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THE IMPACT OF ESTROGEN VS ENERGY STATUS ON BONE BALANCE AND BONE TURNOVER RATE IN YOUNG EXERCISING WOMEN

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ABSTRACT

Energy and estrogen deficiencies are detrimental to bone health in exercising women, but the significance of each independently has not been extensively examined, as energy and estrogen deficiency often occur simultaneously. The purpose of this study was to assess markers of bone formation (procollagen type I N-terminal propeptide) and resorption (serum C-terminal telopeptides) utilized in mathematical models of bone turnover to quantify how energy and estrogen status relate to indices of bone formation, bone resorption, the net balance of bone formation and resorption (bone balance), and bone turnover rate in 109 physically active, premenopausal women (18-35 years old). Energy status was assessed using total triiodothyronine (TT_3) concentration; estrogen status was assessed using self-reported history of menses verified by prospective assessments of urinary estrone-1-glucuronide (E1G) and pregnanediol glucuronide (PdG). Participants were categorized as energy replete (EnR) or energy deficient (EnD) and estrogen replete (E_2R) or estrogen deficient (E_2D), resulting in four subgroups: EnR+E_2R (n=37), EnR+E_2D (n=18), EnD+E_2R (n=22) and EnD+E_2D (n=32). All groups were similar with respect to height and weight ($p>0.05$). Energy deficiency was associated with suppressed TT_3 concentrations (67.1±1.4 ng/dL, $p<0.001$), while estrogen deficiency was associated with decreased E1G and PdG integrated mean concentrations (27.4±2.0 ng/mL and 2.0±0.7 μg/L, respectively, both $p<0.001$). EnD+E_2D women had the lowest bone turnover rate (1.43±0.08, $p=0.045$) driven by a lower index of bone formation (MoMf) (1.00±0.06 μg/L, $p=0.046$) compared to EnR+E_2D women (1.98±0.16 and 1.44±0.14 μg/L, respectively). Energy status impacted the index of bone resorption, such that EnD women had less bone resorption (1.01±0.05 ng/mL, $p=0.019$) than EnR women. There were no effects of estrogen or energy status on bone balance,

yet values were indicative of net bone formation in all groups. Energy status was the main contributor to negative alterations in bone turnover dynamics, especially among estrogen deficient women, highlighting the importance of preventing energy deficiency among exercising women, especially those with menstrual disturbances, in order to maintain skeletal health.

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Chapter 1

Introduction

The Female Athlete Triad, characterized by low energy availability (EA) with or without disordered eating, menstrual dysfunction, and low bone mineral density (BMD), is a syndrome affecting up to 60% of exercising women [1-4]. The Female Athlete Triad impacts not only elite athletes but also recreationally active women [5-7]. EA associated with the Triad is calculated as dietary energy intake minus the energy expended during exercise (corrected for fat free mass or lean body mass (kcal/kgFFM or LBM/d), as defined operationally by Loucks et al. [8-10]. In the Female Athlete Triad model, low EA is caused by inadequate energy intake relative to exercise energy expenditure resulting in inadequate energetic status [3, 4]. If the resultant energy deficit is large enough, the deficit signals that the body does not have sufficient energy to meet the needs of exercise or normal functioning, and physiological compromises ensue [11]. In particular, growth and reproduction are suppressed [12].

The acute short-term effects of reduced EA were demonstrated in young, sedentary but otherwise healthy, menstruating women in a series of experiments conducted by Loucks [10, 13, 14]. The investigators manipulated both diet and exercise, such that a participant was administered a balanced EA prescription for one trial (40kcal/kgLBM/day) and one of three low EA treatments for the second trial, i.e. 30, 20 or 10 kcal/kgLBM/day. Serum markers of bone formation (P1CP and osteocalcin) and urinary markers of bone resorption (uNTx) were measured in both the balanced and low EA conditions [13]. While bone resorption, indicated by uNTX concentration, was elevated only at extreme low EA (i.e., 10kcal/kgLBM/day), bone formation, indicated by

PICP and osteocalcin, was suppressed at moderate and mild levels of energy restriction (i.e., 10, 20 and 30 kcal/kgLBM/day) [13]. A similar pattern was observed for the markers of energetic status, including suppressed total triiodotyronine (TT3) and insulin-like growth factor -1 (IGF-1), such that TT3 and IGF-1 suppression occurred abruptly at an EA of ~30 kcal/kgLBM/day, and worsened at 20 and 10 kcal/kg/LBM/day [10]. Furthermore, luteinizing hormone (LH) pulsatility was decreased even at mildly low EA of 30 kcal/kgLBM/day, and continued to decrease as EA was restricted even further (20 and 10 kcal/kg/LBM/day), indicated by decreased pulse frequency and increased pulse amplitude consistent with suppression of the hypothalamic-pituitary-ovarian (HPO) axis that is associated with disruption of reproductive function [10, 14].

When experiencing chronically low EA for prolonged time periods, perturbations to metabolism and growth can lead to reproductive disturbances and poor bone health [12]. A hallmark sign of energy deficiency and low EA is the presence of menstrual disturbances, the most extreme of which is primary amenorrhea (the failure of menses to occur by age 15) [15] and secondary amenorrhea (the absence of menstrual cycles lasting more than three months after menarche has occurred) [15]. Subclinical menstrual disturbances, including long and irregular cycles (oligomenorrhea), anovulation, or luteal-phase defects, may also occur [16, 17]. Menstrual disturbances are accompanied by hormonal suppression and, specifically in the case of oligo/amenorrhea and anovulation, estrogen deficiency [17]. Estrogen is an important bone-regulating hormone, as it acts to suppress osteoclast formation and activity [18, 19]. In the face of estrogen deficiency, the number of basic multicellular units (anatomical spaces where osteoclast and osteoblast activity occurs) is dramatically increased through upregulation in activation frequency of osteoclast, leading to an expanded remodeling space, increased cortical porosity and enlarged resorption area on trabecular bone [19]. Without adequate concentrations of circulating

estrogen, osteoclast synthesis, recruitment and lifespan increase, all of which contribute to an overall net loss of bone that may not be counteracted by adequate osteoblastic bone formation [19]. Since estrogen is such an important bone-regulating hormone, lifelong estrogen exposure is important for bone health, as well as during the adolescent and pubertal bone accrual period [20].

Studies demonstrating the effect of low estrogen exposure on bone health include multiple cross-sectional reports of exercising women [21-29]. The first investigator to publish data on this was Dr. Barbara Drinkwater in 1984 [22]. She demonstrated the relationship between menstrual history and bone health in a group of young athletes [21]. Exercising women with no history of irregular menstrual cycles had higher BMD in the lumbar spine than women with a history of oligomenorrhea and amenorrhea [21]. Furthermore, the exercising women who had never experienced regular menstrual cycles had the lowest lumbar spine BMD of all three groups [21]. More recently, our laboratory cohort demonstrated that menstrual history in exercising women is a key determinant of bone health, such that accumulation of menstrual disturbances increases the likelihood of lower estimates of femoral neck geometry, as determined by smaller cross-sectional moment of inertia and cross-sectional area [23]. In other studies on adolescent athletes with amenorrhea, significantly lower BMD of the lumbar spine and hip compared to controls and athletes with eumenorrhea (normal menstrual cycle) has been reported [24, 25]. We have published several papers confirming these findings of lower BMD, as well as bone geometry and estimated bone strength, in amenorrheic exercising women compared to menstruating exercising women [26, 27]. Bone geometry is an invaluable parameter to measure because it allows for a more in-depth analysis of bone structure. While it requires the use of peripheral quantitative computed tomography, the analysis of volumetric BMD, as well as cortical and trabecular compartments, separately, are made possible unlike when using DXA [30]. Multiple studies have

revealed that bone geometry has been found to be negatively affected in amenorrheic athletes when compared to healthy counterparts using this technique [28, 29].

Low EA and hypoestrogenism are both associated with altered bone turnover dynamics, characterized by increased bone resorption and decreased bone formation, often referred to as the uncoupling of bone turnover [13]. If energy deficiency and low estrogen exposure are sustained over long periods, the uncoupling of bone turnover can lead to an irreversible reduction in BMD, restricting young women from achieving their genetic potential for peak bone mass (PBM), or a reduction in BMD and bone strength in women who have already achieved their PBM [13, 31].

PBM accrual (i.e., velocity) occurs around age 14 in boys and age 12 in girls [32-35]. In girls, PBM accrual and menarche occur at approximately the same time, preceded by peak height velocity approximately one year earlier [36]. Although PBM occurs by the end of the second decade or early in the third decade of life depending on the skeletal site [37, 38], adolescence is a crucial period for achieving PBM. Disturbances in the timing of maturity among girls, such as late menarche, amenorrhea or poor nutrition, can mitigate the achievement of PBM and increase the risk of experiencing bone-related injuries or diseases. In fact, late maturing females were observed to have 50.7±15.6 grams less total body mineral content than their average maturing peers [39]. Secondary amenorrhea for at least one year during women's teenage years and BMI at present was found to be strongly associated with low BMD in their twenties [40]. Furthermore, women with anorexia nervosa, an eating disorder resulting in severe undernutrition that affects BMD if the disorder persists during adolescence, had impaired bone mineral accrual and progressive spinal osteopenia during the course of one year when compared to healthy age matched counterparts [41, 42]. This is alarming, considering that BMD is observed to remain low and not recover to normal levels even after the recovery from anorexia nervosa [43, 44].

A higher peak bone mass can protect from bone-related injuries and bone-related diseases, such as osteoporosis, later in life. In addition to low BMD, low EA and menstrual dysfunction, which are the other two components of the Female Athlete Triad, influence fracture incidence in female athletes [3, 45]. Barrack et al. [46] examined 259 female adolescents and young adults competing in competitive or recreational exercise activities. The prevalence of a single Triad risk factor variable was associated with a 15% to 20% increased risk for bone stress injury. Not surprisingly, the risk for bone stress injury increased to 30% to 50% if combined Triad risk factor variables were present [46]. The risk of a bone stress injury is also increased in athletes after each subsequent stress fracture that is experienced [47-49].

Dual energy X-ray absorptiometry (DXA) is the clinical tool used to measure BMD. DXA measurements are used to diagnose osteoporosis, assess a patients' risk of fracture and monitor response to treatment [50]. However, there are some limitations to the use of DXA alone to evaluate bone health. Measurements of BMD by DXA represent a static snapshot of bone at one time and do not reflect the trajectory of bone change over time [51]. Furthermore, it can take months or years to detect significant changes in BMD, limiting the ability to assess an individual's response to an intervention [52]. Other methods of assessing bone health, such as measuring markers of bone turnover, allow for the assessment of metabolic changes in bone over short time periods that are observable before detectable BMD changes occur [51, 53, 54].

Markers of bone turnover are biochemical products measured that reflect the metabolic activity of bone during either bone resorption or formation [54]. There are multiple advantages for using markers of bone turnover over methods that assess BMD, such as DXA, including higher responsiveness, usually higher cost and time efficiency [55]. Furthermore, markers of bone turnover provide an overall assessment of skeletal health rather than site specific assessments of

skeletal health that measurement of BMD provide [56]. The International Osteoporosis Foundation and International Federation of Clinical Chemistry recommend the use of one marker of bone formation, type I procollagen carboxy-terminal propeptide (PINP), and one marker of bone resorption, serum C-terminal telopeptide (sCTx) to be used as reference markers and measured by standardized assays [57]. PINP and sCTx are proposed because they have been thoroughly evaluated for fracture prediction and treatment, extensive documentation of biological and analytical variability, as well as sample handling and stability [57].

The utility of PINP and sCTX have been even more enhanced by the development of mathematical models that integrate markers of formation and resorption in order to assess bone turnover dynamics, such as the net balance of bone turnover (i.e. bone balance, BB) and the rate of bone turnover (i.e. bone turnover rate, BTR) [58]. BB is a useful measure to assess if either bone resorption or formation is most prevalent within the bone microenvironment. Both bone resorption and formation are coupled, therefore evaluating each independently can prove erroneous, especially when determining the efficacy of a treatment for osteoporosis [59]. BTR furthers our understanding by indicating whether or not bone resorption or formation is occurring at an elevated or reduced rate. BTR is a useful measure in instances for determining the progression of bone disease in patients.

The purpose of this study was to evaluate the relationship between estrogen, energy, and bone turnover in a group of healthy, exercising women with a range of energy and estrogen status. Energy status was determined by a median split of TT_3 concentration among the sample, such that women with a TT_3 concentration higher than the median were defined as energy replete and those with a TT_3 concentration below the median were defined as energy deficient [60]. Estrogen status was defined by self-reported history of menses verified with prospective assessments of urinary

reproductive hormone metabolites and were subsequently defined as either estrogen replete or estrogen deficient. Using two-way analysis of variance (2-way ANOVA), the main and interactive effects of estrogen and energy were assessed. Levels of PINP and sCTx in each subject will be used in the multiple of medians mathematical to calculate an index of formation, index of resorption, bone balance and bone turnover rate and further our understanding of the bone microenvironment.

