marker β-CTXI, P1NP has a low diurnal variability and does not appear to be affected by food intake. In addition, P1NP has a predictable treatment response, and assays have low intra individual variability (i.e., the variability within a data set from a specific experiment) with Roche intra assay variability measuring between 1.4-2.3% and Roche inter assay variability measuring between 2.1-4.5%[68]. Assay precision, derived from replicated analysis on a data set run on the same day, is also good[69]. Different assays for PINP measure either total or intact PINP. Intact PINP assays measure trimeric PINP, the high molecular weight form, while total P1NP assays measure both trimeric and monomeric forms[68]. The use of different assays across studies makes it difficult to compare results for clinical use. Although studies comparing assays have shown minimal differences in healthy populations, the IOF has assay harmonization as a goal moving forward[68]. PINP is also recommended for its predictability and is also considered a marker that may predict early detection and manage treatment of osteoporosis in some countries including Canada, Poland and Europe, as higher concentrations of P1NP have been measured in some post-menopausal women[70,41,69,64]. These are all factors which make P1NP the bone formation marker of choice.

When measuring bone turnover markers (BTMs), it is important to consider preanalytical and analytical variability of assays. Having standardized procedures including nutrition protocols, timing of samples to control for circadian and seasonal rhythms and following recommendations for proper sample storage are all important factors to adhere to.

2.6 Effects of Menstrual Cycle on Bone Metabolic Markers at Rest

2.6.1 Sclerostin, Estrogen and Menstrual Cycle

Estrogen is a major regulator of sclerostin expression, as evidenced in several studies in postmenopausal women and female athletes[37,71,72]. Estrogen acts through estrogen receptor-alpha (ERα), decreasing sclerostin expression[37]. Decreased sclerostin expression has an osteoanabolic effect on bone through its inhibition of the Wnt pathway[51]. One study investigating the effects of 4 weeks estrogen treatment in postmenopausal women found a 27% reduction in sclerostin ($p < 0.001$) vs +1% in controls ($p < 0.001$) indicating a significant inverse relationship between estrogen and sclerostin in this population[71]. Postmenopausal women have higher levels of circulating sclerostin which can be suppressed with estrogen treatment[73]. Another study comparing sclerostin levels in pre and postmenopausal women, in order to evaluate it's relationship with estrogen, found postmenopausal women had lower values of estradiol ($p < 0.001$), estrone ($p < 0.001$), and free estrogen index (FEI) ($p < 0.001$) and significantly higher sclerostin levels and lower bone mineral density at all sites[74]. FEI was also found to be an independent predictor of sclerostin in postmenopausal women. Further analysis in the premenopausal women in this study were limited as most were taking oral contraceptives.

One in vitro study investigating the effect of Thiazolidinediones (insulin sensitizing medications) on bone mineral density[75], in the presence or absence of 17b-oestradiol in cell

cultures, found significant downregulation of sclerostin expression in the presence of 17β-oestradiol[76]. Human and animal research have both shown increased expression levels of mRNA for ER*a*, an estrogen receptor subtype important to bone biology, as a compensational response to reduced estrogen[77,78]. ER*a* plays a role in early responses to strain in bone. The absence of ER*a* in mice has been shown to attenuate this adaptive response to loading[79]. Although levels of ER*a* are not regulated by strain, rather by estrogen levels, reduced levels of estrogen may still play a role in the early response of osteoblasts and osteoclasts to strain[79]. Research in mice suggests that Wnt/β -catenin signalling plays a role in early responses of bone cells to mechanical strain and the pathway is dependent on ER*a*[80]. Disruption from reduced ER*a* leads to reduced osteoblast differentiation, resulting from interruption of Wnt signalling in the mesenchyme. Therefor reduced bone cell response could be attributed to both reduced estrogen and therefor ER*a*. As a results, there would be an increase in sclerostin binding to the LRP 4/5/6 receptor in the Wnt pathway, resulting in increased bone resorption through the OPG/RANK/RANKL pathway, i.e., by upregulating RANKL leading to more RANK/RANKL binding and increased bone resorption, as well as by inhibiting phosphorylation of β-catenin, therefor inhibiting proliferation and differentiation of mesenchymal cells and osteoblastogenesis.

Although some of the mechanisms remain unclear, evidence does support a relationship between estrogen, sclerostin and bone metabolism through the Wnt signalling and OPG/RANK/RANKL pathways[81].

Since it is well established that estrogen fluctuates naturally during phases of the menstrual cycle, and sclerostin expression has been shown to have an inverse relationship to estrogen levels, it is important to understand if these transient estrogen fluctuations provide enough stimulus to result in fluctuating sclerostin levels. This is important to understand to

establish best practices for research methodology when planning research on bone metabolism in eumenorrheic females. However, limited evidence exists on differences in sclerostin levels between phases of the menstrual cycle. One study investigated data from 31 premenopausal women, mean age 28.6 ± 5.8 years, with regular menstrual cycles, measuring serum sclerostin, free estradiol, testosterone and progesterone during menstruation, late follicular phase, days 6 to 12 post menstruation, and mid-late luteal phases, day 20-23 of the menstrual cycle[82]. Although serum free estradiol was significantly lower during the menstruation phase versus the late follicular and mid luteal phases, there was no significant difference in circulation sclerostin between phases. Additionally no relationship was found between percent changes of sclerostin and estradiol or the other sex hormones measured, despite significant differences in estradiol between phases[38]. Although estrogen is at its lowest during the menstruation phase, this lowest level remains higher than postmenopausal levels of estrogen. It is possible then, that there is a threshold where a low enough level of estrogen could elicit increases in sclerostin expression significant enough to cause an osteocatabolic effect on bone over time. Based on this theory of an 'estrogen threshold', it is also possible that women with healthy menstrual cycles do not reach this lower threshold of estrogen and bone health is preserved throughout a healthy cycle[12].

Another study including fourteen healthy Caucasian premenopausal women with normal menses, ages 33.6 ± 4.5, investigated the relationship between estradiol and gonadotrophins and circulating sclerostin and Dickkopf-1 levels, both which act as antagonists of the canonical Wnt signaling pathway, between the follicular and luteal phases of the menstrual cycle[37,83]. As expected, throughout the cycle significant changes in serum estradiol were present, with its rising levels leading into the mid follicular phase and a peak at ovulation, followed by a drop off post ovulation and a rise again during the mid luteal phase. Late follicular and luteal phase estradiol

levels were significantly higher than both early follicular and luteal phase levels. Despite these significant changes, no relationship was established between estradiol and either sclerostin or Dickkopf-1 levels during either phase of the menstrual cycle[37], which suggests that menstrual cycle fluctuations in estrogen do no effect measured sclerostin concentrations

2.6.2 PTH, Estrogen and Menstrual Cycle

The relationship between PTH and estrogen is important to understand. If estrogen and PTH have a direct or indirect relationship, and significant estrogen fluctuations between menstrual phases can either increase or decrease PTH levels, this would become important for methodological reasons when researching bone metabolism during different menstrual phases. Should transient fluctuations in PTH occur during a normal menstrual cycle, in response to on estrogen fluctuations, it is also of interest to investigate whether these are anabolic or catabolic for bone.

Several studies have investigated the relationship between aging and progressively increasing PTH, as well as aging and decreased estrogen levels and their effects on bone turnover in women. Fewer studies have investigated the relationship between estrogen and PTH on bone metabolism. The studies on aging women have been instrumental in helping us learn more about estrogen's role in bone health, it's relationship to PTH as well as estrogen's relationship with reduced calcium absorption and serum 1,25-dihydroxyvitamin D as we age[84]. These data can help us hypothesize how estrogen fluctuations between menstrual phases, may impact markers of bone metabolism including PTH. One population-based study including 351 women aged 21-94 looked at the relationship between PTH and estradiol[85]. Of the study participants, 47 of the women were on long term estrogen replacement therapy. The authors found that a combined analysis of pre and postmenopausal women measured progressive PTH increases with age, ($p <$

0.001), however these increases in PTH were not seen in women who were on long term estrogen therapy. Interestingly serum 25- hydroxyvitamin D levels were able to show high predictability of PTH levels in both pre and postmenopausal women. This study suggests an inverse relationship between estrogen and PTH[85].

Other studies have investigated PTH levels throughout the menstrual cycle[36,74,24,30,35]. One study included twenty healthy eumenorrheic women, and the authors measured the relationship between endogenous estrogen levels and circulating PTH during the menstrual phases[35]. They hypothesized that there would be an increase in PTH following increases in estrogen during the menstrual cycle, and that this would be in response to calcium demands of the body. Contrary to the hypothesis, authors measured no changes in PTH throughout the menstrual cycle, even despite significant changes in estrogen levels within each cycle. The authors found a negative association between estrone and PTH during the follicular phase, which appeared to be a direct physiological link. Stepwise multiple regressions were able to show a significant relationship between dietary calcium, 25(OH)D, and estrone with circulating levels of PTH, but even despite their contribution to PTH levels a significant regression coefficient between estrone and PTH still remained. The authors remain unclear of the mechanism to explain this relationship, however they did suggest that sharp increases in estrogen could have made correlations with PTH too difficult, whereas estrone levels may have incorporated estrogen fluctuations over time[35]. It is also possible that other physiological mechanisms which are not yet understood are taking place relating to estrone. Although this appears contrary to what the research has shown in postmenopausal women, where progressive increases in PTH occur with age while estrogen is also decreasing, demonstrating an inverse relationship, it seems important to consider that these age-related changes in estrogen levels are long term and of greater

magnitude with estrogen levels below 20 $pg \cdot mL^{-1}$, versus more transient changes seen between menstrual cycle phases in eumenorrheic women with estrogen levels typically above 35 $pg \cdot mL^{-1}$. Evidence from another study measuring serum immunoreactive PTH as well as other markers of bone metabolism during the menstrual cycle in 8 healthy women aged 20-47, showed similar results. In this study, significant fluctuations in estrogen with the peak at ovulation, followed by a sharp drop off were measured, however despite these, PTH levels remained unchanged[24]. One study did find an outcome contrary to the rest, which was a mid cycle increases in iPTH[30]. In this study including 7 women, immunoreactive parathyroid hormone was measured, however estradiol was only measured in two of the 7 women. The study investigators used LH and progesterone to both date the cycle and mark ovulation respectively. This is one of the only studies we found in the literature that showed a progressive increase in PTH, with its peak before the LH. After this point PTH continuously decreased throughout the luteal phase[30]. Of interest was the decreasing ionic calcium (Ca^{++}) in the three to four days leading into ovulation which paired with the timing of increasing PTH. This pattern led the authors to conclude that estrogen could be suppressing PTH, resulting in transient decreases in Ca^{++} and a subsequent rebound of PTH in response. Another theory the authors had was related to calcitonin increases in response to estrogen, which could lead to lower blood calcium and a subsequent increase in PTH. The mechanism of their finding remains unclear. One other small study of 6 women aged 26-39 years aiming to clear up some disparity seen between studies, measured the effects of ovarian hormones on calcium homeostasis and calcitropic hormones, including PTH[36]. Similar to the majority of the aforementioned studies, no significant changes in PTH were observed throughout the menstrual cycle. Although most of the studies suggest no effect of estrogen on PTH

throughout the menstrual cycle, the contradictory results of one study leave it somewhat unclear and make us question what the underlying mechanisms are that led to this result[30].

