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ASSESSMENT AND EVALUATION OF SYSTEMIC BIOMARKERS OF

REPRODUCTIVE AND ENERGETIC STATUS IN ENDOGENOUS AND

EXOGENOUS ESTROGEN ENVIRONMENTS

A Dissertation in

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by

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ABSTRACT

The purpose of this dissertation was to explore how endocrinologic and reproductive health in premenopausal women is influenced by combined hormonal contraceptive (CHC) use and changes in body composition, respectively. In addition, we explored methods to improve the assessment of reproductive health in female populations of varying age. To this end, this dissertation includes four studies that 1) retrospectively assess the agreement between daily and reduced sample collection frequencies of estrone-1-glucuronide (E1G) and pregnanediol glucuronide (PdG) exposure, 2) prospectively assess the compliance to reduced sample collection frequencies, 3) evaluate the impact of combine hormonal contraceptive (CHC) use on hepatic insulin-like growth factor-1 (IGF-1) production, and 4) determine if non-traditional dual x-ray absorptiometry (DXA) measures of body composition predict menstrual recovery in exercising women with functional hypothalamic amenorrhea (FHA). We observed the following main findings: 1) with perfect collection all three reduced sample collection frequencies had good agreement with daily sample collection regarding E1G and PdG exposure and integrated mean; 2) adolescent girls were more compliant to collecting 3 samples/week than to 2 samples/week, which enabled us to observe evidence of luteal activity in 32% of the adolescent menstrual cycles, 3) route of CHC administration suppresses hepatic IGF-1 production, such that a reduction was observed following combined oral contraceptive (COC) use but not contraceptive vaginal ring (CVR) use, 4) the strongest predictor of resumption of menses is post-study body fat percentage in exercising young women with FHA, while post-study leg percent fat was an additional strong predictor of menstrual recovery. Overall, research evaluating reproductive status in adolescent and young, adult women can utilize a reduced sampling frequency; however, use of 2 samples/week is not recommended due to low compliance to the specified collection dates. Additionally, evaluating the long-term effect of CHC use on bone physiology requires continued, vested interest with the development of new therapies. Similar interventions with larger samples and longer in duration are necessary to confirm and expand the findings of

CHC route of administration influence on IGF-1 and bone heath, as well as in understanding whether non-traditional body composition measures are more indicative of the potential for the resumption of menses within the complex interplay of body composition and energetic signals.

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DEDICATION

To Agnes Helmink

Grand-mère, tu as toujours eu une influence de masse dans ma vie, avec ta présence autorité et encourageante. Votre force et détermination à élever 6 enfants a être forts et indépendants a ondulé à travers les générations suivantes. Je suis heureuse de partager l'ADN avec toi, mais plus que cela, je suis bénie de t'avoir dans ma vie et dans mon cœur.

Je t'aime.

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I love you.

Chapter 1

Introduction

Menses is the external sign of reproductive cyclicity in humans. From the onset of

menstruation during the adolescent years (menarche) to reproductive senesce

(menopause), the occurrence of menses at regular intervals (i.e., every 21-35 days) is

important in maintaining bone health [1-3], cardiovascular health [4-6], and reducing the risk

of ovarian [7] and breast cancer [8, 9]. Recent data has demonstrated that since the 1970s,

the average age of onset of thelarche (breast development) has decreased; however the

average age of menarche has remained relatively constant in Caucasian females [10-14],

with late age at menarche being clinically defined by the absence of menarche by age 15,

no pubertal development by age 13, or menarche that does not occur within 5 years of initial

breast development that occurs before age 10 [12, 15, 16]. Although the declining age at

initiation of thelarche is believed to be due to improvements in nutritional and general health

conditions [10-14], the association among a western lifestyle (high dietary fat and low fiber

intake), increased occurrence of obesity, lower levels of physical activity, and unchanged

age at menarche remains elusive [17]. Further, the impact of earlier thelarche on the

maturation of the hypothalamic-pituitary-ovarian axis and propensity for young girls to

experience irregular (long and inconsistent) menstrual cycles is unclear [18, 19].

Involvement in regular physical activity is important for general health; however, many

women who are physically active are at risk for developing menstrual dysfunction [20, 21].

This includes physical activity associated with occupational work and military training or

combat. The failure of physically active women to ingest adequate calories to meet their

energy expenditure needs results in an energy deficit and associated metabolic adaptations,

a process well described by Wade and Schneider [22]. Indeed, energy deficiency or low

energy availability causes compensatory adjustments to energy partitioning such that

energy is sequestered to physiological processes that ensure survival, such as cell

maintenance, thermoregulation, immunity and locomotion, and energy is shunted away from non-essential compartments, such as growth and reproduction [22]. Energy deficiency has been associated with clinical sequelae in a syndrome called the Female Athlete Triad [23]. The Female Athlete Triad symptomology includes the clinical sequelae of infertility (menstrual dysfunction) [20, 21] and poor bone health (skeletal demineralization and stress fractures) [24-27], as well as results in endothelial dysfunction [4, 28].

Chronic energy deficiency promotes shifts in energetic compartments (i.e., suppression of resting energy expenditure (REE)) and metabolic hormones (i.e., reductions in total triiodothyronine (TT_3), leptin, insulin-like growth factor-1 (IGF-1), insulin, and glucose and increases in ghrelin, peptide YY (PYY), adiponectin, cortisol, and growth hormone (GH)) in order to restore a eumetabolic state [29-32]. One or more of the metabolic hormones altered in chronic energy deficient states may be the signal(s) in the cascade of alterations that disrupts gonadotropin releasing hormone (GnRH) pulsatility [33]. Accordingly, a disruption of GnRH due to energetic and metabolic adjustments results in a decrease in the luteinizing hormone (LH) pulse frequency and an increase in the LH pulse amplitude, which impacts the production of the ovarian steroid hormones estradiol and progesterone [34-39]. Indeed, chronic energy deficiency has been linked to disruptions in LH pulsatility [40] and menstrual cyclicity [38, 41] in humans and in animals. Specifically, subclinical (luteal phase defects or anovulation) [42] or clinical menstrual disturbances (oligomenorrhea or functional hypothalamic amenorrhea (FHA)) [21, 43] in exercising women may be induced by an energy deficit.

According to the Female Athlete Triad Coalition, recovery of normal menstrual function is a primary concern for exercising women with FHA, [44, 45], as menstrual recovery is linked to improvement of additional clinical outcomes associated with FHA, including hypercortisolemia, growth hormone resistance, and reduced skeletal mass [46-48]. Increases in energy availability typically precede the recovery of menstrual function and of these additional impacted physiologic systems [31, 32, 38, 49], albeit at varying rates

depending on the severity and duration of the initial energy deficit [50-59]. An increase in energy availability can alter metabolic profiles in days to weeks, with the concomitant increases in body weight and body fat occurring over weeks to months. An increase in body weight and body fat is likely the most clinically significant factor associated with recovery of menstrual function in exercising women [52, 53, 58-60] and in women with anorexia nervosa [47, 54, 55, 61, 62]; however, the target weight recovery range for menstrual resumption has yet to be clearly defined [63, 64]. Additionally, increases in leptin, secondary to increases in fat mass, have been demonstrated to be an important metabolic factor in restoration of LH pulsatility and thus, menstrual recovery [65, 66], while increases in IGF-1 have been demonstrated to be a valuable indicator of recovery from an energy deficit [51, 67, 68]. Women with FHA should be counseled that resumption of menstrual function is dependent on weight recovery [46] and that rather than simply the single occurrence of menses, recovery also includes resumption of consecutive cycles of regular length and recovery of ovulation. As such, menstrual recovery may take months to a year or more to occur in full [50, 54, 69]. Though these more detailed cycle characteristics, such as cycle length and ovulation, have been associated with menstrual recovery, information is not available regarding the progression of menstrual cycle lengths and ovulatory status following the initial menses recovery cycle or how estrogenic activity presents immediately prior to menstrual recovery in exercising amenorrheic women.

Additionally, young women augment menstrual function through use of hormonal contraception. Since the approval of the first oral contraceptive pill in the 1960's millions of women worldwide have used combined hormonal contraception (CHC) [70, 71]. Currently available CHC options include combined oral contraception (COC), transdermal contraceptive patch (TDC), and the contraceptive vaginal ring (CVR) [72]. Between 2011 and 2013 approximately 62% of women age 15-44 in the United States were currently using contraception [73], of which approximately 28% were using some form of hormonal contraception, combined or progesterone only [73]. Efforts to reduce the negative

thromboebolytic side effects to estrogen exposure observed in early COC formulations have resulted in a progressive lowering of the estrogen dose since the 1980's [74]. However, the impact of the lower estrogen exposure on other body systems is unclear or unknown. Specifically, there are concerns that the ultra-low-dose estrogen [<20μg ethinyl estradiol (EE)] formulations in COCs are insufficient to support bone health [75-78]. Since COC preparations are adopted by more women than other forms of hormonal contraception (16% COC, 7.2% long-acting reversible contraception, 4.4% injectable/vaginal ring/patch [73]), understanding the negative outcomes on bone are critically important. Additionally, understanding whether route of CHC administration leads to differences in side effects to other body systems, like bone, is necessary.

Accordingly, this chapter outlines the components of the proposed dissertation, in which the overarching purpose is to evaluate how endocrine and reproductive health in premenopausal women are influenced by CHC use and changes in body composition, as well as evaluating methods of obtaining accurate information of reproductive hormone exposure.

Study One: Reductions in urinary collection frequency for assessment of reproductive hormones provide physiologically representative exposure and mean concentrations when compared with daily collection

<u>Background</u>

Hormonal evaluation of the reproductive potential of women in population based studies most often uses samples of blood [50, 79, 80], urine [21, 81-83], or saliva [81, 84-86], but participant training, compliance, and the cost of the assays generally limit the feasibility of these assessments. Indeed, the optimal method should yield good participant tolerance and compliance with sample collection while still providing reliable, sensitive, and specific information about the participants' reproductive status [85].

In many cases, the use of the gold standard for repeated assessment of reproductive function, i.e., daily serum sampling which involves invasive blood draws, is not feasible; therefore, the collection of daily urine samples is an attractive alternative due to its non-invasive and autonomous nature [87]. Self-collection of daily urine samples during a menstrual cycle or specified monitoring period provides information about reproductive hormone exposure and clinical endpoints of reproductive status, such as ovulation [81, 82, 88-90], pregnancy [91-94], and menstrual cycle status [21, 80, 81, 95-97] in humans and non-human primates. Exposure to reproductive hormones has more recently been shown to be an important predictor of general health and disease risk. Specifically, measures of reproductive hormone exposure from daily urinary samples have been associated with cardiovascular function in amenorrheic exercising women, specifically endothelial dysfunction, bradycardia, low systolic blood pressure, reduced regional blood flow, increased local vascular resistance, and an unfavorable lipid profile [4-6]. Further, reduced exposure to estrogen in amenorrheic exercising women, as assessed by daily urine sample collection, has been associated with increased concentrations of osteoprotegerin, an important regulator of bone resorption [3] and clinical measures of bone health [1, 2]. Risk of

ovarian cancer [98] and breast cancer [9, 99] have also been associated with exposure to reproductive hormones.

Unsupervised participants can easily collect urine samples, thus facilitating monitoring of ovarian function over extended time periods [100]. However, it has been noted that daily urine sample collection presents a substantial participant burden [101], which can contribute to increased non-compliance and study attrition [101]. Compliance with daily urine sample collection is typically high in short-term studies (1-3 months), but the potential for reduced compliance increases over time [101]; however, specific data on participant compliance to urinary collection is scant in publications. For studies lasting between 1 and 3 months, compliance to daily urinary sample collection is in the range of 92-97% [85, 102, 103]. For example, Kesner et al. [85] reported that during a time period of 2 complete menstrual cycles, 97% of all scheduled samples were collected. In the Women's Reproductive Health Study, 93% of all daily urine samples were collected over the course of 2 consecutive menstrual cycles [104].

In studies of 5 and 12 months duration, compliance to urinary sample collection is more variable and somewhat lower, ranging between 50-100% [105-107]. For example, retrospective analysis of the Study of Women Across the Nation Daily Hormone sub-study, only 680 of 848 eligible participants had collected 80% of the required samples [105]. In the Semi-Conductor Health Study, where participants were asked to collect urinary samples daily for 5 cycles, only 57% of all cycles had fewer than 3 days of missing data in any 5-day rolling window [106]. In our laboratory, the participants who completed 4 or more months of a 12-month study collected an average of 90% of the requested samples; however, individual compliance ranged from 61-100% (unpublished data).

Specific Aims, Hypotheses, and Rationale

Aim 1: To evaluate if a reduction in the number of collection days from 7 days per week (i.e., daily sample collection) to 5 (i.e., weekday sample collection), 3 (i.e.,

Monday/Wednesday/Friday), or 2 (i.e., Monday/Thursday) days per week would provide an accurate representation of estrone-1-glucuronide (E1G) and pregnanediol glucuronide (PdG) exposure and mean concentration during an entire menstrual cycle/monitoring period. We also sought to evaluate if the validity of the reduced sample collection frequencies would be affected by cycle type (eumenorrheic or amenorrheic) or variability of cycle lengths (20-45 day range or 26-36 day range). As such, the purpose of this analysis was to explore the average and individual agreement of daily urine sample collection vs. sample collection for 5-days, 3-days, or 2-days per week for the following variables: E1G exposure (area under the curve; AUC), E1G mean concentration, PdG exposure, and PdG mean concentration.

Hypothesis 1i: E1G and PdG cycle AUC and mean concentration would be similar when samples were collected daily vs. 5-days, 3-days, or 2-days per week for a 28-day monitoring period or a complete menstrual cycle with an inter-menstrual interval ranging from 20-45 days.

Rationale: The design of any experiment needs to balance data quantity and quality while reducing participant burden and project cost while ensuring subject compliance. To our knowledge, the only attempt to validate a reduced sampling frequency for use with urine specimens was conducted by O'Connor et al. [90], who evaluated the specificity and sensitivity of reduced collection frequencies to determine the presence of ovulation with PdG based algorithms. The 'every-other-day' reduced collection frequency accurately and precisely detected day of ovulation [90]. Thus, a reduced collection frequency could be useful in conducting research in populations who may be hesitant to participate in research projects that involve daily urine sampling, such as children or adolescents, and may aid in collection of urinary samples in locations with limited cooling and storage capacity. In large-scale and long-term research studies, reduced collection frequencies would not only reduce project cost and participant burden, but would also enable researchers to recruit from a larger geographic area due to the reduced need for storage. We chose to evaluate the impact of reduced collection frequencies on E1G and PdG exposure and mean

concentrations because both measures are important predictors of bone health [1, 2], cardiovascular health [4-6], and ovarian [98] and breast cancer risk [9, 99, 108-110].

<u>Methods, Statistical Analyses, and Expected Findings</u>

Methods: The data for this analysis were obtained from a cross-sectional study of exercising women between the ages of 18-35 years who participated in at least 2 hours of physical activity each week who were grouped according to self-reported history of menses within the past year. Women who reported no menses in the past 3 months were categorized as amenorrheic, women who reported < 10 cycles in the past 12 months were categorized as oligomenorrheic, and those who reported at least 10 cycles in the past 12 months were categorized as eumenorrheic. Participants collected daily urine samples for the duration of individual menstrual cycles (oligomenorrheic and eumenorrheic participants) or 28-day monitoring periods (amenorrheic participants) over the course of 13 months (116 participants and 572 cycles/monitoring periods evaluated for inclusion).

Menstrual cycles will be included in this analysis if they contain no missing samples or no more than 3 missing samples in the first 6 days of the cycle. Monitoring periods will be included if there were no missing samples. E1G and PdG data will be systematically removed from each cycle or monitoring period to mimic a reduced frequency of sample collection for participants with 100% compliance. The reduced collection frequencies to be evaluated are 5 days per week (week days), 3 days per week (Monday, Wednesday, Friday), and 2 days per week (Monday, Thursday). The hormonal variables to be evaluated are E1G and PdG exposure via AUC and mean concentrations during the menstrual cycles and monitoring periods.

Statistical Analyses: Data screening will be conducted prior to all analyses, involving identification of outliers and examination of variable distributions within each of the participant groups, collection frequencies, and hormone variables for normality. Participants will be grouped as all included menstrual cycles and monitoring periods (complete sample

analysis), with sub-analyses of eumenorrheic cycles of 26-36 days in length alone and 28-day monitoring periods alone. A linear mixed model ANOVA will be used to compare all ovarian steroid data (E1G AUC, E1G mean, PdG AUC, and PdG mean) between daily urinary collection and each reduced urinary collection frequency for the complete sample analysis and both sub-analyses. The same individual may provide multiple cycles or monitoring periods and therefore, these data will be considered to be of a nested nature and the participant identifier will be included as a random effect in the linear model. A significance level of alpha = 0.05 will be used to detect differences and for multiple comparisons, alpha will be adjusted using Bonferroni correction. Bland Altman analysis will be performed to determine the 95% limits of agreement and to identify potential mean and proportional bias for both AUC and mean concentration [111]. Errors will be calculated as the difference between daily urinary collection data and each reduced urinary collection data with daily urinary sample collection regarded as the criterion method. Analyses will be conducted using R statistical Software (Revolution Analytics, Palo Alto, CA, USA).

Expected Findings: O'Connor et al. [90] evaluated the sensitivity and specificity of twice-per-cycle, weekly, twice weekly, every-other day, and daily urinary specimen collection designs for estimating the presence of ovulation. It was reported that the accuracy of estimating ovulation within 2 days using the intermittent sampling ranged from 40% with weekly samples to 97% with every-other-day samples. Thus, it is expected that E1G and PdG AUC and mean concentrations will be similar between daily collections and 5-days, 3-days, and 2-days per week collection frequencies for both 28-day amenorrheic monitoring periods and for menstrual cycles ranging from 20-45 days in length.

Study Two: A pilot study on the utilization of reduced urinary collection frequency protocols for the assessment of reproductive hormones in adolescent girls

Background

Assessments of reproductive hormone characteristics across a menstrual cycle or monitoring period in women using daily sampling of blood [50, 79, 80], urine [21, 81-83] or saliva [84-86] are generally limited by participant compliance to and comfort with the daily sampling protocols as well as the cost of the assays. Indeed, the optimal method should yield good participant compliance to sample collection protocols while still providing reliable and sensitive details about menstrual cycle characteristics [85]. In adolescent girls, it is especially difficult to effectively design sampling protocols for large epidemiologic studies that yield good compliance and sufficient details regarding the hormonal characteristics and menstrual function of the peri- and early postmenarcheal time period.

Efforts to characterize different aspects of reproductive function in adolescents in the peri- and early postmenarcheal time period have utilized various methods, including menstrual calendars [112-119], daily blood [113] or saliva [116] samples for a single menstrual cycle, serial 8- [118] or 24-hour blood [120, 121] sampling, first morning urine sampling once per week for 9 weeks semiannually [114] and for 3 months [122], daily first morning urine for two 30 day periods [123, 124] and for 2 years [119], 24 hour urine [115] and blood sample [125] collection every 6 months, and single time point blood [17, 126, 127] or saliva [128] samples spread throughout the year. All current evaluations of reproductive function over 1 or more cycles using daily sampling methods are limited in the generalizability of the findings due to small sample sizes and homogeneity of ethnicity in the sample populations. Additionally, researchers who utilized less than daily sampling had larger sample sizes; however, the collection frequencies were too infrequent to allow for discussions surrounding changes in reproductive function. In an effort to determine the most

appropriate biological collection methodology to expand the literature on the adolescent menstrual cycle and its disorders in future large scale multi-ethnic population studies we conducted two pilot studies to assess feasibility and compliance to two non-daily urine collection protocols across 1-2 menstrual cycles in postmenarcheal girls and collection of once monthly samples for 6 months in premenarcheal girls.

<u>Specific Aims, Hypotheses, and Rationale</u>

Aim 2A: To assess the feasibility and compliance of collecting urinary samples in pre- and postmenarcheal girls and to determine if the abbreviated collection frequency in the adolescent girls is useful for assessing menstrual characteristics. Specifically, in the postmenarcheal participants, the purpose was to assess the feasibility and compliance of collecting 1) two urine samples per week for one menstrual cycle, 2) three urine samples per week for two consecutive menstrual cycles and 3) to compare the compliance observed in the participants that collected 2 versus 3 samples per week for one menstrual cycle.

Hypothesis 2i: There would be no difference in collection compliance between the first and second collection cycles for the participants using the three samples/week collection protocol.

Hypothesis 2ii: There would be no difference in compliance between the two samples/week and three samples/week collection protocols.

Aim 2B: To assess the feasibility of premenarcheal participants collecting once monthly samples for 6 consecutive months.

Aim 2C: To determine if the abbreviated urine sampling collection protocols can be used to assess menstrual characteristics and evidence of luteal activity.

Rationale: Daily sample collection, though the gold standard method for reproductive hormone evaluation, presents a substantial participant burden and contributes to increased non-compliance and increased dropout rates in longitudinal studies [101]. Previously, we demonstrated that daily urinary sampling to assess ovarian steroids is not required to

accurately quantify AUC or mean values of E1G or PdG across an entire menstrual cycle in a young adult population [129]. Indeed, we provided evidence that a less frequent sample collection schedule in young adult women could reduce participant burden while still providing adequate accuracy and precision for the purpose of describing general menstrual cycle characteristics [129]. Inclusion of adolescent girls in studies to evaluate the menstrual cycle, its disorders, and establishment of menstrual regularity during the initial gynecologic maturation period will require a sample collection protocol that young girls and their parents, of all ethnic backgrounds, find feasible and unobjectionable.

<u>Methods, Statistical Analyses, and Expected Findings</u>

Methods: Data for this analysis was obtained from a cross-sectional study wherein young girls between the ages of 8-17 years were recruited from the San Francisco Bay Area over a two-year period from 2013-2014 for two pilot studies. Pilot Study 1 recruited 11 girls who were participants in the California site of the LEGACY Girls Study [130] and Pilot Study 2 included 30 girls recruited through community outreach. At the time of sample collection, participants of Pilot Study 1 were between 13-17 years of age and all girls were postmenarcheal. Pilot Study 2 participants were 8-14 years of age and included 16 premenarcheal and 15 postmenarcheal girls. The Institutional Review Board at the Cancer Prevention Institute of California approved both pilot studies. The participating mother or guardian provided written informed consent and participating girls signed an assent form prior to study participation. Participating mothers or guardians completed online or mail questionnaires on demographic background, medical history, cancer family history, and early-life exposures. Trained study staff conducted a home visit to take anthropometric measurements, including standing height, weight, waist and hip circumference, and body fat percentage, and to explain the urine sampling protocol and completion of the paper calendar. Study staff sent email or text message reminders either weekly to postmenarcheal girls or monthly to premenarcheal girls.

Postmenarcheal participants were asked to collect first morning urine samples. Pilot Study 1 participants (n = 11) were asked to collect 2 samples/week for one menstrual cycle, and Pilot Study 2 participants (n = 15) were asked to collect 3 samples/week for two consecutive menstrual cycles. Participants in the 2 samples/week protocol were asked to collect samples on Mondays and Thursdays for one complete menstrual cycle, beginning with the first Monday or Thursday after the onset of menstrual bleeding [129]. Participants in the 3 samples/week protocol were asked to collect urine samples on Mondays, Wednesdays, and Fridays for two consecutive menstrual cycles, beginning on the first Monday, Wednesday, or Friday after the onset of menstrual bleeding [129]. Participants were provided with a paper calendar for the recording of the days when they collected a urine sample and the days of menstrual bleeding. Premenarcheal participants in Pilot Study 2 (n=16) were asked to collect a first morning urine sample on the same day of the month for 6 consecutive months. Participants were provided with a paper calendar for the recording of the days when a urine sample was collected.

For postmenarcheal participants, we will calculate the total number of possible samples that could have been collected (determined by menses or first/last sample collection if no menstrual calendar was returned) by each participant. The number of samples collected on the correct days of each week for each participant will also be calculated. Compliance will be determined as the number of samples correctly collected divided by the number of possible samples to be collected multiplied by 100. We will also calculate the number of participants who return their paper calendar, as well as calculate the number of participants who properly recorded the occurrence of menses and the date of each urine collection. For the Pilot Study 2 participant compliance for each menstrual cycle will be calculated separately and for both cycles combined. For premenarcheal participants, we will calculate the compliance to collection of 6 samples as well as the percentage of samples collected on the same day each month. Additionally, we will determine how many samples were collected early or late.

Menstrual cycles of postmenarcheal participants will be analyzed for E1G and PdG exposures, which will be determined by calculating the AUC using Kaleidagraph Software (Synergy Software, Reading. PA, USA). We will also calculate mean E1G and PdG concentrations across each menstrual cycle. Evidence of luteal activity will be determined based on peak PdG measured during the second half of the menstrual cycle. If the peak PdG measured is above 2.5µg/mL, we will consider that as evidence of luteal activity [20, 21, 131].

Statistical Analyses: Data screening will be conducted prior to all analyses, involving identification of outliers and examination of variable distributions within each of the groups for normality. Data were analyzed using SPSS for Windows (version 23.0, Chicago, IL) statistical software package (independent and paired T-test, Mann-Whitney U test, and Chi-square). Compliance will be reported as a qualitative variable to summarize the feasibility and usefulness of the urine collection protocols.

Sample Size Calculation: These studies were designed as pilot studies to assess the feasibility of collecting ovarian function data utilizing urinary collection protocols for the development of future large epidemiologic studies in young girls with a family history or familial risk of breast cancer. Most prior studies, using other hormonal assessment methodologies, were based on small sample sizes and produced mixed results [132]. There is a strong ability to study variation in ovarian steroid exposure by familial risk, and other factors, in a large and ethnically diverse sample of young girls given the prospective design of the parent study from which the pilot study participants will be recruited. The participants for the 3 samples/week pilot study will be selected from a pool of 106 girls aged 10-13 years that were identified for the parent study but were not enrolled due to budget limitations. The families of these girls were alerted to future research opportunities at the time of the parent study exclusion. It is estimated that 26 of these girls will be postmenarcheal. From the remaining premenarcheal girls, 26 will be selected. A participation rate of 80% is anticipated, thus the final sample size will be 20 postmenarcheal girls and 20 premenarcheal girls. To

match the anticipated sample size of the 3 samples/week postmenarcheal pilot 26 participants from the parent study will be recruited for the 2 samples/week pilot study, with a participation rate of 80% anticipated there will be a final sample size of 20 participants. The aim for recruitment is to have the girls reflect a mixed race/ethnicity distribution of 46% non-Hispanic white, 27% Latinas, 12% Asian American, 15% African American or mixed race/ethnicity.