Purpose of this Study

The purpose of this study was to evaluate the relationship between energy and estrogen status by assessing markers of bone resorption (sCTx) and formation (P1NP) utilized in mathematical models of bone turnover to quantify the index of bone formation, index of bone resorption, bone balance and bone turnover rate in a population of healthy, exercising women. The study utilized a 2x2 cross-sectional study design in which women were classified as energy replete (EnR) or energy deficient (EnD) and estrogen replete (E_2R) or estrogen deficient (E_2R). This resulted in four groups: EnR + E_2R, EnR + E_2D, EnD + E_2R, EnD + E_2D.

Aim and Hypothesis 1: Interactive Effect of Both Estrogen and Energy Status

Aim: To assess the interactive effects of estrogen status and energy status on the index of bone formation, the index of bone resorption, bone balance and rate of bone turnover in the EnR + E_2R, EnR + E_2D, EnD + E_2R, EnD + E_2D groups.

Hypothesis: There will be an interactive effect of energy and estrogen status such that the EnD + E_2D group will have the lowest bone balance and lowest rate of bone turnover.

Aim and Hypothesis 2: Effect of Estrogen Status

Aim: To assess the main effect of estrogen on the index of bone formation, the index of resorption, bone balance and rate of bone turnover in the EnR + E_2R, EnR + E_2D, EnD + E_2R, EnD + E_2D groups.

Hypothesis: Estrogen status will have a main effect on the index of bone resorption, bone balance, and bone turnover rate such that E_2D women will have a greater index of bone resorption, lower bone balance, and higher bone turnover rate.

Aim and Hypothesis 3: Effect of Energy Status

Aim: To assess the main effect of energy status on the index of bone formation, the index of bone resorption, bone balance, and the rate of bone turnover in the EnR + E_2R, EnR + E_2D, EnD + E_2R, EnD + E_2D groups.

Hypothesis: Energy status will have a main effect on the index of bone formation, bone balance, and bone turnover rate such that EnD women will have a lower index of bone formation, lower bone balance, and a lower bone turnover rate.

Rationale

Both low energy availability and an estrogen deficiency are detrimental to bone, but the significance of each independently have not been extensively examined, as energy and estrogen deficiency most often occur simultaneously. Our laboratory has published two reports of such effects. Firstly, De Souza et al. [27] reported that when both energy deficiency and estrogen

deficiency are present simultaneously, negative alterations in bone turnover are exacerbated. In fact, when compared to exercising women who were energy replete and estrogen replete, energy replete and estrogen deficient and energy deficient and estrogen replete, women who were both energy deficient and estrogen deficient had the lowest levels of PINP, highest levels of U-CTX-I, and the most disrupted metabolic milieu, including high levels of ghrelin and low levels of TT_3 and leptin [27]. Interestingly enough, the degree of estrogen deficiency was similar in both estrogen deficient groups, suggesting that energy deficiency may compound the effect of an estrogen deficiency on bone [27].

More recently, Southmayd et al. [61] reported that energy and estrogen deficiency independently affected volumetric bone mineral density (vBMD), bone geometry and estimated bone strength in distinct ways on load bearing and non-load bearing skeletal sites in exercising women [61]. Specifically, vBMD, bone geometry and estimated bone strength at the tibia (load bearing site) were largely dependent on energy status, while vBMD, bone geometry and estimated bone strength at the radius (non-load bearing site) were largely dependent on estrogen status [61]. Thus, the authors suggested that mechanical loading may counter and compete with the effects of increased bone resorption that takes place during hypoestrogenism in load bearing sites [61]. The effect of loading was not enough to increase the osteogenic effect of bone formation, which is suppressed during an energy deficient state, thus making energy status the main contributor of decreased bone health in the tibia [61]. Building upon this work, we plan to use baseline data from two studies conducted in exercising women with a range of menstrual function and energy status (REFUEL and AWS) to further evaluate the relationship between estrogen, energy and bone turnover by assessing energy status and estrogen status via TT3 and self-reported history of menses

corroborated with urinary hormone metabolites, respectively, and markers of bone formation and resorption, PINP and sCTx.

Currently, DXA is the clinical tool of choice used to asses bone health, but it can take several months to years to detect significant changes in DXA measurements, during which time irreversible bone loss may occur [52]. Serum markers of bone turnover are an attractive alternative, as they allow for more timely and sensitive assessments of metabolic changes in bone than that allowed by DXA [55]. Mathematical models that incorporate formation and resorption markers further clarify the depiction of bone metabolism at a given time [58]. The multiple of medians mathematical approach furthers our understanding by additionally assessing the rate of bone turnover [58]. The bone turnover indices garnered in the multiple of medians approach can be visually depicted on a bone turnover plot (BTP). By plotting the multiple of median for markers of resorption (MoMr) on the x-axis by the multiple of median for formation (MoMf) on the y-axis, bone balance is indicated by the slope of the line through the origin (0, 0) and the point (MoMr, MoMf) [58]. A value of 1 is indicative of balanced bone formation and resorption, whereas a value greater than 1 is indicative of net bone formation and a value less than one is indicative of bone resorption [58]. The rate of bone turnover can be calculated by assessing the length of the vector when plotted on a BTP [58]. A longer vector indicates an increased rate of bone turnover, while a shorter vector indicates a decreased rate of bone turnover [58]. This method was used to assess bone turnover rate and balance over time during daily teriparatide administration and proved useful in visualizing trends between treatment groups throughout the 48 week study period [59].

By understanding the relationship between energy, estrogen and bone turnover, clinicians can more accurately diagnose female athletes and exercising women at risk for bone loss related injuries and diseases. Osteoporosis, a bone related disease, is a “silent” disease that is estimated to

cost the US healthcare system $25 billion in treatment by the year 2025 [62]. The multiple of medians approach [58] will expand our knowledge of the relationship between energy, estrogen and bone turnover and aid in reducing the economic costs of bone loss related injuries and diseases, therefore making it the appropriate method to examine in this study.

Expected Findings

We hypothesized that there would be an interaction effect of energy and estrogen status on bone turnover such that the EnD + E_2D group was expected to have the lowest bone balance. In a previous study, female runners with chronic amenorrhea presented with a bone metabolic state dominated by bone resorption [63]. All of the bone turnover markers tested within the population of amenorrheic female runners were lower when compared to eumenorrheic runners and eumenorrheic sedentary controls, which is indicative of a lower rate of bone turnover [63]. It was unclear whether the reduced rate of bone turnover was attributed to either the estrogen or energy deficit within this population, but it is known that when both an energy and estrogen deficit are combined, bone turnover is reduced even further [27].

In addition to an interaction effect, we hypothesized that there would be a main effect of estrogen status on the index of bone resorption due to its role in suppressing osteoclast formation and activity [18]. In the face of estrogen deficiency, the number of basic multicellular units is dramatically increased through upregulation in activation frequency, leading to an expanded remodeling space too large for osteoblastic bone formation to counteract [19]. This type of uncoupling was expected to result in lower bone balance leading to a state of net bone resorption

and increase the rate of bone turnover as more bone mineral is being resorbed without adequate osteoblastic bone formation.

Finally, we hypothesized that energy status would have a main effect on the index of bone formation. According to a previous study [13], markers of bone formation were suppressed at lower levels of energy restriction in exercising women when compared to markers of bone resorption, which were suppressed only at extreme levels of energy restriction. The larger range of energy availability in which markers of bone formation are affected emphasize their sensitivity to energy status. Furthermore, De Souza et al. [27] reported a main effect of energy status on osteocalcin, a marker of bone formation, in young exercising women. Therefore, an energy deficit will decrease the index of bone formation, lower bone balance and decrease the rate of bone turnover as less bone mineral is being formed.

Statistical Analysis

Descriptive statistics were calculated for basic demographic information, subject characteristics, and bone turnover dynamics within the EnR + E_2R, EnR + E_2D, EnD + E_2R, EnD + E_2D groups. Using two-way analysis of variance (2-way ANOVA), the main effects of both independent grouping factors, energy and estrogen status, on bone turnover dynamics (index of bone formation, index of bone resorption, bone balance, and bone turnover rate) were determined. Energy status was determined by a median split of T_3 concentration among the sample, such that those with a T_3 concentration higher than the median were defined as energy replete (EnR) and those with a T_3 concentration below the median were defined as energy deficient (EnD). Estrogen status was defined by self-reported history of menses corroborated with prospective assessments

of urinary reproductive hormone metabolites. Women with eumenorrheic cycles or oligomenorrhea, but reporting at least 6 menses in the past 12 months, were defined as estrogen replete (E_2R) and women with amenorrhea or oligomenorrhea, but reporting less than 6 menses in the past 12 months, were defined as estrogen deficient (E_2D). If significant interaction effects were present, a simple effects analysis was used to assess at which levels of the independent grouping factors (energy and estrogen status) differences emerged.

Chapter 2

Literature Review

When exercising women fail to consume enough calories to support exercise and all other body functions, low energy availability (EA) results in physiological adaptations to preserve energy [11]. Two common adaptations are suppression of growth and reproduction resulting in menstrual disturbances and poor bone health [12]. The interrelationships between low EA with or without disordered eating, menstrual dysfunction, and low bone mineral density (BMD) is known as the Female Athlete Triad (Triad) [4]. Low EA and hypoestrogenism induced by menstrual dysfunction are independently detrimental to bone, but the degree to which each independently contributes to poor bone health or if the combination of both is especially detrimental is not completely understood to date, as low EA and menstrual dysfunction often occur simultaneously [64]. Low EA and menstrual dysfunction are jointly associated with altered bone turnover dynamics characterized by increased bone resorption and decreased bone formation, and commonly referred to as uncoupling of bone turnover [13]. If energy deficiency and low estrogen exposure are sustained over long periods the uncoupling of bone turnover may lead to an irreversible reduction in BMD, thus increasing the risk of bone-related injuries and bone-related diseases, such as osteoporosis, later in life [13, 20]. The goal of this review is to identify what is known about the independent and combined effects of low EA and hypoestrogenism due to menstrual dysfunction on bone turnover. First, normal physiology of bone turnover in women will be described. Next, the current literature exploring the independent effects of energy and estrogen status on bone physiology will be summarized. Ultimately, this review will serve to summarize

our understanding of relationships between energy, estrogen and bone turnover, while identifying gaps in our knowledge that require further investigation.

Bone Metabolism in Healthy Women

Bone Physiology and the Cells Responsible

Bone is constantly undergoing change through the process of remodeling, which serves to maintain bone mass and size by replacing fatigue-damaged bone with new bone through the coupled actions of bone resorption and formation [65]. The uncoupled actions of bone resorption and formation are termed “resorption modeling” and “formation modeling” by which bone mass is taken away or added, respectively [66]. Both bone remodeling and modeling processes require the coordinated action of bone cells (osteocytes, osteoclasts and osteoblasts) to occur [65].

Osteocytes comprise 90-95% of the total bone cells [67, 68]. Osteocytes are derived from mesenchymal stem cells (MSC) through osteoblast differentiation at the end of the formation phase of remodeling when osteoblasts become encapsulated within the bone matrix and undergo morphological changes thus developing into osteocytes [69, 70]. Osteoblasts lose a number of organelles but gain long cytoplasmic processes [71]. These cytoplasmic processes form gap junctions with other osteocytes, as well as osteoblasts and osteoclasts, to facilitate communication [72]. Once an osteocyte fully matures, it acts as a mechanosensor to detect mechanical loads and translate the mechanical signal to a biochemical signal, which can be sensed by osteoclasts and osteoblasts [73], and is responsive to hormonal and metabolic environments [74].

Osteoclasts are multinuclear cells that originate from mononuclear hemopoietic cells and differentiate under the influence of several of critical components [75]. Initial proliferation of

osteoclast precursors from hemopoietic stem cells and subsequent survival is mediated by monocyte/macrophage colony stimulating factor (M-CSF) [75]. To complete the maturation process, receptor activator of nuclear factor kappa-B ligand (RANKL), secreted by osteoblasts, osteocytes, and stromal cells [76], must bind to its receptor RANK on dedicated osteoclast precursors attached to the bone surface [77]. Conversely, osteoclastogenesis can be inhibited by a decoy receptor, osteoprotegerin (OPG), secreted by osteoblasts and bone marrow stromal cells, binding to RANKL preventing RANKL/RANK interaction on the surface of osteoclasts [78]. Mature osteoclasts resorb bone using ruffled borders that maintain a low-pH environment and secrete proteolytic enzymes and hydrochloric acid to dissolve and degrade inorganic and organic bone within a sealing zone [79].

Osteoblasts are mononuclear cells that arise from MSCs upon the activation of a complex network of pathways that include runt-related transcription factor 2, osterix, Wnt/B-catenin signaling, and bone morphogenetic proteins [80]. Osteoblasts are responsible for filling the resorption cavity that osteoclasts created with newly synthesized bone matrix [81]. The bone matrix is initially secreted by osteoblasts as osteoid, a mixture of non-carboxylated osteocalcin, precursors of collagen 1, calcium salts, and other proteins, which eventually matures and mineralizes [82]. Once newly synthesized bone matrix has completely filled the resorption cavity, a termination signal alerts osteoblasts to cease work and either undergo apoptosis, remain on bone surface and transform into bone lining cells, or become embedded in the bone matrix and differentiate into osteocytes [65]. This completes the bone remodeling process.