2.6.3 CTX, Estrogen and the Menstrual Cycle

Evidence exists showing β-CTXI, the bone resorption marker recommended by the IOF andIFCC), has a relationship with estrogen in relation to bone metabolism. A β-CTXI -estrogen relationship has been studied mostly in postmenopausal women, as β-CTXI has been reported as a sensitive marker of bone resorption for women at this stage of life[86,87,88,89]. One study investigating estrogen's effects on bone regulator Dlk1/FA1 (Delta-like 1/FA1), which has been identified in mice as mediating bone resorption, measured estrogen's effect on β-CTXI and the correlation between Dlk1/FA1 (Delta-like 1/FA1) and β-CTXI [86]. This study looked at pre vs postmenopausal women, as well as postmenopausal women on estrogen replacement. The authors found significant increases in β-CTXI in the estrogen deficient women, compared to premenopausal and estrogen replacement women (both; $p>0.001$), highlighting the inverse relationship between estrogen and β-CTXI. A significant reduction in β-CTXI was found in estrogen replacement therapy women ($p<0.0001$). Both findings related to β-CTXI were significantly correlated with Dlk1/FA1 ($r=0.30$, $p<0.01$), ($r=0.18$, $p<0.05$) respectively[86]. A study of 51 healthy women, free of any diseases that would impact bone metabolism, who underwent surgical menopause were investigated to better understand how a sudden decrease of estrogen levels would effect markers of bone metabolism, including β-CTXI [89]. The investigators compared levels of β-CTXI preoperative and both one and six months post-operative and found mean serum β-CTXI levels were significantly higher at the one- and six-month postoperative times compared to preoperative levels ($p<0.01$). In addition, mean spine BMD was significantly lower at 6 months postoperative in comparison to preoperative ($p<0.001$). Estrogen levels were

significantly lower between preoperative and both one- and six-month postoperative levels ($p<0.001$). The primary activity that drives postmenopausal bone loss is an increase in bone resorption from increased osteoclastic activity. Here we can see the effect of rapid decreases in estrogen levels on bone resorption marker β-CTXI [89]. Although surgical menopause is a rapid and extreme reduction in estrogen levels, these outcomes speak to the relationship between estrogen and β-CTXI. Since estrogen fluctuations are transient within the menstrual cycle and between phases, this information leads us to ask whether these fluctuations can result in fluctuating levels of bone resorption as evidenced by changes in β-CTXI.

Several studies have investigated β-CTXI levels between menstrual cycle phases[32,37,21,22], with contradictory results. In one study including 20 healthy eumenorrheic women, ages 19-32[22], β-CTXI was measured between four time points including; $T_{0,}$ representing the start of the cycle with the lowest estrogen, days 2-4; $T_{14,}$ representing pre ovulation and highest estrogen levels, days 12-14 during the FP; $T_{26,}$ representing the highest progesterone levels in the late LP, days 24-26; and $T_{01,}$ representing the start of the next cycle, days 2-4. The authors found a significant decrease in β-CTXI from its peak at the start of the cycle, T_0, to T_{26} ($p = 0.0455$) as well as significant increases in β-CTXI from $T_{26\ to}$ $T_{01,}$ representing the late luteal phase to beginning of the next cycle ($p=0.0415$). A typical physiological pattern of sex steroid hormone estradiol was seen between menstrual phases with significant variations measured throughout the cycle. Despite these variations in estradiol, no correlation was found between β-CTXI and estradiol. It is important to note that in this study there is no mention of fasting blood draws, refraining from exercise in the 24 hours before blood draws or vitamin D and nutritional status of participants, all factors which can impact β-CTXI measurements. In particular, measures of β-CTXI after food consumption can lower as much as twenty percent[60,90], however β-CTXI samples taken in the

same conditions including time of day and time post meal consumption can be utilized, but cannot then be compared to references tables using fasted morning samples[91]. Other studies have also found higher levels of β-CTXI in the luteal phase[21,40,] and similarly these studies also do not mention refraining from exercise in the 24 hours pre blood draw. On the contrary, one study including 14 healthy eumenorrheic women, investigated β-CTXI levels throughout the FP and LP of the menstrual cycle in relation to sex hormones[37]. Investigators found typical significant variations in estradiol throughout the cycle with its rise in the mid FP and peak at ovulation, followed by a rapid post ovulation decrease and rise in the LP. Significant variations in β-CTXI were seen across the cycle. β-CTXI levels were significantly higher in the FP relative to the LP ($p=0.047$). Significant negative associations were found between estradiol at ovulation and percent change of β-CTXI. Associations were seen at all time points throughout the luteal phase.. In addition to this, and of interest for our current study, there was a positive correlation between sclerostin and β-CTXI, ($r=0.63$, $p<0.05$), even if there were no statistically significant variations in sclerostin throughout the cycle. Although investigators did follow a fasting protocol for this study, no mention refraining from exercise in the 24 hours pre blood draw are mentioned. Authors noted that their participants were not involved in regular exercise or sports and that there were not significant differences in activity levels between each visit. Type of exercise was not accounted for. It is possible the contradictory findings between these studies could be related to different blood sampling protocols and the possible effects of pre visit exercise.

A recent study measuring bone marker concentrations across the menstrual cycle, compared eumenorrheic women and those taking combined oral contraceptives[32]. The study looked at three phases, namely the early follicular (EF), ovulation (OV) and mid luteal (ML). The group of eumenorrheic women included $n=14$. Results differed from both aforementioned

studies. In the eumenorrheic group, typical estradiol fluctuations occurred. Investigators measured significantly lower estradiol concentrations in the EF phase as compared to the OV and ML phases, but no significant differences in β-CTXI despite significant differences in estradiol between phases ($p=0.02$ and $p<0.001$) respectively, with the ML estradiol concentration was significantly higher than that of the OV phase ($p=0.03$). Unlike previous studies, there were no significant variations in β-CTXI concentrations between menstrual phases in the eumenorrheic group, although levels of β-CTXI were higher in the EF phase as compared to both the OV and ML phases for 8 out of 14 women. In comparison to COC users, there were no significant differences in β-CTXI concentrations. It's important to note that unlike some of the other studies mentioned, this study did follow all IOF standardization procedures, including but not limited to fasting, taking measures between 7:30am and 10am and repeating the same procedures at each visit[63]. In addition, investigators in this study had participants refrain from exercise in the 24 hours preceding blood samples, arriving to the lab rested. Of additional interest in this study, were the significant differences in estradiol concentrations between the eumenorrheic and COC participants. Mean estradiol concentrations were significantly higher in the eumenorrheic group, however despite this, there were no differences in β-CTXI concentrations between groups. This brings us back to the theory of an estrogen threshold. Although estradiol concentrations were significantly lower in the COC group, they still did not appear to be low enough to incite significant changes in β-CTXI concentrations. In the COC group, there were significant differences between early and later phases of pill consumption. It is possible that these differences were only seen in this group, because of the overall lower concentrations of estradiol, possibly nearing this threshold, whereas the eumenorrheic group's estradiol levels were significantly higher[92]. Lastly, the authors note the importance of considering variability of bone

markers within individuals as well as variability of responses between menstrual phases for each participant. Combined with the small numbers of participants in some of the studies mentioned, it is possible that this individual variability is a contributing factor to the different results seen in prior research, versus in this study.

2.6.4 P1NP, Estrogen and the Menstrual Cycle

Estrogen and P1NP have a relationship in regards to bone metabolism, which is most apparent when looking at studies comparing pre and postmenopausal women or investigating athletes who are energy or estrogen deplete[93,94,95]. Understanding this relationship can help us to better comprehend whether estrogen fluctuations throughout the menstrual cycle alter P1NP levels and whether this relationship differs between menstrual phases in healthy eumenorrheic women.

Through the lifespan, P1NP is highest in the first year of life and then again during the pubertal spurt, due to increased bone modeling and growth. It has been documented that women have a decrease in P1NP after age 30-35 compared to puberty, followed by a stable period, which likely represents a slowing of bone turnover rate and of bone growth in later ages[96,97]. Data have shown postmenopausal women with osteoporosis have significantly higher P1NP compared to premenopausal women, and significant inverse correlations have been found between P1NP and BMD of the lumbar spine, femoral neck and total hip in postmenopausal women[97]. One study investigating premenopausal women over a 5 year period found that those with osteopenia or osteoporosis had higher P1NP, while all women in the study had significantly increased P1NP from baseline to the end of the five year period showing an increase of P1NP with age[98]. On the contrary, other data have shown no differences in P1NP between premenopausal and postmenopausal women without osteoporosis, indicating a stable period of P1NP[99]. Of interest is the fact that trabecular bone loss has also been reported in the third decade

of life in women in longitudinal studies when estrogen levels remain relatively stable, with losses slowing after this point and increasing in elderly years[100]. In cortical bone, which makes up the largest proportion of the skeleton in adult women, losses are clearly associated with decreases in estrogen[101]. These differences suggest other factors are contributing to bone loss prior to age related decreases in estrogen. Together these data raise questions about the magnitude of the role of estrogen in P1NP levels at different stages of life and make us look to question other factors that may impact P1NP such as oxidative stress and the impact of disease states of osteoporosis/osteopenia themselves.

Estrogen levels drop off significantly in menopause, correlating with age related bone loss in women. Estrogen's protective role in bone health is related to its role in reducing osteoclastic activity through both enhanced OPG production and increased apoptosis of osteoclastic progenitor cells, mediated by estrogen receptor-*a* (ER*a)*[102,14]. In both pre and postmenopausal women, treatment with transdermal estradiol has been shown to significantly increase P1NP levels, with only premenopausal women seeing a decline in this effect with increasing dosage[99]. A study investigating postmenopausal, hysterectomized women taking subcutaneous estradiol found significant increases in P1NP in the first 4 weeks, with the peak at week four of treatment, whereafter there was a significant decrease[103]. This decrease in P1NP over time with estrogen treatment appears to be consistent in the literature. This acute response to estrogen treatment in both pre and postmenopausal women makes us question whether transient fluctuations in estrogen throughout the menstrual cycle could also be osteogenic in nature? However, it is possible that healthy young eumenorrheic women with sufficient estrogen may already be privy to its protective effects?

Another mechanism that estrogen appears to impact P1NP has been discovered through research in primary osteoblastic cells. Data in this area have shown 17β-Estradiol replacement therapy can increase the levels of P1NP through increased cell viability through the Sirt1/NF-κB/MMP-8 pathway. It appears estrogen can therefore effect P1NP through different pathways. Further research into how 17β- Estradiol impacts the Sirt1/NF-κB/MMP-8 pathway in women could provide mechanistic insights about how estrogen may regulate bone throughout the menstrual cycle, via bone formation marker P1NP[104].

Studies comparing P1NP between menstrual cycle phases are limited. Two studies have shown significant differences in P1NP between menstrual phases, both reporting similar findings with one study[37] finding P1NP significantly higher in the mid luteal phase compared to the early and mid follicular phase, while the other found P1NP higher in the luteal compared to follicular phase[21]. Another more recent study found no significant differences in P1NP between menstrual phases. Interestingly in one study which did find differences, there was no association between percent change of P1NP and mid cycle E2 at any time point throughout the luteal phase, nor was there an association with P1NP and luteinizing hormone or follicle stimulating hormone, while P1NP did have a positive significant correlation with β-CTXI. Absolute P1NP also did not correlate with estrogen at any time point[37]. Small study size could play into the lack of correlation between P1NP and estrogen in these studies. Of the two studies that did see phase differences, although fasting was mentioned, there was no mention of standardized food intake the day before or of visits nor was there mention of any pre-visit exercise protocols. These contradicting results leave us with a few things to consider, including; whether other mechanisms exist independent of estrogen that may be leading to changes in P1NP across the menstrual cycle,

the importance of standardized protocols before lab visits, and whether or not such transient changes in P1NP, should they exist, have any long-term physiological relevance.

Table 1: Differences in bone metabolic markers at rest between menstrual cycle phases: current literature and study outcomes.

Bone Metabolic Markers at Rest between Phases

STUDY	β-CTX	P1NP	PTH	Sclerostin
Cidem et al. (2013)				↔ between phases
Liakou et al. (2016)	↑ in FP	↑in LP		↔ between phases
Gas et al. (2008)	↑ in FP	↑ in LP		
Mozzanega et al. (2013)	↑in FP			
Neithheimer et al. (2015)	↑ in FP			
Martin et al. (2021)	↔ between phases	↔ between phases		
Buchanan et al. (1986)			↔ between phases	
Musef et al. (1986)			↔ between phases	
Pitkin et al. (1978)			↑in FP	

FP = Follicular phase LP = Luteal phase ↑ = Increase ↔ = No difference Szulc et al. 2017

Table 2: Strengths and limitations of studies examining differences in resting concentrations of bone markers between menstrual cycle phases.