Expected Findings: To date, there is scant information regarding compliance to longitudinal data collection in adolescents, specifically longitudinal menstrual cycle data. Boros et al. [114] indicated "the most reliable girls provided 36 samples"; however exactly how many participants provided all 36 urine samples (1 sample/week for 9 weeks every 6 months over 2 years) is unclear. Zhang et al. [119] indicated participants were required to provide a minimum of 80% of required samples to continue participation in the study. Of their initial 10 participants there was full 2-year urine collections and informative menstrual calendar data for 6 girls, complete hormone data for an additional girl, and partial hormone data for one additional girl [119]. Bernstein et al. [112] indicated that over their 6-month study with biweekly collection of menstrual cycle calendars and physical activity assessment they had adequate data (meaning no more than 1 missing calendar in the 6-month study) available for 168 of their 174 participants. Additionally, an early morning luteal phase urine sample was collected on day 22 of 2 menstrual cycles a minimum of 3 months apart for 163 of 174 participants and 5 more participants collected a urine sample for 1 menstrual cycle only [112]. Thus, it is anticipated that both the 2 days/week and 3 days/week protocol participants will be equally compliant to sample collection. Additionally, we anticipate that premenarcheal participants will be highly compliant to once monthly sample collection.

Study Three: Oral contraceptives decrease recombinant human GH-stimulated hepatic IGF-1 secretion more than non-oral contraceptives in healthy young women

<u>Background</u>

Optimal bone development in women occurs in the first three decades of life [133-135]. During bone development, many genetic and environmental factors (steroid hormones, nutrition, and exercise) influence bone accrual [135]. Estrogen exerts a complex impact on bone growth and maturation from puberty onward [136], and secretion of this osteogenic hormone varies across the menstrual cycle in women [137]. However, in 2010 approximately 98% of sexually active women reported having ever used some form of combined hormonal contraceptives (CHCs) [138], which contain a combination of an estrogen and progestin, thereby suppressing endogenous estrogen secretion, potentially having downstream effects on bone. Between 2011 and 2013 approximately 62% of women age 15-44 in the United States were currently using contraception [73], of which approximately 28% were using some form of hormonal contraception, combined or progesterone only [73]. Efforts to reduce the estrogenic side effects observed in early COC formulations have resulted in a progressive reduction of the estrogen dose since the 1980's [74]. There are concerns that the ultra-low-dose estrogen [<20μg ethinyl estradiol (EE)] formulations are insufficient to support bone health [75-78]. Since COC preparations are adopted by more women than non-oral forms of hormonal contraception (16% COC vs 4.4% injectable/vaginal ring/patch [73]), understanding the potential differing effects of outcomes on bone are critically important, particularly if these effects have the potential to be negative.

<u>Specific Aims, Hypotheses, and Rationale</u>

Aim 3: To assess the impact of COC and CVR therapy on hepatic insulin-like growth factor-1 (IGF-1) production compared to a non-therapy control group at natural production and in response to recombinant human growth hormone (rhGH).

Hypothesis 3i: There would be a significant decrease in basal IGF-1 concentrations following COC therapy; whereas, CVR therapy will result in less severe decreases or no change in basal IGF-1 concentrations similar to control.

Hypothesis 3ii: There would be a greater reduction in rhGH-stimulated IGF-1 concentrations [area under the curve (AUC) and peak concentration] following COC therapy than CVR therapy compared to baseline values and the control group will not change compared to baseline values.

Hypothesis 3iii: There would be a greater reduction in rhGH-stimulated IGF-1 concentrations (AUC and peak concentration) COC therapy compared to CVR therapy and control; whereas there will be no difference between control and CVR therapy groups.

Rationale: Multiple research teams have reported on prospective and cross sectional data with conflicting results on the association between COC use and bone mineral density (BMD). Increased risk of fracture [139-141] and decreases in areal BMD and volumetric BMD [142, 143] have been reported in COC users compared to never users. Other investigators have observed that BMD did not change among women taking COCs; however, the non-users demonstrated an increase in BMD, indicating that COC use may suppress bone accrual [75, 78]. These negative effects have been observed to be particularly detrimental among women as the dose of oral EE decreases [75, 144]. On the other hand, investigators have also reported no difference in BMD between COC users and non-users at multiple bone sites [145-147], highlighting the conflicting nature of COC use on bone health in young women.

Interestingly, the effects of CHCs on bone may have route of administration-dependent effects [148]. As such, the potential negative effects of COCs on BMD may not be apparent when non-oral forms of CHCs are used, including TDC and CVR. To date,

there have been a limited number of published studies exploring the impact of non-oral forms of CHCs on bone health in young women. The available evidence suggests that adolescents and young women who use TDC or CVR for 12 to 24 months have no difference in BMD or change in BMD compared to non-users [149-151].

The mechanism for the potential harmful effects of route of CHC administration on BMD likely involves the hepatic growth hormone (GH)/IGF-1 axis. The COC pathway of absorption results in the "first pass" effect, where the EE dose is metabolized in the liver; this metabolism is known to affect the synthesis of hormones, growth factors, and binding proteins produced by the liver [152, 153]. Systemic IGF-1 is predominantly synthesized in the liver and the secretion is GH dependent [154-156]. The GH/IGF-1 axis provides the main stimulus for regulation of bone growth [157], thus understanding the impact of CHC administration on the axis is of vital importance. Investigators have observed that oral administration of COCs may decrease IGF-1 synthesis [158, 159]; however, these results have not been consistent [158, 160-162]. As such, it is speculated that the "first-pass" effect of orally administered EE suppresses IGF-1 production, a key osteogenic growth factor, and upregulates the synthesis of binding proteins, further reducing hormone bioavailability [153]. It must be noted, however, that estrogens administered transdermally or vaginally are absorbed directly into the systemic circulation, which circumvents the hepatic portal circulation and may exert less negative effects on liver synthesis than oral estrogens [149, 163, 164].

A second mechanism possibly contributing to the observed suppression of IGF-1 concentrations is a reduced hepatic response to GH. A novel test, the IGF-1 Generation Test, is used to test the hepatic responsiveness to GH. Administration of rhGH has the potential to amplify subtle differences and abnormalities in the liver that are not otherwise detectable with examinations of fasting basal concentrations. Various forms of the IGF-1 Generation Test have been used in disease states [165, 166] and in postmenopausal women undergoing hormone therapy [167, 168] to assess the responsiveness and/or

sensitivity of the liver to GH. In postmenopausal women utilizing various dose and preparations of oral estrogen therapy a minimum of a 20% reduction in peak IGF-1 production in response to rhGH injection has been observed [167, 168], while low-dose transdermal estradiol (50µg/day) therapy had no impact on peak IGF-1 production in response to rhGH injection [168]. These results indicate that the suppression of IGF-1 may be due to reduced liver responsiveness to GH. In premenopausal women, Gleeson et al. [169] demonstrated a decrease in peripheral GH responsiveness with increasing endogenous estradiol in the natural menstrual cycle from mid-cycle onward, using an IGF-1 Generation Test. The IGF-1 Generation Test, however, has never been performed prospectively in premenopausal women taking CHCs.

To date there are no direct comparisons of COC and CVR (or other non-oral) therapies, with a contemporary control group, that explore the influence of contraceptive therapy use on the GH/IGF-1 axis in healthy young women. Additionally, responses to an IGT have never been compared in premenopausal women taking COC and CVR to date.

Methods, Statistical Analyses, and Expected Findings

Methods: This study was a prospective, open label, randomized control study examining the impact of CHC use on hepatic production if IGF-1 in young women aged 18 to 30 years completed at two sites, the Pennsylvania State University (PSU; n = 17) and Purdue University (n = 8). Assessments were completed during a natural menstrual cycle (baseline) and following two contraceptive cycles of either COC or CVR or two natural menstrual cycles (approximately 42 days) in a control group (post-therapy). The impact of COC vs CVRs on IGF-1 production was assessed using a modified IGF-1 Generation Test, where 4 injections of rhGH were utilized as an endocrine probe to amplify effects not observable with fasting concentrations. The Institutional Review Boards of PSU and Purdue University approved the study protocol. All participants signed informed consent prior to initiating screening procedures.

Women not currently using hormonal contraceptives were recruited. Eligibility included: 1) age 18 to 30 years, 2) BMI 18 to 29kg/m^2, 3) non-smoking, 4) naïve to hormonal contraceptives or not using hormonal contraceptives for at least 6 months prior to study entry, 5) not lactating, pregnant or intending to become pregnant in the next 6 months, 6) no apparent metabolic, endocrine, musculoskeletal, or severe psychiatric disease, 7) if physically active the primary mode was required to be weight bearing, 8) able to maintain current exercise training and diet, and remain weight stable (±2kg), and 9) at least 9 menses in the past 12 months.

The study had three periods: baseline, intervention, and post-therapy. *Baseline* occurred during the first natural menstrual cycle. Each participant completed an IGF-1 Generation Test, started taking calcium (up to 1000mg/d) and vitamin D (800IU/d) supplements based on estimated daily calcium intake (Brief Calcium Assessment Tool), completed an aerobic fitness assessment (VO$_{2peak}$), a dual x-ray absorptiometry (DXA) test to assess body composition, weekly body weights, menstrual calendar, and seven-day exercise training log. Baseline testing began between days 2 and 7 of the participants' natural menstrual cycle. During week three of baseline participants desiring contraception were randomized to either COC or CVR treatment groups. The *intervention period* began on the first day of the second or third natural menstrual cycle following the baseline menstrual cycle (due to scheduling the post period testing). The intervention period was completed during one natural menstrual cycle for the control group or during 1.5 contraception cycles if randomized to either COC or CVR groups. The duration of 1.5 contraception cycles was necessary to 1) ensure a minimal yet adequate treatment period, 2) allow ample time to schedule all follow-up testing, and 3) ensure post study testing occurred while COC or CVR therapy was not interrupted with a placebo period. During the intervention period participants had weekly body weights, continued taking calcium and vitamin D supplements, completed a seven-day exercise training log, menstrual/contraceptive therapy calendar. *Post-therapy* testing began between days 2 and 7 of the menstrual cycle following the

intervention period menstrual cycle for the control participants or between days 15 and 17 of the second contraception cycle for the CHC participants. During the *post-therapy* period, participants completed an IGF-1 Generation Test, continued taking calcium and vitamin D supplements, continued taking CHC therapy (COC or CVR if randomized to CHC), completed a seven-day exercise log, and a menstrual/contraceptive therapy calendar.

Participants not desiring hormonal contraception self-selected into the control group (n = 8). Participants desiring hormonal contraception were randomized to use either a monophasic COC (Reclipsen Actavis plc, Parsippany-Troy Hills, NJ; n = 7) or CVR (NuvaRing™, Merck, Kenilworth, NJ; n = 8) for two contraceptive cycles. The COC released 30µg EE and 150µg desogestrel and the CVR contained 15µg EE and 120µg etonogestrel. The first contraception cycle of the intervention period for both COC and CVR participants was used as marketed (21 days of active hormones and a 7 day hormone free interval) and was initiated on day 1 of the menstrual cycle. The second contraception cycle was initiated on the day immediately following a hormone free interval. For the second contraception cycle participants did not have the hormone free interval to ensure hormone exposure during the entire post period of testing. Participants were provided with additional COC or CVR to allow for continued use of the COC or CVR until the end of the post-therapy IGF-1 Generation Test.

Participants underwent a baseline and post-therapy modified IGF-1 Generation Test, with testing occurring before 0930 h for both tests. Four rhGH injections were given subcutaneously into the abdomen at a dose of 0.033mg/kg/day [170], using the weight taken the morning of the first injection. The protocol for the modified IGF-1 Generation Test was 8 days in duration, with the rhGH injections beginning on day 2 of the protocol. The baseline IGF-1 Generation Test was initiated between days 2 and 7 of the baseline menstrual cycle. The post-therapy IGF-1 Generation Test was initiated between days 2 and 7 of the menstrual cycle following the intervention period for the control group or between days 15 and 17 of the second contraception cycle of the COC and CVR groups. Participants were

asked to refrain from resistance exercise for the duration (8 days) of the IGF-1 Generation Test and to not exercise in the morning before any blood draws.

On day 1 of the IGF-1 Generation Test participants had a fasting blood draw. On day 2, participants had a urine pregnancy test performed and body weight was measured to the nearest 0.01kg. If the urine pregnancy test was negative, participants had a fasting blood draw followed by injection 1 of 4 of rhGH (Omnitrope Sandoz, Holzkirchen, Germany). On day 3, participants had rhGH injection 2 of 4. On day 4 participants had a fasting blood draw and injection 3 of 4 of rhGH. On day 5 participants had rhGH injection 4 of 4. On days 6 and 8 participants had a fasting blood draw, while day 7 had no testing. All blood draws were antecubital for the purposes of evaluating IGF-1 concentrations.

Participant compliance was monitored via assessment of daily menstrual calendars on which the participant monitored menstrual symptoms, side effects of the COC or CVR therapy (if randomized to CHC use), as well as time of COC pill ingestion or day of CVR insertion. Compliance was also monitored by way of returned COC and CVR packaging and a fasting blood samples taken to measures sex hormone binding globulin (SHBG), which is known to rise by an average of 40% from baseline with COC therapies not containing levonorgestrel [171, 172]. Samples were obtained between day 10 and 22 of the baseline period menstrual cycle, between days 13 and 21 of the intervention period menstrual cycle/first contraception cycle, and between days 13 to 21 of the second contraception cycle. A third compliance sample was not taken in the control group due to the post-therapy period occurring during the follicular phase not the luteal phase of the menstrual cycle.

Statistical Analyses: Analyses will be performed with SAS (version 9.4, Cary NC). Data will be assessed for normality and outliers prior to analysis. Differences in demographics and body composition between study groups will be assessed with Proc Mixed or Proc NPar1Way. Basal IGF-1 concentrations for the baseline and post-therapy IGF-1 Generation Test are the average of day 1 and day 2 IGF-1 concentrations from each IGF-1 Generation Test. Increment IGF-1 concentrations will be calculated by subtracting basal IGF-1 from the

days 4, 6, and 8 IGF-1 concentrations for each IGF-1 Generation Test. The AUC of the increment IGF-1 concentrations for the baseline and post IGF-1 Generation Tests (basal, day 4, day 6, and day 8) will be calculated using Kaleidagraph Software (Synergy Software, Reading, PA, USA). Data with multiple observations per participant (basal IGF-1, AUC of the increment IGF-1, and peak increment IGF-1) will be analyzed as repeated measures (study period) using Proc Mixed with Tukey-Kramer post hoc analyses. Repeated measures over time will be modeled with the covariance structure with the best model fit. The model will include the fixed effects of group, study period, and group*study period interaction. Denominator degrees of freedom will be estimated with user of the Kenward-Rogers method. Change in basal IGF-1, AUC of the increment IGF-1, and peak increment IGF-1 will be calculated by subtracting the baseline period values from the post period values and analyzed using Proc Mixed with Tukey-Kramer post hoc analysis. Compliance will be assessed for each study period independently utilizing Proc Mixed with Tukey-Kramer post hoc analysis or Proc Npar1way.

Power Analysis: This is a pilot study designed to obtain preliminary data for development of future proposals. Means and standard deviations from published data from other investigators were utilized to perform the sample size calculation needed to have adequate power.

For average change in basal IGF-1 concentrations, published data [153] from 12 postmenopausal women studied before and after using oral EE or transdermal estradiol therapy provide meaningful differences of 0.70U/mL and 0.47U/mL, respectively, with standard errors of 0.09U/mL and 0.04U/mL. A sample size calculation revealed that 6 women per group would provide sufficient power ($1-\beta = 0.80$) to detect differences at $\alpha=0.05$ in basal IGF-1 change. For change in peak IGF-1 concentrations with the IGT, published data [168] from 9 postmenopausal women in a cross-over study of oral estradiol valerate, low dose transdermal estradiol (50μg/d), and high dose transdermal estradiol (200μg/d) were used. Meaningful differences for oral and high dose transdermal compared to control

of 113µg/L and 71µg/L, respectively, with approximate standard deviations of 215.33µg/L and 91.13µg/L were provided. A sample size calculation revealed that 6 women per group would provide sufficient power (1-β = 0.80) to detect differences at α=0.05 in peak IGF-1 change.

Expected Findings: We expect that the young women randomized to use COC therapy will have a greater decrease in basal IGF-1 concentrations compared to those randomized to use CVR therapy. Additionally, we expect that participants using COC therapy will have significant decreased in basal IGF-1 compared to the control group, while the CVR therapy group will not significantly differ from the control group. Massa et al. [162] found no difference in basal IGF-1 concentrations between users of 20-35µg EE COCs compared to non-users. Additionally, Harel et al. [149] demonstrated no change in basal IGF-1 between young women (12-21 years old) using TDC and a control group following 6 or 12 months of TDC use. However, in two cross sectional analyses of past and current COC users, IGF-1 concentrations were greater in past users than current users, but no significant differences were reported between current users and never users [160, 161]. Blackmore et al. [158] demonstrated younger (18-21 years old) women currently using COCs had reduced IGF-1 concentrations compared to never users while older (31-40 years old) women who initiated COC use after age 25 had greater IGF-1 concentrations than never users. Jernstrom et al. [159] observed a clear IGF-1 dose response with EE doses such that the higher the EE dose the lower the basal IGF-1 concentration. Similar suppressed basal IGF-1 concentrations have been reported in postmenopausal women taking oral estrogen therapy [167]. Interestingly, two prospective reports have demonstrated basal IGF-1 concentrations are reduced during the active pill weeks compared to the hormone free interval [159, 173].

We expect that the young women randomized to use COC therapy will have a greater reduction in hepatic response to rhGH stimulation compared to those randomized to use CVR therapy. Additionally, we expect that participants using COC therapy will have significant decreased in hepatic response to rhGH compared to the control group, while the

CVR therapy group will not significantly differ from the control group. Lieberman et al. [167] were the first to demonstrate suppressed peak increment IGF-1 response to a single rhGH injection (0.1mg/kg dose) in a cross sectional study of postmenopausal women. The women on oral estrogen therapy involved in the study had been on therapy for various durations and demonstrated a 56.5% suppressed peak increment IGF-1 compared to postmenopausal women not on estrogen therapy. Similarly, Lissett et al. [168] demonstrated, in a 6 week cross-over study of three different estrogen formulations (oral estradiol valerate, low-dose transdermal estradiol (50μg/d), and high-dose transdermal estradiol (200μg/d)), rhGH (7mg single bolus) stimulated peak IGF-1 concentrations were suppressed by approximately 21% during the oral estrogen treatment period. Additionally, a significant reduction in peak IGF-1 of approximately 13% was observed during the high-dose transdermal estrogen treatment compared to the non-therapy period. The peak IGF-1 response to rhGH stimulation in the low-dose transdermal estrogen period did not differ from non-therapy test [168].

Study Four: The impact of non-traditional DXA measures of body composition

distribution on menstrual recovery of women with functional hypothalamic

amenorrhea

<u>Background</u>

      Exercising women with menstrual disturbances often exhibit inadequate nutritional intake or deliberate food restriction, though to a less severe degree than the observed pathological behaviors in women diagnosed with anorexia nervosa [36, 60, 174-176]. Internal and external cues often drive exercising women to maintain a low body weight, which can manifest in disordered eating and, in some instances, eating disorders [34, 36, 176, 177]. The etiology of FHA in exercising women and women with anorexia nervosa is an energy deficiency, resulting from inadequate caloric intake relative to energy expenditure [36]. The energy deficiency stimulates multiple endocrine and metabolic compensatory mechanisms that translate into hypothalamic suppression of ovarian function, which can result in amenorrhea [34, 36-38]. FHA appears to be reversible with weight gain and recovery of menstrual function is linked to improvement of other clinical outcomes, such as increased BMI and IGF-1 [36, 44-46, 48, 60, 178].

      Resumption of menstrual function is an important indicator of recovery from anorexia nervosa [179-181] and both pediatric and adult psychiatric guidelines highlight the importance of weight recovery and menses resumption in the treatment recommendations for female patients with anorexia nervosa [63, 182]. A primary concern noted in the Female Athlete Triad Coalition Consensus Statement was the necessity of the recovery of menstrual function to be a top priority for athletes with FHA [44, 45]. At present, the best traditionally evaluated anthropometric measurement that predicts functional recovery of the hypothalamic-pituitary-ovarian axis is weight restoration [44, 45, 51-59, 183], but the target weight necessary for menstrual recovery has not yet been clearly defined [63, 64]. In female anorexia nervosa patients, normalization of menstrual function has been associated with the

attainment of a BMI higher than 18 kg/m^2 [184-187]. Other researchers have utilized

expected body weight ranges in evaluating recovery of menstrual function and reported

resumption of menses when 90-95% of expected body weight is achieved [54, 188].

Similarly, Cominato et al. [51] demonstrated resumption of menstrual function in patients

when BMI's were between the 25th and 50th percentiles. Falsetti et al. [67] reported a 24.6%

increased probability in menstrual recovery in patients with anorexia nervosa with each

increase in BMI of 1 kg/m^2. Unfortunately, approximately 15% of patients who increase their

body weight and remain weight stable but do not resume menstrual function [46, 69, 189],

indicating individual susceptibility to menstrual dysfunction and resistance to resumption [51,

54, 190]. Rather than weight gain alone, an understanding of the composition (lean or fat

mass) and/or distribution (central or peripheral) of the weight gain may be important in

determining whether resumption of menstrual function will occur with weight gain.

There is little data available regarding whether the tissue composition of the

recovered weight based on traditional DXA measures of body composition (fat mass, body

fat percentage, and lean body mass) impacts or predicts menstrual recovery. Recently, Al-

Dakhiel Winkler et al. [191] reported body fat percentage was a better predictor of menstrual

recovery than fat mass or lean body mass in adults with a history of anorexia nervosa.

Similarly, El Ghoch et al. [192] demonstrated that body fat percentage at inpatient discharge

was the best independent predictor of menstrual recovery at 1 year of follow up, such that

one unit change in body fat percentage the odds of resumption of menses increased by

14%. A relative sparing of fat mass and body fat percentage has been associated with the

preservation of menstrual function in extremely low weight women who meet all criteria for

anorexia nervosa [193].

In 2013 the ISCD published a position stand on reporting of body composition

variables [194]. Specifically, the ISCD recommended the use of unique measures of

adiposity assessed by DXA that expand beyond the traditionally reported fat mass, body fat

percentage, and lean body mass. The non-traditional variables proposed included

android/gynoid (A/G) ratio, trunk/leg (T/L) ratio, fat mass index (FMI), and lean mass index (LMI) [194], based on the prevailing idea that the pattern of adipose distribution is more clinically relevant than the total quantity of adipose tissue [195]. The A/G and T/L ratios are calculated using percent fat mass of the body regions. FMI and LMI are measures similar to BMI and are total body fat or lean mass in kg divided by height in m^2. There is no data available regarding the newly recommended ISCD variables of body composition and menstrual recovery [194]. These new measures are important as the pattern of body composition distribution is more clinically relevant than the total quantity of tissue in many medical conditions [195]. Miller et al. [193] demonstrated that in addition to fat mass and body fat percentage, sparing of trunk fat mass was associated with preservation of menstrual function in extremely low weight women who meet all criterial for anorexia nervosa. To date, no data is available regarding the impact of body composition on the increase in weight nor the distribution of body composition on resumption of menstrual function in exercising women with FHA.

Specific Aims, Hypotheses, and Rationale

Aim 4a: To determine if traditional measures of anthropometry (weight, BMI, fat mass (FM), body fat percentage (%BF)) or DXA derived non-traditional measures of anthropometry (LMI, FMI, A/G ratio, T/L ratio) are better predictors of menstrual recovery.

Hypothesis 4i: Women with FHA who recover menstrual function will have greater increases in body weight and BMI by the time of recovery than FHA women who do not recover menstrual function by the end of the intervention.

Hypothesis 4ii: Women with FHA who recover menstrual function will have greater increases in gynoid, leg, and FM and %BF by the time of recovery than FHA women who do not recover menstrual function by the end of the intervention.

Hypothesis 4iii: Women who recover menstrual function will have smaller increases in lean mass, based on LMI, compared to fat mass, based on FMI, by the time of recovery than women who do not recover menstrual function by the end of the intervention.

Hypothesis 4iv: Decreases in non-traditional DXA measures of anthropometric distribution (A/G ratio and T/L ratio) will increase the odds of menstrual recovery in women with FHA than increases in other non-traditional DXA and traditional anthropometric measures (FMI, LMI, %BF, FM, BMI, and body weight).

Rationale: The target weight necessary for menstrual recovery has not yet been clearly defined [63, 64]. In female anorexia nervosa patients, normalization of menstrual function has been associated with the attainment of a BMI higher than 18 kg/m^2 [184-187]. Other researchers have utilized expected body weight ranges in evaluating recovery of menstrual function [54, 188]. Golden et al. [54] reported resumption of menses within 6 months when 90% of expected body weight was achieved. Faust et al. [188] reported resumption of menstrual function in 2/3 of patients when 95% of expected body weight was achieved. In adolescents, Cominato et al. [51] demonstrated resumption of menstrual function in patients when BMIs were between the 25th and 50th percentiles. Falsetti et al. [67] reported a 24.6% increased probability in menstrual recovery in patients with anorexia nervosa with each increase in BMI of 1 kg/m^2. However, some patients do not resume menstrual function even with increases in weight and attainment of weight stability [69, 189]. Thus, an understanding of the composition (lean or fat mass) and/or distribution (central or peripheral) of the weight gain may be important in determining whether resumption of menstrual function will occur with weight gain.

Gains in fat mass, determined by percent body fat, have been demonstrated to be associated with menstrual recovery in anorexia nervosa [191, 192], rather than total fat or lean mass. Similarly, in cross sectional analyses, a relative sparing of fat mass (fat mass, trunk fat mass, and body fat percentage) has been associated with preservation of menstrual function in extremely low weight women who meet all criteria for anorexia nervosa

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[193]. Fat mass has been shown to be directly associated with circulating concentrations of leptin, an adipocyte-derived hormone [196-198]. Leptin has been suggested to play a role in the coordinated response of reproductive function to metabolic fuel availability [199], with leptin receptors having been isolated on the hypothalamus [200, 201], anterior pituitary [202], ovary [203], and endometrium [204]. Increases in leptin concentrations above 1.85 ng/mL have been demonstrated to be a metabolic factor important in menstrual recovery [65, 66]. Cominato et al. [51] demonstrated increased leptin, LH, and estradiol, in association with the increase in BMI in adolescent anorexia nervosa patients. Thus, changes in leptin indicate a change in fat mass, which may be associated with resumption of menstrual function in an athletic population. However, increased central adiposity, measured by T/L ratio, has been associated with a reduction in total menstrual cycle estradiol and progesterone in a cross sectional study of eumenorrheic women [205]. The non-traditional DXA LMI variable may be most useful as a discriminatory marker of undernutrition [206-208].