The resorption phase of bone remodeling lasts 30-40 days followed by the formation phase which lasts over 150 days [83, 84]. The duration of the formation phase is dependent on the bone compartment being formed, such that the complete remodeling cycle of trabecular bone lasts

longer than cortical bone [85]. Imbalance between formation and resorption phases affects bone mass either positively or negatively if formation or resorption predominates, respectively. Furthermore, if resorption predominates chronically, pathological conditions may arise and increase the risk of fracture. Therefore, to maintain skeletal integrity, formation and resorption should remain coupled during adult remodeling or formation should predominate, as occurs primarily in growing children and adolescence during adolescent modeling .

Bone Health Measurement Techniques

Various techniques are used to assess bone health and provide a range of bone properties that work toward understanding the bone microenvironment of an individual to prevent bone loss and the subsequent injuries and diseases that accompany excessive bone loss. However, each technique has its own strengths and limitations so it is important to understand them fully in order to accurately choose which one to use in a research or clinical setting.

Dual energy X-ray absorptiometry (DXA) is the clinical tool of choice used to measure BMD. Two X-ray beams with different frequencies are projected through a part of the body to provide 2-dimensional scans at skeletal sites including the hip, spine, forearm, and whole body [86]. BMD is calculated as the ratio of bone mineral content per unit projected area. Additionally, bone geometry can be extracted from skeletal regions of interest (i.e., hip structural analysis). DXA measurements are used to diagnose osteoporosis, assess a patients' risk of fracture and monitor response to treatment [50]. However, there are some limitations to the use of DXA alone to evaluate bone health. Measurements of BMD by DXA represent a static 2-dimensional snapshot of bone at one time and do not reflect the trajectory of bone change over time [51]. Furthermore,

it can take months or years to detect significant changes in BMD, limiting the ability to assess an individual's response to an intervention [52].

Quantitative ultrasound (QUS) is a small, portable, and less expensive device that utilizes two transducers placed on either side of a bone, one acts as a transmitter and the other acts as a receiver, to transmit ultrasound waves through bone mineral [87]. Bone properties measured by QUS are broadband ultrasound attenuation (BUA), ultrasound velocity (SOS), and a stiffness index based on the product of BUA and SOS [30, 88]. These properties relate to bone elasticity, structure of trabeculae, and apparent density; however, mechanisms contributing to observed changes in BUA, SOS, and stiffness index are difficult to interpret [30]. Furthermore, the skeletal sites assessed using QUS are limited due to the sensitivity of ultrasound energy. For instance, the transmitter and transducer need to be nearly parallel and the layer of soft tissue overlaying the bone needs to be relatively thin to obtain accurate measurements [30]. These disadvantages have limited the number of skeletal sites QUS can assess to mainly the calcaneus, the skeletal site of choice in a majority of QUS research [89].

Computed tomography (CT) is an imaging technique that produces a 2-dimensional image of a cross section of bone. More advanced models of CT include peripheral quantitative CT (pQCT) which measures exclusively the peripheral skeleton, and high resolution pQCT (HR-pQCT) that both provide 3-dimensional volumetric images of bone at higher resolutions than that of CT [90]. All CT imaging modalities are capable of measuring whole bone properties and distinguishing between cortical and trabecular compartments at each measured skeletal site, but only pQCT and HR-pQCT can extract volumetric BMD (vBMD) [30]. Cross-sectional geometric properties, strength indices, and structural features can all be computed by placing software-based volume elements, voxels, within bone compartments that x-rays then pass through [91, 92].

Despite these features unique to CT imaging, image acquisition may be limited due to high equipment cost, required operator training, and exposure to high doses of radiation per image [30].

Other methods of assessing bone health besides imaging techniques, such as measuring markers of bone turnover, are available and allow for the assessment of metabolic changes in bone over short time periods that are observable before detectable BMD changes occur [51, 53, 54]. Markers of bone turnover are biochemical products measured that reflect the metabolic activity of bone during either bone resorption or formation [54]. There are multiple advantages for using markers of bone turnover over methods that assess BMD including higher responsiveness, usually lower cost, and improved time efficiency [55]. Furthermore, markers of bone turnover provide an overall assessment of skeletal health rather than site specific assessments of skeletal health that measurement of BMD provide [56]. In 2011, The International Osteoporosis Foundation and International Federation of Clinical Chemistry recommended the use of one marker of bone formation, type I procollagen carboxy-terminal propeptide (PINP), and one marker of bone resorption, serum C-terminal telopeptide (sCTX) to be used as reference markers and measured by standardized assays [57]. PINP and sCTX were jointly proposed because they both have been thoroughly evaluated for fracture prediction and treatment, extensive documentation of biological and analytical variability, as well as sample handling and stability [57]. Of course, using PINP and sCTX to assess bone health is an invasive technique that uses blood serum concentrations of both markers, imparting an important limitation.

Mathematical models have been developed that integrate markers of formation and resorption in order to assess the net balance of bone turnover (i.e. bone balance, BB) and the rate of bone turnover (i.e. bone turnover rate, BTR) [58]. BB measures whether resorption or formation, in a coupled manner, is most prevalent within the bone microenvironment [58]. Both bone

resorption and formation are coupled in healthy adults, therefore evaluating each independently can prove erroneous, especially when determining the efficacy of a treatment for osteoporosis [59]. BTR furthers our understanding by indicating whether bone resorption or formation is occurring at an elevated or reduced rate. BTR is a useful measure in instances for determining the progression of bone disease in patients. The bone turnover indices garnered from these mathematical models can be visually depicted on a bone turnover plot [58]. This method was used to assess bone turnover rate and balance over time during daily teriparatide administration, and has proved beneficial for visualizing group responses to treatment throughout the 48-week study period [59].

It is up to the practitioner's discretion which technique to use in their respective field. Each technique provides unique measures to examine an individual's bone status and should be carefully considered before patient assessment. When interpreting results, it is important to consider other factors that influence bone health in order to understand the root cause of bone loss, such as EA and estrogen status according to the Triad.

Low Energy Availability and Bone Health

EA is operationally defined as the amount of energy remaining for all physiological processes after subtracting the energy that is used for exercise [8-10]. EA is calculated by subtracting exercise energy expenditure from dietary energy intake corrected for fat free mass or lean body mass (kcal/kgFFM or LBM/d) [8-10]. Athletes and exercising women are recommended to keep EA near 45 kcal/kgFFM to remain healthy [93]. However, EA can vary by either decreasing dietary intake or increasing exercise expenditure and result in inadequate energetic status. Low EA, identified as <30 kcal/kgFFM [10, 13], is associated with unfavorable

physiological compromises that redistributes energy necessary for growth and reproduction to more essential metabolic processes such as thermoregulation, cell maintenance, and locomotion [12]. Energetic redistribution is advantageous for survival but detrimental to some aspects of health.

Short Term Low EA and Bone

The acute short-term effects of energy restriction on bone turnover was demonstrated by Loucks et al. [10, 13, 14], who performed a series of experiments in young and sedentary, but otherwise healthy, menstruating women. The investigators manipulated both diet and exercise, such that a participant was administered a balanced EA prescription for one trial (40kcal/kgLBM/day) and was administered one of three low EA treatments for the second trial, i.e. 30, 20 or 10 kcal/kgLBM/day [13]. PICP, osteocalcin, and urinary NTx were measured in both the balanced and low EA conditions [13]. While bone resorption, indicated by uNTx concentration, was elevated only at extreme low EA (i.e., 10 kcal/kgLBM/day), bone formation indicated by PICP and osteocalcin was suppressed at moderate and mild levels of energy restriction (i.e., 10, 20 and 30 kcal/kgLBM/day) [13]. A similar pattern was observed for the markers of energetic status, including suppressed total triiodotyronine (TT3) and IGF-1, such that TT3 and IGF-1 suppression occurred abruptly at an EA of ~30 kcal/kgLBM/day, and worsened at 20 and 10 kcal/kg/LBM/day. The same pattern was observed for insulin concentration [10]. These series of experiments were the first to establish a dose-response relationship between EA and bone turnover.

Papageorgiou et al. [94] have also directly assessed the relation between EA and bone turnover in eleven physically active eumenorrheic women and eleven men to compare the effects

of energy restriction on bone health between sexes. Dietary intake and exercise were manipulated, such that participants achieved an EA of 45 kcal/kgLBM/day and 15 kcal/kgLBM/day during two separate 5-day protocols [94]. In women, P1NP and β-CTx were measured in the blood, and similar directional changes in bone formation and resorption were observed at EA of 15 kcal/kgLBM/day (P1NP: -13%, β-CTX: +19%) as at 30 kcal/kgLBM/day and 10kcal/kgLBM/day by Ihle and Loucks [13]. Sclerostin, a Wnt antagonist protein produced by osteocytes, was unchanged after the induction of low EA in women, indicative of unaltered osteoblast activity that did not account for the reductions in P1NP [94]. Markers of energetic status, TT3 and IGF-1, were also not altered during energy restriction, but leptin and insulin were lower in the restricted EA group compared to the control group [94]. Interestingly enough, bone turnover was not affected by low EA in men, suggesting that bone formation and resorption in women may to be more sensitive to changes in EA [94].

To determine the effects of low EA achieved by diet or exercise individually, Papageorgiou et al. [95] also studied ten physically active eumenorrheic women who completed three 3-day conditions of controlled EA (45 kcal/kgLBM/day), low EA through increasing exercise expenditure (15 kcal/kgLBM/day), and low EA through dietary energy restriction (15 kcal/kgLBM/day). Bone formation decreased significantly (P1NP: -17%) from the beginning of the 3-day trial period without effects on bone resorption [95]. The exercise-induced low EA condition did not demonstrate any significant changes in either bone formation or resorption. Both conditions resulted in lower levels of IGF-1 and leptin, while TT3 decreased only in the diet-induced low EA condition and insulin decreased following the exercise-induced condition [95].

The short-term interventions described above are difficult to compare given the varying degrees of energy restriction, duration of the energy deficit, and choice of bone turnover markers;

however, relationships between energy restriction and bone metabolism do appear to exist. Low EA generally increases bone resorption and decreases bone formation in women [13, 94]. The magnitude of changes depends on the level of EA and how low EA is achieved [13, 95]. Beyond the effect on bone metabolism, hormonal profiles are also altered.

Hormonal Changes Resulting from Low EA

Anorexia nervosa serves as a unique model to study metabolic adaptations to low EA since anorexia nervosa is characterized by severe undernutrition. In response to this severe state of undernutrition, the body alters many endocrine axes in order to stimulate food intake, help maintain euglycemia, and divert available energy for essential bodily functions (i.e. away from growth and reproduction) [96]. Hormonal changes associated with anorexia and other severe cases of low EA contribute both directly and indirectly to low BMD and increased risk of bone related injuries and diseases [97]. Hormone alterations associated with low EA include GH resistance with low IGF-1 [98, 99], elevated ghrelin [100], PYY [101], and adiponectin [102], and suppressed leptin [103], elevated cortisol [104], and suppressed reproductive hormones [10, 14].

GH is secreted by the anterior pituitary gland at a continuous basal level accompanied by secretory bursts that stimulate the production of hepatic IGF-1, an important bone anabolic hormone, in the liver [105]. GH secretion and secretory bursts are augmented in adolescents and adults with anorexia nervosa, but decrease the amount of circulating IGF-1 [98, 99]. GH/IGF-1 axis uncoupling indicates an acquired GH resistance [98, 99], such that GH no longer stimulates adequate production of IGF-1 despite elevated concentrations of GH. GH directly affects osteoblasts by increasing their lifespan; however, GH's total bone anabolic function requires the

presence of IGF-1 to initiate osteoblast proliferation [106]. Therefore, the resulting decrease in IGF-1 will have a direct effect on the bone formation process, specifically by increasing osteoblast apoptosis and reducing osteoblastogenesis [107, 108]. IGF-1 is also synthesized in osteoblasts under the control of parathyroid hormone [109] and increased with estrogen [110] but decreased with cortisol [111]. The origin of IGF-1 production appears to determine the destiny of which bone compartment, cortical or trabecular, IGF-1 primarily maintains [112]. In a review by Giustina et al. [112], the authors noted that systemically produced IGF-1 in the liver is important for cortical bone maintenance while locally produced IGF-1 in the osteoblast is essential for trabecular bone maintenance.