Study Design Strengths and Limitations in Existing Literature at Rest

Study	Standardized Nutrition	Refrain from vigorous Exercise 12-24hrs	Consistent blood draw time	Overnight Fast
Cidem et al. (2013) (Sclerostin)	✗	✗	✗	✗
Liakou et al. (2016) (β-CTX ,P1NP, Sclerostin)	✗	✗	✓	✓
Gas et al. (2008) (β-CTX, P1NP)	✗	✗	✓	✓
Mozzanega et al. (2013) (β-CTX)	✗	✗	✗	✗
Neithheimer et al. (2015) (β-CTX)	✗	✗	✗	✗
Martin et al. (2021) (β-CTX,P1NP)	✓	✓	✓	✓
Buchanan et al. (1986) (PTH)	✗	✗	✓	✓
Musef et al. (1986) (PTH)	✗	✗	✗	✓
Pitkin et al. (1978) (PTH)	✗	✗	✓	✓

2.7 Acute Bone Metabolic Responses to Aerobic Exercise

Acute aerobic exercise can induce a response from bone remodelling markers, regulators and hormones related to bone metabolism including sclerostin, PTH, β-CTXI and P1NP. The current literature shows that the sclerostin response to acute aerobic exercise, including high intensity running and cycling, is an immediate 5-minute post exercise increase in measured sclerostin concentrations, followed by a return to baseline at 1-hour post exercise[105,106]. However, the mechanism for these post-exercise increases in sclerostin are not well understood. Parathyroid hormone typically shows an acute increase following aerobic exercise interventions, however exercise intensity appears to play a role in whether this response is seen[107,108]. Interventions with exercise intensities above 75% of $VO_{2\,max}$, appear to elicit a response in comparison to lower intensity interventions[109]. Factors including serum ionized calcium levels and nutrition may also factor into these outcomes with more calcium perturbations being possible during higher intensity training interventions. Studies have shown serum ionized calcium (iCa) to be a significant factor in post -exercise PTH increases, although iCa did not account for all of the increases in PTH in these studies, which leaves more to be learned about the mechanisms for these PTH increases[107]. In addition calcium intake of 1000mg before aerobic exercise has been shown attenuate increases in PTH, therefor it is important to control for pre exercise calcium intake when possible during interventions including pre-exercise meals with levels of calcium of this magnitude[110].

The existing literature regarding post-exercise response of bone resorption marker β-CTXI predominantly shows post-exercise increases, specifically in studies investigating aerobic exercise such as running and cycling[111]. Exercise duration, intensity as well as pre and during exercise nutrition intake are all possible reasons why some studies have failed to show a post-

exercise β-CTXI response, or even a decrease in β-CTXI post-exercise[60,112]. Although there is no current literature investigating total workload and its effects on post-exercise β-CTXI, it is possible that total workload itself is a factor in β-CTXI post-exercise response[111]. Pre-exercise meal intake has been shown to attenuate β-CTXI post-exercise increases in some studies, while others have shown that pre-exercise differences in fasted versus fed participants (fed participants having lower pre-exercise concentrations of β-CTXI) are diminished at 60-minutes post-exercise, specifically after running 60-minutes at 65% of $VO_{2\ max}$.[112,113]. It is therefore important to control for nutrition intake through standardization of isocaloric meals during acute exercise intervention trials such as our study where we are comparing visits, in order remove nutrition as possible a confounding factor, and to isolate the response of bone resorption marker β-CTXI.

Post-exercise, bone remodelling marker P1NP is often unresponsive to aerobic exercise[106,113]. However some studies have shown acute increases in P1NP in studies including 1-hour long aerobic exercise interventions between 50-75% of $VO_{2\ max}$[113,114,112]. Based on what is understood regarding the process of bone remodelling, where activation precedes bone resorption, which is then followed by bone formation, it is possible that the P1NP response is delayed, however this would not explain why some aerobic interventions showed an acute post-exercise increase in P1NP[15]. In studies where P1NP has increased it has sometimes been paired with a decrease in β-CTXI. This could reflect an osteogenic response, even if it is not the response most commonly seen in the literature[115]. There are inconsistencies in the literature which are currently not well understood and warrant further investigations. It is possible that extended time points, as far as 48 and 72 hours post exercise, may be needed to give us more insights into post-exercise P1NP response.

Although the literature has shown some acute exercise-induced changes in sclerostin, PTH, β-CTXI and P1NP these data do not allow us to extrapolate predictions of long-term bone mineral density, strength and geometry. A more thorough understanding of how acute bone remodeling may impact long term bone health is needed. Many factors are involved in bone remodelling beyond exercise, therefor it is difficult to suggest that findings from acute studies have physiological relevance for long term bone health.

2.8 Effects of Menstrual Cycle on Bone Metabolic Markers in Response to Exercise

2.8.1 Sclerostin Responses

A primary factor that has been shown to regulate sclerostin is mechanical loading of the bones. One study in wild type mice exposed to loading regimens found that they responded with significantly decreased SOST expression, the gene for sclerostin, while those lacking the estrogen-*a* receptor do not have this response, demonstrating the importance of the presence of estrogen in sclerostin expression[78]. In humans, more physically active adults have significantly lower circulating sclerostin concentrations compared to the least physically active adults, suggesting that chronic exercise may be leading to lower circulating sclerostin[116]. However, studies investigating sclerostin's response to acute exercise consistently show a transient increase in sclerostin 5 minutes post-exercise followed by a return to baseline by 1-hour post-exercise[105].

To date, no evidence exists examining the relationship between sclerostin and estradiol in healthy eumenorrheic women between menstrual phases, at rest and in response to vigorous running. Although two studies investigated the relationship between menstrual phases at rest and after resistance training, they were looking at the relationship between estradiol and hormones progesterone, testosterone and growth hormone, not bone metabolism[117,118]. One study did

measure markers of bone metabolism at rest and after acute resistance exercise, however sclerostin was not measured[28]. In this study the authors did however find a significant increase in bone formation marker BAP immediately post-exercise ($p < 0.05$) as compared to pre-exercise levels in both the follicular and luteal phases ($p < 0.05$). Additionally, BAP significantly decreased in the luteal phase at 1hour and 24hours post-exercise. Bone resorption marker ICPT significantly increased immediately post-exercise in the follicular phase ($p < 0.05$) while increasing 1hour and 24hours post-exercise in the luteal phase ($p < 0.05$). Post-exercise bone markers shifted to indicated increased resorption in the luteal phase[28]. There are no studies to our knowledge that have measured sclerostin at rest and post vigorous running between menstrual cycle phases.

2.8.2 PTH Responses

Only one study was found in the literature investigating differences in PTH levels between menstrual cycle phases at rest and in response to exercise, specifically to resistance exercise. Although the paper was predominantly written in Japanese, it appears that in both phases of the cycle, PTH significantly decreased immediately post and 1 hour post-exercise, as compared to pre-exercise levels, whereas 24-hour post-exercise levels were no longer significantly different from baseline values[119]. This is contrary to what has been seen in several exercise intervention studies in both men and women, where PTH has been investigated at baseline and post-exercise.

Indeed, evidence from research investigating males pre and post moderate intensity cycling and running interventions until exhaustion at 75% of $\dot{V}O_2$max have shown increases in PTH in response to exercise. In one study, increases in PTH led to increased bone resorption indicated by increase levels of CTX[107], while another found no significant increases in CTX, despite significant increases in PTH when males ran at 75% of $\dot{V}O_2$max for sixty minutes[108]. Other

studies investigating females during moderate intensity running at 50% of $\dot{V}O_2max$ have found no significant changes in PTH at 1 hour post running while significant increases in PTH were measured at the 24 and 72 hour post intervention time periods[120]. Notably, the intensity of this intervention was lower than the first two studies mentioned, and previous studies have shown that higher exercise intensity at 75% of $\dot{V}O_2max$, compared to 55% and 65% of $\dot{V}O_2max$, is necessary to incite significant changes in PTH response post-exercise[109]. One hypothesis for the increases in PTH is that it is in response to disturbed calcium homeostasis.

Post-exercise PTH increases have been hypothesized to be in response to a decline in serum ionized calcium (iCa)[121]. Increases in PTH stimulate bone resorption. Mobilized calcium, as a response to PTH increases, can then attenuate, or prevent additional serum calcium losses. More recent research in cyclists investigated this hypothesis. In one study, a calcium infusion was provided to prevent a decline in iCa[122]. PTH response to exercise was attenuated by 65% with the infusion[107]. It is unclear what mechanism led to the remaining 35% increase in PTH, but this raises questions about the possibilities of other mechanisms, including a relationship with sclerostin. Another study looked to gain more insight and investigated the relationship between iCa, dermal calcium losses, PTH and β-CTXI response, to see the magnitude to which dermal calcium losses contributed to iCa, PTH and β-CTXI changes. Participants included n =14 (men) and n = 14 (women). Participants cycled in either a cool or warm exercise condition at 75% of $VO_{2\ Peak}$. Dermal calcium sosses were 50% higher in the warm condition, but this was not reflected by larger increases in PTH[107]. The authors note that decreases in serum iCa concentration are the driver of PTH changes during exercise, and that changes in iCa were seen in the first 15 minutes of exercise, well before significant sweat loss occurred. Essentially, dermal sweat loss was not driving the change in serum iCa, while iCa was driving much of the

PTH response, however their previous study showed that infusion only attenuated 65% of the PTH response. Within sex changes of iCa and PTH were not significantly different. These data are important to note in exercise interventions where hematocrit changes are not measured, as this study suggests the main driver of PTH change is occurring early into exercise, versus in response to increased sweat losses. These are all important factors to consider when comparing PTH between menstrual phases as these data shed light on the importance or lack thereof, of dermal sweat losses on calcium, in relation PTH responses, in healthy young females. More research is needed investigating healthy eumenorrheic women and the different levels of PTH between menstrual cycle phases, at rest and after an acute vigorous bout of running.

2.8.3 CTX Responses

We did not come across any data investigating pre and post-exercise β-CTXI concentrations between menstrual cycle phases. Of the data that exist comparing at rest versus post-exercise bone metabolism between menstrual phases, the study protocols have used resistance training exercise, versus vigorous running as in our current study[28,118], with one investigating cycling[123]. None are specific to β-CTXI. Several studies unrelated to the menstrual cycle have shown contradicting results in regard to β-CTXI levels to intermittent exercise or prolonged endurance exercise, paired with minimal response of bone formation marker P1NP. This makes it difficult to assess what the long term net effects of these results may be[113,124,108,125,126,127].

Although data specific to β-CTXI were not found in the literature, there are data about estradiol levels as well as alternative markers of bone resorption from menstrual cycle studies. These could give us some insights about the effect of menstrual phase on post-exercise bone resorption[28,118]. One small study investigated the effects acute resistance exercise on markers of bone metabolism in $n = 7$ eumenorrheic women, however they used Type I collagen degradation

product (ICTP) as the marker of bone resorption[28]. The authors found significant increases in ICTP in follicular phase post-exercise, ($p < 0.05$), while ICTP significantly decreased at both 1 and 24 hours post-exercise in the luteal phase, indicating lower bone turnover in the luteal phase. The authors noted the importance of nutrition status, which they did not account for, and training load, as factors that need to be taken into consideration in future studies. Another study investigated levels serum levels of estradiol pre and post resistance exercise between eumenorrheic and amenorrheic or oligomenorrheic young women in the early follicular (EF) and mid luteal (ML) phases[118]. They found significant increases in estradiol and progesterone in the mid luteal phase post-exercise, compared to at rest in the eumenorrheic group, whereas no changes were seen in the women with menstrual disorders. Whether these fluctuations could affect bone resorption marker β-CTXI is unknown as it was not measured in this study. It is interesting to note that the women with menstrual disorders did not respond with increased estradiol post-exercise, because if estradiol does have an inverse relationship with β-CTXI, and this is suppressed in these women, this could represent suppression of healthy, necessary bone resorption, which is part of the normal bone remodelling process. There is a gap in the literature regarding the effects of menstrual cycle phase at rest versus post vigorous running, and its effect on bone resorption maker β-CTXI.