Methods, Statistical Analyses, and Expected Findings

Methods: This study is a longitudinal analysis of exercising women with amenorrhea who participated in a randomized controlled trial (RCT) designed to investigate the effects of a 12-month intervention of increased caloric intake on menstrual function and bone health among women with severe exercise-associated menstrual disturbances. For this RCT, women with amenorrhea (no menses in the past 3 months) who participated in at least 2 hours of physical activity per week were randomized into one of two groups. The exercise associated menstrual disturbances (EAMD)+Calories group was instructed to increase caloric intake and maintain their habitual exercise habits, while the EAMD Control group was instructed to maintain their habitual eating and exercise habits during the intervention. Confirmation of amenorrhea was obtained from assessment of urinary metabolites of reproductive hormones in daily urine samples collected over a 28-day monitoring period

during the baseline period, prior to the start of the intervention. Participants will be grouped as achieving menstrual resumption (FHA-R) or not achieving menstrual resumption (FHA-NR) during the study intervention. Resumption will be defined as occurrence of menses with the subsequent menstrual cycle being less than 90 days in length.

Participant anthropometrics were measured during the study, weight was measured to the nearest 0.01kg, and height was measured to the nearest 0.1cm. Body weight was taken every week during baseline and averaged for the baseline value and repeat body weights were taken at the beginning of every 4-week intervention period. DXA scans of the total body were performed at baseline and at every three months during the intervention to assess changes in body composition. Participants from the University of Toronto cohort were scanned using a GE Lunar Prodigy (enCORE 2002 software version 6.50.069; n=8), early participants in The Pennsylvania State University cohort were scanned using a Hologic QDR45000W DXA scanner (Hologic Inc. Bedford MA; n=5), and later participants in the Pennsylvania State University cohort were scanned using a GE Lunar iDXA (enCORE 2008 software version 12.10.113; n=19). A cross calibration study was performed to remove systematic bias between the systems, consistent with the ISCD guidelines, and all systems were calibrated to the iDXA. Equations were derived using simple linear regression to remove biases. Additional measures evaluated will be fat mass, lean body mass, and trunk and leg fat percentage. The T/L ratio will be calculated as percent trunk fat/percent leg fat. The FMI and LMI variables will be calculated as fat mass in kg/height in m^2 and lean body mass in kg/height in m^2.

Participants collected daily urine samples for the assessment of estrogen and progesterone exposure and completed menstrual calendars to associate menstrual status with hormone exposure. Urine samples were analyzed for E1G and PdG concentrations. Participant exposure to estrogen and progesterone were calculated as AUC of E1G and PdG, respectively, for the entire menstrual cycle and for days 2-12 for E1G. Peak E1G and

PdG concentration and day of peak concentration, as well as mean concentrations across the menstrual cycle of E1G and PdG, and for days 2-12 of E1G, were calculated.

Statistical Analyses: Analyses will be performed with SPSS for windows (version 23, Chicago, IL). Data will be assessed for normality and outliers prior to analysis. Baseline demographic characteristics will be analyzed with independent Student t-tests or Wilcoxon Signed-Rank test. Independent and paired Student t-tests, with a Bonferroni adjustment for multiple t-tests (p=0.025), will be used to assess reproductive hormones between groups. Change and percent change in anthropometrics will be calculated as change to resumption for FHA-R (measurement at resumption minus baseline measurement) and change to post-study for FHA-NR (measurement at end of study minus baseline measurement). Student t-test will be used to assess differences in anthropometry between FHA-R and FHA-NR at baseline and post-study. Correlation analyses will be performed to determine the associations among baseline, post-study, or change in continuous anthropometry measures with recovery of menstrual function. Forward stepwise logistic regression (entry p=0.05, exit p=0.10) will be used to determine predictors of menstrual recovery and odds ratios calculated from parameters of covariates. Predictors will be chosen based on the literature as well as those that had significant correlation to resumption of menses in our sample.

Power Analysis: Means and standard deviations from published data from other investigators were utilized to perform the sample size calculation needed to have adequate power.

For FM and FMI, published data [192] from 50 anorexia nervosa patients pre and post inpatient treatment provided a meaningful difference of 8.1 kg and 3.1 kg/m^2, respectively and standard deviations of 4.55 kg and 1.7 kg/m^2. A sample size calculation revealed that 5 women per group provide sufficient power (1-β = 0.80) to detect differences at α=0.05 in FM and FMI. Similarly, Winkler et al. [191] reported a meaningful difference of 2.3kg and standard deviation of 0.24kg for FM between 52 amenorrheic and 61 menstrual

recovered anorexia nervosa patients. A sample size calculation revealed that 4 women per group provide sufficient power $(1-\beta = 0.80)$ to detect differences at $\alpha=0.05$ in FM.

For body fat percentage, trunk fat mass, android fat mass, and gynoid fat mass meaningful differences of 13.9%, 14.8kg, 18.3kg, and 18.6kg, respectively, and standard deviations of 8.8%, 9.05kg, 10.7kg, and 11.6kg were observed [192]. A sample size calculation revealed that 6 women per group provide sufficient power $(1-\beta = 0.80)$ to detect differences at $\alpha=0.05$ in body fat percentage, trunk fat mass, android fat mass, and gynoid fat mass. In 52 amenorrheic and 61 menstrual recovered anorexia nervosa patients a difference of 3.7% was reported for body fat percentage with a standard deviation of 0.43% [191]. A sample size calculation revealed that 4 women per group provide sufficient power $(1-\beta = 0.80)$ to detect differences at $\alpha=0.05$ in FM and FMI.

Expected Findings: We expect that FHA women who experience menstrual recovery will have greater increases in body weight, BMI, lower body (leg) fat mass, overall fat mass and body fat percentage than FHA women who do not recover menstrual function. The increase in overall fat mass will be demonstrated by increased body fat percentage and FMI, as well as a decreased LMI. Since fat mass is strongly associated with leptin concentrations and leptin has been associated with the coordinated response of reproductive function to metabolic fuel availability [199], we expect that menstrual recovery will be predicted by the addition of adipose tissue in the leg region compared with addition of adipose tissue in the trunk region and overall increases in body mass, adipose tissue, or lean mass. These expected findings are in agreement with the findings of menstrual recovery in female patients with anorexia nervosa [51, 54, 67, 184-188]. These findings will assist in further defining concrete clinically assessable variables that are predictive in menstrual recovery.

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Chapter 2: Part 1

Allaway HCM, Southmayd EA, De Souza MJ. The Physiology of Functional Hypothalamic Amenorrhea associated with Energy Deficiency in Exercising Women and in Women with Anorexia Nervosa. *Horm Mol Biol Clin Investig.* 2016; 25(2):91-119.

Abstract

An energy deficiency is the result of inadequate energy intake relative to high energy expenditure. Often observed with the development of an energy deficiency is a high drive for thinness, dietary restraint, and weight and shape concerns in association with eating behaviors. At a basic physiologic level, a chronic energy deficiency promotes compensatory mechanisms to conserve fuel for vital physiologic function. Alterations have been documented in resting energy expenditure and metabolic hormones. Observed metabolic alterations include nutritionally acquired growth hormone resistance and reduced insulin-like growth factor-1 concentrations; hypercortisolemia; increased ghrelin, peptide YY, and adiponectin; and decreased leptin, triiodothyronine, and kisspeptin. The cumulative effect of the energetic and metabolic alterations is a suppression of the hypothalamic-pituitary-ovarian axis. Gonadotropin releasing hormone secretion is decreased with consequent suppression of luteinizing hormone and follicle stimulating hormone release. Alterations in hypothalamic-pituitary secretion alters the production of estrogen and progesterone resulting in subclinical or clinical menstrual dysfunction.

Introduction

Since the early 1980's, much has been learned about the association of energy deficiency and functional hypothalamic amenorrhea (FHA) in young women with under-nutrition or deliberate starvation [1-8]. Exercising women with menstrual disturbances often exhibit inadequate nutritional intake or deliberate food restriction, though to a less severe degree than the pathological eating behaviors observed in women with anorexia nervosa (AN) [7-10]. Exercising women are often driven by both internal and external cues to maintain a low body weight, often manifesting in disordered eating and in some cases, eating disorders [4, 8, 11, 12]. The etiology of amenorrhea in both exercising women and anorexic women with FHA is inadequate caloric intake resulting in energy deficiency and, in the case of AN, severe under-nutrition [8]. The energy deficiency, in turn, stimulates compensatory mechanisms, such as weight loss and energy conservation, that translates to hypothalamic suppression of ovarian function and resultant amenorrhea amidst a constellation of endocrine and metabolic adaptations [4, 8, 13, 14]. FHA is associated with infertility that largely appears reversible with weight gain and resumption of menses [7, 8, 15]. The clinical consequences of FHA and the associated endocrine alterations include low bone density and bone strength and an increase in the risk of fractures [7, 16, 17]. This review aims to synthesize the available data and provide a comprehensive understanding of the endocrinological alterations associated with amenorrhea in exercising women and in women with AN. This review examines the impact of altered metabolism, to include changes in resting energy expenditure (REE), thyroid hormone, gastrointestinal peptides, adipokines, cortisol, and the growth hormone (GH)/insulin-like-growth factor (IGF) axis, on induction of reproductive dysfunction. Additionally, the role of disordered eating psychopathology in exercising women to include behaviors such as cognitive dietary restraint and a high drive for thinness, in the development of metabolic alterations associated with FHA will be examined.

The Normal Menstrual Cycle and Hypothalamic-Pituitary-Ovarian Activity

The Basics: To understand the physiological changes observed with menstrual

disturbances, such as amenorrhea, an appreciation of the normal menstrual cycle and

optimal reproductive function is required (**Figure 2.1**). The menstrual cycle is defined as the

time from the day of the onset of menses to the day before the next onset of menses [18].

During the menstrual cycle the recruitment of small antral follicles into a growing phase

occurs at regular intervals [19-22]. A eumenorrheic menstrual cycle is defined as the

occurrence of menses at consistent intervals every 21-35 days, with the average menstrual

cycle lasting 28 days [23-27]. The menstrual cycle is divided into two main phases: the

follicular phase and the luteal phase [18]. Ovulation is the mid-cycle event that separates the

follicular and luteal phases, with the follicular phase beginning at the onset of menses and

the luteal phase beginning the day after ovulation. In women between the ages of 19-42 it

has been demonstrated that the average length of the follicular phase is 14.6 days and the

average length of the luteal phase is 13.6 days [28, 29]. Menstruation, the external sign of

menstrual cyclicity, occurs at the end of the luteal phase and the beginning of the follicular

phase (the luteo-follicular transition). The duration of menstrual bleeding last from 3-6 days

in 80% of menstrual cycles, and the heaviest menstrual flow is usually reported on the

second day [24, 30]. During each menses, the average loss of blood is 43.4 mL [31].

Neuroendocrine control of the normal menstrual cycle begins with the secretion of

gonadotropin releasing hormone (GnRH) from the arcuate nucleus of the hypothalamus

[32], which stimulates the pulsatile release of luteinizing hormone (LH) and follicle

stimulating hormone (FSH) from the gonadotroph cells in the anterior pituitary gland [32, 33].

Both FSH and LH stimulate the ovarian production of estrogen (in the follicular and luteal

phases) and progesterone (in the luteal phase) [32]. The rhythmic and acute secretory

actions of GnRH have been demonstrated in classic experiments conducted in *rhesus*

monkeys [34, 35]. The release of GnRH typically occurs every 60-90 minutes, leading to
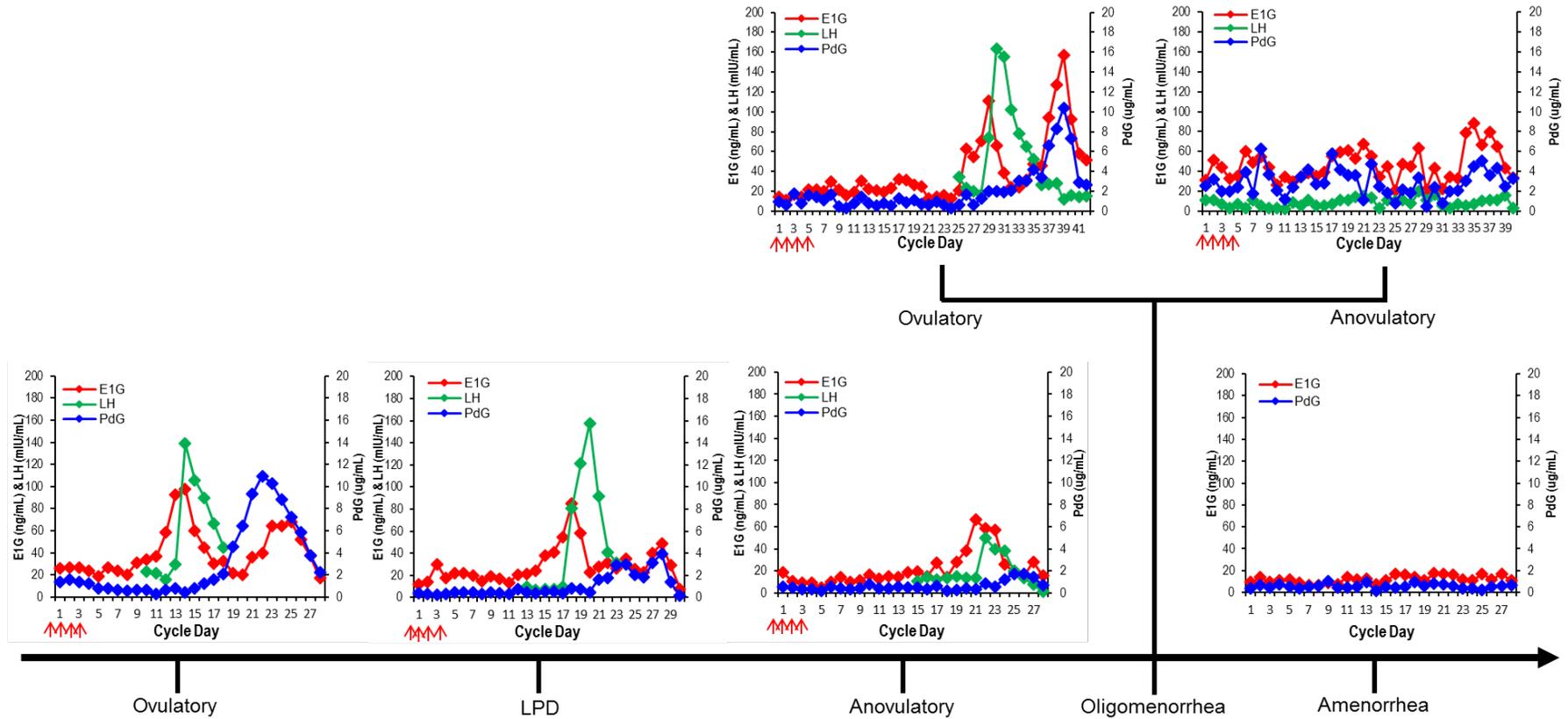
Figure 2.1: Representative examples of daily urinary reproductive hormone excretion in premenopausal women across the spectrum of menstrual function and dysfunction. The mean daily E1G and PdG concentrations are displayed in eumenorrheic ovulatory, LPD, and anovulatory menstrual cycles, oligomenorrheic ovulatory and anovulatory menstrual cycles, and in amenorrheic monitoring periods of 28 days. LPD = luteal phase defect; E1G – estrone-1-glucuronide, PdG = pregnanediol glucuronide; red arrow = menses

secretion of gonadotropins from the anterior pituitary approximately once every hour [18, 32, 36].

Follicular Growth and Development: During the menstrual cycle women exhibit wave patterns of follicle development, which can be defined as minor or major waves [21, 22, 37, 38]. Most women exhibit two waves of follicle growth (68%) while the remaining 32% exhibit three waves of follicle growth during the menstrual cycle [19, 37]. Each wave of follicle growth is preceded by an elevation in circulating FSH [19]. Major waves are defined as waves in which a dominant follicle is selected at 10mm in diameter to continue growth while all other follicles undergo atresia [19]. In minor waves, the selection of a dominant follicle does not occur and none of the follicles grow larger than 10mm in diameter [19]. Major waves that are initiated during the luteo-follicular transition usually end in ovulation, while major and minor waves that are initiated between ovulation and menses are anovulatory [19, 39]. Women with 2-wave menstrual cycles exhibit ovulatory wave emergence, dominant follicle selection, and the pre-ovulatory LH surge earlier in the menstrual cycle than women with 3-wave menstrual cycles, leading to a shorter average menstrual cycle length compared to women with 3-wave cycles [19]. The growth of antral follicle waves consists of up to 4 stages, depending on whether the wave is major or minor, ovulatory or anovulatory.

During the luteo-follicular transition, GnRH pulsatility, and thus FSH production [19, 36], increases, stimulating the synchronous recruitment and growth of a cohort of 3-20 follicles, each between 2 and 5mm in diameter [40-44]. The recruitment of follicles into this wave requires FSH to rise 10-30% above basal concentrations [22, 38, 45-49]. During the early follicular phase, FSH pulsatility maintains a relatively high frequency [36] due to the absence of negative feedback from estradiol (E_2) and progesterone. The rise in FSH at the time of wave emergence induces expression of FSH receptors (FSHr), the aromatase enzyme, and activin, which regulate follicle growth following wave emergence [38, 43, 50-52]. Production of E_2 through the synergistic efforts of theca and granulosa cells of the ovarian follicles increases gradually during the follicular phase [36], upregulating the number

of FSHr in the maturing follicles, thus increasing the bioactive effect of FSH and the production of E_2 [36, 53]. The visible growth of ovarian follicles is largely due to the growth of the antrum, a protein and fluid filled space surrounding the oocyte and encapsulated by the layers of theca and granulosa cells [54].

The expression of aromatase increases in 6-8 mm diameter follicles [55-64], which is integral in the correlation between increasing concentrations of serum E_2 and the size of the dominant follicle [63, 65]. The selection of the follicle that will become the dominant follicle of the ovulatory wave occurs in the early to mid-follicular phase [37, 66, 67] at a diameter of approximately 10mm (occurring between menstrual cycle days 6-9) [19, 43, 63, 67]; however, selection can occur in any of the follicular waves. The number of LH receptors (LHr) on the granulosa cells of the dominant follicle increases allowing the follicle to become more responsive to LH and less FSH-dependent following selection [68-70]. At the same time that follicle selection is occurring, the secretion of FSH declines due to the negative feedback of E_2 and inhibin B production from the growing follicles, mainly the newly selected follicle, on the anterior pituitary and hypothalamus [32, 71-73]. The decline in FSH concentrations leads to a reversal in the LH:FSH ratio, which is critical to the mechanism underlying the difference between the continued growth of the dominant follicle and initiation of a static phase and/or atresia of the subordinate follicles (the remaining follicles in the recruited cohort not selected for preferential growth) [19, 41, 63, 70, 74, 75]. Serum concentrations of LH begin to increase in the mid-follicular phase assisting in the continued growth of the dominant follicle [70, 76, 77]. The dominant follicle is considered mature upon reaching pre-ovulatory size (16-28 mm), at which time the production of E_2 by the follicle increases rapidly [32, 34, 36, 78]. The rapid increase in E_2 exerts positive feedback on the anterior pituitary gonadotroph cells, sensitizing the cells to GnRH and stimulating the bolus release of LH after the E_2 exceeds a serum concentration threshold, which varies by individual, for at least 36 hours [32, 34, 36, 78].

Ovulation occurs in response to a bolus release of LH, triggered by an elevation of E2, around day 14-16 of the follicular phase [38, 79]. The LH surge typically occurs 24-36 hours after peak E_2 secretion has been attained and the surge lasts for 24-48 hours after initiation [36]. The pre-ovulatory follicle takes approximately 36-38 hours to ovulate following the onset of the LH surge in humans [80] due to the complex biochemical, morphological, and physiological changes in and around the dominant follicle that accompany ovulation [19, 37, 38, 56, 80]. One critical step of the process is the secretion of plasminogen activator by the theca and granulosa cells [81-83], which initiates the conversion of plasminogen to plasmin within the follicular fluid to generate collagenases for digestion of the follicular wall [82, 84], allowing the release of the oocyte from the follicle. The final stages of the ovulatory process are the expansion, loose reorganization, and increased vascularization of the follicular wall [85, 86]. The deep internal portion of the follicle wall thickens and the apex (a small portion of the follicle wall where enzymes digest the collagen until the tensile strength is barely able to withstand the intra-follicular pressure [87]) thins 3 hours prior to ovulation [85, 86]. A stigma forms at the weakest point of the follicular apex approximately 15-20 minutes before follicle rupture [85, 88]. The oocyte and surrounding cumulus cells release from the side of the follicle wall and float freely in the follicular fluid just prior to rupture [89]. The rupture of the follicle occurs at the stigma and within 15 seconds, approximately 50% of the follicular fluid is released [85], with complete evacuation of follicular fluid requiring 6 seconds to 18 minutes [85]. The LH surge also stimulates the initiation of progesterone synthesis in the theca and granulosa cells [90, 91].

Luteinization and the Corpus Luteum: Follicle rupture and the formation of the corpus luteum (CL) marks the beginning of the luteal phase. Luteinization is the process of CL formation through structural and functional remodeling of the theca and granulosa cells. The theca and granulosa cells transform into two populations of steroidogenically active lutein tissue [92]: thecal cells become theca-lutein while the granulosa cells become granulosa-

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lutein [36]. Additionally, an extensive capillary network forms in the layer of transforming granulosa cells [92]. The LHr on the cellular surface of transforming theca and granulosa cells allow for normal CL function [36, 92, 93]. The theca- and granulosa-lutein fill the follicle's former antral space during the process of involution and cellular hypertrophy [92, 94]. Tonic secretion of low circulating levels of LH occurs during the early luteal phase, stimulating production of progesterone and small amounts of E_2 [36, 92, 93]. To facilitate the production of progesterone, the luteinization process increases the number of cellular receptors for low density lipoprotein allowing for the intracellular transport of cholesterol [93]. The production of angiogenic factors (vascular endothelial growth factor and vascular permeability factor) by the luteinized follicle allows for the peak in vascularity to occur 7 days after ovulation, which is the approximate time of maximum progesterone secretion (10-18 ng/mL) [95, 96].

In the absence of a rise in human chorionic gonadotropin induced by pregnancy, the CL begins to regress approximately 7 days after ovulation, defining the mid to late luteal phase [30, 36, 97]. The regression of the CL is referred to as luteolysis and results in formation of the corpus albicans [36]. In luteolysis, there is a loss of progesterone production leading to declining circulating progesterone concentrations and a removal of negative feedback on the anterior pituitary [36]. Subsequently, FSH begins to rise, initiating follicular wave emergence for the beginning of the next menstrual cycle.

The Uterine Cycle: The uterus has a cycle of endometrial growth that coincides with the follicular and luteal phases of the ovarian cycle. These phases are the proliferative and secretory phases, respectively. The proliferative phase of the uterine cycle involves a rebuilding of the stratum functionalis (the functional layer of the endometrium) following its shedding during menstruation [36, 98]. The rebuilding is possible due to cells of the stratum basalis, which proliferate in response to rising concentrations of E_2 during the follicular phase and cause a thickening of the endometrium [36, 99]. By the end of the proliferative

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phase, the endometrium reaches approximately 6-10mm in thickness due to hyperplasia of the endometrial cells [36, 99]. Thereafter, stimulation by CL secretion of progesterone causes secretion of glycogen from the endometrial glandular tissue, found in the stratum spongiosa, and increases in the vascularization of the endometrial lining to support embryonic implantation should fertilization occur [36]. By the end of the secretory phase of the uterine cycle, the endometrium reaches 7-14mm in thickness [99]. In the absence of fertilization there is a decline in progesterone and E_2 production, which deprives the endometrium of hormonal support [36]. The deprivation of hormonal support causes the constriction of the spiral arteries that support the endometrium resulting in destruction of the functional layer of the endometrium [36]. Decomposition of the stratum functionalis begins at the luminal edge of the endometrium and extends to the deeper stratum spongiosum [98] as a result of ischemia and necrosis of the endometrial tissues [36]. Blood and necrotic epithelium are shed into the lumen of the uterus and discharged via the cervix during menstruation.

Proper functioning of the hypothalamic-pituitary-ovarian (HPO) axis is required to support adequate follicular growth and development, normal ovarian and uterine cyclicity, and normal reproductive function. Among exercising women with menstrual disturbances and women with AN and FHA, under-nutrition compromises the metabolic environment, altering the HPO axis to affect both the ovarian and uterine cycles, thus disrupting the menstrual cycle. This disruption in turn influences the woman's reproductive potential.

Types of Menstrual Disturbances Associated with Energy Deficiency

Sustained energy deficiency in exercising women and in women with AN leads to suppression of growth and reproduction, physiological processes considered least essential for survival [100]. Reproductive dysfunction occurs along a spectrum of disturbances ranging from subclinical menstrual disturbances, which include luteal phase defects (LPD) and anovulation, to severe clinical menstrual disturbances, which include oligomenorrhea

and FHA [101], whereby estrogen and progesterone exposure decreases as the menstrual disturbance increases in severity. **Figure 2.1** displays the spectrum of menstrual disturbances observed secondary to energy deficiency.

Subclinical Menstrual Disturbances: Subclinical menstrual disturbances are the least severe of the menstrual disturbances on the spectrum. Subclinical menstrual disturbances include LPD and anovulation and can occur in the presence of regular cycle length [102], so they are not readily apparent without detailed hormonal assessment of an entire cycle [101, 103]. LPD are characterized by a short luteal phase (<10 days in length), an inadequate (or insufficient) luteal phase (suppressed progesterone concentrations) or both [101, 104]. Anovulatory cycles, which represent the more severe form of subclinical menstrual disturbances (compared to LPD), are characterized by the absence of an LH surge, the absence of ovulation, and the absence of a CL and thus luteal phase progesterone production [102, 105].