Ghrelin concentrations are elevated in conditions of low weight and fat mass (i.e. low EA) [113, 114] and stimulates the release of GH by binding to growth hormone secretagogue receptor (GHS-R) at the hypothalamus and pituitary level [115]. The connection between ghrelin and the GH/IGF-1 axis demonstrates ghrelin's indirect effect on bone, but GHS-R is also expressed in osteoblasts establishing a potential direct effect on bone [116]. In fact, ghrelin modulates osteoblast proliferation and differentiation, as well as inhibit tumor necrosis factor (TNF)-α induced apoptosis in rat osteoblasts in vitro and in vivo [116-118]. Furthermore, ghrelin administration was observed to increase femoral BMD in normal and dwarf rats [118]. The effects of ghrelin on human osteoblasts are not as well defined, but ghrelin appears to stimulate proliferation and differentiation in a manner dependent upon the maturity of osteoblast cells, and independent of GHS-R likely via an autocrine/paracrine mechanism [119, 120].

PYY is elevated in adolescent girls and women with anorexia nervosa [101, 121] and associated with diminished BMD in women with anorexia nervosa [121], as well as premenopausal exercising women [122]. Furthermore, PYY is associated with decreased markers of bone

formation and resorption [101]. Using PYY knockout and PYY transgenic mice, Wong et al. [123] were able to demonstrate that PYY exerts a negative influence on bone mass via osteoblast and osteoclast activity. An absence of PYY resulted in increased osteoblast activity, measured by mineral apposition rate, but no change in osteoclast activity [123]. Conversely, PYY over-expression decreased osteoblast activity through the Y1 receptor, and increased osteoclast activity by increasing osteoclast number in female only mice and expanding bone surface coverage in both male and female mice [123]. Osteoclasts did not express any Y receptors, suggesting that osteoclasts respond to PYY indirectly through osteoblast signaling rather than directly [123]. Additionally, the skeletal effects of PYY were sexually dimorphic and more notable in female transgenic mice [123].

Adiponectin's role in maintaining energy homeostasis includes the activation of fatty acid oxidation, promotion of glucose uptake, inhibition of gluconeogenesis, and elevation of insulin sensitivity through 5'-AMP-activated protein kinase (AMPK) specifically in the liver of animal models [124, 125]. Adiponectin receptors are present in the liver but have also been confirmed within human hypothalamus [126], pituitary gland [127], and reproductive tissues, including the ovaries [128] and endometrium [129]. Additionally, osteoblasts and osteoclasts both express adiponectin and its receptors [130-132], but adiponectin's function on bone homeostasis has not fully been elicited. Regardless of the function, studies demonstrated a negative relation between serum adiponectin levels and BMD [130-132], indicating an anti-osteogenic effect. This negative relation has been displayed in premenopausal women with energy deficiency associated amenorrhea, independent of gonadal status, providing further evidence to support adiponectin's direct role in bone homeostasis [133].

Leptin functions as a feedback mechanism to inhibit food intake during periods of adequate energy availability [134, 135], and serum leptin concentration is lower in women with anorexia nervosa [136] and with the Triad [114]. Early studies that examined the relationship between leptin and BMD were confusing, which may have been due to the differential effects of leptin on the axial and appendicular skeleton, as demonstrated in mice [137]. Using leptin-deficient, leptin receptor-deficient, and control mice, Turner et al. [138] determined that bone formation was lower in leptin-deficient and leptin receptor-deficient mice suggesting that leptin directly increases bone formation by increasing osteoblast number and activity primarily through peripheral pathways. Leptin may also indirectly influence bone metabolism through central pathways via the hypothalamic control of estrogen, GH/IGF-1, and cortisol [139].

Cortisol is a steroid hormone released from the adrenal glands in a daily pattern and regulated by the hypothalamic-pituitary-adrenal axis [140]. In states of low EA (i.e., anorexia nervosa and the Triad), cortisol secretory patterns are altered leading to elevated cortisol concentration [141, 142]. In women with anorexia nervosa, higher concentrations of cortisol were negatively correlated with BMD and markers of bone formation (PICP and osteocalcin) [104, 143]. These negative correlations are intuitive as cortisol inhibits bone formation by decreasing the number of osteoblasts through altered osteoblast differentiation and induced apoptosis [144, 145]. Cortisol has differing effects on bone resorption, and in vitro, cortisol increased osteoclastogenesis by increasing the expression of RANKL and decreasing OPG expression [146]. In contrast, cortisol increased the lifespan of trabecular osteoclasts while not changing RANKL or OPG expression in vivo [147]. Despite the action of cortisol on osteoclasts, Misra et al. [104] did not find any correlation, positive or negative, between cortisol and uNTx, a marker of bone resorption, in

anorexic girls, A lack of correlation suggests a stronger influence of hypercortisolemia on the bone formation process.

The previously mentioned hormones all affect bone metabolism by influencing bone formation, bone resorption, or both. However, adiponectin and leptin also influence reproductive function [148-151]. Specifically within the hypothalamus of mice, adiponectin inhibited gonadotropin releasing hormone, an important regulator of follicle stimulating hormone and luteinizing hormone (LH) that stimulate estrogen production by binding to the ovaries, secretion through the AMPK pathway [148], as well as the upstream signal, kisspeptin [149]. Leptin fluctuates in accordance with stages of the menstrual cycle and has been shown to peak at the time of the LH surge before ovulation [152, 153], indicating a role in the control of the menstrual cycle. Furthermore, leptin administration has proven effective in inducing ovulation and LH pulsatility in women with hypothalamic amenorrhea [150, 151]. These findings establish a secondary pathway in which low EA can influence both health, through estrogen production.

Estrogen Deficiency and Bone Health

A hallmark sign of energy deficiency and low EA is the presence of menstrual disturbances, the most extreme of which is primary amenorrhea (the failure of menses to occur by age 15) [15], and secondary amenorrhea (the absence of menstrual cycles lasting more than three months after menarche has occurred) [15]. Subclinical menstrual disturbances, including long and irregular cycles (oligomenorrhea), anovulation, or luteal-phase defects, may also occur [16, 17]. Menstrual disturbances, specifically in the case of oligo/amenorrhea and anovulation, are accompanied with estrogen deficiency [17]. In the face of estrogen deficiency, the bone microenvironment shifts

toward a state of overall net bone loss. Several studies have demonstrated the negative effect of amenorrhea on BMD [21, 22, 24, 154-159] and bone geometry [28, 29, 160-163] of Triad-affected exercising women when compared to healthy counterparts.

The first investigator to publish data on menstrual dysfunction and bone health was Dr. Barbara Drinkwater in 1984 [22]. The important relationship between menstrual history and bone health was demonstrated in a group of young athletes [21]. Exercising women with no history of irregular menstrual cycles had higher BMD in the lumbar spine than women with a history of oligomenorrhea and amenorrhea [21]. Furthermore, exercising women who had never experienced regular menstrual cycles had the lowest lumbar spine BMD of all three groups [21]. As imaging techniques advanced to include pQCT and HR-pQCT, bone geometry, a parameter providing a more in-depth analysis of bone structure, was also shown be negatively affected by amenorrhea [26, 28, 29]. Cortical area, thickness, perimeter and trabecular density have all been found to be negatively affected by amenorrhea resulting in decreased ability to withstand mechanical loading and increased risk of fracture in amenorrheic women [162]. Therefore it can be concluded that estrogen is an important bone-regulating hormone. Specifically, estrogen influences osteocytes, osteoblasts, and osteoclasts through pathways that regulate apoptosis, differentiation, and activity regarding the rate of bone formation and resorption [164].

Estrogen's Effect on Osteocytes

Early studies examining the effect of estrogen on osteocytes observed a role in apoptotic signaling [165]. Tomkinson et al. [165] investigated the effect of GnRH analogue-induced estrogen withdrawal in six premenopausal women after six months of GnRH analogue therapy,

and the resultant estrogen deficiency increased the proportion of nonviable osteocytes by 375%. Osteocyte nonviability was determined by the presence of DNA fragmentation, typical of apoptosis, using a DNA nick translation stick [165]. Tomkinson conducted a similar study in mice [166], but used DNA laddering, nuclear morphology, and nick translation to more accurately identify apoptotic osteocytes via DNA fragmentation in the tibia. In ovariectomized mice, the overall proportion of nonviable osteocytes in both cortical and trabecular bone increased by 400% [166]. Interestingly enough, the addition of estrogen after ovariectomy completely blocked the increase in nonviable osteocytes so that the proportion of nonviable osteocytes was equal to that of sham mice [166]. The inhibition of osteocyte apoptosis on cortical bone after ovariectomy in mice was also demonstrated by treatment with a pan-caspase inhibitor and blocked the subsequent increase in resorption observed in ovarectomized mice without treatment with pan-caspase inhibitor [167]. Windahl et al. [168] and Kondoh et al. [169] studied the effects of estrogen receptor alpha (ERα) on osteocyte signaling to osteoblasts and osteoclasts, although found contradicting results in male and female mice and did not account for osteocyte apoptosis. Hence, it is difficult to definitively state the effects of estrogen on osteocyte signaling but a function is clearly evident [168, 169].

Estrogen's Effect on Osteoblasts

Estrogen has been shown to inhibit osteoblast apoptosis and increase osteoblast lifespan [166, 170]. Upon further investigation, Almeida et al. [171] found that apoptosis was driven by estrogen binding to ERα on mature osteoblasts. Augmented apoptosis was not associated with altered femoral bone mass in ERα deleted female mice, which suggests that estrogen-mediated

mature osteoblasts alone are not enough to induce bone loss [171]. In contrast, Maata et al. [172] and Melville et al. [173] found impaired bone mass when ERα was deleted from mature osteoblasts in female mice. However, this discrepancy has been attributed to the fact that the control mice in these studies were from a different genetic background than the experimental mice [172, 173]. Bone loss, specifically in cortical bone, was achieved by deleting ERα in mesenchymal and osteoblast progenitors such that differentiation to mature osteoblasts was reduced [171]. Unliganded ERα in mesenchymal and osteoblast progenitors were found to potentiate the Wnt/B-catenin signaling necessary for complete maturation, while estrogen-bound ERα attenuated differentiation [171, 174]. Therefore, in an estrogen deficient environment, bone formation increases due to enhanced osteoblast differentiation, yet the magnitude of compensatory bone formation compared to bone resorption is attenuated by greater osteoblast apoptosis along with increased production of inflammatory cytokines, IL-7 and TNF, in the absence of estrogen that limit the activity of mature osteoblasts [175, 176].

Estrogen's Effect on Osteoclasts

Estrogen acts to suppress osteoclast formation and activity [18, 19]. Nakamura et al. [177] and Martin-Millan et al. [178] were able to demonstrate that elevated osteoclast activity and lifespan is mediated by estrogen receptor alpha (ERα) expressed by osteoclasts and that female mice lacking osteoclastic ERα exhibited trabecular bone loss but not cortical bone loss. Estrogen was also shown to directly downregulate RANKL and M-CSF-induced osteoclast differentiation of bone marrow cells isolated from the tibia and femur of ovariectomized mice [179, 180] and increase the production of OPG [181], all of which decrease the number of osteoclasts present on

bone. Therefore in the face of estrogen deficiency, osteoclast activity and differentiation increase and contribute to an overall net loss of bone that is not counteracted by adequate osteoblastic bone formation [19].

Overall, an estrogen deficiency leads to the uncoupling of bone formation and resorption such that resorption prevails. If the deficiency is left unchecked, net bone loss ensues. However, bone loss appears to be compartment specific. Elevated osteoclastic resorption affects trabecular bone while osteoblastic formation affects cortical bone. Both actions are detrimental to bone and increase the risk of bone related injuries and diseases.

Independent vs Combined Effects of Energy and Estrogen Deficiency

Using pQCT, Southmayd et al. [61] reported that energy and estrogen deficiency independently affected vBMD, bone geometry and estimated bone strength in distinct ways on load bearing and non-load bearing skeletal sites in exercising women [61]. Specifically, vBMD, bone geometry and estimated bone strength at the tibia (load bearing site) were largely dependent on energy status, while vBMD, bone geometry and estimated bone strength at the radius (non-load bearing site) were largely dependent on estrogen status [61]. Thus, the authors suggested that mechanical loading may counter and compete with the effects of increased bone resorption that takes place during hypoestrogenism in load bearing sites [61]. The effect of loading was not enough to increase the osteogenic effect of bone formation, which is suppressed during an energy deficient state, thus making energy status the main contributor of decreased bone health in the tibia [61].

De Souza et al. [27] reported that when both energy deficiency and estrogen deficiency are present simultaneously, negative alterations in bone turnover are exacerbated. In fact, when compared to any other combination of energy and estrogen status, women who were both energy deficient and estrogen deficient had the lowest levels of PINP, highest levels of U-CTX-I, and the most disrupted metabolic milieu, including high levels of ghrelin and low levels of TT_3 and leptin [27]. Interestingly enough, the degree of estrogen deficiency was similar in both estrogen deficient groups, suggesting that energy deficiency may compound the effect of an estrogen deficiency on bone [27]. When translating bone marker findings to BMD outcomes measured by DXA, an estrogen deficiency was associated with lower lumbar spine BMD and increased resorption, evidenced by elevated U-CTX-I concentrations [27]. However, there was no clear association between an energy deficiency and any measure of BMD [27]. Furthermore, in contrast with bone turnover results, BMD was not lowest in energy deficient and estrogen deficient women [27]. This discrepancy could be attributed to DXA's insufficiency to detect the trajectory of bone change over time and attest to the use of bone turnover markers for the assessment of bone metabolic activity.