2.8.4 P1NP Responses

To our knowledge, studies investigating differences in P1NP both at rest and post-exercise between menstrual phases do not exist in the literature. One study looked at bone formation marker BAP at rest compared to post resistance training exercise and found a significant increase in BAP immediately post-exercise in both the follicular and luteal phase with a significant decrease at 1 hour and 24 hours post only in the luteal phase. Based on translation of this

document, it appears participants were taking oral contraceptives. Studies investigating P1NP changes in response to exercise, independent of menstrual cycle phase, have found contradictory results. Some studies have shown P1NP to be mostly nonresponsive to acute exercise, including resistant training and exhaustive running[108,128]. On the other hand, other studies involving aerobic and/or jumping exercise, have shown significant post-exercise increases in P1NP[113,129]. Although it is possible that there is a small contribution to post-exercise P1NP from muscle or tendinous synthesis, the literature suggests that these increases would be similar across the menstrual cycle[67,66].

Although there are currently discrepancies between studies in regard to changes in PTH, β-CTXI and P1NP between phases of the menstrual cycle at rest, we hypothesize that there will be no differences between phases for any of these markers in our study. There are currently no studies investigating sclerostin, PTH, β-CTXI and P1NP after vigorous running to compare to.

The estrogen threshold theory suggests that estrogen levels above 20 $pg \cdot mL^{-1}$ are protective of bone[12]. Although it is typical to see fluctuations of estrogen with lower estrogen in the follicular phase and higher estrogen in the luteal phase, in a healthy eumenorrheic population, we hypothesize that the estrogen levels will remain high enough across the menstrual cycle to have a bone protective effect. Should there be an increase in estrogen post exercise, there is no physiological reason that know of that would suggest this transient increase would impact these markers of bone metabolism or impact them differently between menstrual phases. An increase in estrogen, does not necessarily indicate there will be a sudden increase in estrogen receptors or of bone cells, nor a higher affinity for estrogen at the cellular level. Lastly, from an evolutionary perspective it does not compute that our skeletal system would be designed to be so delicate that transient fluctuations in estrogen at each menstrual phase would pose a risk to bone health at

each cycle. In addition, our study has a rigorous study design and methods for confirming menstrual phase are robust, which will be important factors in removing confounding factors which may otherwise interfere with isolating the physiological response of bone to both the menstrual cycle phase and vigorous running.

CHAPTER 3. Methods

3.1 Participants

Participants included 10 healthy, eumenorrheic females between the ages of 18 and 30 years, who volunteered for the study from a convenient university population. All participants were 'healthy', 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. Participants were eumenorrheic, a minimum 3-year post-menarche, and were not taking hormonal contraceptives. Eumenorrhea was defined as regularly occurring menstrual cycles lasting 24-35 days for a minimum of 1 year, and a cycle length of 3-7 days. Participants not actively tracking their menstrual cycles were asked to do so for three months to insure they fit the inclusion criteria. Exclusion of participants occurred 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 in 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. Participant baseline characteristics can be found in Table 1.

Table 3: Baseline characteristics of eumenorrheic participants

	Participants $n = 10$
Age (y)	21.7 ± 3.2
Height (cm)	164.2 ± 3.4
Body Mass (kg)	62.6 ± 10.4
BMI ($kg \cdot m^{-2}$)	23.2 ± 3.4

3.2 Study Design

The study used a randomized, repeated measures, counterbalanced design. A random computer-based number generator was used to determine which phase the participants started with. The study protocol included a 1-hour 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 follicular phase (FP) and the luteal phase (LP) of the menstrual cycle.

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 and email. Ovulation was assumed when a participant had a sustained increase of ~0.3°C[130]. 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. For the FP, testing occurred on day 8 ± 1.4, which corresponds to mid-late FP. The LP sessions were scheduled 1 week after positive test from a LH detection kit. With LH spiking ~36 h before ovulation, the LP session was timed near the mid LP. Specifically, for the LP, testing occurred on day 22 ± 2.5, which would typically correspond to the mid LP.

3.3 Experimental Procedures

3.3 Familiarization

Familiarization and information collection occurred 1-3 weeks before the running trials, depending on the order of the participant's sessions. During this time participants were screened using the Get Active Questionnaire (GAQ)[131], to assess health status, as well as the Godin Leisure Time Exercise questionnaire[132], to get a baseline of activity levels. Participants provided informed consent and had time to familiarize themselves with the motorized treadmill (4Front, Woodway, WI, USA) that they would be using for the exercise sessions. 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.

The graded running test to exhaustion was done on a motorized treadmill (4Front, Woodway, WI, USA). Participants were fitted with silicon facemask (7400 series Vmask, Hans Rudolph Inc. KS, USA), to prevent leaking and ensure comfort. 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). Participants did a warmup walking at 3.5mph for 5 minutes, after which point, they began running at their own selected pace between 5-7mph 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-minute rest period. During this test participants ran at 110% of their maximal work rate, until volitional fatigue. $\dot{V}O_2max$ 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 bmp 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.

3.4 The vigorous intensity continuous running trials

All participants arrived at the lab at 0800 hours after a 12-hour overnight fast. Participants also refrained from caffeine and alcohol for 12 hours prior to all visits. A standardized breakfast was provided to each participant, which consisted of a Chip Clif bar, 29 $kJ \cdot kg^{-1}$ of the participant's body weight. Fifteen minutes was allotted for consuming the bar and 45 minutes to digest it, while water was consumed *at libitum* during this time. From 0800 to 0845 hours, participants sat quietly while resting gas exchange was measured.

Each running trial began at 0910 hours on a motorized treadmill (4Front, Woodway, WI, USA), and consisted of the same standardized 5-minute warm up at 3.5 mph followed by 30 minutes of high intensity, continuous running at 80% of $\dot{V}O_2max$, and a 5-minute cool down. Heart rate and gas exchange ($\dot{V}O_2$ and $\dot{V}CO_2$) were continuously measured throughout the trial. By calculating the $\dot{V}O_2$ reserve, a pre-determined work rate was established[133]. 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.

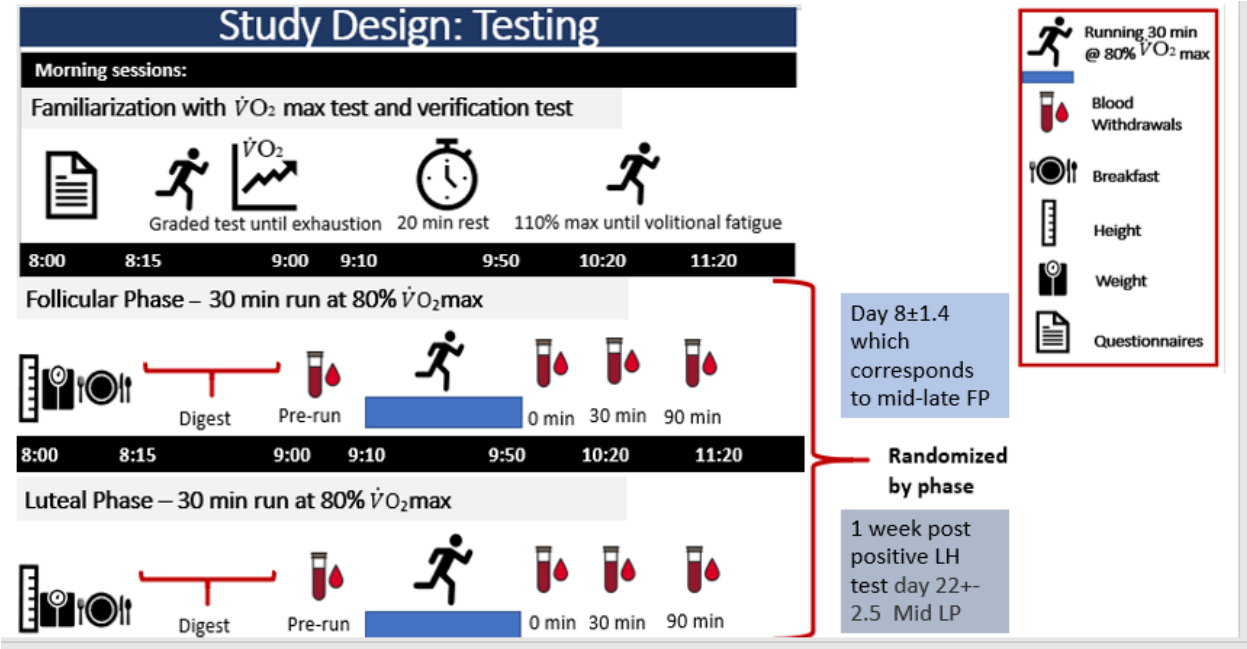

Figure 6: Study Design – Exercise Trials

3.5 Measurements

3.5.1 Anthropometrics, Activity and Dietary Measures

Upon arrival to the laboratory, each participant's height (to the nearest 0.1 cm) and weight (to the nearest 0.1 kg) were recorded using a mechanical scale (Health-o-meter Professional, Sunbeam Products Inc., Ill., USA). 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-day 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.

3.5.2 Blood Collection and Analysis

Blood samples were obtained at four time points: pre-exercise (0900 h), immediately post-exercise (0950 h), 30 min post-exercise (1020 h) and 90 minutes (1120 h) post-exercise. Participants were positioned in supine position during sample collection, and blood was collected from an antecubital vein. 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 samples were stored at -80 °C until further analysis.

Estradiol and progesterone were measured using standard enzyme-linked immunosorbent (ELISA) single assay kits (DiaMetra, Spello, Italy) at both rest and post-exercise. The in-house, intra-assay coefficients of variation were 9.1% for E2, and 10.5% for P4. Sclerostin was measured using two ELISA assay kits (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-CTX, and P1NP were measured at the Mount Sinai Hospital Core Laboratory (Toronto, Ontario) using a Roche Cobas e602 automated analyzer for β-CTX and PTH, and a Roche e411 Elecsys automated analyzer (Roche Diagnostics, Rotkreuz, Switzerland) for P1NP. 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-CTX, PTH, and P1NP.

3.6 Statistical Analysis

All statistical analysis was done using a Statistical Package for Social the Scientist (SPSS IBM version 25, USA). From a total of 80 blood samples (10 participants x 2 phases x 4 sampling times), there were a few missing values due to either a missed blood draw or an insufficient sample volume to run all assays. Three participants were excluded from the analysis (2 from the

β-CTX analysis and 1 from the PTH analysis) because they had more than two missing values. If a participant one or two missing values, each of the missing values (5 of 80 for sclerostin, 2 of 72 for PTH, 6 of 64 for β-CTXI and 3 of 80 for P1NP) 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[134]. 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 P1NP 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 (estrogen and progesterone) the Greenhouse-Geisser correction was used.

Resting and post-exercise data 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 for time, pairwise comparisons were performed. Effect sizes are reported as partial eta squared (η_p^2) for ANOVA and were interpreted based on the Cohen criteria for partial η^2 : 0.01=small, 0.06=moderate, 0.14=large effect[135,136]. In results, data are reported as means ± SE while figures present means ± 1 SD. For all statistical tests, significance was assumed at an alpha level of <0.05.