Clinical Menstrual Disturbances: Clinical menstrual disturbances are the most severe menstrual disturbances, and include oligomenorrhea and FHA. Both oligomenorrhea and FHA are easily detected since they are associated with obvious changes in menstrual cyclicity [101, 106]. Oligomenorrhea is characterized by long and inconsistent intermenstrual intervals of 36-90 days in length [101, 106]. Oligomenorrhea is difficult to study due to its inconsistent intermenstrual intervals and hormonal characteristics [101, 106]. Oligomenorrhea may present with erratic, often suppressed, estrogen production and can occur with or without ovulation [106]. The cause of oligomenorrhea may or may not be hypothalamic in origin [107]. Oligomenorrhea is often associated with hyperandrogenism and polycystic ovarian syndrome (PCOS), which is the most common cause of infertility in women [108, 109]. A thorough screening of oligomenorrheic women, perhaps by a

reproductive endocrinologist, may be necessary to complete an appropriate differential diagnosis to rule out the presence of PCOS [107].

FHA, the most severe menstrual disturbance, is associated with serious clinical sequalae due to chronic hypoestrogenism [5, 6, 101], which include stress fractures, decreased bone density and strength, and endothelial dysfunction [15, 25]. FHA is defined as the absence of menses and is classified as either primary or secondary. Primary amenorrhea is defined as the failure to menstruate by age 15 in girls with secondary sex characteristics [110] whereas secondary amenorrhea is the abnormal cessation of menses after menarche for at least 90 days [101]. The exclusion of all other causes of amenorrhea, such as hyperandrogenism, hyperprolactinemia, thyroid dysfunction, or other systemic diseases, is required for diagnosis of FHA [111]. FHA associated with chronic energy deficiency originates in the arcuate nucleus of the hypothalamus and is associated with reduced LH pulsatility and chronically suppressed ovarian steroid hormones. Hyperandrogenism may provide an alternative mechanism underlying FHA in some exercising women. In women with FHA, classical polycystic ovarian morphology (at least one ovary greater than 10cm^3 and at least 12 peripheral cysts between 2-9mm [112]) is occasionally observed in association with increased ovarian stroma [111]. Wang et al. (2008) did not observe hypoandrogenism in their baseline evaluation of women with FHA and polycystic ovarian morphology; however, with recovery from FHA a clinical picture of irregular cycles and some symptoms of hyperandrogenism emerged [111]. Additionally, GnRH administration has led to hyperandrogenism in some women with FHA [111, 113]. The findings of Wang et al. (2008) suggest in the presence of polycystic ovarian morphology the ovaries of women with FHA may be inherently hyperandrogenic; however, in the suppressed gonadotropin environment the ovaries are quiescent and may exhibit characteristic features of PCOS with recovery of menstrual function [111]. Whereas hyperandrogenism has been proposed as the primary mechanism underlying PCOS-related oligomenorrhea [108], the proposed mechanism underlying exercise associated

oligomenorrhea or amenorrhea has typically been presumed to be secondary to hypothalamic inhibition related energy deficiency, although many investigators have neglected to separately group and characterize oligomenorrheic and amenorrheic exercising women [106]. Recent evidence suggests that hyperandrogenism, though not as severe as demonstrated in women with PCOS, may be an alternative mechanism underlying oligomenorrhea in some exercising women [114-117]. Given the potential for oligomenorrhea to be associated with hyperandrogenism, it may be that this subgroup does not present with the typical energy deficiency related menstrual disturbances observed in exercising women.

While the presence of secondary amenorrhea [118] was previously used as a diagnostic criterion for AN, the American Psychiatric Association recently removed this requirement in the updated Diagnostic and Statistical Manual of Mental Disorders (DSM-5). The removal of amenorrhea as a necessary diagnostic criterion was due to the recognition that a person's vulnerability to neuroendocrine dysregulation is highly variable and many women with very low body weight and variable degrees of body fat continue to have menstrual cycles of regular length [119]. Additionally, the criterion of secondary amenorrhea cannot be applied to the diagnosis of AN in males, pre-menarcheal females, females taking hormonal contraceptives, or post-menopausal females. Although no longer a requirement for AN diagnosis, amenorrhea is still prevalent among pre-menopausal women with the disorder. The current diagnostic criteria for AN includes a distorted view of one's body weight and shape and very low body weight, a pathological fear of becoming fat is observed, even with a significantly low weight, and excessive energy restriction leading to severe weight loss resulting in a significantly lower body weight than what is expected for sex, age, height, growth trajectory, and physical health [118, 120].

Changes in Hypothalamic-Pituitary-Ovarian Activity associated with Amenorrhea

The etiology of amenorrhea in exercising women and women with AN is secondary to energy deficiency, under-nutrition or deliberate starvation, in the case of AN [4, 6]. The development of an energy deficit is a key factor involved in the induction of alterations in metabolic and reproductive hormone secretion. In a wide number of mammalian species, physiologic processes, including reproductive function, are dependent on the availability of oxidizable metabolic fuels at the cellular level [100]. Wade et al. [100] demonstrated support for the energy availability (EA) hypothesis, which states that energy intake is oxidized to metabolic fuel and subsequently partitioned to facilitate the function of five major physiologic processes (thermoregulation, locomotion, cellular maintenance, reproduction, and growth) [100]. In the face of a chronic energy deficit, a cascade of energy conservation mechanisms are initiated (**Figure 2.2**) [3, 4, 9, 121, 122], attenuating energy expenditure through suppression of REE [3, 123], total triiodothyronine (T_3) [3, 123, 124], IGF-1 and IGF binding proteins (IGFBP) [125, 126], leptin [127] and insulin concentrations [125, 126], and elevation of cortisol [126, 128, 129], GH [125, 126], peptide YY (PYY) [130], and ghrelin [131]. These alterations in indicators of energetic and nutritional status modulate the five major physiologic processes, shunting energy away from growth and reproduction in order to facilitate those processes vital for survival of the individual. As such, growth and reproduction are not adequately fueled and are henceforth suppressed. Thus, energy deficiency results in menstrual disturbances characterized by altered production and release of hormones from the level of the hypothalamus to the ovaries [18, 34].

Slow GnRH pulsatile secretion is a characteristic of FHA. The altered GnRH secretion results in a larger decline in LH compared with FSH and low ovarian activity. The LH pulsatility pattern observed in FHA consists of either low amplitude pulses or erratic pulses at night with increased pulse amplitude, resembling an early pubertal pattern [129, 132, 133]. The responsiveness of the hypothalamus to GnRH administration is intact; however, responsiveness to GnRH administration is exaggerated. This finding of intact
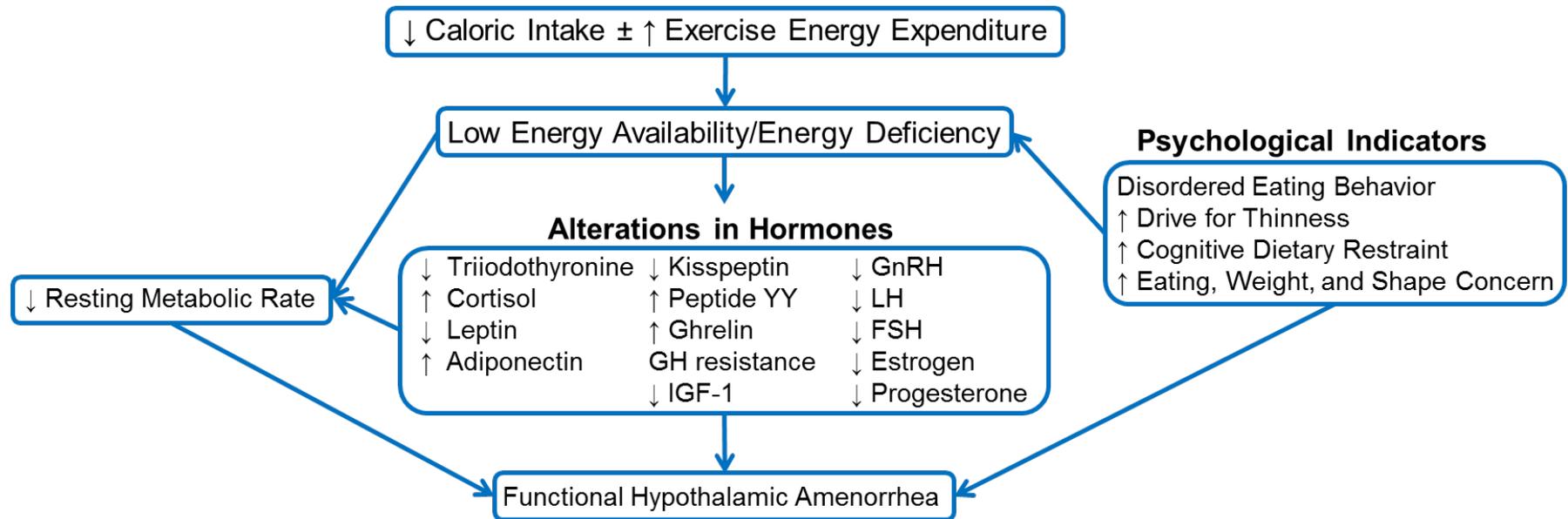
Figure 2.2: Metabolic and psychologic factors contributing to menstrual dysfunction in anorexic and exercising women with FHA. GH = growth hormone; IGF-1 = insulin-like growth factor 1; GnRH = gonadotropin releasing hormone; LH = luteinizing hormone; FSH = follicle stimulating hormone.

responsiveness to GnRH administration confirms the hypothalamic origin of the amenorrhea [132-134].

GnRH secretion, thus reproduction, may be linked to nutrition/metabolic status through multiple signals including neurotransmitters, hormones, and growth factors which convey hypogonadism and EA [135]. Specifically, KNDy neurons, GABA neurons, and leptin provide signals leading to alterations in GnRH secretion [135-140]. With food deprivation, GnRH and gonadotropin secretion are impaired, with leptin playing a role in this inter-regulation by stimulating LH release [140, 141]. In humans with mutations in the leptin or the leptin receptor gene hypogonadism is observed [142]. Additionally, periods of fasting and longer term caloric restriction decrease LH pulse frequency and increase pulse amplitude [121, 143, 144]. In women with FHA, administration of recombinant leptin resulted in increased LH pulse frequency [145]. The ability of metabolic signals provided by leptin to signal the HPO axis is a result of the expression of leptin receptors on KNDy and GABA neurons, which interconnect with GnRH neurons where leptin receptors are absent [139, 146-151]. In addition, estrogen receptors are located on KNDy, GABA, and GnRH neurons [34, 139, 146, 148]. The presence of estrogen receptors on KNDy and GABA neurons indicates changes in the sex steroid milieu alters GnRH secretion directly and through decreasing stimulatory neurotransmitter signals from KNDy and GABA neurons [140, 152, 153]. Kisspeptin binding to GnRH neurons increases gonadotropin release, with a preferential stimulatory effect for the release of LH [140, 154-161]. In addition to reduced GnRH secretion with food deprivation, kisspeptin secretion is reduced [162-165]. In women with FHA, kisspeptin54 administration stimulates increases in LH pulse frequency [157, 160, 161]. Additionally, KNDy neurons communicate with POMC, NPY, and CART expressing neurons (discussed further in the gastrointestinal peptides section) and are able to modulate the expression of proteins in these neurons [150, 166].It is important to note that GnRH secretion is not readily measureable in the periphery; however, LH pulses are measureable with frequent blood sampling and reflect GnRH pulsatility. In 1974 Boyar et al. [132]

described altered 24 hour LH pulsatility in women with AN and re-established normal LH pulsatility with weight regain and subsequent return of menses. The observations of Boyar et al. [132], however, do not reveal the cause of altered LH pulsatility. Causal evidence of the effect of an energy deficit on LH pulsatility has been demonstrated in acute [121, 167] and longitudinal models [168]. Loucks et al. [121] demonstrated that inducing a low EA (≤ 20 kcal/kg FFM/d) for 5 days in sedentary, regularly menstruating women resulted in a suppressed LH pulse frequency and increased pulse amplitude compared to women who remained energy replete (45 kcal/kg FFM/d). Similarly, Williams et al. [167] demonstrated a significant decrease in LH pulse frequency in pre-menopausal women undergoing a 60% decrease in energy intake for 7 days concomitant with a 3 day increase in exercise training volume. In an animal model, LH pulsatility responded rapidly (within minutes) to shifts in energy availability [100]. Scheid et al. [168] conducted a 3 month study examining LH pulsatility following 3 months of dietary restriction (-30 to -60% of baseline energy needs) combined with 5 days per week of aerobic exercise (70-80% of maximal heart rate). Women exposed to the highest energy deficit experienced a significant decline in 24 hour LH pulse frequency compared to LH secretion before the intervention, while the women in the control group (no exercise or dietary restriction) demonstrated no change in LH pulse characteristics [168].

While FSH secretion is impacted by an energy deficit and contributes to menstrual dysfunction, FSH pulsatility and release has not been well characterized [121]. Significantly lower FSH concentrations have been observed during the luteo-follicular transition in exercising women with LPD and low EA [103]. The reduced FSH secretion during the luteo-follicular transition impacts follicular wave emergence and dominant follicle selection, thus contributing to the low E_2 concentrations observed during days 6-12 of the menstrual cycle of exercising women with LPD [103]. However, Loucks et al. [121] did not observe any changes in FSH concentrations following the imposition of low EA for 5 days, despite changes in LH and E_2 concentrations, thus suggesting FSH responds differently to acute

versus chronic energy deficits. In women with AN, FSH secretion patterns have been reported to be exaggerated, normal, and impaired [169, 170].

As previously stated, a reduction in FSH secretion impacts follicular wave emergence and dominant follicle selection leading to a decrease in estrogen concentrations/secretion [103]. The disruption of LH pulsatility, on the other hand, may be a contributing factor to the observed reduction in progesterone secretion, as LH stimulates progesterone production in thecal cells [101, 171]. The suppression of ovarian hormone concentrations varies with the severity of the menstrual disturbances from LPD (least severe) to amenorrhea (most severe) [101]. The characteristic ovarian hormone profile of FHA is a chronic suppression of E_2 and progesterone, with a complete absence of the normal peaks of these ovarian hormones (**Figure 2.1**). Therefore, menstrual dysfunction associated with exercise and AN, wherein the underlying etiology is a chronic or acute energy deficit, is the result of a sequence of changes in metabolic and reproductive hormones, beginning with peripheral energy signals and affecting each level of the HPO axis.

Metabolic Considerations in Amenorrheic Women

Resting Energy Expenditure (REE): REE is a major contributing factor to total energy expenditure and comprises 60-75% of total energy expenditure [172]. REE has been defined as the energy necessary to maintain physiologic function and homeostasis [172]. As previously mentioned, in the event that energy intake fails to compensate for energy expenditure, a series of energy conservation mechanisms are initiated including a reduction in REE, with concomitant adaptations in metabolic hormones [3, 4, 9, 121, 122]. Thus, REE is an indicator of energy status and evidence in exercising and anorexic women with FHA demonstrates an association between suppressed REE and hormonal indicators of chronic energy deficiency [3, 130, 173]. For example, comparing exercising women with FHA to sedentary and exercising ovulatory control women, De Souza et al. [3] observed that a

decrease in REE controlled for fat free mass (FFM) was associated with changes in three key metabolic hormones: total T_3, ghrelin, and leptin. Similarly, a lower REE/FFM was observed in exercising women with subclinical menstrual disturbances (LPD and anovulation) compared to sedentary, ovulatory women [3].

Suppressed REE has been demonstrated in patients with AN in a similar manner; however, such suppression is often more severe than that observed in exercising women with FHA [174-176]. For example, Obarzanek et al. [175] demonstrated an average reduction of 400 kcal/d REE in low weight AN patients compared to healthy volunteers. Kosmiski et al. [174] likewise documented significant differences in REE/FFM between AN patients and controls (>200 kcal/kg FFM/d). In 2000, Polito et al. [176] demonstrated a 21% reduction in absolute REE in AN patients compared to a comparable group of healthy women. Adjusting for FFM accounted for 48% of the difference in REE between the groups, while the remaining difference in REE between AN and controls suggested a reduction in the metabolic activity of the active tissue [176].

A ratio of measured REE (mREE) to Harris-Benedict predicted REE (pREE) provides an estimate of the energy deficiency associated with nutritional restriction [3, 9, 10, 130, 173, 174, 176-178]. In women with AN, during periods of low body weight and prior to refeeding, a reduced ratio of mREE to Harris-Benedict pREE of 0.60-0.80 has been observed [176-178]. In 2014, Kosmiski et al. [174] demonstrated an average ratio of mREE to pREE of 0.79 in AN patients using measures of body composition from DXA and specific metabolic rates for body compartments to calculate the pREE more rigorously than the Harris-Benedict prediction allows. The ratio of 0.79 observed in AN patients was significantly different than the ratio of 1.03 observed in the lean control women [174]. In our laboratory, we have calculated the ratio of mREE to Harris-Benedict pREE in amenorrheic and eumenorrheic exercising women and have observed that a cut-off of <0.90 best discriminates energy status (energy deficient vs. energy replete) and menstrual status (amenorrheic vs. ovulatory) [3, 9, 10, 130, 173]. Reflective of less severe nutritional

restriction, the typical mREE to pREE ratio observed in exercising women with FHA ranges from 0.80-0.88 [130, 173], which is in considerably less alarming than the ratio of 0.60-0.80 observed in AN [174, 176-178].

Triiodothyronine (T_3): The most active form of thyroid hormone is T_3 and it is produced mostly in peripheral tissues from thyroxine (T_4) [179]. Physiologic processes, such as growth, metabolism, body temperature, and heart rate, are regulated by T_3. REE, total daily energy expenditure, and oxygen consumption are tightly coupled with measures of total T_3 [180]. There is evidence from animal and human experiments that supports the influence of general energy and macronutrient intake directly on thyroid hormone status and indirectly on REE [181-183].

Measures of total T_3 are often used to indicate the presence of an energy deficiency, as reductions in serum total T_3 concentrations are suggested to initiate energy conservation mechanisms to restore homeostasis in underweight individuals [181, 183]. A series of eloquent experiments in sedentary, regularly menstruating women demonstrated the effect of manipulating EA (through both increased exercise energy expenditure and decreased caloric intake) on total T_3 [124, 184]. In response to the induction of a low EA, reductions in total T_3 were observed and were subsequently prevented when extra calories were provided to compensate for the induced energy deficiency [124]. In a follow-up study, Loucks et al. [184] demonstrated reductions in total T_3 concentration when EA was between 19 and 25 kcal/kg FFM in initially sedentary, ovulatory women. Thus, total T_3 represents a sensitive marker of changes in EA, whether associated with changes in energy restriction and/or changes in exercise training in women [124, 184].

In the 1970s, many authors demonstrated low basal levels of T_3 in FHA women with AN [185-187]. Wakeling et al. [188] were the first to identify a positive correlation between body weight and T_3 concentration with 4-6 weeks of weight regain in a women with AN. More recently, it has been demonstrated that low plasma total T_3 is observed in conjunction

with a reduction in REE in women with AN [189], in a manner similar to that observed in exercising women with suppressed REE [3, 123, 173, 190]. Additionally, women with AN who regained weight exhibited increases in total T_3 with concomitant increases in REE independent of changes in FFM [189].

Causal links between reproductive dysfunction and energy status, as indicated by thyroid hormones, have been demonstrated in both animal and human experiments. In 1991, Michaud et al. [191] demonstrated that thyroidarche (maturation of the thyroid axis) precedes menarche (maturation of the ovarian axis) in healthy adolescents. The induction and reversal of amenorrhea in female monkeys has been correlated with changes in circulating total T_3 [14]. During a period of restricted dietary intake, total T_3 concentrations in female monkeys decreased by 27% while a significant 18% increase in T_3 was observed during resumption of regular menses [14]. In humans, low concentrations of total T_3 have been linked to reproductive dysfunction in exercising women with amenorrhea [1, 3, 192] and women with AN [193]. In 1992, Loucks et al. [192] demonstrated amenorrheic female athletes had suppressed total T_3 concentrations compared to their eumenorrheic counterparts. In a study from our laboratory, amenorrheic exercising women had significantly lower total T_3 concentrations compared to eumenorrheic exercising and sedentary women [3]. The findings from our laboratory support the premise of a high correlation between REE and total T_3 [3].

Taken together findings in human and animal experiments [1, 3, 14, 192] demonstrate agreement that menstrual dysfunction is linked to mechanisms of energy conservation that are observed with underlying energy deficiency. Key markers of the energy conservation mechanisms are REE and total T_3 [1, 3, 14, 192]. Across the continuum of menstrual disturbances, changes in REE and total T_3 present in a dose response manner, such that increases in energy conservation (thus reduced REE and total T_3) are observed with increasing severity of menstrual disturbances [3]. Recovery of

menstrual function is associated with restoration of an adequate energy intake relative to energy expenditure [13, 14], which can be observed by increases in total T_3.

Gastrointestinal peptides: Ghrelin and Peptide YY (PYY): Ghrelin and PYY are gastrointestinal peptides that are released into systemic circulation where they are able to influence the central mechanisms that regulate energy homeostasis [194] and reproductive function [168, 195-200] at the level of the hypothalamus (**Figure 2.3**). Both ghrelin and PYY modulate the actions of neuropeptide Y (NPY)/agouti-related protein (AgRP) co-expressing neurons, pro-opiomelanocortin (POMC)/cocaine and amphetamine-regulated transcript (CART), and GnRH neurons in the arcuate nucleus and preoptic area of the hypothalamus [199]. Neuropeptides secreted from POMC/CART neurons exert a potent anorexigenic effect (decreasing food intake thus body weight), while NPY/AgRP neurons exert an orexigenic effect (inducing food intake) [199].

Ghrelin is an orexigenic peptide hormone produced by the oxyntic glands of the gastric fundus [201-203]. It is the only peripheral gut hormone known to simulate appetite, with blood concentrations of ghrelin rising with increased sensations of hunger [203-207], while being a GH secretagogue [202, 208, 209]. The mechanism by which ghrelin regulates food intake and satiety acts via the activation of NPY/AgRP and suppression of POMC/CART neurons in the hypothalamus [210-213]. Changes in circulating ghrelin concentrations act as a metabolic signal for initiation and termination of a meal by rising in the fasted state and falling in the fed state [205]. PYY is a well-recognized anorexigenic peptide hormone [214]. The active form of PYY, PYY_{3-36} [215, 216], binds to Y2 receptors activating POMC/CART neurons and suppressing NPY/AgRP neurons in the hypothalamus [217]. The cascade of events initiated by PYY binding at the level of the hypothalamus leads to a decrease in energy intake and can lead to a decrease in body weight [217]. Recently, there has been an emerging interest in the role of ghrelin and PYY in the etiology of
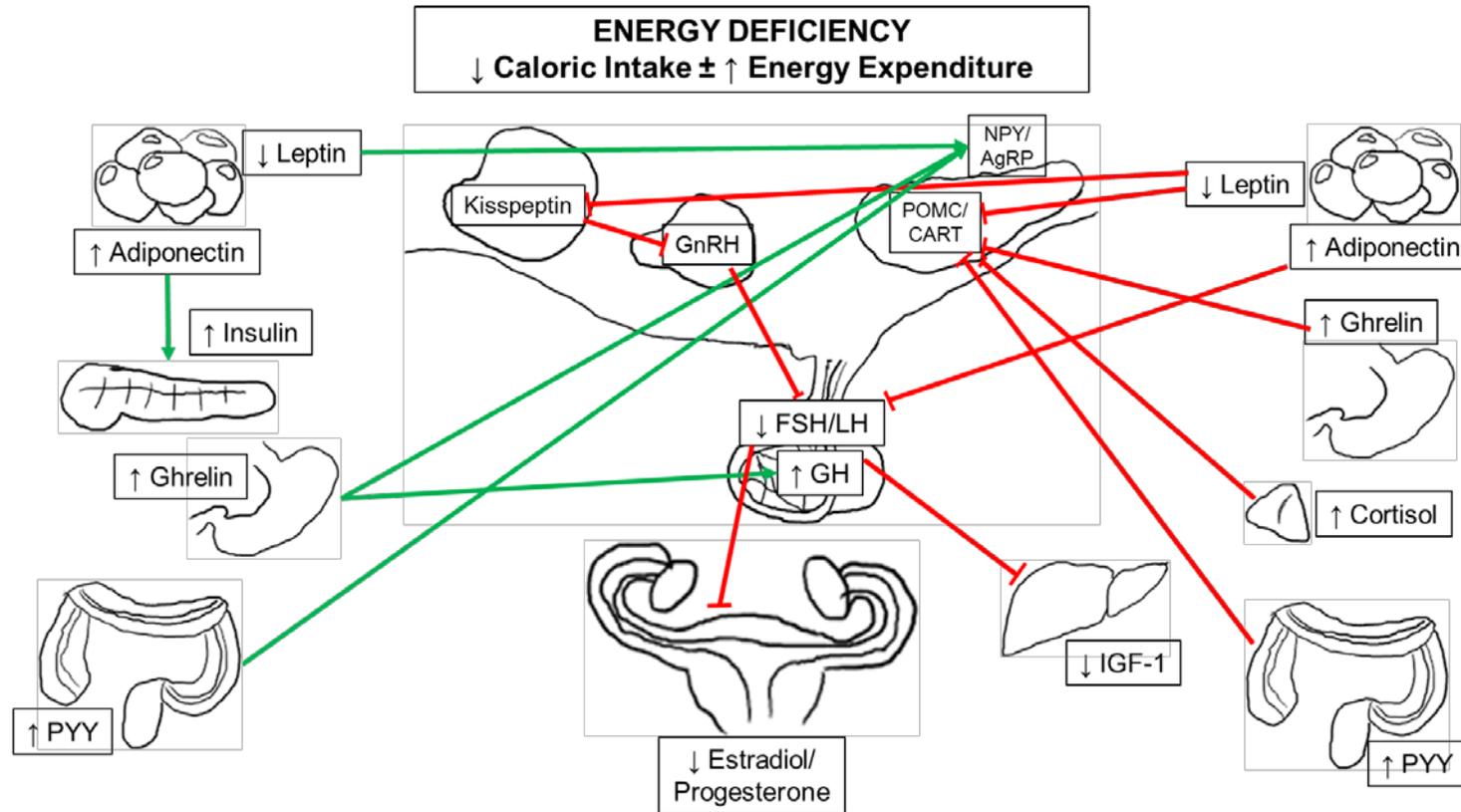
Figure 2.3: The impact of an energy deficiency on the HPO axis. Peripheral hormones such as leptin, adiponectin, ghrelin, PYY, and cortisol cross the blood-brain barrier and exert varying effects on the regulatory mechanisms within the hypothalamus. Additionally, many of these peripheral hormones control activation and inhibition of appetite through neuronal activity in the ARC of the hypothalamus. Likewise, pulsatility of GnRH neuron secretion within the PVN and ARC is modulated by these peripheral hormones. Consequently, FSH and LH production and secretion from the anterior pituitary is impacted, thus resulting in reduced ovarian steroid production. HPO = hypothalamic-pituitary-ovarian; NPY = neuropeptide Y; AgRP = agouti-related peptide; CART = cocaine and amphetamine-regulated transcript peptide; POMC = pro-opiomelanocortin; GnRH = gonadotropin releasing hormone; FSH = follicle stimulating hormone; LH = luteinizing hormone; GH = growth hormone; IGF-1 = insulin-like growth factor 1; PYY = peptide YY; ARC = arcuate nucleus; PVN = paraventricular nucleus.

menstrual disturbances in association with a chronic energy deficiency in exercising women and women with AN [218-222].