Conclusion

Low EA and hypoestrogenism induced by menstrual dysfunction are independently detrimental to bone, but the degree to which each independently contributes to poor bone health or if the combination of both is especially detrimental is not completely understood to date, as low EA and menstrual dysfunction often occur simultaneously. Generally, low EA increases bone resorption and decreases bone formation in women, but the magnitude of effect depends upon the

severity of energy deficiency, duration of energy deficiency, and how the energy deficiency is achieved. An estrogen deficiency affects osteocytes, osteoblasts, and osteoclasts independently resulting in increased bone resorption and bone formation. However, uncoupling between the two results in net bone loss as bone formation does not adequately counter the effects of increased bone resorption. Southmayd et al. [61] found that energy and estrogen deficiency affect load bearing skeletal sites and non-load bearing sites, respectively. De Souza et al. [27] suggest that energy deficiency may be more detrimental to bone health when both energy and estrogen deficiencies are present in women. It would prove useful to assess markers of bone turnover because of their apparent sensitivity and utilize mathematical models to examine bone turnover dynamics according to energy and estrogen status in women. These findings would further our knowledge of how bone metabolic activity react in the face of a combined energy and estrogen deficiency.

Chapter 3

Materials and Methods

Study Design

This study was a cross-sectional study consisting of 109 healthy, exercising women that included data from two studies: (1) baseline data from a 12-month randomized control trial investigating the effects of increasing caloric intake on reversal of energy deficiency and recovery of menses and bone health in exercising women [182] and (2) data from a cross-sectional, observational study that aimed to assess estimated bone strength in young exercising women as a function of energy and menstrual status. Anthropometric data were collected, including height, weight and body mass index (BMI). Body composition and BMD were measured via DXA. PINP and sCTx were measured in blood samples as markers of bone formation and resorption, respectively. Bone Balance and Bone Turnover Rate were calculated using the multiple of medians mathematical approach [58] and visually depicted on a bone turnover plot (BTP). Energy status was assessed using TT_3 concentrations. Estrogen status was assessed using self-reported history of menses and verified by prospective assessments of urinary reproductive hormone metabolites (estrone-1-glucuronide (E1G) and pregnanediol glucuronide (PdG)). Participants were categorized as energy replete (EnR) or energy deficient (EnD) and estrogen replete (E_2R) or estrogen deficient (E_2D), resulting in four subgroups: EnR + E_2R (n=37), EnR + E_2D (n=18), EnD + E_2R (n=22) and EnD + E_2D (n=32).

Subject Characteristics

Participants for both studies were recruited through newspaper advertisements, email listserv announcements, website postings, and flyers posted on the Pennsylvania State University, University Park and/or University of Toronto campuses and in the surrounding communities. Inclusion criteria were (1) age 18-35 years, (2) body mass index (BMI) between 16-30 kg/m^2, (3) non-smoker, (4) not taking any hormonal medication for at least 6 months, (5) no known metabolic disease or bone disorder, (6) not pregnant or lactating and (7) at least 2 hours of purposeful exercise per week.

Anthropometrics

Height was measured without shoes using a wall-mounted stadiometer. Weight was measured to the nearest 0.1 kg on a digital scale. Body mass index (BMI) was calculated from height and weight (kg/m^2).

Body Composition

DXA scans of the total body were used to assess body composition (body fat percentage, fat mass, lean body mass, and fat free mass). Depending on the location and time the scan took place, participants were measured on one of three machines: Lunar Prodigy (GE Lunar Corporation, Madison, WI, enCORE 2002 software version 6.50.069) (n=29), Hologic QDR4500W (Hologic Inc., Bedford, MA) (n=4), or Lunar iDXA (GE Lunar Corporation, Madison, WI, enCORE 2008 software version 12.10.113) (n=76). Cross-calibration studies were

performed per the International Society for Clinical Densitometry guidelines and measurements from the Lunar Prodigy and Hologic QDR4500W were converted to the Lunar iDXA for analyses to remove any system bias. DXA scans were performed and analyzed by technologists certified by the International Society for Clinical Densitometry.

Blood Sampling and Storage

Blood samples were obtained via venipuncture in a 12-hour fasted state for the assessment of TT_3 and bone turnover markers in serum. Subjects were instructed to refrain from vigorous exercise, caffeine, and alcohol for 24 hours prior to blood sampling. Samples were collected between 0630 h and 1000 h and allowed to clot for 30 minutes at room temperature, then centrifuged at 3000 rpm for 15 minutes at 4 °C. Blood samples were then aliquoted into 1 ml polyethylene tubes and stored at -80 °C until analysis.

Assessment of Energy Status

Energy status was determined by a median split of TT_3 concentration among the sample. Women were categorized as energy replete (EnR) if their TT_3 concentration was higher than the median and energy deficient (EnD) if their TT_3 concentration was below the median. TT_3 was analyzed in serum using a chemiluminescence-based immunoassay analyzer (Diagnostic Products Corporation, Mountainview, CA). Analytical sensitivity for the TT3 assay was 0.54 nmol/L. The intra-assay and inter-assay coefficients of variation were 10.3% and 13.3%, respectively.

Serum Bone Marker Analysis

PINP was measured using a radioimmunoassay (Orion Diagnostica, Oslo, Finland). The sensitivity of the PINP assay was 2 μg/L. The intra-assay and inter-assay coefficients of variation were both 10%. sCTx was measured using an enzyme-linked immunoassay (ELISA). The sensitivity of the sCTx assay was 0.02 ng/ml. The intra-assay and inter-assay coefficient of variation were 3% and 10%, respectively.

Assessment of Bone Turnover Dynamics

A multiple of medians mathematical approach was used to calculate the index of formation (MoMf), index of resorption (MoMr), bone balance (BB), and the bone turnover rate (BTR). The calculation of MoMf and MoMr required the calculation of the median PINP and sCTx concentrations from an in-house database of healthy, eumenorrheic, ovulatory exercising women, which served as the reference group. The MoMf for each participant was calculated by dividing the individual's PINP concentration by the median PINP concentration in the reference group ($\frac{[PINP]}{Median\ [PINP\}_{OV\ Refernce\ Group}}$). The MoMr for each participant was calculated by dividing the individual's sCTx by the median sCTx concentration in the reference group ($\frac{[sCTx]}{Median\ [sCTx]_{OV\ Reference\ Group}}$). BB was then determined as the ratio of formation to resorption by dividing the index of formation by the index of resorption ($\frac{MoMf}{MoMr}$). BTR was quantified by taking the square root of the sum of the formation and resorption indices ($\sqrt[2]{MoMf^2 + MoMr^2}$).

Bone turnover plots were created to depict the average group formation and resorption indices, bone balance, and bone turnover rate. For the bone turnover plots, the resorption index

was represented on the x-axis and the formation index was represented on the y-axis. BB was represented by the slope of the line from the origin (0, 0) to the point (MoMr, MoMf). A slope of 1 is indicative of balanced bone formation and resorption, whereas a slope greater than 1 is indicative of net bone formation and a slope less than one is indicative of net bone resorption. The BTR was indicated by the length of the line from the origin to the point (MoMr, MoMf), calculated as the hypotenuse of a right triangle in which the indices of formation and resorption served as the adjacent sides. A longer vector indicated an increased rate of bone turnover, while a shorter vector indicated a decreased rate of bone turnover

Assessment of Menstrual Function and Estrogen Status

Estrogen status was defined by self-reported history of menses confirmed by prospective assessments of urinary reproductive hormone metabolites (estrone-1-glucuronide (E1G), pregnanediol glucuronide (PdG), and luteinizing hormone (LH)). Women with eumenorrheic cycles or oligomenorrhea but reporting ≥6 menses in the past 12 months were defined as estrogen replete (E_2R) and women with amenorrhea or oligomenorrhea but reporting <6 menses in the past 12 months were defined as estrogen deficient (E_2D). Normal ovulatory cycles were considered between 24-35 days in length, and women were deemed eumenorrheic if they self-reported a history of ≥9 menstrual cycles in the last 12 months. Oligomenorrheic cycles were those that were between 39-90 days in length, and women were considered oligomenorrheic if they self-reported a history of at least one menses in the last 90 days and <9 menses in the last 12 months. Women were determined to be amenorrheic if they failed to menstruate for a minimum of 90 days.

Urine Collection and Storage

First-morning void urine samples were collected for the duration of a complete menstrual cycle if subjects were menstruating or for one 28-day monitoring period if subjects were amenorrheic in order to assess reproductive hormone profiles to corroborate estrogen status. Samples were frozen upon collection before being returned to the laboratory. Upon receipt of the urine samples, the samples were thawed, aliquoted into 2 ml polyethylene tubes and stored at -20 °C until analysis.

Urinary Metabolite Analysis

All daily urine samples were assayed for E1G and PdG using microtiter plate competitive enzyme immunoassays. The E1G (R522-2) and PdG (R13904) assays use a polyclonal capture antibody supplied by the Coralie Munro University of California (Davis, CA). The inter-assay coefficients of variation for high and low internal controls for the E1G assay are 14.7% and 13.1%, respectively, and the PdG intra and inter-assay variability was determined in-house as 15.68% and 17.7%, respectively. Urinary LH was measured using a coat-a-count immunoradiometric assay (Siemens Healthcare Diagnostics, Deerfield, IL). The intra-assay and inter-assay coefficients of variation were 1.6% and 7.1%, respectively. To account for hydration status, specific gravity of each urine sample was determined using a hand refractometer (NSG Precision Cells) [183]. In order to compare estrogen and progesterone exposure among participants, Kaleidagraph (software Version 4.1.1, Synergy Software, Reading, PA) was used to calculate area under the curve (AUC) for E1G and PdG across the entire menstrual cycle or 28-day monitoring period. Mean E1G and

PdG concentration was also calculated for each menstrual cycle or 28-day monitoring period to further assess estrogen and progesterone exposure.

Statistical and Data Analysis

The main outcome variables of this study were the index of bone formation, index of bone resorption, bone balance, and bone turnover rate assessed via the multiple of medians approach discussed above. Before any statistical analyses were performed, data screening was conducted in order to identify whether the data met assumptions required by two-way analysis of variance (ANOVA). Data screening included the inspection for homogeneity of variance among the four groups (Levene's statistic) and testing for normality within each of the four groups (Shapiro Wilk test). If either assumption was not met, the variable was transformed and re-tested until both assumptions were satisfied. Raw values are reported, while transformed variables were used for analyses.

Descriptive statistics were calculated for basic demographic information, subject characteristics, and bone turnover dynamics within the EnR+E_2R, EnR+E_2D, EnD+E_2R, EnD+E_2D groups. Using two-way ANOVA, significance was tested for main and interaction effects of both independent grouping factors (energy and estrogen status) on demographic information and bone turnover dynamics (index of bone formation, index of bone resorption, bone balance, and bone turnover rate). Main effects were only interpreted in the absence of a significant interaction effect. No post-hoc analyses were required in the presence of significant main effects because each independent grouping factor only contained two levels (replete and deficient). If a significant interaction effect was present, a simple effects analysis was used to assess at which

levels of the independent grouping factors differences emerged. Significance level of $p<0.05$ was used to identify significant differences. All statistical analyses were performed using SPSS for Windows (version 12.0; Chicago, IL) software.

Chapter 4

Manuscript

Introduction

The Female Athlete Triad is a medical condition observed in physically active girls and women characterized by low energy availability (EA) with or without disordered eating, menstrual dysfunction and low bone mineral density (BMD) [3]. EA associated with the Triad is defined as the amount of energy remaining for all physiological processes after subtracting the energy that is used for exercise and calculated as dietary energy intake minus exercise energy expenditure corrected for fat free mass or lean body mass (kcal/kgFFM or LBM/d) [8-10]. If the resultant energy deficit is large enough, the deficit signals that the body does not have sufficient energy to meet the needs of exercise and normal physiological function and physiological adaptations ensue to conserve energy [11]. In particular, growth and reproduction are suppressed [12].

The acute short-term effects of reduced EA were demonstrated in young, sedentary but otherwise healthy, menstruating women in a series of experiments conducted by Loucks and colleagues [10, 13]. A dose response relationship between degree of energy deficiency and markers of bone turnover was illustrated, such that uNTX concentration, a marker of bone resorption, was elevated at extreme low EA (i.e. 10kcal/kgLBM/day), and PICP and osteocalcin, markers of bone formation, were suppressed at moderate to mild levels of low EA (i.e. 20 and 30 kcal/kgLBM/day) [13]. Furthermore, luteinizing hormone (LH) pulsatility was suppressed at mild low EA (30 kcal/kgLBM/day), indicating alterations in the hypothalamic-pituitary-ovarian (HPO) axis and disrupted reproductive function [10].