CHAPTER 4 Results

4.1 Exercise Outcomes

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% $\dot{V}O_2$max) and 29.42 ± 3.10 mL·kg^{-1}·min^{-1} (79.6 ± 0.02% $\dot{V}O_2$max), with no significant difference between phases (t(10)=0.324, p=0.753, d=-0.10). Likewise, there were no significant differences in the average heart rate between the trials performed in the FP (171 ± 15 b·min^{-1}) and LP (171 ± 12 b·min^{-1}; t(10)=0.127, p=0.902, d=-0.01), or in RER achieved in the running trials between the FP (1.01 ± 0.05) and the LP (1.00 ± 0.04; t(10)=2.13, p=0.058, d=0.64). $\dot{V}O_2$-derived estimates of energy expenditure (assuming a relationship of 5 kcal·L^{-1} O2) were 270.9 ± 36.0 kcal and 272.5 ± 45.5 kcal in the FP and LP, and were not significantly different between phases (t(10)=0.662, p=0.523, d=-0.20).

4.2 Hormones

The measured estradiol concentrations were 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), in line with previous research[32]. Estradiol was lower in the follicular phase than in the luteal phase in 8 of the 10 participants (Figure 7A). Resting progesterone was lower in the follicular than in the luteal phase, on average (4.11 ± 0.74 ng·mL^{-1} versus 11.86 ± 1.42 ng·mL^{-1}, p=0.0001), as well as individually (Figure 7B). All participants had resting estradiol levels within adult premenopausal reference values in both phases, with the reference range being 15-350 pg·mL^{-1}. Similarly, resting progesterone fell within the reference range of ≤ 0.89 ng·mL^{-1} in the follicular phase for all participants, except for one who had a value of 0.21 ng·mL^{-1}. For the luteal phase, all participants levels fell within the reference range

of 1.8 – 24 ng·mL^{-1}, with only two participants under 12 ng·mL^{-1}. Thus, data for sclerostin, P1NP, PTH and of β-CTXI were analyzed with and without these two participants who did not have an increase in estradiol from the follicular to luteal phase. References ranges were derived from Mayo Clinic Laboratories[137]. Moreover, estradiol showed significant main effects for time ($F=3.56$, $p=0.038$; $_p\eta^2=0.16$) and menstrual phase ($F=5.28$, $p=0.034$; $_p\eta^2=0.23$), but no interaction ($F=0.65$, $p=0.529$; $_p\eta^2=0.03$). Specifically, estradiol increased from pre- to immediately post-exercise in both the follicular and luteal phases (15 ± 4% and 7 ± 10%). Significant main effects for time ($F=5.20$, $p=0.013$; $_p\eta^2=0.22$) and menstrual phase ($F=16.65$, $p=0.001$; $_p\eta^2=0.48$), with no interaction ($F=2.37$, $p=0.114$; $_p\eta^2=0.12$), were also shown in progesterone. Specifically, progesterone increased from pre- to immediately post-exercise in both the follicular and luteal phases (24 ± 10% and 18 ± 5%).

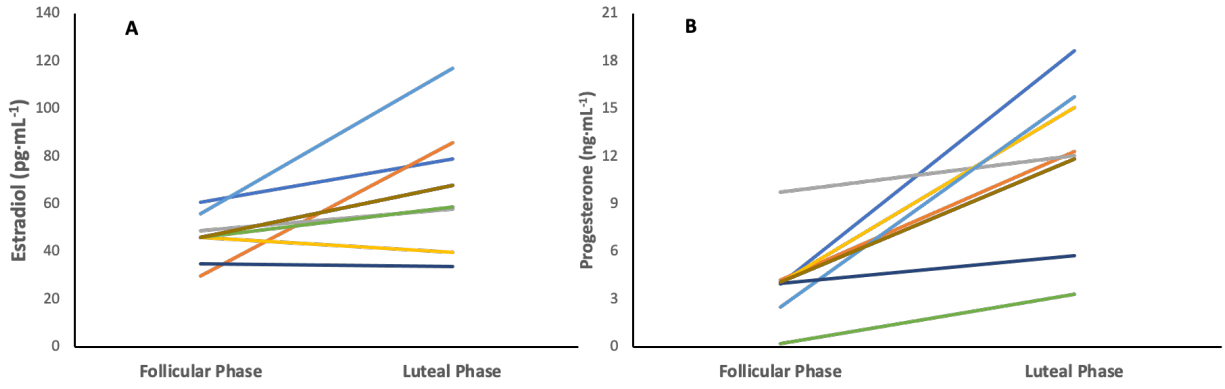

Figure 7 – Plasma concentrations of Estradiol (A) and progesterone (B): *Both the follicular and luteal phases of the menstrual cycle are shown for each female participant* (n=10). *Note: each participant is presented in a different color.*

4.3 Bone Markers

Sclerostin showed no effect for menstrual phase ($F=0.46$, $p=0.507$; ${}_p\eta^2=0.02$) and no interaction ($F=0.99$, $p=0.404$; ${}_p\eta^2=0.05$), but there was a significant main effect for time ($F=4.65$, $p=0.006$; ${}_p\eta^2=0.20$). At rest, sclerostin was not different between follicular and luteal phases (266.5± 48.6 $pg \cdot mL^{-1}$ and 296.0 ± 37.5 $pg \cdot mL^{-1}$). Following running, sclerostin increased immediately post-exercise in both the follicular and luteal phases (46 ± 8 %), then returned to near its pre-exercise levels at 30 min and 90 min post-exercise (Figure 8). These results were similar also when the 2 participants who did not show an increase in estradiol from the follicular to the luteal phase were excluded from the analysis. The results were similar with and without the 2 participants with progesterone within but in the lower end of the range.

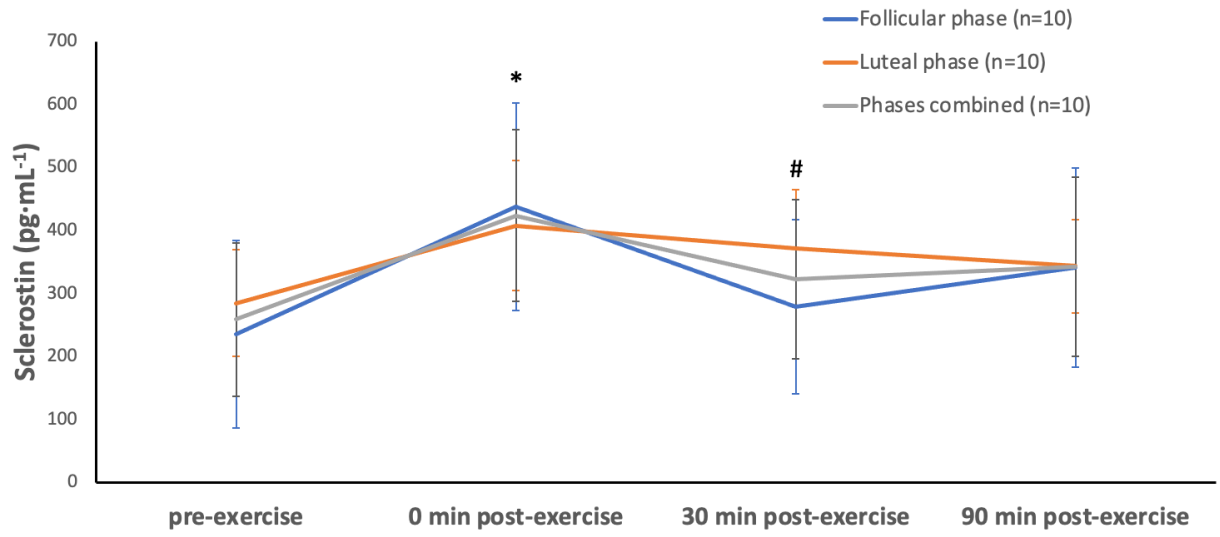

*Figure 8 – Sclerostin: Changes in plasma concentrations (mean ± SD) of sclerostin from pre- to post-exercise in the follicular and luteal phase of menstrual cycle in young adult, eumenorrheic females. There was no effect for phase and no interaction; *denotes significant difference from pre- to 0 min post-exercise ($p<0.0001$) in pairwise post-hoc comparisons for both phases combined. #denotes significant difference from 0 min to 30 min post-exercise ($p=0.023$) in pairwise post-hoc comparisons for both phases combined.*

Raw PTH concentrations showed no main effects of menstrual cycle phase ($F=0.51$, $p=0.485$; $_p\eta^2=0.029$) or time ($F=1.05$, $p=0.397$; $_p\eta^2=0.058$) and no interaction ($F=0.47$, $p=0.707$; $_p\eta^2=0.027$). Resting PTH was not different between follicular and luteal phases (1.00 ± 0.22 pmol·L^{-1} and 0.71 ± 0.16 pmol·L^{-1}), and, although it was higher immediately after running, this difference was not statistically significant. However, this could be due either to large variability in the PTH response to exercise or a few outliers. Indeed, after careful inspection of the data we found two outliers (using the criteria that outliers were greater than 2.5 SD outside of the mean), which we then removed from subsequent analysis. In doing so, we found a significant time effect for PTH ($F=4.14$, $p=0.011$; $_p\eta^2=0.217$), with no phase effect ($F=1.082$, $p=0.315$; $_p\eta^2=0.067$) and no interaction ($F=0.615$, $p=0.585$; $_p\eta^2=0.039$), reflecting an overall increase ($+43 \pm 25$ %) in both phases (Figure 9). These results were similar also when the 2 participants who did not show an increase in estradiol from the follicular to the luteal phase were excluded from the analysis.

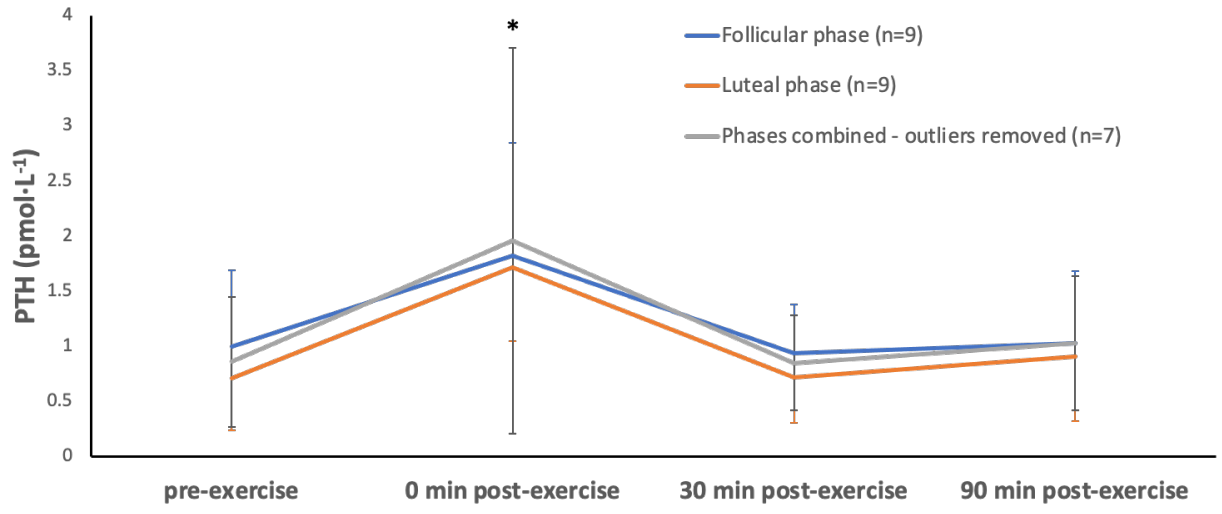

*Figure 9 – Parathyroid Hormone (PTH): Changes in plasma concentrations (mean ± SD) of PTH from pre- to post-exercise in the follicular and luteal phase of menstrual cycle in young adult, eumenorrheic females. There was no effect for phase and no interaction. *denotes significant difference from pre-exercise to 0 min post exercise (p=0.011) in pairwise post-hoc comparisons for both phases combined. Values are absolute concentrations, not log-transformed.*

For β-CTXI we showed 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 follicular and luteal phases (243.1± 52.7 ng·L^{-1} and 202.4±30.8 ng·L^{-1}). Following running, β-CTXI decreased significantly immediately post-exercise (20 ± 7 %), then remained below its pre-exercise levels at 30 min and 90 min post-exercise (12 ± 11 % and 17 ± 13 %) with no difference between phases (Figure 10). These results were also similar when the 2 participants who did not show an increase in estradiol from the follicular to the luteal phase were excluded from the analysis.