Fluctuations in ghrelin and PYY are a proposed homeostatic feedback mechanism involved in the regulation of appetite and energy intake and promotion of changes in body weight [194, 223-225]. Thus, changes in the secretion of these gut peptides are considered pivotal in weight loss and weight maintenance in healthy populations [223-225]. Additionally, both hormones are implicated as contributory factors in the development of reproductive dysfunction [218], as observed in models of severe weight loss-associated chronic energy deficiency (women with AN [219-222]) and moderate weight loss (exercising women with FHA [4, 130]).

Ghrelin has been associated with 24 hour energy intake [206]. When intravenously administered in non-obese men and women, ghrelin has been correlated with a subsequent large increase in energy intake [214]. Additionally, elevated fasting ghrelin concentrations have been consistently observed in women with AN [219, 221, 222] and exercise associated FHA [4, 130]. Interestingly, women diagnosed with AN fail to demonstrate the meal induced decline in ghrelin [219, 222, 226]. Furthermore, in women with AN, total ghrelin secretion over 12 hours following a meal is elevated compared to healthy controls [222]. In exercising women with FHA, De Souza et al. [131] observed fasting ghrelin concentrations that were 85% higher in exercising women with LPD/anovulation than exercising and sedentary ovulatory women. In pre-menopausal normal-weight women undergoing a three-month diet and exercise intervention to result in a negative energy balance, an elevated 24-hr ghrelin was observed [223]. Additionally, elevations in fasting mealtime peaks and nocturnal peaks of ghrelin were observed following weight loss in pre-menopausal normal-weight women [223], demonstrating circulating ghrelin concentrations are sensitive to changes in body weight. The chronic elevation in fasting ghrelin concentrations in exercising and anorexic women with FHA is perplexing, as these women consistently report suppressed levels of hunger, lower energy intake, and/or weight loss [131, 225, 227, 228].

In addition to elevations in ghrelin, elevations in PYY have been observed in exercising and anorexic women with FHA [130, 220, 229, 230]. In these women, it has been proposed that the anorexigenic effects of elevated PYY may mask the expected orexigenic effects of elevated ghrelin [130, 220]. Additionally, a negative correlation between fasting PYY concentrations and REE/FFM has been observed, indicating an association between appetite regulating hormones and mechanisms of energy conservation [130, 220] that may play a role in the eating behaviors in these populations.

In attempts to better understand the regulation of energy balance in non-obese and obese populations, the impact of acute exercise on ghrelin and PYY has been explored [231-233]. In response to an acute bout of exercise, suppression of appetite has consistently been observed [231-234] and thus, has been suggested to promote a decrease in relative energy intake. Hubert et al. [234] demonstrated differential changes in hunger, as an energy deficit induced by diet increased hunger while induction by exercise did not increase hunger. These experimental findings suggest a biological drive regarding the lack of initiative to match energy intake with exercise energy expenditure [235]. Additionally, the routinely prescribed high carbohydrate diets for endurance athletes may perpetuate the deficit in energy intake following exercise [236-238] through a mechanism known as "exercise-induced anorexia", with appetite-related hormones being proposed as mitigating factors [232, 233]. A suppression of acylated ghrelin during aerobic exercise has been demonstrated [231-233] with no difference between ad libitum energy intake following an exercise bout or a rest condition [231]. The suppression of ghrelin with exercise appears to be temporary, thus it is likely that the elevations in circulating PYY concentrations, opposing the actions of ghrelin to signal satiety, promote eating behaviors that result in a chronic energy deficit [232, 233]. Suppression of ghrelin and elevation of PYY results in an anorexigenic hormonal profile and has been proposed to be the mechanism by which gut hormones suppress appetite following exercise [232] and cause the observed uncoupling between energy intake and expenditure. If the uncoupling of intake and expenditure is

maintained for a prolonged period it may be the cause of "inadvertent under eating", whereby exercising women simply do not consume enough energy to compensate for exercise energy expenditure, and may lead to a chronic energy deficit [235].

In the complex network of mechanisms that underlie the disruption and/or restoration of energy homeostasis and reproductive function, ghrelin and PYY have been proposed to be key metabolic signals [168, 218]. The induced changes in ghrelin and PYY associated with an energy deficiency are important factors involved in the suppression of the HPO axis [217, 218]. Notably, ghrelin has been linked to reproductive function through direct and indirect actions that alter GnRH pulsatility [217, 218] and lead to the suppression of LH secretion and pulsatility [168, 200, 219, 239]. Vulliemoz et al. [200] demonstrated a reduced LH pulse frequency, not amplitude, with peripheral ghrelin infusion in adult ovariectomized rhesus monkeys. Similarly in an ovariectomized rat model, ghrelin injection inhibited LH secretion. In humans, the impact of weight loss induced by diet and exercise on reproductive function in premenopausal women was examined by Scheid et al. [168], who demonstrated decreased LH pulse frequency with increased 24hr mean ghrelin and lunch peak ghrelin. Similarly in AN, elevated ghrelin concentrations predict reductions in LH concentrations measured in a single fasted morning serum sample [219]; however, the impact of ghrelin on LH pulse frequency and amplitude in AN remains to be evaluated.

Adipokines: Leptin and Adiponectin: The adipokines leptin and adiponectin are two additional factors that have been proposed to be involved in the hormonal regulation of reproductive function and energy homeostasis in humans. Leptin is a white adipocyte secreted protein that is the product of the ob gene [240] and leptin concentrations are directly correlated with fat mass [241, 242]. Adiponectin is also a white adipocyte secreted protein that circulates at high concentrations in healthy populations [243-246] and functions to increase peripheral sensitivity to insulin [247-249]. Circulating concentrations of adiponectin are inversely associated with fat mass [249-253].

Leptin is considered a satiety factor and has displayed opposing action to ghrelin [222]. Leptin has been demonstrated to inhibit NPY/AgRP neurons, which subsequently activates POMC/CART neurons to decrease food intake (**Figure 2.3**) [254]. It has been suggested that leptin plays a role in the coordinated response of reproductive function to metabolic fuel availability [255]. Leptin can cross the blood brain barrier and can activate cells in the brain that express an isoform of the leptin receptor [254, 256]. Leptin receptors have been isolated in the hypothalamus [257, 258], anterior pituitary [259], ovary [260], and endometrium [261]. Leptin concentrations are sensitive to energy deprivation. A reduction in leptin concentrations to 10-30% of baseline concentrations have been observed after 2-3 days of caloric deprivation, before major changes in bodyweight or fat mass have occurred [262, 263]. Additionally, diet-induced weight loss also results in a reduction in circulating leptin concentrations [141, 242, 264]. Activation of the leptin receptor in the hypothalamus leads to changes in the expression of hypothalamic neuropeptides and thus regulation of energy homeostasis [257, 258].

Low concentrations of leptin are important in signaling an energy deficit to the hypothalamic-pituitary axes [263, 265]. In leptin deficient animals, impaired GnRH pulsatility has been observed [266, 267]. Suppressed leptin concentrations have been consistently observed in exercising and anorexic women with FHA [127, 190, 222, 268, 269]. Additionally, Ackerman et al. [270] demonstrated a positive association between leptin and LH secretion. Taken together, these results suggest circulating leptin concentrations may act as a metabolic signal communicating nutritional inadequacy to the hypothalamus. In fact, the administration of recombinant leptin for several months in women with FHA resulted in increased follicle number and size, but concomitantly caused weight and fat loss due to a reduction in food intake [145, 271]. As such, although recombinant leptin has been demonstrated to positively signal the HPO axis, its resultant impact on body weight and fat mass makes this therapeutic option unattractive in an already low weight population.

Adiponectin plays an important role in the regulation of food intake and energy homeostasis [272, 273]. Normal concentrations of adiponectin reduce post-prandial fatty acid concentrations and hepatic glucose output [274] and reduced circulating adiponectin concentrations are associated with increased prevalence of atherosclerosis [275]. Adiponectin concentrations have been observed to be elevated in AN compared to control women [253, 276] and reduced in very severe AN [277]. In contrast, concentrations of adiponectin were not different between adolescent amenorrheic or eumenorrheic athletes nor their sedentary eumenorrheic controls [278]. In young adults between 18-35 years old, fat mass adjusted log adiponectin concentrations were significantly elevated in exercising women with FHA compared to exercising and sedentary ovulatory women [279]. O'Donnell et al. [279] demonstrated that 41% of the variance in fat mass adjusted log adiponectin was explained by truncal fat mass. A role for adiponectin in female reproductive function has been suggested.

The influence of adiponectin on GnRH neuronal activity was examined in female mice [280]. Klenke et al. [280] demonstrated reduced GnRH neuronal activity in a subpopulation of GnRH neurons. Similarly, adiponectin inhibited LH release in rat pituitary cell cultures [281]. Adiponectin receptors have been located in female peripheral reproductive tissues, including the ovaries, oviduct and endometrium [282, 283]; however, the role of adiponectin in peripheral reproductive tissues is unclear. The role of adiponectin in ovarian function may include the modulation of steroidogenesis within the granulosa and thecal cells [284-290]. Researchers examining the influence of adiponectin on ovarian steroidogenesis have demonstrated inconsistent findings, with enhancement, suppression, and neutral impacts on steroidogenesis being reported depending on the model system evaluated [284-290]. Though there is certainty to the exposure of peripheral reproductive tissues to adiponectin, the understanding surrounding adiponectin signaling and reproductive organ function requires further investigation. Additionally, adiponectin has been

demonstrated to activate osteoblasts and osteoclasts [291, 292], thus may be an important regulator of bone health in exercising and anorexic women with FHA.

Growth Hormone and Insulin-Like Growth Factor-1: Growth hormone and IGF-1 are pivotal metabolic agents [293-296] controlled by metabolic and other peripheral hormones [294-299]. Nutritional status influences the GH/IGF-1 axis at the level of the hypothalamus all the way down to the level of binding proteins [300-303]. Systemic GH concentrations are higher in adolescents and adults with AN and exercise-associated FHA [126, 304, 305] and women with the lowest BMI and fat mass have been observed to have the highest circulating GH concentrations [304, 305]. Laughlin et al. [126] demonstrated alterations to the GH secretion profile in amenorrheic exercising women, including increased GH pulse frequency, elevated inter-pulse GH concentrations, and unaltered GH pulse amplitudes. Similarly, Stoving et al. [305] and Misra et al. [304] demonstrated that women with AN display increased basal GH secretion, a greater number of GH pulses, and elevated GH concentration. Stoving et al. [305] demonstrated the altered 24-hour GH secretion patterns is significantly correlated with BMI. Recently, leptin, ghrelin, and NPY have been under increased investigation as the nutritional signals that control GH secretion [203, 297-299, 306]. The importance of these three factors in controlling the endocrinological and metabolic responses to changes in nutritional status are of interest due to the well-known influence of these hormones on controlling food intake [299]. Additionally, leptin has been demonstrated to have a stimulatory effect on GH through NPY in the hypothalamus [299]. Further investigation into the impact of hormones produced by the stomach and adipocytes on GH secretion will lead to new insights regarding the alterations in the GH/IGF-1 axis in AN and in less severe states of malnutrition [307].

Prolonged starvation also modifies GH receptor activation and function [300, 308], resulting in an acquired peripheral GH resistance, which explains the documented reduction in IGF-1 synthesis in such patients [301, 302, 304, 305, 309, 310]. Low circulating IGF-1 is

directly due to inhibition of hepatic IGF-1 production due to under-nutrition and GH

resistance [301, 302, 305, 311, 312]. The low IGF-1 concentrations, through negative

feedback, in association with increased ghrelin, augment GH secretion [8, 304].

Administration of GH, even for 12 weeks, is ineffective in increasing IGF-1 concentrations

[313]. These differences in secretion suggest a nutritionally-acquired GH resistance [313]. In

the state of low EA associated with FHA, high GH secretion is consistent with the need for

maintaining euglycemia despite under-nutrition [8]. GH has a gluconeogenic role through

increasing lipolysis [314, 315]. The GH impact on lipolysis is not mediated through IGF-1,

thus allowing preservation of euglycemia in exercising and anorexic women with FHA [314,

315].

Additionally, a growing body of evidence demonstrates the ability of GH to directly

modulate reproduction through gonadotropin-dependent and gonadotropin-independent

actions. This topic has recently been reviewed by Hull et al. [316]. In relation to

reproduction, it appears that GH acts at both the pituitary and ovaries to modify the actions

of GnRH. At the level of the pituitary gonadotroph cells contain GH receptors and secretion

of LH/FSH is reduced in GH-deficient/resistant rats [317]. At the level of the ovary the in vitro

evidence suggests GH modulates estradiol and progesterone production from granulosa

cells and luteinized granulosa cells and this effect varies throughout the ovarian cycle [318-

323]. Additionally, it appears that GH is necessary for optimal follicular maturation and

survival [324, 325]. GH receptor knock out mice have more primordial follicles, fewer primary

to antral follicles and corpora lutea, and increased follicular atresia [324, 325]. Further, GH

resistance in cows resulted in a complete blockage of dominant follicle development [326].

IGF-1 receptors are abundant in follicles [327, 328] and IGF-1 has been demonstrated to

augment the action of gonadotropins in steroidogenesis [329-332].

Cortisol: Mild to severe hypercortisolism is observed in both exercising [128, 129, 333-337]

and anorexic [312, 338-343] women with FHA. In women with AN, the elevation in mean

cortisol concentrations is rarely observed to exceed twice the upper limit of normal [8]. In an early investigation, elevated 24-hour plasma cortisol concentrations in anorexic women with FHA were associated with an increased cortisol half-life and a decreased cortisol clearance rate, rather than an increase in cortisol production [338]. In addition, Lawson et al. [344] demonstrated higher mean pooled 12-hour overnight plasma cortisol in both women with FHA and women with AN compared to healthy controls. Interestingly, women with AN trended towards having higher cortisol than women with FHA, suggesting an intermediate hypercortisolemic phenotype in women with less severe energy deficiency [344]. Cortisol has been further investigated using both urine and saliva sampling, with investigators demonstrating associations between AN and elevated urinary free cortisol [345, 346] and late night salivary cortisol [343]. Explorations of the pathophysiology of hypercortisolemia in AN has been accomplished through administration of exogenous ovine corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) to observe the cortisol response. This investigatory probe demonstrated that while women with AN had an attenuated ACTH response compared to normal weight control subjects, cortisol secretion was elevated with AN [346], suggesting an increased sensitivity of the adrenal cortex to ACTH. In addition, the increased ghrelin secretion observed in women with AN has been positively associated with cortisol burst frequency [219] as well as CRH release from the hypothalamus, which stimulates ACTH secretion from the anterior pituitary [209, 347].

Cortisol dynamics in amenorrheic and eumenorrheic athletes generally represent mild hypercortisolism [128, 129, 333-337]. Elevated 24-hour mean plasma cortisol has been reported in exercising women with FHA compared with healthy control women [334] and Rickenlund et al. [116] reported increased 24-hour mean serum cortisol concentrations in amenorrheic athletes compared with eumenorrheic athletes and non-athletes. Additionally, De Souza et al. [335] demonstrated a mild hypercortisolism in amenorrheic runners compared to eumenorrheic runners and non-athletes. The amenorrheic runners exhibited a blunted exogenous ACTH-stimulated cortisol response, which was restored following

dexamethasone suppression of endogenous cortisol prior to ACTH administration [335]. On the other hand, Loucks et al. [129] reported no differences in ACTH secretion patterns or cortisol pulse *frequency* between amenorrheic athletes, eumenorrheic athletes, and non-athletes; however, Loucks et al. [129] did observe higher overnight cortisol concentrations in both amenorrheic and eumenorrheic athletes compared to non-athletes. Additionally, the cortisol pulse *amplitude* decreased over the course of the day in the eumenorrheic athletes but remained elevated in the amenorrheic athletes, resulting in a higher 24-hour cortisol measurement in the amenorrheic athletes [129]. Ackerman et al. [348] further investigated cortisol dynamics in female athletes and reported increased cortisol pulse amplitude, pulse mass, area under the curve, and cortisol half-life in amenorrheic athletes compared to eumenorrheic athletes and non-athletes. However, in 1996 Laughlin et al. [126] demonstrated higher 24-hour serum cortisol in *both* amenorrheic and eumenorrheic athletes compared to non-athletes. Based on these inconsistencies, it is evident that several factors contribute to differences in cortisol secretion in female athletes with varying menstrual function.

Mechanistically, physiologic and psychologic stress act through the HPA axis to modulate the HPO axis and ultimately impact reproductive function. In animal models, activation of CRH is an adaptive response to a state of low energy availability and has the potential to inhibit the activity of the GnRH pulse generator [349-352]. In animal models of stress where cortisol causes a decrease in GnRH secretion, the responsiveness of gonadotrope cells of the pituitary to available GnRH is further impaired [353, 354]. This alteration in gonadotrope sensitivity is apparently mediated through type II glucocorticoid receptors in the anterior pituitary [353]. Similar alterations in gonadotrope responsiveness have been observed in women with FHA. Berga et al. [333] demonstrated an association between the elevated cortisol secretion and reduced GnRH secretion, thus reduced LH pulsatility, in women with FHA. Additionally, hypercortisolemia in amenorrheic athletes has been associated with a decrease in LH pulse frequency [116, 126, 129]. Similarly, in

adolescent and young adult athletes, Ackerman et al. [348] reported overnight cortisol area under the curve was inversely correlated with LH area under the curve, a finding that remained significant after controlling for fat mass, ghrelin, and leptin. Additionally, diurnal secretion of cortisol was negatively associated with the number of menses in the past 12 months in endurance athletes with varying menstrual status and healthy sedentary controls [116], indicating a key role of cortisol and the HPA axis in suppressing menstrual/reproductive function.

Consideration of the diurnal rhythm of cortisol secretion is warranted, as little is definitively known regarding alterations in these dynamics in exercising or anorexic women with FHA. In healthy individuals, the cortisol awakening response is recognized as a 50-100% increase in cortisol concentration in the first 30-45 minutes after waking [355-357]. This peak is followed by a steady decline over the course of the day, reaching a nadir during the first half of the night [356]. Various physical and psychological factors contribute to the characteristic cortisol awakening response [355, 358-360] and thus may be altered in exercising or anorexic women with FHA. While some investigators have reported preservation in the circadian rhythm, others have reported a suppression of the rhythm [338, 343, 361, 362]. Early work by Boyar et al. [338] demonstrated a normal circadian cortisol rhythm in young anorexic women, yet Putignano et al. [343] measured salivary cortisol and demonstrated a flattened but maintained cortisol rhythm in women with AN compared to healthy controls. In 2007, dos Santos et al. [362] reported 1/3 of the women with AN demonstrated an absence of a salivary cortisol rhythm, while 2/3 of the women with AN had a preserved cortisol rhythm and an area under the curve that was significantly *higher* than healthy controls. Monteleone et al. [363, 364] have demonstrated that in women with AN and an average BMI in the 17kg/m^2 range, the cortisol awakening response was significantly elevated compared to a healthy-weight control group. Wild et al. [365] added to this finding, reporting that in women with AN, the cortisol awakening response was significantly correlated with the severity of AN symptomology. Specifically, the cortisol awakening

response was significantly lower in severely ill women with AN while women with higher BMIs showed a trend toward higher initial cortisol awakening response [365]. There is no evidence to suggest the diurnal rhythm of cortisol secretion is altered in amenorrheic athletes.

Overall, the available data in exercising and anorexic women with FHA suggest hypercortisolemia is due to the state of low energy availability, and perhaps some factors related to psycho-social stress [1, 333]. One proposed reason for the adaptive upregulation of cortisol in women in a state of low energy availability is to maintain a euglycemic state [341], working in conjunction with GH [8, 314, 315]. Consistent with the proposed mechanism, Rickenlund et al. [116] and Ackerman et al. [348] have reported an inverse association between cortisol concentrations and fat mass in athletes and non-athletes, indicating a mobilization of lipid stores. Additionally, in women with AN, individuals with the lowest BMI, fat mass, and fasting glucose concentrations were observed to have the highest cortisol concentrations [341, 342]. In contrast, Laughlin et al. [126] reported no association between cortisol concentrations and fasting glucose.

Hypercortisolemia associated with FHA has also demonstrated correlations with body composition [342, 366-368], bone density [341, 344], and psychopathologies [342, 344]. Lawson et al. [344] demonstrated a positive correlation between cortisol and symptoms of anxiety and depression, while being negatively associated with bone mineral density in women with FHA. In women with AN, high concentrations of cortisol predicted a reduced percentage of extremity lean mass [367] and bone mineral density [341, 344]. Additionally, a "U" shaped relationship has been reported between BMI and both overnight serum cortisol and urinary free cortisol corrected for creatinine clearance in women with AN [368].

Little has been reported regarding changes in hypercortisolemia in response to recovery from FHA or AN. In 1986, Gold et al. [346] demonstrated hypercortisolemia only resolved after long term recovery (6 months to 2 years) in AN, indicating a relatively slow

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physiological time course to adapt to the increase in body weight. In AN, there is a positive correlation between measures of baseline urinary free cortisol and the percent increase in truncal fat with weight gain [366]. The normalization of cortisol concentrations with weight gain have not been associated with BMI or body composition [339, 346]. In 2006, Misra et al. [342] demonstrated a link between increased baseline cortisol concentrations in girls with AN and increases in fat mass with recovery from AN, which was, in turn, associated with resumption of menses. Future studies to further investigate and refine the time-course for normalization of cortisol dynamics following recovery from AN or FHA are warranted.

Psychopathology

Another pathway to evaluate the potential of developing a chronic energy deficit is disordered eating attitudes [9, 10]. Eating attitude psychopathologies in athletes and eating disorders have recently been reviewed [369-372]. Self-administered questionnaires, such as the eating disorders examination questionnaire (EDE-Q), eating disorders inventory (EDI-2 and 3), eating attitudes test (EAT), and three factor eating questionnaire (TFEQ), are often used to measure eating disorder/disordered eating psychopathology in research settings. The EDE-Q indexes behavior along 4 dimensions, including dietary restraint, eating concern, shape concern, and weight concern [373]. The EDI-2 and EDI-3 are divided into 11 main subscales, including drive for thinness, bulimia, body dissatisfaction, ineffectiveness, perfectionism, interpersonal distrust, interoceptive awareness, maturity fears, asceticism, impulse regulation, and social insecurity [374]. The EAT total score measures the existence of abnormal eating behaviors and severity of existing pathological thoughts concerning food, dieting, and body image through three factors, including dieting behavior, bulimic behavior, and oral control [375, 376]. The TFEQ measures three dimensions of human eating behavior, which include cognitive dietary restraint, disinhibition, and hunger [377]. In a recent examination using the EDI-2 in adolescents with AN, FHA, and healthy controls, a 2 fold higher drive for thinness score was demonstrated in AN compared to girls with FHA,

while girls with FHA demonstrated a 5 fold higher drive for thinness score than healthy controls [378]. The relationship between core measures of eating disorder psychopathology (i.e., drive for thinness, cognitive dietary restraint, eating concern, weight concern, and shape concern (**Figure 2.2**)) and appetite-related hormones (i.e., ghrelin, PYY, and leptin) in amenorrhea secondary to an energy deficiency is not clear.

Evidence linking subclinical and clinical elevations in restrictive eating behaviors and appetite hormone dysregulation indicative of an energy deficit have been observed in exercising and anorexic women with FHA [9, 10, 200, 379-381]. In AN, serum PYY concentrations are positively associated with the degree of disordered eating thinking and behavior assessed by self-administered questionnaires [379, 381]. Additionally, Scheid et al. [130] demonstrated a positive correlation between fasting PYY and drive for thinness score in women categorized by exercise status (exercise vs. sedentary) and menstrual function (amenorrhea vs. ovulatory). De Souza et al. [9] demonstrated significantly elevated ghrelin concentrations in exercising women with a high drive for thinness compared to women with a normal drive for thinness. Additionally, Schneider et al. [382] demonstrated a positive association between elevated EAT scores and ghrelin concentrations in women with FHA compared with exercising and sedentary eumenorrheic control participants. However, ghrelin concentrations were not associated with core measures of eating disorder pathology in women with AN [381]. Leptin was moderately, negatively associated with core measures of eating disorder thinking and behavior in women with AN [379-381].

These findings suggest that disordered eating psychopathology may play a role in suppression of energy intake in the presence of elevated ghrelin and PYY concentrations and suppressed leptin concentrations. Thus, elevated PYY concentrations are suggested to prevent compensatory increase in energy intake normally associated with elevated ghrelin concomitant with psychological markers of subclinical disordered eating and increased exercise energy expenditure [9, 130]. Similarly, Eddy et al. [379] have suggested that high PYY concentrations drive the persistent restrictive eating patterns in AN. Thus, it is possible

there is a psychopathologic phenotype with a greater susceptibility to developing a chronic energy deficiency and subsequent menstrual dysfunction.

Resumption of Menstrual Function

In AN treatment resumption of menses is an important indicator of recovery from the disorder [383-385], with pediatric and psychiatric guidelines highlighting the importance of menses resumption in treatment recommendations for women with AN [386, 387]. Additionally, according to the Female Athlete Triad Coalition Consensus Statement, recovery of menstrual function is a primary concern for athletes with FHA [15]. Recovery of menstrual function in all women with FHA is linked to improvement of other clinical outcomes associated with FHA [15, 342, 388, 389]. At present, it appears that the most important clinical parameter that predicts functional recovery of the HPO axis is weight restoration [15, 390-399].