A hallmark sign of energy deficiency and low EA is the presence of menstrual disturbances which range across a spectrum, the most extreme of which is primary amenorrhea (the failure of menses to occur by age fifteen) [15]. Less severe disturbances include long and irregular cycles (oligomenorrhea), anovulation, or luteal phase defects characterized by short luteal phases or inadequate progesterone concentrations during the luteal phase [16, 17]. Menstrual disturbances are accompanied by hormonal suppression and, specifically in the case of oligo/amenorrhea and anovulation, estrogen deficiency [17]. Estrogen is an important bone-regulating hormone, as it functions to suppress osteoclast formation and activity [18, 19]. Without adequate concentrations of circulating estrogen, osteoclast synthesis, recruitment and lifespan increase, all of which contribute to an overall net loss of bone that may not be counteracted by adequate osteoblastic bone formation [19]. Poor bone health in the face of an estrogen deficiency has been demonstrated in multiple cross sectional studies that have reported lower BMD at the lumbar spine and hip in amenorrheic athletes compared to eumenorrheic athletes and healthy control subjects [21, 24, 25].

Dual energy X-ray absorptiometry (DXA) is the clinical tool used to measure BMD in order to diagnose low bone mass and osteoporosis, assess a patient's risk of fracture and monitor changes in BMD over time [50]. However, measurements of BMD using DXA represent a static snapshot of bone at one time and do not reflect the metabolic activity of bone that could be occurring [51]. Furthermore, it can take months or years to detect significant changes in BMD via DXA, limiting the ability to assess an individual's short-term response to an intervention [52]. Other methods of evaluating bone health, such as measuring markers of bone turnover, allow for the assessment of metabolic changes in bone over short time periods that are observable before detectable BMD changes occur [51, 53, 54].

Individual markers of bone turnover reflect the metabolic activity of bone resorption and formation and provide an overall assessment of skeletal health, rather than site specific assessments of skeletal health that measurements of BMD provide [54, 56]. The International Osteoporosis Foundation and International Federation of Clinical Chemistry recommend the use of one marker of bone formation, type I procollagen carboxy-terminal propeptide (PINP), and one marker of bone resorption, serum C-terminal telopeptide (sCTx), to be used as reference markers and measured by standardized assays because of their thorough evaluation for fracture prediction and treatment, extensive documentation of biological and analytical variability, as well as sample handling and stability [57]. A multiple of medians mathematical approach has been developed that incorporates markers of formation and resorption to assess the net balance of bone formation and resorption (i.e. bone balance, BB) and the rate of bone turnover (i.e. bone turnover rate, BTR) [58]. While evaluating formation and resorption independently can lead to incomplete conclusions about the overall state of bone metabolism, BB is a useful measure to assess if either bone resorption or formation is predominating since BB assesses bone resorption and formation together [59]. BTR furthers our understanding by indicating whether or not bone resorption or formation is occurring at an elevated or reduced rate, which may have implications for bone gain or loss and fracture risk [58].

The purpose of this study was to assess markers of bone formation (Procollagen type I N-terminal propeptide (P1NP)) and resorption (Serum C-terminal telopeptides (sCTx)) utilized in mathematical models of bone turnover to quantify how energy and estrogen status are related to bone formation, bone resorption, bone balance and bone turnover rate in premenopausal exercising women. Energy status was defined as energy replete (EnR) or energy deficient (EnD) using a median split of total triiodothyronine (TT_3) concentration among the sample. Estrogen status was

defined as estrogen replete (E_2R) or estrogen deficient (E_2D) using self-reported history of menses verified with prospective assessments of urinary reproductive hormone metabolites. The hypotheses were threefold: (1) there would be an interaction effect of energy and estrogen status such that the women who were both energy and estrogen deficient (EnD + E_2D group) would have the lowest bone balance and lowest rate of bone turnover compared to women who were either energy or estrogen deficient; (2) there would be a main effect of estrogen status on the index of bone resorption, bone balance, and bone turnover rate such that E_2D women would have a greater index of bone resorption, lower bone balance, and a higher bone turnover rate; and (3) there would be a main effect of energy status on the index of bone formation, bone balance, and bone turnover rate such that EnD women would have a lower index of bone formation, lower bone balance, and a lower bone turnover rate.

Materials and Methods

Study Design

This study was a cross-sectional study consisting of 109 healthy, exercising women that included data from two studies: (1) baseline data from a 12-month randomized control trial investigating the effects of increasing caloric intake on reversal of energy deficiency and recovery of menses and bone health in exercising women [182] and (2) data from a cross-sectional, observational study that aimed to assess estimated bone strength in young exercising women as a function of energy and menstrual status. Anthropometric data were collected, including height, weight and body mass index (BMI). Body composition and BMD were measured via DXA. PINP and sCTx were measured in blood samples as markers of bone formation and resorption,

respectively. Bone Balance and Bone Turnover Rate were calculated using the multiple of medians mathematical approach [58] and visually depicted on a bone turnover plot (BTP). Energy status was assessed using TT_3 concentrations. Estrogen status was assessed using self-reported history of menses and verified by prospective assessments of urinary reproductive hormone metabolites (estrone-1-glucuronide (E1G) and pregnanediol glucuronide (PdG)). Participants were categorized as energy replete (EnR) or energy deficient (EnD) and estrogen replete (E_2R) or estrogen deficient (E_2D), resulting in four subgroups: EnR + E_2R (n=37), EnR + E_2D (n=18), EnD + E_2R (n=22) and EnD + E_2D (n=32).

Subject Characteristics

Participants for both studies were recruited through newspaper advertisements, email listserv announcements, website postings, and flyers posted on the Pennsylvania State University, University Park and/or University of Toronto campuses and in the surrounding communities. Inclusion criteria were (1) age 18-35 years, (2) body mass index (BMI) between 16-30 kg/m^2, (3) non-smoker, (4) not taking any hormonal medication for at least 6 months, (5) no known metabolic disease or bone disorder, (6) not pregnant or lactating and (7) at least 2 hours of purposeful exercise per week.

Anthropometrics

Height was measured without shoes using a wall-mounted stadiometer. Weight was measured to the nearest 0.1 kg on a digital scale. Body mass index (BMI) was calculated from height and weight (kg/m^2).

Body Composition

DXA scans of the total body were used to assess body composition (body fat percentage, fat mass, lean body mass, and fat free mass). Depending on the location and time the scan took place, participants were measured on one of three machines: Lunar Prodigy (GE Lunar Corporation, Madison, WI, enCORE 2002 software version 6.50.069) (n=29), Hologic QDR4500W (Hologic Inc., Bedford, MA) (n=4), or Lunar iDXA (GE Lunar Corporation, Madison, WI, enCORE 2008 software version 12.10.113) (n=76). Cross-calibration studies were performed per the International Society for Clinical Densitometry guidelines and measurements from the Lunar Prodigy and Hologic QDR4500W were converted to the Lunar iDXA for analyses to remove any system bias. DXA scans were performed and analyzed by technologists certified by the International Society for Clinical Densitometry.

Blood Sampling and Storage

Blood samples were obtained via venipuncture in a 12-hour fasted state for the assessment of TT_3 and bone turnover markers in serum. Subjects were instructed to refrain from vigorous exercise, caffeine, and alcohol for 24 hours prior to blood sampling. Samples were collected between 0630 h and 1000 h and allowed to clot for 30 minutes at room temperature, then centrifuged at 3000 rpm for 15 minutes at 4 °C. Blood samples were then aliquoted into 1 ml polyethylene tubes and stored at -80 °C until analysis.

Assessment of Energy Status

Energy status was determined by a median split of TT_3 concentration among the sample. Women were categorized as energy replete (EnR) if their TT_3 concentration was higher than the median and energy deficient (EnD) if their TT_3 concentration was below the median. TT_3 was analyzed in serum using a chemiluminescence-based immunoassay analyzer (Diagnostic Products Corporation, Mountainview, CA). Analytical sensitivity for the TT3 assay was 0.54 nmol/L. The intra-assay and inter-assay coefficients of variation were 10.3% and 13.3%, respectively.

Serum Bone Marker Analysis

PINP was measured using a radioimmunoassay (Orion Diagnostica, Oslo, Finland). The sensitivity of the PINP assay was 2 μg/L. The intra-assay and inter-assay coefficients of variation were both 10%. sCTx was measured using an enzyme-linked immunoassay (ELISA). The sensitivity of the sCTx assay was 0.02 ng/ml. The intra-assay and inter-assay coefficient of variation were 3% and 10%, respectively.

Assessment of Bone Turnover Dynamics

A multiple of medians mathematical approach was used to calculate the index of formation (MoMf), index of resorption (MoMr), bone balance (BB), and the bone turnover rate (BTR). The calculation of MoMf and MoMr required the calculation of the median PINP and sCTx concentrations from an in-house database of healthy, eumenorrheic, ovulatory exercising women, which served as the reference group. The MoMf for each participant was calculated by dividing

the individual's PINP concentration by the median PINP concentration in the reference group ($\frac{[PINP]}{Median\ [PINP]_{OV\ Refernce\ Group}}$). The MoMr for each participant was calculated by dividing the individual's sCTx by the median sCTx concentration in the reference group ($\frac{[sCTx]}{Median\ [sCTx]_{OV\ Reference\ Group}}$). BB was then determined as the ratio of formation to resorption by dividing the index of formation by the index of resorption ($\frac{MoMf}{MoMr}$). BTR was quantified by taking the square root of the sum of the formation and resorption indices ($\sqrt[2]{MoMf^2 + MoMr^2}$).

Bone turnover plots were created to depict the average group formation and resorption indices, bone balance, and bone turnover rate. For the bone turnover plots, the resorption index was represented on the x-axis and the formation index was represented on the y-axis. BB was represented by the slope of the line from the origin (0, 0) to the point (MoMr, MoMf). A slope of 1 is indicative of balanced bone formation and resorption, whereas a slope greater than 1 is indicative of net bone formation and a slope less than one is indicative of net bone resorption. The BTR was indicated by the length of the line from the origin to the point (MoMr, MoMf), calculated as the hypotenuse of a right triangle in which the indices of formation and resorption served as the adjacent sides. A longer vector indicated an increased rate of bone turnover, while a shorter vector indicated a decreased rate of bone turnover

Assessment of Menstrual Function and Estrogen Status

Estrogen status was defined by self-reported history of menses confirmed by prospective assessments of urinary reproductive hormone metabolites (estrone-1-glucuronide (E1G), pregnanediol glucuronide (PdG), and luteinizing hormone (LH)). Women with eumenorrheic

cycles or oligomenorrhea but reporting ≥6 menses in the past 12 months were defined as estrogen replete (E_2R) and women with amenorrhea or oligomenorrhea but reporting <6 menses in the past 12 months were defined as estrogen deficient (E_2D). Normal ovulatory cycles were considered between 24-35 days in length, and women were deemed eumenorrheic if they self-reported a history of ≥9 menstrual cycles in the last 12 months. Oligomenorrheic cycles were those that were between 39-90 days in length, and women were considered oligomenorrheic if they self-reported a history of at least one menses in the last 90 days and <9 menses in the last 12 months. Women were determined to be amenorrheic if they failed to menstruate for a minimum of 90 days.

Urine Collection and Storage

First-morning void urine samples were collected for the duration of a complete menstrual cycle if subjects were menstruating or for one 28-day monitoring period if subjects were amenorrheic in order to assess reproductive hormone profiles to corroborate estrogen status. Samples were frozen upon collection before being returned to the laboratory. Upon receipt of the urine samples, the samples were thawed, aliquoted into 2 ml polyethylene tubes and stored at -20 °C until analysis.

Urinary Metabolite Analysis

All daily urine samples were assayed for E1G and PdG using microtiter plate competitive enzyme immunoassays. The E1G (R522-2) and PdG (R13904) assays use a polyclonal capture antibody supplied by the Coralie Munro University of California (Davis, CA). The inter-assay coefficients of variation for high and low internal controls for the E1G assay are 14.7% and 13.1%,

respectively, and the PdG intra and inter-assay variability was determined in-house as 15.68% and 17.7%, respectively. Urinary LH was measured using a coat-a-count immunoradiometric assay (Siemens Healthcare Diagnostics, Deerfield, IL). The intra-assay and inter-assay coefficients of variation were 1.6% and 7.1%, respectively. To account for hydration status, specific gravity of each urine sample was determined using a hand refractometer (NSG Precision Cells) [183]. In order to compare estrogen and progesterone exposure among participants, Kaleidagraph (software Version 4.1.1, Synergy Software, Reading, PA) was used to calculate area under the curve (AUC) for E1G and PdG across the entire menstrual cycle or 28-day monitoring period. Mean E1G and PdG concentration was also calculated for each menstrual cycle or 28-day monitoring period to further assess estrogen and progesterone exposure.

Statistical and Data Analysis

The main outcome variables of this study were the index of bone formation, index of bone resorption, bone balance, and bone turnover rate assessed via the multiple of medians approach discussed above. Before any statistical analyses were performed, data screening was conducted in order to identify whether the data met assumptions required by two-way analysis of variance (ANOVA). Data screening included the inspection for homogeneity of variance among the four groups (Levene's statistic) and testing for normality within each of the four groups (Shapiro Wilk test). If either assumption was not met, the variable was transformed and re-tested until both assumptions were satisfied. Raw values are reported, while transformed variables were used for analyses.