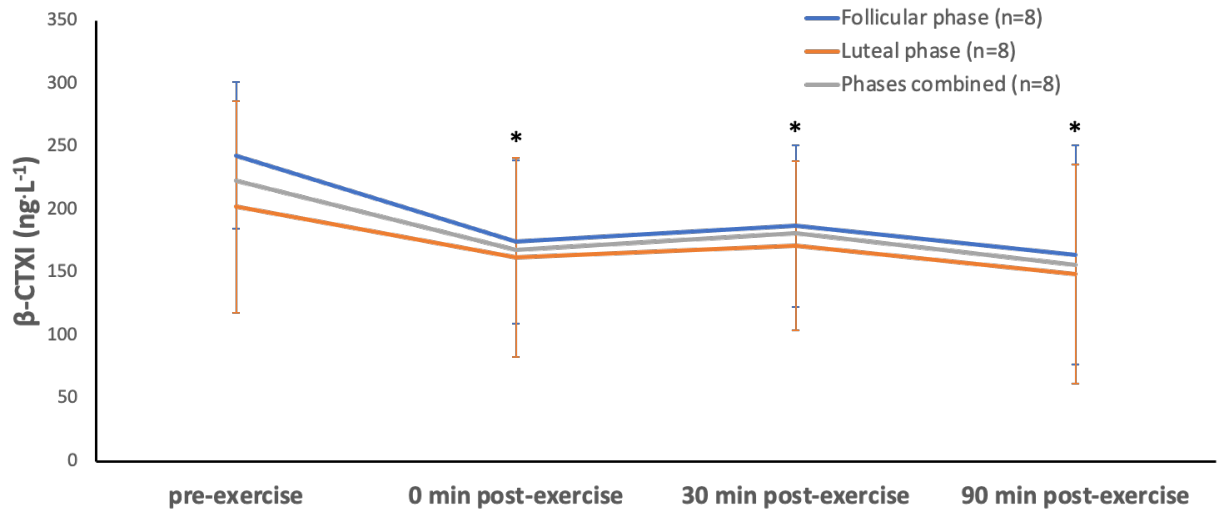

*Figure 10 – ß-isomerized-C-terminal-cross-linking-telopeptides (ß-CTXI): Changes in plasma concentrations (mean ± SD) of ß-CTXI from pre- to post-exercise in the follicular and luteal phase of menstrual cycle in young adult, eumenorrheic females. There was no effect for phase and no interaction; *denotes significant difference from pre-exercise to 0 min post-exercise (p=0.004), 30 min post-exercise (p=0.039) and 90 min post-exercise (p=0.002) in pairwise post-hoc comparisons for both phases combined.*

There was no main effect of menstrual cycle phase (F=2.47, p=0.133; $_p\eta^2$=0.121) and no interaction (F=1.16, p=0.330; $_p\eta^2$=0.061) for P1NP, but there was a significant main effect of time (F=26.50, p=0.000; $_p\eta^2$=0.595). At rest, P1NP was not different between follicular and

luteal phases (56.9 ± 11.30 µg·L^{-1} and 64.30 ± 18.32 µg·L^{-1}). Following running, P1NP increased significantly immediately post-exercise (29 ± 18.9 %), then returned to its pre-exercise levels at 30 min and 90 min post-exercise with no difference between phases (Figure 11). These results were also similar when the 2 participants who did not show an increase in estradiol from the follicular to the luteal phase were excluded from the analysis.

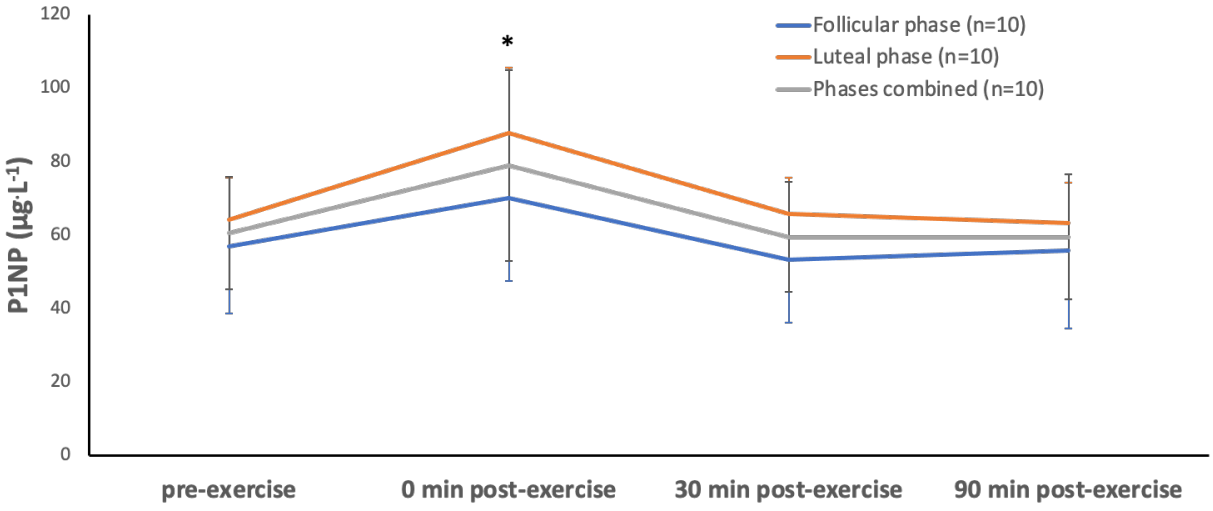

*Figure 11 – Procollagen type 1 N-terminal propeptide (P1NP): Changes in plasma concentrations (mean ± SD) of P1NP from pre- to post-exercise in the follicular and luteal phase of menstrual cycle in young adult, eumenorrheic females. There was no effect for phase and no interaction; *denotes significant difference from pre- to 0 min post-exercise ($p<0.0001$) in pairwise post-hoc comparisons for both phases combined. Values are absolute concentrations, not log-transformed.*

CHAPTER 5 Discussion

5.1 Most Significant Results

We found no significant differences in plasma concentrations of sclerostin, PTH, β-CTXI and P1NP at rest or in response to exercise between the follicular and luteal phases of the menstrual cycle, despite a significantly lower concentration of estradiol and progesterone in the follicular compared to the luteal phase in 8 of the 10 participants. Sclerostin, PTH and P1NP concentrations were significantly higher immediately post-exercise at both phases of the menstrual cycle, while β-CTXI was significantly lower for up to 90 min post-exercise.

5.2 Markers of Bone Metabolism at Rest and in Response to Exercise

5.2.1 Sclerostin at rest and in response to exercise

At rest, sclerostin did not show significant fluctuations across the menstrual cycle, which is in line with other research investigating sclerostin concentrations between menstrual cycle phases at rest[37,38]. These results are despite typical and significant fluctuations in estradiol between baseline (menstruation) concentrations and those in the mid-follicular phase in all three studies[38,37].

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. The estrogen threshold theory suggests that certain tissues have different sensitivities to estradiol, and in relation to bone, it notes that a concentration of estradiol (30-45 $pg \cdot mL^{-1}$) can partially prevent bone loss. Evidence suggests calcium and gonadotropin hormones are the two most sensitive tissues to estradiol, and that concentrations at or above 25 $pg \cdot mL^{-1}$ are protective of bone[12]. According to this theory, estradiol concentrations below 20 $pg \cdot mL^{-1}$ and between 10-20 $pg \cdot mL^{-1}$

are osteocatabolic. Estrogen deficiency in postmenopausal women is a major determinant of bone loss[138]. Estrogen deficiency increases serum sclerostin and osteoclast formation, leading to increased bone resorption. In addition, estrogen deficiency is associated with an increased bone resorption and decreased bone formation response, leading to an imbalance, which results in increased negative bone turnover. This is mediated by restraining estrogen's typical anabolic actions on estrogen receptors in both osteoblasts and osteoclasts[139,140,74,141]. On the contrary, estrogen treatment in postmenopausal women is associated with lower bone sclerostin mRNA levels[142]. Based on these data, estrogen and sclerostin have an inverse relationship[71]. However, in our study although estrogen levels differ significantly between menstrual phases, they do not drop low enough to elicit significant changes in sclerostin levels. It has been shown that serum levels of estrogen during the menstrual cycle remain higher than those seen post menopause[143]. Therefore, the cyclical decrease in estrogen levels seen during the follicular phase in our young eumenorrheic females does not appear to compromise bone metabolism, particularly through antagonism of the Wnt pathway. As the theory suggests, there may be a enough estradiol present to offer a protective effect on bone[12].

Sclerostin concentrations were significantly higher immediately post-exercise in both phases with no difference between phases. This was expected based on previous research investigating sclerostin responses to running [105,144] and other forms of loading in females[145]. Although we did not correct for exercise induced plasma volume changes in the currently study, a previous study in females that corrected for exercise induced plasma volume shifts also showed significant increases in sclerostin concentrations 5 min post-exercise[105]. It is important to note that participants in this previous study[105] were all on birth control. Previous studies have shown that young healthy oral contraceptive users have significantly lower estrogen levels than

eumenorrheic women[92], with similar sclerostin response immediately post exercise, which suggests that these differences in estrogen levels are not of large enough magnitude to impact sclerostin and that the lower range of estrogen may still be enough to be protective of bone. The exact mechanism leading to immediate post-exercise increases in sclerostin concentrations in our study as well as previous studies is unknown, although it seems to be in response to the loading from vigorous exercise protocols of both high and low impact[105]. Most importantly, there were no differences in sclerostin concentration between phases, which is important, since it suggests that despite significantly higher estrogen levels in the luteal phase, bone metabolism in response to exercise does not appear to differ between phases, therefor when considering the research protocol in studies investigating bone metabolism and sclerostin in females throughout the menstrual cycle, phase does not appear to effect sclerostin response post-exercise.

5.2.2 PTH at Rest and in Response to Exercise

There were no significant fluctuations in resting PTH across the stages of menstrual cycle measured, which is in line with previous studies, despite significant fluctuations in estrogen and progesterone[24,35,36]. Contrary to these results, a significant increase in PTH, with its peak just before the LH peak, followed by a subsequent decrease in levels throughout the luteal phase, has been previously documented[30]. A possible explanation for this is that estrogen inhibits PTH induced bone resorption, lowering serum calcium, resulting in an increase in PTH. The theory does not align with what we see in postmenopausal women, which is that as estrogen decreases there is either an increase or no change in PTH levels[85]. Based on this theory, we should also have seen an increase in PTH in both the follicular and late luteal phases of this study, where estrogen spikes typically occur, although this is not what was shown. A study following up on this theory found no significant changes in PTH across the same three phases used in the

previous study[35], which falls in line with our study. It is possible that the use of different assays in these two studies may have played a role in yielding different results, since lack of harmonization between assays has been reported in the literature[146]. In addition, there are large individual biological variations in PTH, up to twenty percent in healthy people, which could factor into differences in results between studies[146].