Increases in EA will result in recovery of physiological systems [14, 124, 143, 184] at varying rates depending on the severity of the energy deficit and translate into menstrual recovery [390-399]. An increase in EA can alter metabolic hormone profiles positively within days to weeks [15]. A concomitant increase in body weight occurs over weeks to months and is likely the most clinically significant factor associated with resumption of menstrual function in both exercising women [392, 393, 398, 399] and in women with AN associated FHA [394, 395, 397, 400, 401], which can take weeks to months to years [15]. In women with FHA, Arends et al. [390] demonstrated a mean time to resumption of one year (range 8-33 months), while Cominato et al. [391] and Dueck et al. [392] demonstrated resumption in 5-6 months and Kopp-Woodroffe et al. [396] demonstrated menstrual resumption in 9-12 weeks with nutritional therapy. Additionally, the target weight necessary for menstrual recovery has yet to be clearly defined [386, 402]. In women with AN, a BMI higher than 18kg/m^2 is a relevant factor associated with normalization of menstrual function [403-406]. Other researchers have found that 2/3 of patients resume menstrual function when they

reach 95% of their expected body weight [407] and if 90% of expected body weight is reached, 86% of patients resumed menses within 6 months [394]. Cominato et al. [391] demonstrated 64% of patients resumed menses when their BMI's were between the 25th and 50th percentiles. Falsetti et al. [408] demonstrated each increase in BMI of 1kg/m^2 resulted in an increased probability of menstrual recovery of 24.6%. However, some patients who increase their weight and remain weight stable do not resume menstrual function [409, 410]. This indicates that there is individual susceptibility to menstrual resumption [391, 394, 411] and that the success of non-pharmacologic treatment that targets weight gain is influenced by additional factors, including neuroendocrine and metabolic factors [125, 131, 398].

Increases in leptin concentrations greater than 1.85 ng/mL has been demonstrated to be an important metabolic factor necessary for the restoration of increased LH pulsatility and menstrual recovery [412, 413]. Increased IGF-1 has also been demonstrated to be a valuable metabolic factor indicative of nutritional recovery. Cominato et al. [391] demonstrated increased BMI, LH, estradiol, leptin, and IGF-1 with nutritional recovery; however, only increases in IGF-1 significantly distinguished women who resumed menses from those who did not resume menstrual function. Additionally, Falsetti et al. [408] demonstrated reductions in cortisol and increases in LH and IGF-1 in women who recovered menstrual function. Increased IGF-1 with nutritional recovery of patients with FHA has been associated with normal, high, and low GH concentrations [304]. Women with FHA should be counselled that resumption of regular menstrual function may take longer than 1 year to occur [390, 394, 409]. However, non-pharmacologic treatment strategies that reverse the energy deficit that lead to the menstrual dysfunction should be implemented under the supervision of a multidisciplinary team consisting of a physician, dietician, and a mental health practitioner [15].

Conclusion

There are distinct and undeniable physiological interactions between energy status and reproductive function that become overt in many exercising women and women suffering from AN. Disruption of reproductive function in these two populations presents in the form of menstrual disturbances that occur along a spectrum, ranging from subclinical disturbances, including LPD and anovulation, to clinical perturbations, including oligomenorrhea and amenorrhea. Amenorrhea, the most severe menstrual disturbance, may be indicative of a profound energy deficiency in exercising and anorexic women. Classic and contemporary work in animal and human models has illustrated the link between an energy deficiency and alterations in REE and metabolic hormones (i.e. T_3, ghrelin, PYY, leptin, adiponectin, GH, IGF-1, and cortisol). These adaptations, in turn, may contribute to the suppression of the HPO axis, supporting the hypothesis by Wade et al. [100] that, in response limited fuel availability, energy conservation will take place in order to preserve the most essential physiologic functions at the cost of less vital functions, namely growth and reproduction. In the case of AN, deliberate starvation can rapidly create a state of low EA resulting in menstrual dysfunction. In exercising women, hormonal responses to exercise, especially altered gut peptide concentrations, may result in an energy deficiency due to appetite suppression and inadvertent undereating. Additionally, exercising women may exhibit elevated levels of dietary cognitive restraint leading to the development of an energy deficiency. Indeed, observed relationships between psychological disordered eating traits and altered metabolic hormone secretion indicate the possibility of a psychopathological phenotype that renders an individual more susceptible to the development of an energy deficit. Thus, in exercising or anorexic women with FHA, resumption of reproductive function will likely follow restoration of optimal EA.

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Chapter 2: Part 2

Allaway HCM, Mallinson RJ, De Souza MJ. Impact of Combined Oral Contraceptive Use on Exercise and Health in Female Athletes. IN *Exercise and Human Reproduction: Induced Fertility Disorders and Possible Therapies*. Eds. Vaamonde D, du Plessis SS, Agarwal A. Springer Science+Business Media, New York. 2016; p287-302.

Abstract

Nearly half of girls and women between the ages of 15 and 24 years use hormonal contraceptives. The overall health and well-being of girls and women of any age is benefited by routine exercise; however, the impact of hormonal contraceptive use on the capacity to exercise has yet to be conclusively addressed. Since the development of combined hormonal contraceptives, the concentration of the estrogen component (ethinyl estradiol; EE) has been decreased and further development of the progesterone component has occurred to reduce side effects and increase the safety profile. There is currently no information available regarding the impact of transdermal or vaginal combined hormonal contraceptives on exercise performance variables; therefore, this review focuses on the impact of oral contraceptive (OC) preparations on factors influencing exercise performance. Current findings do not indicate consistent changes in substrate utilization during aerobic or anaerobic exercise in women using OC preparations. In addition, the influence of exogenous steroid concentrations used in monophasic and triphasic OC preparations on endurance performance variables of ventilation and oxygen uptake (VO_2) is unclear. Short-term duration of OC use, as well as the androgenic activity of the progestin, appears to cause a decrease in VO_2 and increased ventilatory response, with the observed effects of exogenous steroids dissipating with long-term use. It appears that modern OC formulations do not have enough androgenic potency across a cycle to influence muscular strength, however these formulations may have the potential to increase anaerobic capacity. A negative impact on reactive strength has been documented with monophasic OC use. Long-term use of OCs can lead to an increase in body fat percentage, and may compromise bone health in female athletes. Aerobic and anaerobic performance could be negatively impacted

by these changes in body composition. Among female athletes, OCs may be prescribed to treat menstrual cycle disturbances, such as amenorrhea and oligomenorrhea, which occur as a result of an energy deficit. It must be emphasized that the use of OCs alone are not effective in treating these menstrual cycle disturbances and should be administered in combination with non-pharmacologic treatment when non-pharmacological treatment alone has not been effective.

Introduction

In a recent report from the Centers for Disease Control and Prevention, 44% of women between the ages of 15 and 24 years use hormonal contraceptives [1]. Of these women, 49% use oral contraceptive (OC) preparations and approximately another 14% use other forms of hormonal contraceptives [1]. The prevalence of OC use among athletic women is increasing and is now estimated to match the prevalence of use in the general population [2, 3]. The increased use of combined hormonal contraceptives (containing both an estrogen and progestin) in the athletic community is likely due to the reduction in cycle-length variability and the consistent 28-day "cycle" that is achieved with administration of exogenous sex steroids and subsequent systematic control of endogenous sex hormone concentrations [2]. The effect of OC preparations on athletic performance and health has not been conclusively answered as early investigators utilized a diverse number of OC preparations, range of participant fitness levels, and timing of experiments within the OC pill cycle. Most research studies have been cross sectional and were not carried out using the most commonly prescribed contraceptives today (low and ultra-low OC preparations and the vaginal ring).

Modern combined hormonal contraceptives can be provided in a variety of types and formulations. These include, but are not limited to, OC pills (monophasic, biphasic, and triphasic), the contraceptive transdermal patch, and the contraceptive vaginal ring. Current combined hormonal contraceptives typically contain one of two types of synthetic estrogen, ethinyl estradiol (EE) or mestranol, while the progestin component is typically one of eight different forms. The biological properties of the progestogen derivatives used in contraceptives relate to the potency and the relative binding affinity [4]. Progestins with a higher relative binding affinity for the progesterone receptor exert their progestational effects with smaller doses. Progestins with high relative binding affinity for the androgen receptor cause undesirable side effects that counteract the positive effects produced by the synthetic estrogen [4].

Since the introduction of the OC pill, the dose of the estrogen component has decreased. The high dose OC preparations contain 50µg/d or more of the estrogen component, low dose OC preparations contain less than 50µg/d (generally 20-35µg/d of the estrogen component), and ultra-low dose contain 15µg/d of the estrogen component [5, 6]. Low and ultra-low OC pills are the most commonly prescribed today [6].

The progestin component has undergone four developmental generations since the introduction of the OC pill. The first generation was the first to demonstrate the desired activity on the reproductive axis and include norethindrone and medroxyprogesterone acetate [7-9]. The successive generations were designed to minimize the side effects and increase the safety profile (i.e., to decrease risk of blood clots). The second generation includes levonorgestrel and norgestrel, the third generation includes norgestimate and etonogestrel, and the fourth generation includes drospirenone and nomegestrol acetate [7-9]. All conventional 28-day hormonal contraceptive regimens provide exogenous steroids for 21 days (active pill phase) followed by a 7-day hormone free phase. The hormone free phase, which allows for breakthrough bleeding, can either consist of inactive/placebo pills or the cessation of taking any pills for 7 days. Monophasic OC formulations contain the same dose of estrogen and progestin in all active pills; whereas, biphasic OC formulations contain a constant estrogen dose but the progestin dose increases in the second half of the active pill phase [10]. Triphasic OC formulations have two doses of estrogen (higher in the second week of the active pill phase than the first or third week) and the progestin increases in three steps during the active pill phase [10]. The contraceptive transdermal patch delivers 20µg of EE daily with a third generation progestin, norelgestromin [11], and the contraceptive vaginal ring delivers 15µg EE daily with a third generation progestin, etonogestrel [12].

The purpose of this brief review is to elucidate the current understanding of the effects of OC preparations on factors that influence exercise performance and the health of athletes. Information regarding the impact of the contraceptive transdermal patch and vaginal ring on exercise performance are not currently available. This review will focus on

the effects of OC preparations on the factors that affect exercise performance, including substrate utilization, aerobic and anaerobic capacity, ventilatory capacity, and anaerobic strength, and factors that affect the health of female athletes, including body composition, bone health, and menstrual function.

Factors that Affect Exercise Performance

Substrate Utilization: The new low and ultra-low dose and third generation progestin OC preparations minimize the side effects of early preparations on insulin resistance, decreased glucose tolerance, high plasma cholesterol and triglyceride levels [2]. However, current formulations of ultra-low dose OC preparations have not been extensively evaluated to determine the differences in substrate utilization throughout the active pill phase or hormone-free phase and during exercise. It has been hypothesized that during the active pill phase, with high EE, there would be a glycogen sparing effect and increased lipid use during exercise [13-16]. However, progestins are hypothesized to oppose the lipolytic effects induced by EE [13-16]. These hypotheses were based on observations of increased lipolysis and reduced carbohydrate oxidation during exercise with high estrogen in the follicular phase of the natural menstrual cycle and an opposition to lipolysis in the luteal phase with high progesterone and estrogen [17-19]. However, in rats and humans, the use of exogenous sex steroids have been shown to interfere with substrate utilization [13, 20]. A few researchers have utilized various modes (cycle ergometer and treadmill) and intensities of exercise to investigate changes of fuel utilization during exercise when combined with OC use.

Inconsistent results have been reported with regard to substrate availability following endurance exercise between *monophasic* OC users and non-users. For example, Bonen et al. [14] investigated substrate utilization during exercise in monophasic OC users compared to non-OC users. During 30 minutes of exercise at 40% of maximal oxygen consumption (VO_{2max}), a shift to high plasma free fatty acids and decreased plasma glucose

concentrations were observed in monophasic OC users compared to non-OC users; however, no differences between the active pill and hormone free phases were observed [14]. Similarly, Bemben et al. [13] reported a decrease in carbohydrate utilization and lower plasma glucose in the 40th and 50th minutes of a 90 minute treadmill test at 50% VO_{2max} in monophasic (35µg EE) and multi-phasic (35µg EE) OC users during the active pill phase compared to the luteal phase in non-OC users. However, no difference in fat oxidation was observed between OC users and non-OC users [13]. In contrast, a cross-sectional analysis by Sunderland et al. [21] investigated the impact of long term (12 months) monophasic (20-30µg EE) OC use on substrate utilization in regularly active women and failed to observe any changes in blood glucose concentrations between the active pill and hormone free phases, and between OC users and non-OC users following 30 second sprint treadmill exercise [21].

In support of the proposed hypothesis that high EE would spare glycogen and encourage lipid utilization but high progestin concentrations would oppose lipolysis, Redman et al. [22] investigated the impact of the third week of the active pill phase and the hormone free phase of *triphasic* OC use on metabolism in five elite female rowers. During three consecutive triphasic OC cycles increased glucose (37% and 8%) and reduced plasma triglyceride (8% and 31%) concentrations were reported at rest and post-anaerobic exercise in the hormone free phase compared to the active pill phase [22]. Similarly, in the menstrual cycle before and following 4 months of triphasic OC use, Casazza et al. [15] evaluated lipid utilization during exercise in eight active, eumenorrheic women and reported greater triglyceride mobilization during 60 minutes of leg ergometer exercise at 45% of VO_{2max} in the third week of the active pill phase (21.6%) and at 65% of VO_{2max} during the active pill (20.4%) and hormone free phase (21.6%) compared to pre-OC use [15]. However, no differences were observed in free fatty acid oxidation, but re-esterification of free fatty acids was not investigated [15]. In addition, Jacobs et al. [23], observed increased free fatty acid appearance, disappearance, and oxidation during exercise at 45 and 65% of peak oxygen

uptake (VO_{2peak}) compared to during rest in eight active women during the natural menstrual cycle prior to OC initiation and following 4 months of triphasic OC use. They also observed an increase in free fatty acid re-esterification and a decrease in the proportion of plasma free fatty acid rate of disappearance at rest and during exercise with triphasic OC use compared to pre-OC use. Suh et al. [24] investigated the effects of four months of triphasic OC use on substrate metabolism in eight active women; no difference in the rate of glucose appearance was observed between the third week of the active pill phase and the hormone free phase at rest or during exercise at 45 and 65% VO_{2peak}. Further, Suh et al. [24] did not observe any differences in glucose or lipid oxidation rates between the natural menstrual cycle and triphasic OC use or between the third week of the active pill phase and the hormone free phase of the OC cycle. A decrease in rate of glucose appearance was observed at rest and during exercise at 45 and 65% VO_{2peak} during triphasic OC use compared to the menstrual cycle prior to OC use [24]. The investigations of the effect of *triphasic* OC use on substrate metabolism to date used differing modes and durations of exercise [15, 22-24], and there appears to be a shift toward increased free-fatty acid mobilization, while substrate oxidation does not show a clear difference between phases of the OC cycle (active pill and hormone free phases) or cross-sectionally between triphasic OC users and non-OC users.

Limited research has been conducted to evaluate differences in substrate utilization with OC use in various prandial states. Tremblay et al. [25] investigated measures of substrate oxidation during 120 minutes of cycle ergometer exercise at 57% of VO_{2max} following ingestion of 2g/kg of glucose between triphasic OC users and non-OC users and reported no differences in exogenous and endogenous carbohydrate oxidation rates. However, Issaco et al. [26] reported a greater reliance on lipids during 45 minutes of exercise at 65% of VO_{2max} in the fasting state compared to in the post-prandial state in 21 active women using a monophasic (20-30μg EE) OC preparation. However, a greater reliance on lipids during exercise in the fasted state compared to the post-prandial state was

also observed in non-OC users, and there were no differences in lipid reliance between OC users and non-OC users [26].

In summary, current research findings do not indicate consistent changes in substrate utilization during exercise in women using monophasic and triphasic OC preparations. It is possible that during shorter exercise tests, where there is less reliance on glycogen and lipid utilization, less opportunity is available for the exogenous steroids to exert an influence over substrate flux [10, 27]. An insufficient number of studies have been conducted at various durations and intensities of exercise and durations of OC use to definitively determine whether OCs have an impact on substrate utilization during aerobic and anaerobic exercise. Further research is also needed to determine the effects of transdermal and vaginal hormonal contraceptives on substrate utilization during exercise in athletic women.

Exercise Capacity: The availability and use of glucose and lipids as a fuel source during exercise are known to impact aerobic and anaerobic capacity [28]. With the use of OC pills, early reports indicate an alteration of 1) fat and carbohydrate metabolism, as previously described, 2) movement of glucose into muscle (glucose flux), and 3) insulin sensitivity [13-16, 20]. These early observations of alterations in the proportion of fuel availability, glucose flux, and insulin sensitivity led to theories of the impact of OC use and phase of OC cycle on both aerobic and anaerobic capacity.

During the active pill phase, EE may exert a glycogen sparing effect, which is proposed to enhance sustained aerobic capacity [29]. During the hormone-free phase, when sex steroid hormone concentrations are lowest, the carbohydrate metabolism is up-regulated and may enhance anaerobic capacity [29, 30]. It is noteworthy that this review will focus on the effects of OC use on factors influencing exercise capacity, which are commonly assumed to correlate with athletic performance [31, 32], and will NOT focus on athletic

performance per se, which is defined as the complex interaction between physiological, tactical, technical, and psychological factors [31].

Aerobic Capacity: The large majority of investigators have evaluated the effects of monophasic OC preparations on aerobic capacity in both trained [33-37] and untrained [36, 38, 39] women. Few investigators have evaluated the influence of biphasic or triphasic OC preparations on aerobic capacity [23, 24, 40, 41]. Differences in aerobic capacity have been evaluated in both cross-sectional [33, 35-37] and longitudinal studies [23, 24, 40, 41]. Researchers have also focused on evaluating the variation in aerobic capacity between the active pill phase and the hormone free phase [33, 38].

In *untrained* women using *monophasic* OC pills, study results have been inconsistent [36, 38, 39]. Early work indicated a significant increase in VO_{2peak} for a standardized workload in untrained women following two cycles of high dose (50μg EE) monophasic OC use when compared with baseline measures (assessed during the menstrual cycle prior to OC initiation) [39]. However, more recently, investigators have suggested that low dose (20-35μg EE) monophasic OC use for 12 months had no effect on oxygen consumption (VO_2) in untrained women during the hormone free phase or the final week of the active pill phase [38]. Similarly, Rebelo et al. [36] reported no difference in VO_{2peak} in sedentary women who had been using a low dose (20μg EE) monophasic OC for 18 months compared to non-OC users.

Varying results have also been reported in studies evaluating the impact of *monophasic* OC pill use in *trained* women, both in recreational and in elite athletes [33, 34, 36, 37, 42]. In recreationally active women who had been using a low-dose (20-30μg EE) monophasic OC for a minimum of 18 months, submaximal VO_2 was reduced by 3-6% during the active pill phase compared to the hormone free phase [33]. Other reports in recreationally active women using OCs for a minimum of 12 months demonstrate a reduced VO_{2peak} and a lower VO_2 at the anaerobic threshold, with no differences reported in time to

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exhaustion during a submaximal aerobic test compared to non-OC users [35]. Likewise, Notelovitz et al. [43] described a 7-8% decrease in VO_{2peak} following 6 months of low-dose (35μg EE) OC use in trained women, while the non-OC user control group had a 7.5% increase in VO_{2peak} during the same timeframe. However, in 2010, Rebelo et al. [36] did not observe a significant difference in VO_{2peak} nor VO_2 at the anaerobic threshold of trained women who had been using a low-dose (20μg EE) monophasic OC for a minimum of 18 months compared to non-OC users. Similarly, in trained rowers, who had been on an low dose (20μg EE) OC preparation for a minimum of 3 months, no significant differences in measurements of VO_{2max} and VO_2 at the aerobic-anaerobic transition were observed between day 8 or day 20 of a monophasic OC pill cycle [37]. Studies of monophasic OC use in highly trained women also indicate that there is no significant effect of OC preparations on aerobic capacity [34].

Due to the relatively equal proportion of studies that have shown a decrease or no significant change in VO_2 with OC use in *untrained* and *trained* women, the impact of *monophasic* OC use on aerobic capacity remains unclear. In summary, the conflicting results in VO_2 among *untrained and trained* women using a *monophasic* OC have been reported to be secondary to a wide variety of causes, that include 1) differences in progestational and androgenic activity of the progestins within the contraceptives in the studies, 2) duration of OC use (no use, 3, 6, 12 or 18 months of use prior to study initiation), 3) varying training status of the study participants, 4) small sample sizes that may not power studies adequately for primary study outcomes, and 5) variation in the exercise protocols utilized in each research study [10, 29]. Further research will be necessary to resolve this issue.

Very few investigators have evaluated the impact of *triphasic* OC preparations on aerobic capacity in *trained* women [23, 24, 40, 41]. In a study of trained athletes over two triphasic OC pill cycles, a 4.7% decrease in VO_{2peak} but no change in endurance performance, as measured by time to exhaustion, was observed [41]. Similarly, a study of

triphasic OC pills over 4 [24] and 6 [23] cycles demonstrated a decrease in VO_{2peak} of 11-15% in active women, agreeing with results from a study conducted by Casazza et al. [40] which demonstrated an 11% decrease in VO_{2peak} among recreationally active women using a triphasic OC for 4 months. In summary, triphasic OC preparations appear to decrease VO_2 in trained athletes during use of up to 6 months. No studies, to date, have investigated the impact of triphasic OC use 1) of long duration (> 6 months) or 2) in untrained women.

Ventilatory Capacity: Early research on the menstrual cycle has shown that the natural rise in progesterone during the luteal phase increases chemosensitivity of the lungs to hypoxia and hypercapnia [44]; thus monophasic OC preparations with high doses of progestin and the third phase of the triphasic OC may also increase the chemosensitivity of the lungs and increase ventilatory capacity. In agreement with this hypothesis, a few studies conducted in trained women using monophasic OC preparations demonstrated an increase in ventilation (VE) [37, 45]; however, these results have not been consistent, particularly when considering the ventilation response to OC preparations in untrained women or with a different OC formulation, i.e. triphasic OC preparations.

For example, during a one hour endurance test performed by *trained* cyclists taking *monophasic* OC preparation mean VE and mean ventilation per oxygen consumption (VE/VO_2) were 7 and 5% higher, respectively, during the active pill phase compared to measures taken early and late in the hormone free phase [45]. However, in trained rowers administered a monophasic OC, there was a tendency toward an increased ventilatory response during the hormone free phase demonstrated by increased VE and VE/VO_2 at VO_{2max} (1.6 and 6.3%, respectively) compared to the active pill phase [37].

On the other hand, no significant differences in VE were observed in *untrained* women, who had been using a *monophasic* OC preparation (20-30µg EE) for a minimum of 18 months and performed submaximal treadmill exercise during any phase (active pill and hormone-free) of a monophasic OC pill cycle [33]. Further, Redman et al. [46] evaluated

ventilatory measures in untrained women who had been using contraceptives for a minimum of 6 months. Participants changed their OC use in a cross-over design to a 35μg EE monophasic OC with high (1000μg norethisterone) or low (500μg norethisterone) progesterone concentration. VE and VE/VO$_2$ measured at VO$_{2peak}$ were not different between progesterone concentrations; however, VE and VE/VO$_2$ were 10.5 and 8.5% higher, respectively, at rest during use of the high progesterone OC compared to the low progesterone OC [46]. Rebelo et al. [36] evaluated VE in active and sedentary OC users (20μg EE; minimum use 18 months) and non-OC users and did not observe any differences among the four groups. Further, Joyce et al. [35, 42] did not observe any differences in VE between *untrained or trained* OC users (minimum use 12 months) and non-users.

Contrary to the hypothesis that the third week of the *triphasic* OC preparation may increase ventilatory capacity due to the high progestational content, the majority of studies have demonstrated no effect of triphasic OC preparation use on VE in both *untrained and trained* women. For example, in *untrained* women who utilized a triphasic OC for 4 months, VE measured in the active pill and hormone free phases were not significantly different compared to measurements taken during the follicular or luteal phase of the menstrual cycle prior to OC initiation [40]. Similarly, in *trained* women, VE did not differ between measurements taken following 4 months of triphasic OC use during the active pill and hormone free phases nor compared to the follicular and luteal phases in the menstrual cycle prior to OC initiation [24]. Further, VE did not change with a single cycle of a triphasic OC in trained women compared to the menstrual cycle prior to OC initiation [41].

Overall, OC use (monophasic or triphasic formulations) does not appear to have an effect on ventilatory capacity. The conflicting results observed with monophasic OC use can be attributed to the initial fitness level of the participants, the intensity and duration of the exercise, and/or the androgenic activity of the progestin in the OC preparations used. Further research is necessary to confirm the results from studies evaluating triphasic OC use.

Anaerobic Exercise: Few studies have been conducted on the influence of OC preparations on anaerobic performance, including capacity and strength. Variation in anaerobic performance during an OC cycle could be caused by the impact of EE and progestins on substrate utilization, buffering capacity, and neuromuscular function [47].

Anaerobic Capacity: During short-duration maximal exercise, the maximal amount of ATP resynthesized via anaerobic metabolism (phosphocreatine and glycolysis) is considered anaerobic capacity [29]. During a menstrual cycle, a low estrogen environment and increased circulating aldosterone following a drop in progesterone (as observed at the end of the luteal phase) upregulates carbohydrate metabolism, which is necessary for the anaerobic production of ATP [29]. Further, increases in circulating aldosterone may increase body fluid and electrolyte retention, thus increasing buffering and anaerobic capacity [48]. As such, in accordance with the influence of endogenous steroid hormones on substrate utilization and buffering capacity, it is plausible that anaerobic capacity would be greatest during the hormone free phase of the OC cycle [29]. Currently, available literature does not provide hypotheses surrounding the impact of OC use on anaerobic capacity when compared to non-OC users. Production of lactate, a by-product of anaerobic metabolism, has been evaluated in women using monophasic and triphasic OC preparations, thus serving as an indicator of the impact of OC use on anaerobic capacity

The majority of studies, however, do not support the proposed hypothesis that anaerobic capacity is enhanced during the hormone free phase. For example, Bonen et al. [14] evaluated lactate concentrations following a walk to elicit 85% VO_{2max} in *monophasic* OC users (30-50µg EE) and observed no differences between phases of the OC cycle [14]. Similarly, Bernardes et al. [18] investigated anaerobic capacity in a mixed group of women using *monophasic* and *triphasic* OC preparations of a similar EE dose (30-40µg EE) and found no variation in blood lactate between the active pill phase and the hormone free phase following intermittent exercise (80% VO_{2max}) tests. Likewise, Lynch et al. [49]

investigated the impact of low dose (30-35μg EE) *monophasic* OC administration on intermittent exercise (five by 20s treadmill runs at increasing speed on a 10.5% incline) in *recreationally active* women and observed no significant difference in peak blood lactate between OC users and non-OC users; however, peak blood lactate was significantly higher during week 1 compared to week 2 (11.2 vs. 9.6 mmol/L) of the active pill phase of OC users. Notably, in contrast to their previous report, Lynch et al. [38] observed no difference in blood lactate concentrations between week 1 and week 3 of the active pill phase after repeating the intermittent exercise test on *untrained* women who had been using a low dose OC preparation (20-35μg EE) for at least 12 months. Interestingly, neither of the studies by Lynch et al. [38, 49] evaluated differences between the active pill and hormone free phases. Following high intensity, intermittent exercise in the heat, no differences in blood lactate concentrations were observed between OC cycle phases in *trained* women using *monophasic* (20-35μg EE) OC preparations [50]. In a study by Redman et al. [22], conducted among 6 highly *trained* rowers who had been taking a *triphasic* OC for a minimum of 12 months, a significant increase in glucose and decrease in plasma triglyceride with no difference in lactate concentrations were observed in the hormone free phase compared to the third week of the active pill phase following a 1000m simulated row test. The participants were tested in three consecutive OC cycles and the results were consistent between the active pill phase and hormone free phase [22]. In 2011, Sunderland et al. [21] also evaluated the impact of *monophasic* OC use (20-30ug EE) on blood lactate concentration following an all-out 30s sprint. Blood lactate concentrations were not different between the active pill phase and hormone free phase in OC users [21]. These results indicate that there is no difference in carbohydrate metabolism between OC cycle phases during tests of short, intense activity when compared with studies of aerobic and submaximal endurance exercise [27].