Descriptive statistics were calculated for basic demographic information, subject characteristics, and bone turnover dynamics within the EnR+E_2R, EnR+E_2D, EnD+E_2R, EnD+E_2D groups. Using two-way ANOVA, significance was tested for main and interaction effects of both independent grouping factors (energy and estrogen status) on demographic information and bone turnover dynamics (index of bone formation, index of bone resorption, bone balance, and bone turnover rate). Main effects were only interpreted in the absence of a significant interaction effect. No post-hoc analyses were required in the presence of significant main effects because each independent grouping factor only contained two levels (replete and deficient). If a significant interaction effect was present, a simple effects analysis was used to assess at which levels of the independent grouping factors differences emerged. Significance level of $p<0.05$ was used to identify significant differences. All statistical analyses were performed using SPSS for Windows (version 12.0; Chicago, IL) software.

Results

Demographics

Demographic characteristics are presented in Table 1. There were no main effects of energy status on age or BMI. There was a main effect of estrogen on age ($p=0.020$) and BMI ($p=0.026$), such that the E_2D women were younger (21.3±0.5 vs 22.7±0.6 years) and had a lower BMI (20.7±0.3 vs 21.8±0.3 kg/m^2) than E_2R women. There was a significant interaction effect of estrogen and energy status on fat mass ($p=0.039$) and percent body fat ($p=0.027$). Within the EnD women, E_2R women had significantly greater fat mass ($p=0.002$) and percent body fat ($p=0.002$) than E_2D women (15.8±0.9 vs 12.5±0.6 kg and 22.4±0.8 vs 26.4±0.8%, respectively). Also, within

the E_2D women, EnR women had significantly greater fat mass ($p=0.002$) and percent body fat ($p<0.001$) than EnD women (16.1±1.1 vs 12.5±0.6 kg and 22.4±0.8 vs 27.3±1.3%, respectively). There were no significant main effects of energy and estrogen on fat free mass, lean body mass, height, or weight ($p>0.05$).

Menstrual Characteristics and Urinary Ovarian Steroids

Menstrual characteristics and urinary ovarian steroid data are presented in Table 1. There was no main effect of energy on age of menarche, but there was a main effect of estrogen on age of menarche ($p<0.001$), such that E_2D women had an older age of menarche compared to E_2R women (13.8±0.2 vs 12.6±0.2 years). There were main effects of estrogen and energy status on gynecological age ($p<0.001$ and $p=0.015$, respectively), such that E_2D women had a younger gynecological age than E_2R women (7.6±0.5 vs 10.2±0.6 years), and EnD women had an older gynecological age than EnR women (9.4±0.6 vs 8.6±0.6 years).

There were no main effects of energy status on PdG AUC or mean concentration, yet there was a main effect of estrogen status on PdG AUC and mean concentration (both $p<0.001$), such that E_2D women had a lower PdG AUC (38.8±4.2 vs 78.3±6.9 μg/L) and mean concentration (2.0±0.7 vs 2.8±0.2 μg/L) than E_2R women. There was an interaction effect between energy and estrogen on E1G AUC ($p=0.020$). Within the EnD women, E_2D women had a lower E1G AUC ($p<0.001$) than E_2R women (728.4±76.4 vs 1460.0±139.9 ng/ml). There was a main effect of estrogen status on E1G mean concentration ($p<0.001$), such that E_2D women had a lower mean concentration than E_2R women (27.4±2.0 vs 45.1±2.6 ng/mL).

Metabolic Hormones

Metabolic hormone data are shown in Table 1.There was a main effect of energy status on TT_3 concentration ($p<0.001$), such that EnD women had a lower TT_3 concentration than EnR women (67.1±1.4 vs 100.3±1.7 ng/dL). There was no main effect of estrogen on TT_3 concentration.

Bone Turnover Dynamics

Bone turnover data are presented in Table 1. There was a significant interaction effect of energy and estrogen status on PINP concentration and the index of formation (both p=0.046), and bone turnover rate (p=0.045). Within the E_2D women, EnD women had a lower PINP concentration (p=0.002), index of formation (p=0.002), and bone turnover rate (p=0.001) than EnR women (53.64±3.14 vs 77.93±7.34 μg/L, 1.00±0.06 vs 1.44±0.14 μg/L, and 1.43±0.08 vs 1.98±0.16, respectively). There was no main effect of estrogen, but a significant main effect of energy on sCTx concentration and the index of resorption (both p=0.019), such that EnD women had a lower sCTx concentration and index of bone resorption than EnR women (0.65±0.03 vs 0.76±0.04 ng/mL and 1.01±0.05 vs 1.17±0.06 ng/mL, respectively). There were no main or interaction effects of estrogen and energy status on bone balance, but bone balance was greater than 1 in all groups, indicative of net bone formation.

Table 1. Demographic and menstrual characteristics, urinary ovarian steroid and metabolic hormones, and bone turnover dynamics of the study groups categorized by the combination of energy and estrogen status

	EnD+E_2D n=32	EnR+E_2D n=18	EnD+E_2R n=22	EnR+E_2R n=37	p En X E_2	p En	p E_2
Demographic characteristics							
Age (years)	22.1±0.6	19.9±0.5	22.9±0.9	22.6±0.7	0.195	0.094	0.020
Weight (kg)	55.8±1.2	58.2±1.7	59.6±2.0	59.2±1.0	0.344	0.494	0.105
Height (cm)	166.2±1.1	164.0±1.5	164.7±1.2	164.7±1.1	0.403	0.402	0.794
BMI (kg/m^2)	20.2±0.4	21.6±0.4	21.9±0.5	21.8±0.3	0.082	0.117	0.026
Body fat (%)	22.4±0.8[a,b]	27.3±1.2	26.4±0.8	27.2±0.7	0.027	-	-
Fat mass (kg)	12.5±0.6[a,b]	16.1±1.1	15.8±0.9	16.2±0.6	0.039	-	-
Fat free mass (kg)	43.3±0.9	42.1±1.0	43.8±1.3	42.9±0.7	0.887	0.291	0.469
Lean body mass (kg)	40.9±0.9	39.8±0.9	41.4±1.3	40.5±0.7	0.933	0.277	0.527
Menstrual characteristics							
Age of menarche (years)	13.5±0.2	14.3±0.4	12.6±0.3[*]	12.6±0.3	0.187	0.160	<0.001
Gynecological age (years)	8.7±0.6	5.6±0.7	10.5±1.0[*]	10.0±0.7	0.051	0.015	<0.001
Urinary ovarian steroid hormones							
PdG AUC	39.5±5.4	37.6±6.8[†]	84.7±14.5[*]	74.6±7.0[‡]	0.935	0.847	<0.001
PdG mean (µg/L)	2.4±1.1	1.2±0.2[†]	2.8±0.5[*]	2.7±0.3[‡]	0.472	0.670	<0.001
E1G AUC	728.4±76.4[a]	1019.9±189.7[†]	1460.0±139.9[*]	1172.6±75.5[‡]	0.020	-	-
E1G mean (ng/mL)	25.1±1.9	31.9±4.6[†]	48.9±4.4[*]	42.9±3.1[‡]	0.095	0.850	<0.001
Metabolic hormones							
TT3 (ng/dL)	65.7±1.6	100.5±3.1	69.2±2.4	100.1±2.1	0.401	<0.001	0.490
Bone Turnover Dynamics							
PINP (µg/L)	53.64±3.14[b]	77.93±7.34	64.43±5.77	65.79±3.27	0.046	-	-
sCTx (ng/mL)	0.64±0.05	0.85±0.07	0.66±0.05	0.71±0.05	0.093	0.019	0.351
MoMf (µg/L)	1.00±0.06[b]	1.44±0.14	1.19±0.11	1.21±0.06	0.046	-	-
MoMr (ng/mL)	1.00±0.07	1.32±0.11	1.03±0.08	1.10±0.07	0.093	0.019	0.351
BB	1.09±0.07	1.12±0.10	1.18±0.08	1.19±0.07	0.836	0.838	0.223
BTR	1.43±0.08[b]	1.98±0.16	1.59±0.13	1.67±0.08	0.045	-	-

Values are mean±SEM

En X E_2 = interaction effect, En = main effect of energy status, E_2 = main effect of estrogen

[a] Energy Deficient+Estrogen Deficient vs Energy Deficient+Estrogen Replete

[b] Estrogen Deficient+Energy Deficient vs Estrogen Deficient+Energy Replete

[*] n=21, [†] n=16, [‡] n=36

Discussion

This study made use of unique mathematical models of bone turnover dynamics to assess markers of bone resorption (sCTx) and formation (P1NP) in relation to energy and estrogen status. Specifically, indices of bone formation, bone resorption, bone balance and bone turnover rate were explored in a population of healthy, exercising women. The results demonstrated that the combination of energy and estrogen deficiency was associated with the lowest bone turnover rate driven by a lower index of bone formation compared to energy replete and estrogen deficient women, indicating the importance of energy status in exercising women with menstrual disturbances. Contrary to our hypotheses, energy status impacted the bone resorption index, such that bone resorption was lower in both groups of energy deficient exercising women compared to energy replete exercising women, suggesting that lower energy availability suppresses overall bone turnover.

Energy deficiency within the energy and estrogen deficient women was corroborated by a lower body fat percentage and fat mass compared to the energy deficient and estrogen replete exercising women as well as the energy replete and estrogen deficient exercising women. This finding suggests that the exercising women who were both energy and estrogen deficient were likely in a more severe state of energy deficiency than the exercising women who were energy deficient by TT3 concentration, but still estrogen replete. Consequently, the detriment to bone turnover was more severe in the exercising women who experienced the combination of energy and estrogen deficiency.

Estrogen deficiency within the energy and estrogen deficient women was indicated by a lower E1G AUC compared to the energy deficient and estrogen replete exercising women. Additionally, both groups of estrogen deficient exercising women demonstrated suppressed E1G

mean concentrations and PdG AUC and mean concentrations compared to estrogen replete exercising women. Furthermore, estrogen deficient exercising women were younger, had a lower BMI, older age of menarche, and younger gynecological age than estrogen replete exercising women. Gynecological age was also affected by energy status such that energy deficient exercising women had a younger gynecological age than energy replete exercising women. Loucks et al. [184] demonstrated that reduced LH pulsatility in response to energy restriction (i.e., 10 kcal/kgLBM/day) was dependent on gynecological age. Specifically, women with younger gynecological ages ranging from 5-8 years experienced reduced LH pulse frequency as opposed to women with gynecological ages ranging from 14-18 years [184]. Reduced LH pulse frequency is associated with amenorrhea [185, 186] and in turn, low BMD [21, 22, 24, 154-159]. Therefore, gynecological age may contribute to altered bone turnover dynamics in energy deficient exercising women.

Our results are consistent with previously reported results from De Souza et al. [27], as PINP concentration, and thus the bone formation index, was driven by energy status and lowest when accompanied by an estrogen deficiency. Indeed, acute-short term effects of energy restriction on bone formation have been well documented [13, 94]. Ihle and Loucks [13] demonstrated that in healthy sedentary women, bone formation, measured using PICP concentration, was suppressed at moderate and mild levels of energy restriction (ie, 10, 20 and 30 kcal/kgLBM/day). In addition, Papageorgiou et al. [94] observed similar directional changes in bone formation of women who participated in 5 days of prescribed low EA of 15 kcal/kgLBM/day. Amenorrheic athletes with an estrogen deficiency have also been exhibited lower than normal bone formation than their eumenorrheic counterparts [24, 63]. Therefore, it is likely that both energy and estrogen deficiency

synergistically act on the bone microenvironment to exacerbate the negative alterations in PINP and subsequently, the index of bone formation.

Alternatively, the synergistic effect of energy and estrogen deficiency was not observed to affect sCTx concentration. Rather, only energy status had a main effect on bone resorption, such that sCTx concentration, and thus the index of bone resorption, was decreased in an energy deficient state. This relationship is in contrast to previous studies [13, 94], which demonstrated that bone resorption increased in response to short-term energy restriction in women. Furthermore, De Souza et al. [27] reported that uCTx was related to both energy and estrogen status and increased among energy and estrogen deficient women. This discrepancy may be due to disparate use of markers of bone resorption. For instance, Ihle and Loucks [13] measured bone resorption via uNTx, Papageorgiou et al. [94] via β-CTx, and De Souza et al. [27] via uCTx. Consistency among reference markers between studies is absolutely necessary to properly compare results. Nevertheless, Ihle and Loucks [13] observed that bone resorption was more resistant to energy restriction than bone formation giving rise to the possibility that our subjects did not achieve a low enough EA in order to increase sCTx. Alternatively, the discrepancies between our results and those of previous investigations may reflect short-term vs. long-term energy deficiency. For instance, the data presented by Ihle and Loucks [13] reflected a short-term energy deficit, which may transiently increase bone resorption, while the current study reflected an overall suppression of bone turnover (formation and resorption) among women in whom energy deficiency was chronic and severe enough to elicit menstrual dysfunction.