Despite significant fluctuations in estrogen during our study, and three other studies[36,24,35], the inverse relationship seen between estrogen and PTH in postmenopausal women[85] does not seem to be present in our eumenorrheic population. A possible explanation is that the magnitude of estrogen fluctuation is not large enough during a healthy eumenorrheic menstrual cycle, to result in low enough estrogen levels to compare to those shown in postmenopausal women, therefore, we are not seeing the increase in PTH. This falls in line with the estrogen threshold theory, which suggests that once estrogen levels drop below 20 $pg \cdot mL^{-1}$ this becomes catabolic to bone, while levels above 25 $pg \cdot mL^{-1}$ are likely protective of bone[12]. It is possible that the transient nature of the fluctuations during a menstrual cycle are also not long enough to elicit the PTH response compared to when estrogen is chronically lower during menopause or after an ovariectomy. The calcium dysregulation that is seen post menopause, may also contribute to the increases in PTH seen in this older population, which would not be seen in our younger female participants.

PTH increased by 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[28]. Contrary to our study, PTH was increased immediately post-exercise in the follicular phase only, and 1 hour post-exercise in both the luteal and follicular phases[28], although participants were taking oral contraceptives. Oral contraceptive

users have been found with significantly lower estrogen levels than eumenorrheic women[32]. Thus, it is possible that the use of oral contraceptives in Suzuki et al.[119] played a role in their different PTH response to exercise, compared to ours. However, the estrogen levels remained above 30 $pg \cdot mL^{-1}$ in both phases, which is not low enough to be problematic for bone. It is also possible that the 30 minute running exercise protocol used in our study led to a different PTH response than the resistance training protocol used by Suzuki et al.[28]. Even if the type of exercise impacted the PTH response, this still would not explain why PTH only increased in the follicular phase immediately post exercise in their study. It is possible that the individual variability between phases was large in the Suzuki et al. study, and that this contributed to the differences they saw immediately post exercise between phases, which we did not see in ours. High individual variability in the PTH response to exercise was also large in our study, with two outliers that needed to be removed in order to control this variability.

Previous studies not focused on menstrual phase have also shown conflicting post-exercise PTH responses, with some showing increases immediately post-exercise, followed by a return to or below baseline at one hour post[109,108,145,121], while others have shown no significant post-exercise PTH response. 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. Calcium ingestion pre and during exercise has been shown to attenuate increases in PTH[147,148,149,150,110]. Although we did not measure calcium in our study, it is important to note since participants consumed a bar that contained 294 mg of calcium per serving before exercising. The average 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. Future studies

investigating PTH between menstrual phases should aim for larger sample size and should consider the impact of biological variations in PTH when analyzing results.

5.2.3 β-CTXI at Rest and in Response to Exercise

Despite significant fluctuations in estrogen and progesterone throughout the menstrual cycle at rest, there were no significant differences in β-CTXI levels between menstrual phases in our study. This is in line with recent research that investigated β-CTXI in eumenorrheic and oral contraceptive users between phases[32]. Contrary to this, several studies have reported significant increases in β-CTXI concentrations in the FP as compared to the LP of the menstrual cycle[22.21,40,37]. Several factors could explain why our study didn't see these differences including, standardized participant and blood sample preparations, pre-analytical storage, differences in standardized nutrition protocols, participant age, refraining from vigorous exercise prior to testing, measuring at different time points and circadian timing of blood draws. In our study, participants refrained from vigorous activity 12 hours prior to lab visits, which was not the case for all previous studies with contradictory results[37,21,22,40], although one did note consistent activity levels throughout all visits, without the type of activity being specified[37]. Controlling for pre visit high intensity exercise, potentially allowed us to show a more accurate representation of β-CTXI levels at rest. Our participants completed an overnight fast and followed a standardized nutrition protocol prior to and on the day of each visit. While fasting is mentioned in two of the four previous studies reporting inconsistent results compared to ours[21,37,] there is no mention of a standardized nutrition protocol before or during visits. Previous studies have shown how nutrition can modulate β-CTXI levels[151,152]. It is possible that the pre-exercise meal in our study reduced β-CTXI levels; however, our nutrition protocol was consistent between visits and no differences in β-CTXI were measured between phases. It is difficult to know if nutrition in these

previous studies contributed to the differences we see with our results. For example, if participants fasted during the FP and ate something the morning of the LP visit, it would be likely that β-CTXI levels would be acutely lower during visit where nutrition was consumed[151]. Our blood samples were drawn between 9 and 9:10am, in accordance with the range recommended by the National Bone Health Alliance[63], based on circadian rhythms. Since only one of the studies with contradicting results drew blood within the recommended 8-10am timeframe[63], which in and of itself is a broad timeframe, it is possible the timing factored into our result being different. Specifically, β-CTXI levels tend to decrease throughout the day with its nadir in the afternoon[60,151,90]. Measuring within a tight and consistent, recommended time frame could have factored into our results being consistent between phases in comparison to previous contradictory results.

The average age in our study was 21.7 years, whereas several previous studies had women aged 40[40] and 45[37]. It is possible that since vitamin D synthesis and intestinal absorption of calcium can be lower in this older population compared to ours, that this could have indirectly affected bone marker concentrations[153]. Another factor to consider are the assays used between studies. We measured β-CTXI using the Roche Cobas e602 automated analyzer, which was not consistent across studies, and for some studies assay type was not mentioned. Significant discrepancies between assays have been reported in the literature with limited commutability between assays[154]. This makes comparing our results to previous studies using different assays problematic, and is an area that should be addressed in order to allow for legitimate comparisons between assays[155]. Future studies should aim to use consistent standardized nutrition protocols, refrain from pre-visit vigorous exercise and use the same assays where possible in order to have

more consistent baseline protocols which would lead to a β-CTXI representation with fewer potentially confounding factors.

Post-exercise β-CTXI concentrations were significantly lower than pre-exercise concentrations at all three time points, however no differences were shown between menstrual phases. Importantly, no studies have examined the β-CTXI responses to exercise in different phases of the menstrual cycle. Thus, we will reference research done outside of the menstrual cycle realm to compare our results to in the context of post-exercise β-CTXI response. Previous studies have shown conflicting results when measuring β-CTXI concentrations from pre- to post-exercise[108,109,106,113,156,147,124], with many showing significant increases and some showing decreases or no change. An increase or decrease in β-CTXI could both be osteogenic, with increases representing an upregulation of bone remodelling and reductions indicating a decrease in osteoclastic activity and reduced bone loss, with more potential for bone formation.

There are several possible explanations as to why we did not see increases in β-CTXI in our study. Several studies in men running at similar intensities as our study, have measured increases in β-CTXI one hour post-exercise[108,112]. These studies investigated fasted participants, with the latter result referencing the placebo arm of a study with three nutrition protocols[112]. If we look at the other two arms in this study[112], which included participants consuming a carbohydrate/protein solution either immediately post-exercise, or later post-exercise, we see that those who consumed nutrition immediately post-exercise had lower β-CTXI at 1 hour post-exercise compared to baseline, despite increased β-CTXI immediately post-exercise. This makes us ask about the importance of nutrition. Evidence has shown that consumption of carbohydrates results in decreased markers of bone resorption, namely β-CTXI, as do consumption of protein and fats[151,114]. Based on the literature investigating carbohydrate or mixed nutrient consumption

pre, during or post-exercise, these reductions in bone resorption marker β-CTXI appear to be acute, lasting one up to as many as 6 hours post-exercise, indicating that food consumption seems to acutely attenuate exercise induced increases in β-CTXI. This could be one explanation for our reduced post-exercise β-CTXI levels.

Studies that directly compared fed vs fasted exercise responses, have also shown that despite significantly lower pre-exercise β-CTXI levels in the fed group, these differences diminished in the acute post-exercise period, when no additional nutrition was consumed during exercise[113]. Based on this study, it is possible that the mixed meal our participants consumed pre-exercise contributed to the reduction in the β-CTXI levels we saw post-exercise, but we cannot be certain that post-exercise β-CTXI would have differed compared to fasted participants. In regard to nutrition, ingestion of specific minerals such as calcium, pre and during exercise have also been shown to attenuate increases in β-CTXI[147]. It is possible the calcium in our pre-exercise meal also contributed to the decrease in β-CTXI seen in our participants. In any case, for our study's purpose, it's most important to note that participants underwent a controlled nutrition protocol including the exact same meal and meal timing before both exercise sessions in each menstrual phase. In addition, participants followed the same diet the day prior to laboratory visits. Therefore, regardless of whether β-CTXI concentrations increased or decreased, there were no significant differences between the response both at rest and post-exercise with this controlled nutrition protocol.

Exercise duration and intensity may also have contributed to differences in β-CTXI measures between ours and other studies. If we look at duration first, in our study, women ran for 30 minutes at 80% of their maximal aerobic capacity, whereas in the two studies mentioned above, with fasted participants, the men ran until exhaustion[112,108]. Two previous studies in

women, which also included 30 minute exercise interventions, showed similar results to our study, with one showing significant post-exercise decreases in β-CTXI from baseline[145] and the other showing no significant changes in β-CTXI concentrations[143]. It's possible the shorter exercise duration did not produce enough strain to lead to an increase in β-CTXI, or that the timing of blood sampling missed the peak in β-CTXI in both studies. Exercise induced β-CTXI increases could also have been attenuated by pre-exercise nutrition intake. With regard to intensity, previous research comparing different exercise intensities, despite same overall workload, has shown the ability for differing intensities to elicit different β-CTXI responses, with the highest intensity protocol increasing β-CTXI for up to two hours compared to lower intensities only increasing β-CTXI for up to one hour[124]. In addition, research comparing males and females cycling at different intensities, found only males had significant increases in β-CTXI post-exercise, which raises the question of exercise intensity and sex differences[157]. Our study was done at eighty percent of maximal aerobic capacity. In combination with the shorter duration, intensity could have been too low to elicit the strain required to increase β-CTXI levels. The literature also suggests that exercise type is not likely a major factor in post-exercise β-CTXI response when intensities are matched between running and cycling[106], therefore type is not likely the reason for the differences we see between ours and other studies.

Despite estrogen not being an apparent factor in β-CTXI concentrations in our study, it is possible β-CTXI concentrations are being mediated by other hormones or factors not yet understood. Sex differences could be playing a role, time specific changes may be too transient to encapsulate the full process of bone metabolism and individual variation in bone markers may play a role in the different results seen between studies. Future studies should consider a longer time frame to capture a longer window of bone metabolism, while standardizing nutrition, assay

type and following recommended standard protocols for β-CTXI. In relation to our objective, there were no differences between menstrual phases in our study and this remains important for methodological considerations for future research.

5.2.4 P1NP at rest and in response to exercise

There were no significant differences in P1NP at rest between menstrual phases in our study, despite significant differences in estrogen and progesterone. Results from previous studies have been inconsistent with two studies showing higher P1NP in the luteal versus follicular phase and one showing no differences between phases[21,37,92]. Reasons why we may have shown different results in our study compared to those with increased P1NP in the luteal phase include individual variability of bone marker responses, race and pre-visit exercise protocols. In our study, participants refrained from any vigorous activity for at least 12 hours before arriving at the laboratory. Within the basic multicellular unit, bone is remodelled first by the initiation of bone resorption which is followed by bone formation and mineralization[15]. Therefore, the potential lag of P1NP response to exercise could affect resting P1NP levels if standardization of exercise prior to lab visits isn't the same during both phases of testing. In our study, we did control for exercise pre-visit factors, however several previous studies do not mention controlling for this[21,37]. This difference between studies could impact resting P1NP levels. Our study had a small sample which could affect our result. It is possible that the individual variation in P1NP and the different responses to menstrual phases could factor into the different results in our study compared to previous studies[21,37], however a recent study which found results similar to ours would have also contended with these factors[92].