Recently, however, findings by Rechichi et al. [27] do support the hypothesis that anaerobic capacity is greater during the hormone free phase of the OC cycle. For example,

Rechichi et al. [27] measured a 23% decrease in peak blood lactate following a 200m time trial in the hormone free phase compared to late in the active pill phase in competitive swimmers using a monophasic OC (30µg EE) preparation, indicating an increase in buffering capacity.

In addition to comparing differences between phases of the OC cycle, three teams of investigators also reported comparisons between OC users and non-OC users. In 1991, Bonen et al. [14] evaluated lactate concentrations following a walk to elicit 85% VO_{2max} in monophasic OC users (30-50µg EE) and non-OC users. No differences in blood lactate concentrations were observed between OC users and non-OC users [14]. Sunderland et al. [50] investigated high intensity intermittent exercise in the heat in trained women on monophasic (20-35µg EE) OC preparations and non-OC users and found no differences in blood lactate concentrations. However, in 2011, Sunderland et al. [21] found significantly higher blood lactate concentrations following an all-out 30-second sprint in OC users compared to non-OC users. Of three available reports comparing OC users and non-OC users, this was the first report demonstrating a difference in blood lactate concentrations between OC users and non-OC users. The differences in the available data are due to the large variability in EE concentrations as well as the variation in the exercise evaluated and the conditions of the test.

The majority of available data to date do not support the hypothesis that anaerobic capacity would be greater during the hormone free phase compared to the active pill phase in OC users. The limited comparisons of anaerobic capacity between OC users and non-OC users indicate the exogenous hormones in OC preparations have a diminished capacity to alter aldosterone and lactate buffering capacity; however, further research is needed to conclude if there is a general impact of OC use (compared to non-OC users) on anaerobic capacity. Data regarding blood lactate accumulation following anaerobic exercise during use of monophasic or triphasic OC preparations suggest the impact of aldosterone on buffering capacity is not consistent with changes observed in the natural menstrual cycle. Thus, future

studies should investigate the alterations in the progesterone/aldosterone ratio at more than one time point during the active pill and hormone free phases. It is also possible that duration of activity is a significant factor in the ability of exogenous hormones from OC preparations to impact anaerobic capacity. With shorter duration tests the influence of sex steroids on lipid and glycogen utilization is limited; thus there may be less opportunity for the exogenous steroids to exert their influence [10, 29].

Anaerobic Strength: It has been proposed that endogenous estrogens, when highest, may have a positive impact on muscle strength, while progesterone inhibits the effects of estrogen [51]. However, EE may not influence the estrogen receptors within the skeletal muscle in the same way as endogenous estrogen or the progestin consumed with OC use may influence the interaction of EE with the neuromuscular pathway [47]. Rechichi et al. [47] evaluated reactive strength with a 45cm drop height test in team sport *athletes* taking a *monophasic* OC preparation and found reactive strength was approximately 11% lower during the hormone free phase (early or late) compared to the active pill phase. In contrast, Sarwar et al. [52] observed no differences with handgrip strength or isometric quadriceps strength between active pill and hormone free phases of a monophasic OC cycle. Similarly, Elliot et al. [53] found no differences in dynamic or isometric leg strength between the active pill and hormone free phases in 14 women using a monophasic OC (30-35µg EE) preparation. In addition, Elliot et al. [53] did not observe differences in dynamic or isometric leg strength between the 14 monophasic OC users and 7 eumenorrheic women. A study by Peters et al. [54] on trained athletes using monophasic OC (30µg EE) preparation showed no difference in maximal leg isokinetic strength through extension or flexion between the active pill and hormone free phases of the OC cycle. Similarly, Ekenros et al. [55] did not observe any differences in isokinetic knee extensor strength or isometric handgrip strength between monophasic (20-35µg EE) OC users and non-OC users nor between phases of an OC cycle. Further testing is required to determine if there is an effect of OC use on reactive strength; however, the available data indicates that isometric strength

is not influenced by *monophasic* OC use or phase of the monophasic OC cycle. Further

research is needed to determine the impact of *triphasic* OC use on all aspects of muscular

strength.

Competitive Exercise Performance: As previously discussed, factors that influence

performance may be altered by use of OC preparations; however, what is the cumulative

effect on competitive performance? There are currently few studies that have evaluated

field measures of performance, such as a time trial or competitive event [27]. As such,

limited information is known about how OC use will ultimately affect the outcome of an

individual athlete's competitive performance. Overall, of the studies that have assessed

more generally applicable measures of performance, it is found that OC use does not

appear to benefit athletic performance. For example, in five female rowers who had been

using a triphasic OC preparation for 12 months, Redman et al. [22] found 1000m simulated

rowing times were 3s faster during the hormone free phase compared to the third week of

the active pill phase in 3 consecutively tested OC cycles. Rechichi et al. [27] also

demonstrated this apparent lack of beneficial influence of OC use on athletic performance

among six competitive swimmers and water polo players using a monophasic OC (30µg EE)

preparation; they observed no significant difference in 200-meter swim time between the

active pill phase and the hormone-free phase. Likewise, a few investigators have evaluated

time to exhaustion as a proxy measure of endurance performance and have shown no

difference in time to exhaustion between OC users and non-OC users [35, 41, 56], nor

between phases of the OC cycle [56].

Conflicting results have been reported by investigators during tests of peak power

output. Casazza et al. [40] reported that four months of OC use in six moderately-active

women revealed a significant 8% decrease in peak power output as evaluated on a cycle

ergometer, indicating that OC use may impair peak exercise capacity. On the other hand,

Redman et al. [46] reported improved peak power output (1.5%) as evaluated on a cycle

ergometer in 26 sedentary women using a monophasic OC preparation with a high progesterone content (1000µg norethisterone) compared with a low progesterone preparation (500µg norethisterone), indicating that OC preparations with higher progesterone content may enhance peak exercise capacity. However, the lack of large studies that assess field measures of performance highlights the need for more research in this area and limits knowledge about how OC use may ultimately impact competitive performance in female athletes.

Factors that Affect the Health of Athletes

<u>Body Composition and Bone Health:</u>

Body Composition: In athletic girls and women, changes in body composition, particularly in the fat mass compartment, caused by OC use would be important in determining changes in athletic performance. Available research has focused on a broad population of women, while there is limited information available specifically regarding changes in body composition in female athletes using OC preparations. In general, investigators have demonstrated an increase in body mass, in particular fat mass, with OC use among female athletes. For example, Coney et al. [57] observed a non-significant increase of ≥ 1kg in body mass over 6 months of *monophasic* OC use in 40% of participants in a pooled analysis of two phase III randomized, placebo-controlled trials. Further, use of monophasic OC for 6 months by active women showed a 2kg increase in body mass compared to active controls [43]. However, following a month without use of OC pills the participants returned to their baseline weight [43]. Rickenlund et al. [58] observed an increase in body weight and fat mass following 10 months of monophasic OC use in female, endurance athletes who had oligomenorrhea/amenorrhea prior to OC use. Likewise, a 3% and 9% increase in body weight and fat mass, respectively, were observed following 4 months of *triphasic* OC use in active women, while no change in fat-free mass was observed [40]. Similarly, Jacobs et al. [23] and Suh et al. [24] demonstrated significant increases in body mass (1.7-2.5%) and

percent body fat (5.6-10%) over 4-6 months of triphasic OC use in active women. Lebrun et al. [41] observed a significant increase in body mass and percent body fat with use of a triphasic OC in trained women over 2 pill cycles.

In contrast, results from a few studies, reveal a decrease or no change in body mass or fat mass. In adolescent girls administered OC preparations for the first time, a significant decrease in body fat percentage (0.1%) and a non-significant increase (0.6%) in lean body mass after 6 months of use was observed [59]. Thai women using two different *monophasic* OC pill types (35μg/d or 30μg/d EE) showed a trend of loss in body mass of 1.5kg and 1.1kg, respectively, over 6 months [60]. On the other hand, in collegiate cross-country runners randomized to use of a monophasic OC there was little difference in weight or fat mass gain compared to no treatment following a minimum of 6 months of OC use [61]. Women who had regular menses prior to initiation of OC use showed a significant increase in lean body mass compared to no treatment; however, there was no association of OC use and lean body mass change among women with oligomenorrhea prior to OC initiation [61]. The differences observed between studies on the general population and specific athletic populations are most adequately explained by the difference in training status of the participants; however, differences in progestational or androgenic activity of the progestin and EE concentrations in the monophasic and triphasic OC pills used in each study may also explain some of the variation in results.

In a review by Gallo et al. [62] no large effect of OC preparations on weight gain was evident; however, there was insufficient evidence to rule out an association. The changes observed over long duration monophasic and triphasic OC use, though small, are meaningful differences in an athletic performance context. An increase in body fat, even small, could be viewed as detrimental to performance in many sports, most notably aesthetic sports where body shape is imperative (i.e., gymnastics, distance running, and dance) and in weight-classification events where a pre-event weigh in determines the competition category (i.e., rowing and weightlifting). Overall, the afore mentioned studies suggest that

athletic women may experience an increase in body mass and percent body fat during long term use of any type and formulation of OC. The weight gain observed in studies have been hypothesized to be due to fluid retention, increased subcutaneous fat, anabolic effects on appetite, and androgenic effects on muscle mass; however, no dose-response relationship with weight gain has been observed between the EE concentration of the various oral contraceptive formulations that have been examined [62].

Bone Health: Published data on the influence of OC preparations on bone mass, another component of body composition, among physically-active girls and women are limited and inconclusive. However, results from a few published studies suggest that OC preparations may have detrimental effects on bone health, especially when combined with exercise, thereby initiating concern about the use of OC preparations in female athletes.

In the only prospective study to date that assessed bone mineral density (BMD) and content (BMC) among women taking OC preparations and participating in a 24-month exercise training program, Weaver et al. [63] reported a significant decrease in BMD and BMC at the lumbar spine after 6 months of OC use and exercise training. Interestingly, this change was significantly different when compared with the women participating in the exercise training program but not taking an OC preparation and the women taking an OC preparation but not exercising who both demonstrated a significant gain in lumbar spine BMD after 6 months [63]. After 24 months of training, lumbar spine BMD of the women who were taking an OC preparation and exercising had returned to baseline but the women who were taking an OC preparation and not exercising demonstrated a continued gain in lumbar spine BMD [63]. Although not statistically significant, women who were taking OC preparations and participating in the exercise program had smaller gains in femoral neck cross-sectional moment of inertia and cross-sectional area, two important indicators of bone strength, when compared with women who were neither taking OC preparations nor exercising [64]. Further, in a cross-sectional study, Hartard et al. [65] explored the history of

OC use and regular physical activity on BMD among young women; those who had a long history of both OC use and physical activity had lower BMD than those with a short history of OC use and a long history of physical activity. In agreement with these findings, a retrospective analysis of female endurance athletes aged 18-35 years revealed that BMD at both the hip and the lumbar spine was significantly lower in the athletes who reported OC use for greater than 3 years or greater than 50% of the time since menarche compared with control athletes [65, 66]. Further, age at initiation of OC use was a strong positive predictor of BMD at the lumbar spine, indicating the potentially negative effects that OC use may have on BMD during key years of bone mineral accrual [66].

It appears that OC use may compromise the skeletal benefits of regular physical activity; however, the underlying reason why OC use in combination with exercise may be particularly detrimental to bone health is unclear. It must be noted that even within the general population of women, the influence of OC preparations on bone health lacks clarity, with studies demonstrating an increase [67], decrease [68, 69], and no change [6, 68, 70-74] in BMD during OC use lasting 12-36 months.

The skeletal consequences that may be associated with OC use are proposed to stem from the hepatic first pass effect of EE. When administered orally, doses of estrogen greater than what is naturally in the circulation must be given due to the reduced bioavailability of the hormone after active metabolism by the liver [75]. The synthesis of hormones, clotting factors, growth factors, and binding proteins by the liver can be affected by this first-pass effect through the liver [75]. Thus, it is speculated that the first-pass effect of exogenous estrogen through the liver suppresses hepatic production of insulin-like growth factor-1 (IGF-1) and up-regulates the synthesis of certain binding proteins, such as IGF binding protein-1 (IGFBP-1), which bind to IGF-1 further reducing its bioavailability [75]. IGF-1 is a protein known have strong anabolic effects on bone tissue and may stimulate bone formation, as evidenced by the positive association between circulating IGF-1 concentrations and a marker of bone formation during childhood and adolescence [76]. In

support of the proposed negative effect of OC administration on hepatic production of IGF-1, Hansen et al. [77] reported significantly lower serum concentrations of IGF-1 and P1NP (amino terminal propeptide of type I collagen), a marker of bone formation, 24 hours after an exercise bout in women who were taking OC preparations compared to women who were not using an OC.

Current studies indicate that long term use of OC preparations may increase body weight and percent body fat, while causing a decrease in bone mineral density and content in athletic women. Changes in both bone and fat composition are likely to have a negative impact on aerobic and anaerobic performance. Further research is necessary to confirm available results, determine mechanisms of action on changes in body composition, and evaluate the effects of all forms of hormonal contraceptives on body composition and bone health.

Menstrual Function and Contraceptive use in Female Athletes: Up to 60% of female athletes present with the most severe menstrual disturbance, amenorrhea [78]. The underlying cause of exercise-associated amenorrhea is low energy availability, or insufficient energy to perform the daily physiological and locomotive functions of life [79]. The energy deficit contributes to a cascade of physiological adaptations to conserve fuel, including metabolic adaptations that suppress the reproductive axis. (For a detailed explanation of the characteristics and consequences of an energy deficiency in female athletes, please refer to chapters 11 and 12.) The metabolic and reproductive suppression that occurs in an energy deficient environment contributes to low bone mass. The combination of low energy availability, amenorrhea, and low bone mass is referred to as the Female Athlete Triad [80]. Correcting the low energy availability that leads to the menstrual dysfunction and poor bone health, via an increase in energy intake or a decrease in energy expenditure, should be the mainstay of clinical practice [81, 82]. However, the increase in energy intake and/or decrease in energy expenditure that often leads to weight gain can be

a challenging treatment strategy for many clinicians and female athletes. As such, clinicians may prescribe OC preparations to female athletes who present with amenorrhea in order to "regulate" the menstrual cycle [83].

As described in the 2014 Female Athlete Triad Consensus Statement [81, 82], pharmacological treatment, such as OC preparations, should only be considered for a female athlete with the Triad if there is no positive response to one year of non-pharmacological treatment (increasing energy intake and/or decreasing energy expenditure) and if the athlete experiences a new fracture. More specifically, pharmacological treatment may be considered in athletes who have undergone non-pharmacological treatment but continue to experience health consequences of amenorrhea such as infertility, vaginal dryness and dyspareunia, or impaired bone health [81, 82].

However, the efficacy of OC preparations in improving bone health in female athletes with the Triad is unclear [81, 82, 84]. Further, spontaneous menses is not restored with the use of OC preparations; rather, the induced withdrawal bleeding that occurs with OC preparations instigates a false sense of security to the athlete [81, 82]. The root cause of the menstrual disturbances and poor bone health, i.e. the low energy availability, is not addressed, but masked, with administration of OC preparations. For this reason, OC preparations alone are not effective for treating menstrual disturbances that occur as a result of an energy deficit, and if used, should be administered in combination with non-pharmacological treatment [81, 82]. It must be emphasized that the cornerstone of treatment of the female athlete triad is reversal of the low energy availability that leads to the menstrual dysfunction and poor bone health. The engagement of a multidisciplinary team that includes a physician, sports dietician, and mental health professional is necessary to guide the athlete through recovery to restoration of a healthy energy state and menstrual status, which are important to overall and long-term health and well-being.

Conclusions and Future Directions

The purpose of this brief review was to elucidate the current understanding of the effects of combined oral contraceptives on factors that influence exercise performance and the health of athletes. The scope of the review was limited to OC preparations as there is not currently data available regarding the impact of the transdermal patch or vaginal ring contraceptive use on physiologic factors associated with exercise performance. The effects of OC preparations on physiologic factors related to performance are complicated due to the large variation in OC formulations available. Older formulations used in the earliest studies available will have had a greater physiologic impact on athletic performance compared to the newer, low dose formulations.

Available data examining physiology and performance within a single OC cycle and across multiple OC cycles, regardless of duration of prior OC use, indicates the potential for variation in: 1) aerobic performance due to an altered ventilatory response [37, 45], 2) anaerobic performance, due to alterations in substrate utilization and buffering capacity [21, 27]; and 3) reactive strength [29, 47]. Most data that are available on the effects of OC preparations on factors influencing performance includes data on substrate utilization [2, 13-26], aerobic capacity [23, 24, 33-41], and body composition [23, 24, 41, 43, 57, 59-62], while less is understood regarding the effects of OC use on other factors that influence exercise performance including core body temperature, cardiovascular responses, anaerobic capacity, muscle strength or power, and post exercise recovery [10]. There is little evidence to suggest that the acute exogenous hormonal fluctuations observed across a single OC cycle have a significant effect on variables that impact aerobic performance. The variations in types of OC preparations used and the dosage of progesterone within the available studies relate to the observed variability in ventilation, VO_2 and substrate metabolism [29].

Results in the small number of studies to date are conflicting and disagreements abound between investigators regarding the effects and mechanisms involved in the variation in physiology and performance at different phases (active pill and hormone free

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phases) within a single OC cycle. The current cross-sectional studies with small sample sizes should be viewed with caution due to the absence of randomization and variation in formulations used by participants [10]. Further studies on monophasic, triphasic, transdermal, and vaginal contraceptives are needed to evaluate the impact of exogenous steroids on all aspects of athletic performance. Future trials should be appropriately randomized and controlled [10]. There will likely be high inter-individual variability in response to the exogenous hormones contained in each hormonal contraceptive evaluated.

Variation between the experimental findings in available studies are likely caused by: 1) the use of different types of OC agents (monophasic vs. triphasic preparations); 2) differences in exogenous hormone concentrations of the different OC formulations (as well as the androgenicity and potency of the progestin); 3) variation in defining the phases of an OC cycle and determination of testing days within an OC cycle; 4) small sample sizes in individual studies; 5) variation in the training status of participants; 6) variation in the exercise protocols used to test aerobic and anaerobic performance between investigations; and 7) variation in OC exposure prior to study initiation [10, 29]. The day of testing during a hormonal contraceptive cycle is important especially due to variation in dosing of exogenous steroids across a cycle [10]. In monophasic OC preparations, as well as transdermal and vaginal contraceptives, the same dose of EE and progestin is delivered across the entire active hormone phase followed by 7 days free from hormones. However, in triphasic OC preparations there is a need to evaluate the impact of all 3 portions of the active pill phase and during the hormone free phase to ensure observed effects do not vary across the OC cycle [10]. There is also a need to account for the duration of hormonal contraceptive use when comparing the effects of a single OC cycle or multiple cycles observed in studies. The effects of OC usage on physiologic variables impacting exercise performance may change with the duration of OC use [10]. It is quite possible the effects are more strongly observed within the first 2 hormonal contraceptive cycles, and thereafter the impact of OC use on exercise performance is negligible.

More research is necessary to determine the effects of the most commonly prescribed contraceptives (monophasic and triphasic OCs and the vaginal ring) on endurance performance. The current findings are unclear as to whether variation in oxygen uptake, ventilation, and exercise economy are influenced by changes in exogenous steroids used in monophasic and triphasic OCs. The influence of training status (sedentary, recreationally active, or elite), dose of exogenous steroids, and the duration of OC use on endurance performance still requires research.

Future studies should address the afore mentioned issues in comparability, as well as examine differences in effects of short term and long term consumption on athletic performance, off label extension of cycles, and the effects of transdermal and vaginal contraceptives on performance. Expanding research to examine the differences in duration of use, off label extension of cycles, as well as transdermal and vaginal contraceptives will assist in determining the mechanisms behind the impact of exogenous steroids on physiologic variables of performance. Strong consideration of the training status of participants, sample size, type of hormonal contraceptive, and days of testing during the hormonal contraceptive cycle should be made in all future studies. A wide variety of training statuses (sedentary, recreationally active, trained, and elite) have been evaluated to various degrees, however the effects of OCs on athletic performance may be more important to elite athletes. Therefore, interpretation of results based on statistics and meaningful differences that are not statistically significant should also be considered. To allow determination of meaningful and statistical differences, future studies should ensure appropriate randomization for the design while maintaining adequate power when assessing the effects of hormonal contraception on performance. Evaluation of a single formulation within each study is important to assess the impact of specific EE and progestin potencies and androgenicities on exercise performance and will be necessary in the development of guidelines of OC use for athletes.

It is prudent that any female athlete considering hormonal contraception should talk with the general practitioner and consider not only the hormonal contraceptive formulation but its potential impact on overall health and performance variables. The type of hormonal contraceptive (oral contraceptive, transdermal contraceptive or vaginal contraceptive) and the potency and androgenicity of the progestin determine the potential effects on performance and should be considered when deciding which hormonal contraceptive, if any, should be taken and when during the training period a hormonal contraceptive regimen should be initiated. The correct hormonal contraceptive choice for each athlete may be different; therefore, trying various formulations available on the market in an individualized manner for each athlete in conjunction with a general practitioner may be necessary. Further, it must be emphasized that OC use may not promote optimal health in athletes with the Female Athlete Triad. As such, allowing a wide range of information to be available to athletes and support staff is important in allowing guidelines for OC use in athletes to be developed and based on sound scientific evidence.

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Chapter 3: Study 1

Abstract

To determine if reducing the frequency of urinary sample collection from daily to 5-, 3- or 2-days per week during a menstrual cycle or 28-day amenorrheic monitoring period provide accurate representations of the reproductive hormone metabolites estrone-1-glucuronide (E1G) and pregnanediol glucuronide (PdG) exposure and mean concentrations. Exercising women presenting with eumenorrhea or exercise-associated menstrual disturbances collected daily urine samples for the assessment of E1G and PdG concentrations. After enzyme immunoassay analysis of the daily samples, E1G and PdG data were systematically removed from each menstrual cycle or amenorrheic monitoring period to mimic three reduced collection frequencies, representing 5-, 3-, and 2-days per week. Exposure and mean concentration were calculated for both hormones and all 4 urinary collection frequencies. E1G and PdG exposure and mean cycle concentrations derived from reduced collection frequencies were not different from daily collection (p>0.05), independent of whether menstrual cycles and monitoring periods were analyzed together or separately. Bland-Altman analysis indicated acceptable agreement between each reduced collection frequency and daily collection. Compared to daily urinary collection reduced collection frequency of 5-, 3-, or 2-days each week provides accurate E1G and PdG profiles of collection periods of various lengths and types of menstrual function. Reduction of urinary sample collection frequency may enable researchers to reduce participant burden and costs, increase compliance, and study a wider range of study populations.

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Introduction

Hormonal evaluation of the reproductive potential of women in population based studies most often uses samples of blood [1-3], urine [4-7], or saliva [5, 8-10]. These assessments are generally limited by participant training, compliance, and the cost of the assays. Indeed, the optimal method should yield good participant tolerance and compliance with sample collection while still providing reliable, sensitive, and specific information about the participants' reproductive status [10].

In many cases, the use of the gold standard for repeated assessment of reproductive function, i.e., daily serum sampling which involves invasive blood draws, is not feasible; therefore, the collection of daily urine samples is an advantageous alternative due to its non-invasive and self-collectable nature [11]. Daily collection of urine samples during a menstrual cycle or specified monitoring period provides information about reproductive hormone exposure and clinical endpoints of reproductive status, such as ovulation [5, 6, 12-14], pregnancy [15-18], and menstrual cycle status [3-5, 19-21] in humans and non-human primates. Exposure to reproductive hormones has more recently been shown to be important predictors of general health and disease risk. Measures of reproductive hormone exposure from daily urinary samples have been associated with cardiovascular function in amenorrheic exercising women, specifically endothelial dysfunction, bradycardia, low systolic blood pressure, reduced regional blood flow, increased local vascular resistance, and an unfavorable lipid profile [22-24]. Further, reduced exposure to estrogen in amenorrheic exercising women, as assessed by daily urine sample collection, has been associated with increased concentrations of osteoprotegerin, an important regulator of bone resorption [25], and clinical measures of bone health [26, 27]. Risk of ovarian cancer [28] and breast cancer [29, 30] have also been associated with exposure to reproductive hormones.

Unsupervised participants can easily collect urine samples, thus facilitating monitoring of ovarian function over extended time periods [31]. However, it has been noted

that daily urine sample collection presents a substantial participant burden [32]. The substantial cost of time and participant burden can contribute to increased non-compliance and higher dropout rates [32]. Compliance with daily urinary sample collection is typically high in short-term studies (1-3 months). The potential for reduced compliance increases over time [32]; however, specific data on participant compliance to urinary collection is scant in publications. For studies lasting between 1 and 3 months, compliance to daily urinary sample collection is in the range of 92-97% [10, 33, 34]. For example, Kesner et al. [35] reported that during a time period of 2 complete menstrual cycles, 97% of all scheduled samples were collected. In the Women's Reproductive Health Study, 93% of all daily urine samples were collected over the course of 2 consecutive menstrual cycles [36].