Despite a decreased index of bone formation within the energy and estrogen deficient women, bone balance was not different between any groups. In fact, bone balance was greater than 1 in all groups and representative of net bone formation. This is in contrast with a number of studies

that have observed net bone loss in amenorrheic women [21, 22, 24, 154-159] and in women with anorexia nervosa [41, 42], an eating disorder characterized by extreme undernutrition resulting in severe energy deficiency. Although bone balance across all groups was indicative of net bone formation, bone turnover rate was decreased when energy deficiency was adequate enough to suppress reproductive status, suggesting a downregulation of overall bone turnover. Using our data, it is unclear how a downregulation of bone turnover is related to clinically relevant diagnostic criteria, such as BMD. In order to elucidate this relationship, future studies are encouraged to investigate how decreasing or increasing bone turnover rate impacts BMD in our population.

One limitation of this study is that the mathematical models used to assess bone turnover dynamics are not validated. To improve the usefulness of the algorithms in women with the Triad, future studies should aim to relate bone turnover dynamics with BMD outcomes. Specifically, investigations should be completed using DXA to measure BMD at weight-bearing and non-weight-bearing skeletal sites, as well as the whole body and lumbar spine for premenopausal women $\geq$ 20 years and $<$ 20 years , respectively, per the recommendations by the Female Athlete Coalition [45], alongside mathematically derived bone turnover dynamics to evaluate the relationship between measures of BMD and bone balance and bone turnover rate. In doing so and with further investigation, the mathematically derived bone turnover dynamics used in this study could provide an additional way in which clinicians identify female athletes and exercising women at risk for bone loss related injuries and diseases associated with the Triad, in addition to DXA-measured BMD [45]. Indeed, De Souza et al. [27] demonstrated that bone turnover markers were a more sensitive measure of bone metabolic activity compared to DXA-measure BMD. Corroborating bone turnover markers with mathematical models to assess bone turnover dynamics could identify at risk women sooner than allowed by DXA.

The novelty of our study lies in the use of mathematical models to determine bone balance and bone turnover rate, as well as the index of bone formation and resorption from PINP and sCTx concentrations, respectively, in our group of healthy, exercising women with a range of energy and estrogen status. These mathematical models provided a more thorough and complete interpretation of bone metabolism, such that we were able to distinguish the relationship between energy and estrogen status on bone turnover dynamics. The combination of energy and estrogen deficiency was associated with the lowest bone turnover rate driven by a lower index of bone formation compared to energy replete and estrogen deficient women, yet, only energy status impacted the index of bone resorption, such that bone resorption was lower in both groups of energy deficient women compared to energy replete women. Therefore, energy status appears to be an important contributor to altered bone turnover dynamics in women with menstrual disturbances, underlining the importance of maintaining an adequate EA for the benefit of a healthy skeleton.

Chapter 5

Conclusion

Energy and estrogen deficiencies are detrimental to bone health in exercising women, but the significance of each independently has not been extensively examined, as energy and estrogen deficiency often occur simultaneously. The purpose of this study was to assess markers of bone formation (PINP) and resorption (sCTx) utilized in mathematical models of bone turnover to quantify how energy and estrogen status related to the index of bone formation, index of bone resorption, bone balance and bone turnover rate in n=109 premenopausal exercising women age 18-35 years old. The study utilized a 2x2 cross-sectional study design by which women were categorized by energy status and by estrogen status. Specifically, exercising women were defined as energy replete (EnR) or energy deficient (EnD) using a median split of total triiodothyronine (TT_3) concentration among the sample. Exercising women were defined as estrogen replete (E_2R) or estrogen deficient (E_2D) using self-reported history of menses verified with prospective assessments of urinary reproductive hormone metabolites. This resulted in four groups: EnR+E_2R (n=37), EnR +E_2D (n=18), EnD+E_2R (n=22), EnD+E_2D (n=32).

Our hypotheses were threefold: (1) there would be an interaction effect of energy and estrogen status such that the women who were both energy and estrogen deficient (EnD+E_2D group) would have the lowest bone balance and lowest rate of bone turnover compared to women who were either energy or estrogen deficient; (2) there would be a main effect of estrogen status on the index of bone resorption, bone balance, and bone turnover rate such that E_2D women would have a greater index of bone resorption, lower bone balance, and a higher bone turnover rate; and

(3) there would be a main effect of energy status on the index of bone formation, bone balance, and bone turnover rate such that EnD women would have a lower index of bone formation, lower bone balance, and a lower bone turnover rate.

In accordance with De Souza et al. [27], our results demonstrated that the combination of energy and estrogen deficiency was associated with the lowest bone turnover rate, driven by a lower index of bone formation compared to energy replete and estrogen deficient women, indicating the importance of adequate energy status in exercising women with menstrual disturbances. Contrary to our hypotheses and previous studies assessing short-term energy restriction in women [13, 94], energy status impacted the bone resorption index, such that bone resorption was *lower* in both groups of energy deficient women compared to energy replete women. The discrepancies between previous investigations and our results may reflect the effects of short-term energy deficiency in previous investigations vs. long-term energy deficiency in the present study, which may have been severe enough to elicit menstrual dysfunction and suppress overall bone turnover (formation and resorption).

Energy deficiency within the energy and estrogen deficient women was corroborated by a lower body fat percentage and fat mass compared to the energy deficient and estrogen replete women as well as the energy replete and estrogen deficient women. This suggests that the women who were both energy and estrogen deficient were likely in a more severe state of energy deficiency than the women who were energy deficient by TT_3 concentration, but still estrogen replete. Consequently, the detriment to bone turnover was more severe in the women who experienced the combination of energy and estrogen deficiency.

Estrogen deficiency within the energy and estrogen deficient women was indicated by a lower E1G AUC compared to the energy deficient and estrogen replete women. Additionally, both

groups of estrogen deficient women demonstrated suppressed E1G mean concentrations and PdG AUC and mean concentrations compared to estrogen replete women. Furthermore, estrogen deficient women were younger, had a lower BMI, older age of menarche, and younger gynecological age than estrogen replete women. Gynecological age was also affected by energy status such that energy deficient women had a younger gynecological age than energy replete women. In a separate study [184], a younger gynecological age was observed to suppress LH pulsatility in response to energy restriction, and therefore may have contributed to altered bone turnover dynamics in our energy deficient women since suppressed LH pulsatility is associated with amenorrhea [185, 186]and in turn, low BMD [21, 22, 24, 154-159].

In contrast with previous studies in amenorrheic women [21, 22, 24, 154-159] and women with anorexia nervosa [41, 42] that observed net bone loss, bone balance was greater than 1 in all of our groups and representative of net bone formation despite a decreased index of bone formation within the energy and estrogen deficient women. Although bone balance across all groups was indicative of net bone formation, bone turnover rate was decreased when energy deficiency was adequate enough to suppress reproductive status, suggesting a downregulation of overall bone turnover. It is unclear how a downregulation of bone turnover is related to clinically relevant diagnostic criteria, such as BMD, so future studies are encouraged to investigate how decreasing or increasing bone turnover rate or bone balance impact BMD.

One limitation of this study is that the mathematical models used to assess bone turnover dynamics are not validated. To improve the usefulness of the algorithms in women with the Triad, future studies should aim to relate bone turnover dynamics with BMD outcomes. Specifically, investigations should be completed using DXA to measure BMD at weight-bearing and non-weight-bearing skeletal sites, as well as the whole body and lumbar spine for premenopausal

women ≥ 20 years and < 20 years , respectively, per the recommendations by the Female Athlete Coalition [45], alongside mathematically derived bone turnover dynamics to evaluate the relationship between measures of BMD and bone balance and bone turnover rate. In doing so and with further investigation, the mathematically derived bone turnover dynamics used in this study could provide an additional way in which clinicians identify female athletes and exercising women at risk for bone loss related injuries and diseases associated with the Triad, in addition to DXA-measured BMD [45]. Indeed, De Souza et al. [27]demonstrated that bone turnover markers were a more sensitive measure of bone metabolic activity compared to DXA-measure BMD. Corroborating bone turnover markers with mathematical models to assess bone turnover dynamics could identify at risk women sooner than allowed by DXA.

The novelty of our study lies in the use of mathematical models to determine bone balance and bone turnover rate, as well as the index of bone formation and resorption from PINP and sCTx concentrations, respectively, in our group of healthy, exercising women with a range of energy and estrogen status. These mathematical models provided a more thorough and complete interpretation of bone metabolism, such that we were able to distinguish the relationship between energy and estrogen status on bone turnover dynamics. According to our results, energy status appears to be an important contributor to altered bone turnover dynamics in women with menstrual disturbances, underlining the importance of maintaining an adequate EA for the benefit of a healthy skeleton.

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Academic Vita of Andrew Philip Oneglia

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EDUCATION

Bachelor of Science in Kinesiology, **Pennsylvania State University**, May 2019
Concentration: Movement Science
Minor: Nutrition
Honors in Kinesiology, **Schreyer Honors College, Penn State University**
Thesis*: The Impact of Estrogen vs Energy Status on Bone Balance and Bone Turnover Rate in Young Exercising Women*

CERTIFICATIONS

Certified EKG Technician (CET)
National Healthcareers Association

AWARDS, FELLOWSHIPS, GRANTS

2017 Erickson Discovery Grant ($3,500), *The Pennsylvania State University*

RESEARCH SKILLS AND EXPRIENCES

Proficient in the conduction of biomedical human subject research to include the following skills and experiences:

Research Study Conduction
Scheduling and conducting research subject study appointments

Laboratory Skills
Conducting VO_2 Max exercise testing
Handling and processing biological samples
Performing a chemiluminescent assay on an automated iSYS platform
Analyzing flow cytometry data via FlowJo Software

Shadowing Experience
Mouse sacrificing and dissection
Assisted in an Iodine 125 isotope radioimmunoassay

ABSTRACTS-*NOT PUBLISHED*

Oneglia, A.P., Southmayd, E.A., De Souza, M.J. (2018) *The Impact of Estrogen vs Energy on Bone Balance and Bone Turnover Rate in Young Exercising Women*

Southmayd, E.A, **Oneglia, A.P.**, Mallinson, R.J., Williams, N.I., De Souza, M.J. (2018) *Low T3 is associated with decreased bone turnover rate in exercising women with eumenorrhea and amenorrhea*

PAPERS IN PREPARATION FOR REFEREED PUBLICATION

Koltun, K.J., Aurigemma, N.C., Southmayd, E.A., **Oneglia, A.P.**, Williams, N.I., De Souza, M.J. (2018) *Comparison of Female Athlete Triad Coalition and RED-S Risk Assessment Tools.* ***IN REVIEW***

CURRENT RESEARCH PROJECTS AND COLLABORATION

Comparison of Plasma vs in vitro Assessment of Inflammatory Cytokine Production – Pennsylvania State University

Probiotics and Gut Health – Pennsylvania State University

Comparison of Athlete Eligibility Decisions Based on the Female Athlete Triad Coalition and RED-S Cumulative Risk Assessment Scoring Tools – Pennsylvania State University

The Effect of Oral vs. Transdermal Contraceptive Therapy on Bone Turnover Using 41Ca Methodology – Pennsylvania State University

The Impact of Estrogen vs Energy Status on Bone Balance and Bone Turnover Rate in Young Exercising Women – Pennsylvania State University

Randomized Control Trial of Dietary Supplementation with Dried Plums on Bone Density, Geometry and Estimated Bone Strength in Postmenopausal Women – Pennsylvania State University

AWS-PSU: Active Women's Study – Pennsylvania State University

CONFERENCES

May 2017 American College of Sports Medicine Annual Meeting, *Denver, CO*

November 2016 Mid-Atlantic Regional Chapter of the American College of Sports Medicine, *Harrisburg, PA*

PROFESSIONAL EXPERIENCES

Fall 2018 Penn State Women's Soccer Team, Data Analyst

- *Working alongside coaching staff to utilize Catapult GPS units, Polar Heart Rate Monitors and Rating of Perceived Exertion in order to monitor player performance across the season and reduce the expected declines in performance and associated injuries as the season progresses*
- *Real time monitoring and analysis of heart rate and velocity data during practice and game sessions*
- *Attend coaching meetings to present data and discuss translational uses for application to the team*

LEADERSHIP SKILLS AND EXPERIENCES

2017-2019 College of Health and Human Development Ambassador

5 semesters Pennsylvania State University

Responsibilities*: Represent Health and Human Development students at events involving prospective students, alumni and other friends of the college by promoting a positive image for Health and Human Development and to develop strong relationships that enhance the overall mission of the College of Health and Human Development.*

SERVICE

2015-2019 THON, Operations Committee Member and Lieutenant

THON is a student-run philanthropy committed to enhancing the lives of children and families impacted by childhood cancer. Our mission is to provide emotional and financial support, spread awareness and ensure funding for critical research – all in pursuit of a cure.

Responsibilities: *Setting up equipment in the Bryce Jordan Center prior to THON weekend, maintaining a clean environment to keep THON running smoothly and efficiently, and tearing down equipment in the Bryce Jordan Center at the conclusion of THON weekend*