There were no differences in post-exercise P1NP between menstrual phases, however there was a significant increase in P1NP immediately post-exercise in both phases. There are no

previous studies that we are aware of that have investigated changes in P1NP at rest compared to post-exercise between menstrual cycle phases. If we look at the literature investigating P1NP changes in response to exercise, independent of menstrual cycle phase, there are inconclusive results. Many studies have shown P1NP to be mostly nonresponsive to acute exercise, including resistance training and exhaustive running[108,128,158]. On the contrary, some studies including aerobic exercise and/or jumping, have shown significant post-exercise increases in P1NP, as we did in our study[113,129]. One study found a post-exercise decrease in P1NP[159]. It is unclear which factors are contributing to differences in response, although it is possible that longer durations of aerobic activity may elicit a greater post-exercise P1NP response. Although post-exercise P1NP has been shown to increase from tendinous and muscular collagen synthesis, these increases would likely be minimal and the current literature suggests they would not be affected by menstrual cycle phase[67,66]. It is difficult to interpret what the changes in P1NP could mean long term in relation to bone mineral density and strength as it is unclear what the relationship is between acute changes in bone formation markers and long-term bone health. More research in this area is needed, as ours is the first study to investigate pre to post-exercise P1NP changes between menstrual phases. Future studies should aim to minimize confounding variables that may cloud the results. For our study, what is most important is that there were no significant differences in P1NP between menstrual cycle phases. This is important when considering research methodology in studies investigating bone metabolism in women.

5.4 Strengths and Limitations

The strengths of our study include the methods used to confirm eumenorrhea and the standardization of nutrition, exercise, and blood draw protocols. By minimizing changes in the testing and nutrition environments between lab visits, our data comparisons both at rest and in response to exercise were representative of physiological responses.

Limitations include the small sample size, which limits the generalizability of our results, and not accounting for exercise induced shifts in plasma volume. It is also possible that the study was underpowered, which could increase the likelihood of type 2 error. In addition, we used a recreationally active population, and it is possible that a trained population could have different adaptations and hormonal responses to exercise. Lastly, acute sampling time points may have missed time specific changes outside of these windows and extending sampling time points up to 24- and 48-hours post-exercise could help better characterize post-exercise changes in bone metabolism.

CHAPTER 6 Conclusion

6.1 General Conclusion

There were no significant differences in levels of β-CTXI, PINP, PTH or sclerostin between menstrual phases, despite significant differences in estrogen between the follicular and luteal phases. There was a significant exercise-induced reduction in β-CTXI for up to 90 min post-exercise, and a significant transient increase in sclerostin, PTH and P1NP immediately post-exercise in both phases of the menstrual cycle.

6.2 Implications and Future Directions

Currently it is unclear whether menstrual phase effects bone metabolism at rest and in response to vigorous exercise, which may be important when designing studies in this area. Although previous literature has reported conflicting findings regarding β-CTX and PTH levels at rest between menstrual phases, these may have been due to methodological differences. Our study adds to the literature, supporting the tenant that there are no significant differences in P1NP, β-CTX, PTH and sclerostin concentrations between phases in young healthy recreationally active eumenorrheic women at rest. The literature on bone metabolism in response to exercise between menstrual phases is minimal. Our study communicates that the acute response to running does not differ between menstrual phases, therefore both phases of the menstrual cycle have the same bone response to 30 minutes of running at 80% of $\dot{V}O_2max$ in healthy eumenorrheic women. In addition, women are often excluded from research as the menstrual cycle is often cited as a barrier of entry, making research more complicated and time consuming. Our study shows that we can confidently remove this barrier of entry when investigating sclerostin, P1NP, PTH and

β-CTXI in women, since menstrual cycle phase will not interfere with measured concentrations of these markers of bone metabolism at rest or after an acute bout of running.

Future studies in this area should aim to include sclerostin as the data are minimal in this area both at rest and in response to exercise. Measuring longer post-exercise time frames to gain a better understanding of bone metabolism over time should be a consideration in studies moving forward. Larger samples sizes would be an important consideration since inter-individual variability, which can be large when measuring hormones such as PTH, can make it more difficult to detect statistical significance and therefor increase the potential for type 2 error.

Larger sample sizes can help to distinguish the role of individual variation in bone markers, which may or may not be contributing to trends seen in the current literature. Future studies should aim to have consistency regarding lab protocols for standardized nutrition, assay type, sample timing due to circadian rhythms, pre visit exercise restraint protocols and methods for confirming menstrual phase.

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APPENDICES

Appendix 1: Ethics Approval

Brock University
Office of Research Ethics
Tel: 905-688-5550 ext. 3035
Email: reb@brocku.ca

Health Science Research Ethics Board

Certificate of Ethics Clearance for Human Participant Research

DATE:	2/24/2021
PRINCIPAL INVESTIGATOR:	KLENTROU, Nota - Kinesiology
CO-INVESTIGATOR(S):	Nigel Kurgan (nk10gw@brocku.ca); Joshua Stoikos (js17up@brocku.ca); Malcolm Sanderson (ms16ot@brocku.ca); Tom Hazell (thazell@wlu.ca)
FILE:	20-218 - KLENTROU
TYPE:	Masters Thesis/Project STUDENT: Anne Guzman SUPERVISOR: Nota Klentrou
TITLE:	Menstrual cycle related fluctuations in circulating markers of bone metabolism at rest and in response to running in eumenorrheic females

ETHICS CLEARANCE GRANTED

Type of Clearance: NEW Expiry Date: 2/1/2022

The Brock University Health Science Research Ethics Board has reviewed the above named research proposal and considers the procedures, as described by the applicant, to conform to the University's ethical standards and the Tri-Council Policy Statement. Clearance granted from **2/24/2021** to **2/1/2022**.

The Tri-Council Policy Statement requires that ongoing research be monitored by, at a minimum, an annual report. Should your project extend beyond the expiry date, you are required to submit a Renewal form before 2/1/2022. Continued clearance is contingent on timely submission of reports.

To comply with the Tri-Council Policy Statement, you must also submit a final report upon completion of your project. All report forms can be found on the Office of Research Ethics web page at https://brocku.ca/research-at-brock/office-of-research-services/research-ethics-office/#application-forms.

In addition, throughout your research, you must report promptly to the REB:

a) Changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) All adverse and/or unanticipated experiences or events that may have real or potential unfavourable implications for participants;
c) New information that may adversely affect the safety of the participants or the conduct of the study;
d) Any changes in your source of funding or new funding to a previously unfunded project.

We wish you success with your research.

Approved:

Craig Tokuno, Chair
Health Science Research Ethics Board

Note: Brock University is accountable for the research carried out in its own jurisdiction or under its auspices and may refuse certain research even though the REB has found it ethically acceptable.

If research participants are in the care of a health facility, at a school, or other institution or community organization, it is the responsibility of the Principal Investigator to ensure that the ethical guidelines and clearance of those facilities or institutions are obtained and filed with the REB prior to the initiation of research at that site.

Appendix 2: Consent Form

CONSENT TO PARTICIPATE IN RESEARCH Date:
LETTER OF INFORMATION

Title of Study: **Examining the potential involvement of sex hormones on appetite regulation (REB #5856)**

Dear _____:

You are being invited to participate in a research study conducted by Tom J. Hazell (PhD) and Sara Moniz (BA Kin) from the Energy Metabolism Research Laboratory. In order to participate it is important you are: a) between 18-30 years old, b) recreationally active <3/wk, c) healthy, d) normally menstruating, e) not currently pregnant, do not have plans to become pregnant during the duration of the study, and have not been pregnant (>3 months) within the last 3 years, and e) not using birth control or using no form of hormonal contraceptive as this project is designed to determine how sex hormones influence appetite hormones.

PURPOSE OF THE STUDY
The primary purpose of this study is to examine the effects of vigorous-intensity continuous running on concentrations of sex hormones and examine their associations with post-exercise alterations in appetite-regulating hormones in women during the follicular phase (FP) and luteal phase (LP) of the menstrual cycle.

PROCEDURES (#7)
This study requires you to visit the Energy Metabolism Research Laboratory 3 separate times, one for a familiarization session (~1 h) and then for 2 testing sessions (~4h, one session in both the FP and LP) for a total time commitment of ~9 hours. The FP constitutes days ~1-11 of the menstrual cycle, including time of menses, and is the period of the cycle where levels of female sex hormones (estradiol and progesterone) are at the lowest concentration. The LP constitutes days ~16-28 of the menstrual cycle, and is the period of time following ovulation (days ~12-15) where levels of estradiol and progesterone are increased relative to the FP. Menstrual phase will be subject-monitored through oral body temperature taken daily upon awakening, and reported each morning to the researcher via text or email. The familiarization session will include a test of cardiorespiratory fitness ($\dot{V}O_{2max}$ test), consisting of a graded running test performed until exhaustion. Following a 5 min warm up, you will begin the test running at a speed between 5-7 mph at 0% incline. Every 2 minutes thereafter, incline on the treadmill will increase by 2%. The test will continue until volitional fatigue. All experimental sessions (separated by 1 week minimum) will include a standardized breakfast (consisting of a chocolate chip Clif bar) and 40 min exercise session (5 min warm-up, 30 min continuous running, and 5 min cool-down) followed by 2 hours post-exercise where participants rest comfortably and quietly in the laboratory (i.e. reading). A measurement of resting gas exchange will occur for 15 minutes before the exercise session, 30 minutes post-exercise, and for the final 15 minutes of the remaining 2 hours post-exercise. This measurement involves wearing a sanitized mask that is connected to a metabolic cart. Blood samples will also be drawn from the forearm pre- and post-exercise (two 3 mL samples per draw, 4 total blood draws). Blood draws will be taken from the inner elbow while lying supine by a trained researcher. All equipment will be sterile one-time use. Blood draw site will be cleaned prior to each sample and researcher will wear clean gloves to ensure cleanliness. You will also be asked several questions before and after exercise to determine your feelings of hunger and satiety as well as record dietary intake the day before the trial, on trial day and the day after. You will also be required to

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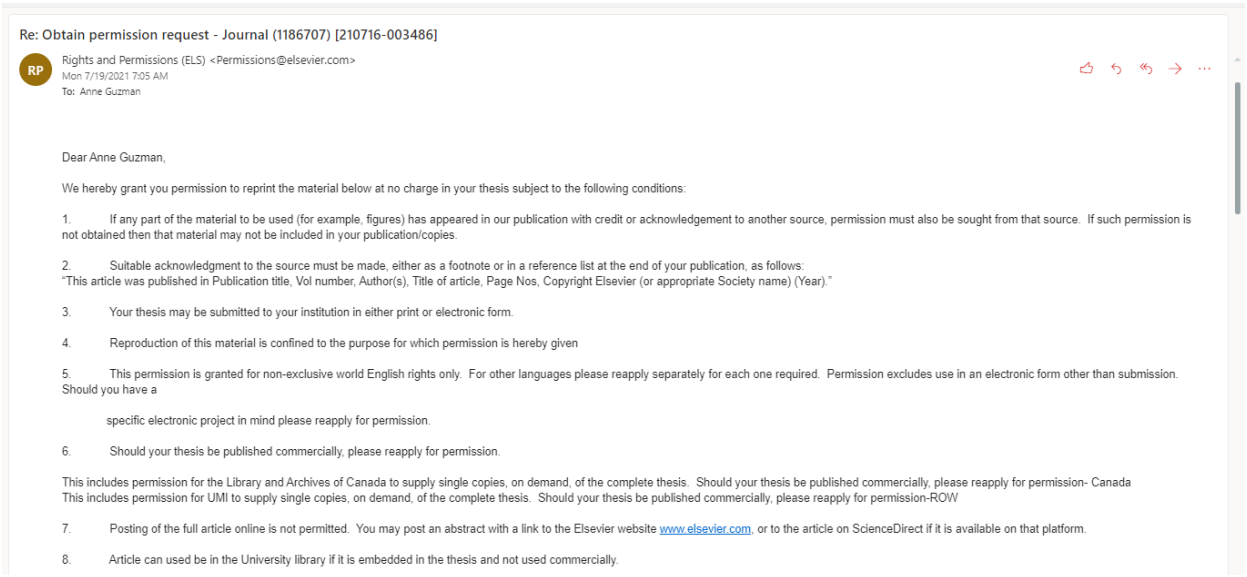

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