In studies lasting between 5 and 12 months, compliance to urinary sample collection is more variable and somewhat lower, ranging between 50-100% [37-39]. For example, retrospective analysis of the Study of Women Across the Nation Daily Hormone sub-study, only 680 of 848 eligible participants had collected 80% of the required samples [37]. In the Semi-Conductor Health Study, where participants were asked to collect urinary samples daily for 5 cycles, only 57% of all cycles had fewer than 3 days of missing data in any 5-day rolling window [38]. In our laboratory, the participants who completed 4 or more months of a 12-month study collected an average of 90% of the requested samples; however, individual compliance ranged from 61-100% (unpublished data).

The design of any experiment needs to balance data quantity and quality while reducing participant burden and project cost and increasing compliance. To our knowledge, the only attempt to validate a reduced sampling frequency for use with urine specimens was conducted by O'Connor et al. [40], who evaluated the specificity and sensitivity of reduced collection frequencies to determine the presence of ovulation with progesterone glucuronide based algorithms. The every other day reduced collection frequency accurately and precisely detected day of ovulation [14]. Thus, a reduced collection frequency could be useful in conducting research in populations who may be hesitant to participate in research

projects that involve daily urine sampling, such as children or adolescents, and may aid in collection of urinary samples in locations with limited cooling and storage capacity. In large-scale and long-term research studies, reduced collection frequencies would not only reduce project cost and participant burden but would also enable researchers to recruit from a larger demographic area due to the reduced need for storage.

To reduce the burden of collecting daily urinary hormone specimens and reduce project costs, our goal was to evaluate if a reduction in the number of collection days from 7 days per week (i.e., daily sample collection) to 5 (i.e., weekday sample collection), 3 (i.e., Monday/Wednesday/Friday), or 2 (i.e., Monday/Thursday) days per week would provide an accurate representation of estrone-1-glucuronide (E1G) and pregnanediol glucuronide (PdG) exposure and mean concentration during an entire menstrual cycle/monitoring period. We chose to evaluate the impact of reduced collection frequencies on E1G and PdG exposure and mean concentrations because both measures are important predictors of bone health [26, 27], cardiovascular health [22-24], and ovarian [28] and breast cancer risk [29, 30, 41-43]. We also sought to evaluate if the validity of the reduced sample collection frequencies would be affected by cycle type (eumenorrheic or amenorrheic) or variability of cycle lengths (20-45 day range or 26-36 day range). As such, the purpose of this analysis was to explore the average and individual agreement of daily urine sample collection vs. sample collection for 5-days, 3-days, or 2-days per week for the following variables: E1G exposure (area under the curve; AUC), E1G mean concentration, PdG exposure, and PdG mean concentration. We hypothesized that E1G and PdG cycle AUC and mean concentration would be similar when samples were collected daily vs. 5-days, 3-days, or 2-days per week for a 28 day monitoring period or a menstrual cycle with an inter-menstrual interval ranging from 20-45 days.

Materials and Methods

Experimental Design: This study utilizes menstrual cycle data from subjects participating in a study conducted at two sites, University of Toronto (UT) and the Pennsylvania State University (PSU) over 8 years. Subjects included women with severe exercise-associated menstrual disturbances (EAMD), including oligomenorrhea (long and inconsistent menstrual cycle lengths of 36-90 days) and functional hypothalamic amenorrhea (the absence of menses for > 90 days). The study also included a eumenorrheic exercising group (EU) that served as a control group. Concentrations of reproductive hormone metabolites were assessed in daily urinary sample collections. The study was approved by the Research Ethics Board at the UT and the Institutional Review Board at the PSU. All participants signed an approved informed consent document.

Participants: Women reporting regular menstrual cycles of 26-35 days for the previous 6 months prior to the study were recruited for the EU group, while women reporting no menses in the previous three months, or <6 cycles in the previous 12 months were recruited for the EAMD group. Eligibility criteria for the study included, 1) age 18-35 years; 2) weight stability (± 2 kg) for at least 3 months; 3) body mass index (BMI) 16-25 kg/m^2; 4) good health as determined by medical exam and no history of any serious medical conditions; 5) no chronic illness, including hyperprolactinemia and thyroid disease; 6) not currently dieting; 7) no current clinical diagnosis of an eating disorder or psychiatric disorder; 8) non-smoking, 9) not taking any form of hormonal therapy for at least 6 months; 10) currently participating in ≥ 2 hours/week of purposeful exercise; 11) no history of a clinical diagnosis of polycystic ovarian syndrome (PCOS); 12) not pregnant, lactating or planning a pregnancy; 13) no medication use that would alter metabolic or reproductive hormone concentrations; and 14) no other contraindications that would preclude participation in the study.

Participant Grouping Categories: Classification of participant menstrual status (eumenorrheic, oligomenorrheic or amenorrheic) was based on self-reported menstrual histories and menstrual calendars. Menstrual status was confirmed by urinary

concentrations of the reproductive hormone metabolites, E1G, PdG, and luteinizing hormone (LH).

Demographic Assessment: Height (to the nearest 1.0 cm) and weight (to the nearest 0.1 kg) were measured and participants completed questionnaires to assess medical history, exercise and menstrual history, eating behaviors and psychological health. A physical exam and blood sample were performed to determine overall health.

Urinary Collection Procedures: Participants in the EAMD group collected daily urinary samples for a 28-day monitoring period and EU participants collected daily specimens for an entire menstrual cycle. The EAMD group initiated urinary collection on an arbitrary day in the study, while the EU group initiated urinary collection on day 1 or 2 of the menstrual cycle subsequent to demographic assessment. All participants utilized calendars to record menses and time of urine collection. All urine specimens were labeled with calendar date, cycle/monitoring period number and cycle/monitoring period day. Participants stored urine specimens in their household freezers between drop offs at the laboratory. Frozen ice packs and insulated lunch packs were used to keep samples cold during transport to the laboratory. In the laboratory urine samples were stored in a -20C freezer until analyzed.

Urinary Measurement of E1G and PdG: Microtiter plate competitive enzyme immunoassays (EIA) were used to measure E1G and PdG, as previously described [4]. The secretion of these estrogen and progesterone metabolites in the urine parallels serum concentrations of the parent hormones [7, 44]. Urinary concentrations of E1G and PdG were corrected for specific gravity, determined using a hand refractometer (NSG Precision Cells, Inc., Farmingdale, NY), to account for hydration status [45-47].

Selection of eligible cycles: A flow chart is presented (**Figure 3.1**) to describe the design of the study and the contribution of participants and cycles from each study site. A total of 116 participants and 572 cycles/monitoring periods were evaluated for eligibility for this analysis. There were 63 amenorrheic participants with 267 28-day monitoring periods and 79 eumenorrheic participants with 305 menstrual cycles within the range of 20-45 days.

Menstrual cycle length was defined as the number of days from day 1 of menses to the day before the first day of the next menses. From this data set, 79 complete menstrual cycles and 70 28-day monitoring periods (contained no missing samples) that were collected during the 12-month study were used in this analysis. In addition, 41 menstrual cycles with no more than 3 missing days in the first 6 days of the cycle were also used. In these cases, concentrations of E1G and PdG for the missing days were estimated by averaging the concentrations from the day before and after the missing day. If the missing day was the first day of the cycle, days 2-4 of the cycle were averaged to estimate the concentration for the missing day. Menstrual cycles included in the complete sample analysis were a combination of ovulatory (n=23), luteal phase defect (LPD; n=43), and anovulatory (n=54) cycle classifications. Classifications of menstrual cycles were completed from the original daily specimens and conducted to ensure inclusion of all types of menstrual cycles in the analysis. Specific hormonal criteria for classification of ovulatory, LPD, and anovulatory cycles has been described previously [4]. Sixty-one participants and their 382 menstrual cycles/monitoring periods were excluded from the analysis (**Figure 3.1**). Monitoring periods (used for amenorrheic women) were excluded if there were any missing samples. Menstrual cycles (used for eumenorrheic and oligomenorrheic women) were excluded if there were more than 3 missing days in the first 6 collection days or any missing days beyond the first 6 days. Menstrual cycles were also excluded if the cycle length was outside the 20-45 day range included in this analysis. The UT site contributed 27 participants and 90 menstrual cycles/monitoring periods to this analysis while the PSU site contributed 28 participants and 100 menstrual cycles/monitoring periods to this analysis (**Figure 3.1**).

Simulation of Reduced Number of Sample Collection Days: The daily samples per menstrual cycle/monitoring period (n=190) collected by the participants were referenced to day of the menstrual cycle/monitoring period and collection calendar date. To determine if fewer days of urine collection would provide accurate and precise data for E1G and PdG
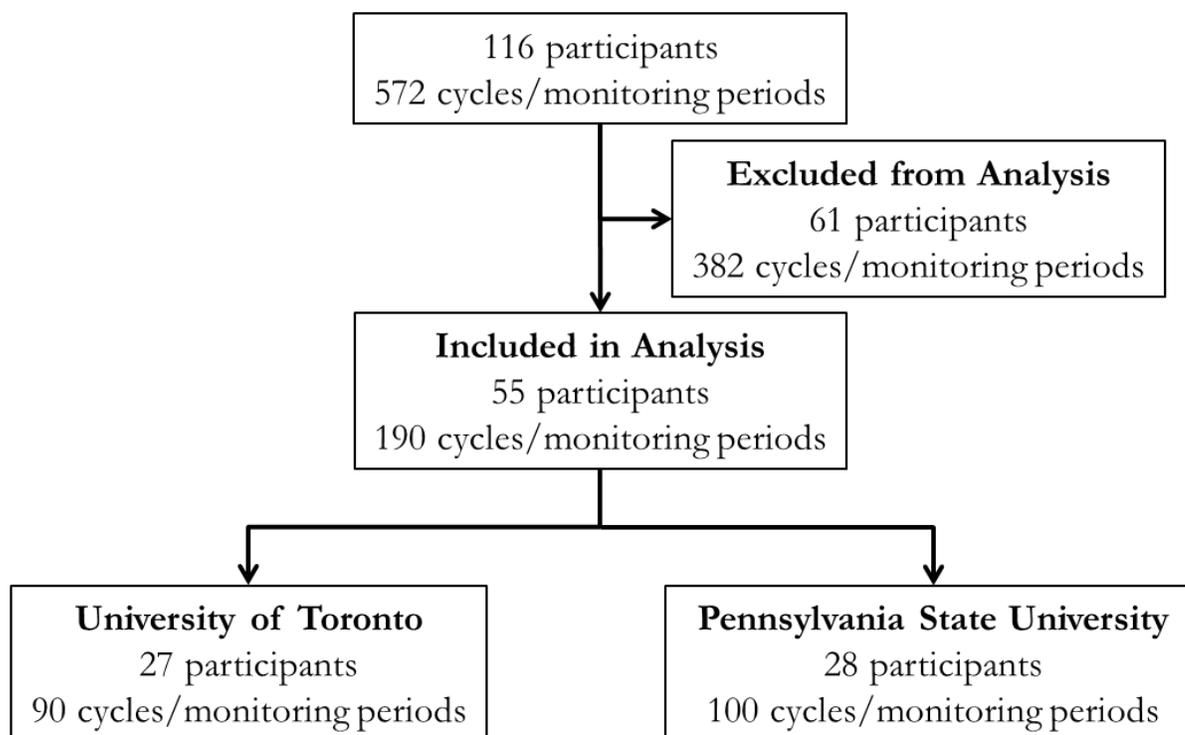
Figure 3.1: Number of participants and menstrual cycles/monitoring periods, which contributed to and were excluded from the current analysis. Data from participants recruited at two study sites, University of Toronto (UT) and the Pennsylvania State University (PSU), were included. Initially there were 572 menstrual cycles/28-day monitoring periods from 116 participants evaluated for complete sample collection. 382 menstrual cycles/monitoring periods from 61 participants were excluded from the analysis due to missing collection days. Included in the analysis were 190 menstrual cycles/monitoring periods from 55 participants, which included 90 menstrual cycles/monitoring periods from 27 participants enrolled at the UT site and 100 menstrual cycles/monitoring periods from 28 participants enrolled at the PSU site.

exposure and mean concentration during the menstrual cycle/monitoring period, E1G and

PdG data were systematically removed from each menstrual cycle or 28-day monitoring

period to mimic a reduced frequency of sample collection for participants with 100%

compliance to daily collection. The reduced sample collection frequencies were selected to

reduce participant burden and represented three different collection frequencies as follows:

5 days of urine collection each week, 3 days of urine collection each week, and 2 days of

urine collection each week. Specifically, for the simulation of collecting 5 urinary samples per

week, E1G and PdG data for Saturday and Sunday each week of the menstrual

cycle/monitoring period were systematically removed, leaving only the E1G and PdG

concentrations from the weekdays for analysis. For the simulation of collecting 3 urinary samples per week, E1G and PdG data were systematically removed for Tuesday, Thursday, Saturday and Sunday each week of the menstrual cycle/monitoring period leaving E1G and PdG concentrations for Monday, Wednesday, and Friday for the analysis. For simulation of collecting 2 urinary samples per week, E1G and PdG data were systematically removed for Tuesday, Wednesday, Friday, Saturday, and Sunday each week of the menstrual cycle/monitoring period leaving only E1G and PdG concentrations for Monday and Thursday for analysis.

Urinary Hormone Assessment Calculations: E1G and PdG exposures across the menstrual cycle or monitoring period were determined by calculating the AUC for daily, 5-days, 3-days, and 2-days per week collection frequencies using Kaleidagraph Software (Synergy Software, Reading. PA, USA). Mean E1G and PdG concentrations across the cycle or monitoring period for all collection frequencies were also calculated.

Statistical Analyses: The data presented were obtained at two different locations, the UT and PSU, over 8 years. E1G and PdG data were analyzed as a merged group of eumenorrheic cycles of 20-45 days in length and 28 day amenorrheic monitoring periods (complete sample analysis; n=190). Sub-analyses of eumenorrheic cycles of 26-36 days in length (which is the most common range of inter-menstrual intervals among regularly menstruating women; n=94) alone and 28 day amenorrheic monitoring periods (n=70) alone. Data screening was conducted prior to statistical analysis in order to identify whether the data met the assumptions required by the specific statistical techniques in this analysis. Data screening involved examination of variable distributions within each of the three analysis groupings for all four collection frequencies (daily, 5-day, 3-day, and 2-day) and all four hormone variables (E1G AUC, PdG AUC, E1G mean, and PdG mean). All hormonal variables were found to be not normally distributed. However, logarithmic transformation did not improve normality of these variables. In addition, logarithmic transformation was not considered as a practical approach for Bland-Altman analysis, as the limits of agreement

(LOA) are expressed as multiples of the measured concentration following logarithmic transformation [48]. All data are presented as means ± SD, unless otherwise indicated. Linear mixed model ANOVA was used to compare all ovarian steroid (E1G AUC, E1G mean, PdG AUC, or PdG mean) data between daily urinary collection and each reduced urinary collection frequency for the complete samples analysis and the eumenorrheic and amenorrheic sub-analyses. Since the same individual provided multiple cycles and/or monitoring periods these data were considered to be of nested nature; therefore, the participant identifier was included as a random effect in the linear model. A significance level of alpha = 0.05 was used to detect differences, and for multiple comparisons, alpha was adjusted using Bonferroni correction. Bland Altman analysis was performed to determine the 95% LOA and to identify potential mean and proportional bias for both AUC and mean concentration [49]. Errors were calculated as the difference between daily urinary collection data and each reduced urinary collection data since daily urinary sample collection was regarded as the criterion method. For convenience, mean error and lower and upper LOA are also reported as percent of the average of daily and each reduced urinary collection values. All analyses were conducted using R statistical software (Revolution Analytics, Palo Alto, CA, USA).

Results

1. Complete Sample Analysis

In total, there were 55 participants and 190 menstrual cycles (20-45 days in length) and 28-day monitoring periods with complete data. There were 120 menstrual cycles and seventy 28-day amenorrheic monitoring periods. The participants were aged 22.6±4.3 years, weighed 57.0±6.6 kg, were 164.3±6.6 cm tall, and had a BMI of 21.1±2.0 kg/m^2. The average age at menarche was 13.2±1.6 years and the mean gynecologic age was 9.5±4.6 years.

Mixed Model Analysis: Composite graphs of the average E1G and PdG concentrations, respectively, across the entire cycle/monitoring period for daily, 5-day, 3-day, and 2-day collection frequencies, shown in **Figures 3.2A and B**. The average AUC and cycle mean concentration for each urinary collection frequency are displayed in inset bar graphs within the composite graphs and in **Table 3.1**. There were no significant differences detected between daily collection and each reduced collection frequency (p>0.99) with regard to E1G mean concentration; however, E1G AUC for 2-days per week collection was significantly lower when compared to E1G AUC for daily collection (p>0.046). There were no significant differences detected between daily collection and each reduced collection frequency (5-day, 3-day, or 2-day per week) with regard to PdG AUC (p>0.050) or mean concentration (p>0.99).

Bland Altman Analysis: On average, the reduced urine collection frequencies for the complete sample analysis under-estimate the daily E1G AUC by 1.4% for the 5-day collection frequency, 3.2% for the 3-day collection frequency and by 8.2% for the 2-day collection frequency. The 5-day collection frequency demonstrated the lowest degree of under-estimation and 2-day collection frequency demonstrated the greatest degree of under-estimation when compared to daily sample collection. The E1G AUC for all reduced collection frequencies demonstrate good agreement with daily urine collection as indicated by the inclusion of zero in the 95% LOA for the Bland Altman analyses. The best agreement was observed with 5 day collection, indicated by the smallest range for the 95% LOA (see **Figure 3.3A-C**). A proportional bias was observed in all reduced collection frequencies (p<0.010) indicating larger AUC values are, on average, under-estimated more than smaller AUC values in all reduced collection frequencies compared to daily urine collection (see **Table 3.2**).

On average, reduced sample collection under-estimated the daily PdG AUC by 0.6% for the 5-day collection frequency, 2.9% for the 3-day collection frequency and by 10.8% for the 2-day collection frequency (see **Figure 3.3D-F** and **Table 3.3**). The 5-day collection

frequency demonstrated the lowest degree of under –estimation of daily sampling, while the 2-day collection frequency demonstrated the greatest degree of under-estimation. The PdG AUC for all reduced collection frequencies demonstrate good agreement with daily urine collection as indicated by the 95% LOA including zero. The best agreement was observed with 5-day collection frequency, indicated by the smallest range for the 95% LOA. A proportional bias was not observed in the 5-day and 3-day collection frequencies (p=0.10), while a proportional bias was observed in the 2-day collection frequency (p<0.000) indicating larger AUC values are, on average, under-estimated more than smaller AUC values in the 2-day collection frequency compared to daily urine collection (see **Table 3.3**).

On average, daily E1G mean concentration was over-estimated by 0.5% for the 5-day collection frequency, 0.8% for the 3-day collection frequency and by 0.4% for the 2-day collection frequency (see **Figure 3.4A-C** and **Table 3.4**). The 2-day collection frequency demonstrated the lowest degree of over-estimation of daily sampling, while the 3-day collection frequency demonstrated the greatest degree of over-estimation. The E1G cycle mean concentration for all reduced collection frequencies demonstrate good agreement with daily urine collection as indicated by the 95% LOA including zero. The best agreement was observed with the 5-day collection frequency, indicated by the smallest range for the 95% LOA. A proportional bias was not observed in the 5-day and 3-day collection frequencies (p>0.60), while a proportional bias was observed in the 2-day collection frequency (p=0.040) indicating greater mean cycle concentrations are, on average, over-estimated more than smaller mean cycle concentrations in 2-day collection frequency compared to daily collection (see **Table 3.4**).

Daily PdG mean concentration was over-estimated by 2.2% for the 5-day collection frequency and 2.1% for the 3-day collection frequency, while the 2-day collection frequency under-estimated the daily PdG mean concentration by 0.2% (see **Figure 3.4D-F** and **Table 3.5**). The 5-day collection frequency demonstrated the greatest degree of over-estimation of daily collection, while the 2-day sample collection frequency demonstrated under-estimation

of daily sample collection. The PdG cycle mean concentration for all reduced collection frequencies demonstrate good agreement with daily urine collection as indicated by the 95% LOA including zero. The best agreement was observed with the 5-day collection frequency, indicated by the smallest range for the 95% LOA. A proportional bias was observed in the 5-day and 3-day collection frequencies ($p<0.010$) indicating greater cycle mean concentrations are, on average, over-estimated compared to daily urine collection, while a trend toward a proportional bias was observed in the 2-day collection frequency ($p=0.060$; see **Table 3.5**).

2. Sub-Analysis of Eumenorrheic Cycles:

A sub-analysis of eumenorrheic menstrual cycles of 26-36 days in length included 31 participants and 94 menstrual cycles with complete data. This sub-analysis included anovulatory (n=14), LPD (n=31), and ovulatory (n=49) menstrual cycles, which were classified using daily collection frequency hormonal measurements. The participants were aged 24±4.5 years, weighed 57.1±6.0 kg, were 164.5±6.5 cm tall, and had a BMI of 21.1±1.7 kg/m^2. The average age at menarche was 12.5±1.3 years and the mean gynecologic age was 11.5±4.3 years.

Mixed Model Analysis: Composite graphs of the average E1G and PdG concentrations, respectively, across the entire cycle for daily, 5-day, 3-day, and 2-day collection frequencies shown in **Figures 3.5A and B**. The classic characteristics of ovulatory cycles, i.e. the mid-cycle E1G peak and luteal phase PdG peak, are evident. The average AUC and cycle mean concentration for each urinary collection frequency are displayed in inset bar graphs within the composite graphs and in **Table 3.1**. There were no significant differences detected between daily collection and each reduced collection frequency (5-day, 3-day, or 2-day per week) with regard to E1G AUC ($p>0.46$) or mean concentration ($p>0.99$). There were no significant differences detected between daily collection and each reduced collection frequency (5-day, 3-day, or 2-day per week) with regard to PdG AUC ($p>0.27$) or mean concentration ($p>0.99$).
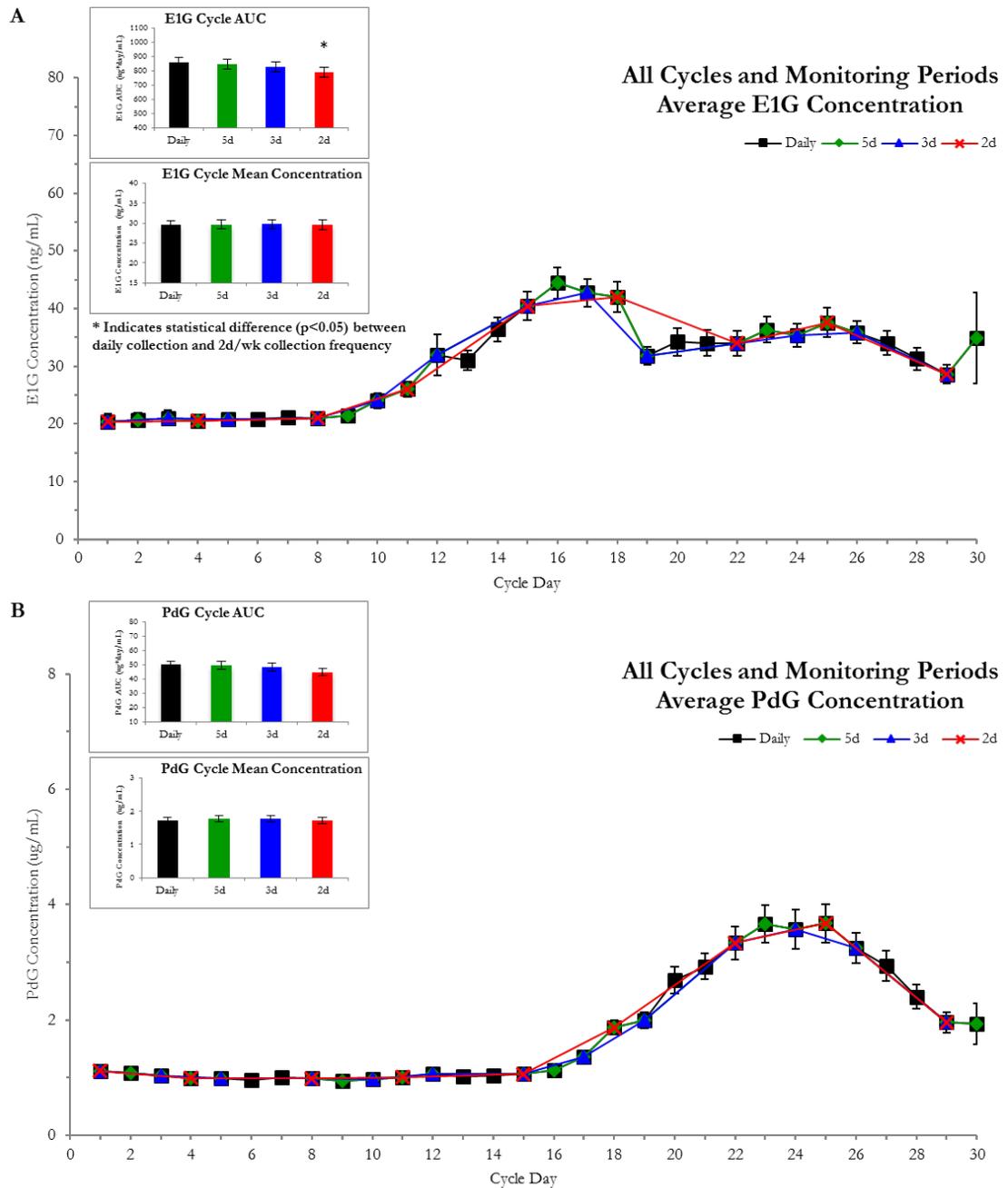
Figure 3.2: Composite graphs of urinary reproductive hormone excretion of E1G (A) and PdG (B) for eumenorrheic cycles of 20-45 days in length and 28 day amenorrheic monitoring periods for daily, 5-day/week, 3-day/week, and 2-day/week urinary collection frequencies. The inset bar graphs depict the cycle AUC and mean concentration for E1G and PdG for daily, 5-day/week, 3-day/week, and 2-day/week collection frequencies. Cycle day one represents the first day of menses. The data points for the central cycle/monitoring period days have an n=190. Cycle/monitoring period days at the beginning and end of the composite graphs have a variable 'n' due to the varying lengths of the cycles included in the sample. E1G, estrone-1-glucuronide; PdG, pregnanediol glucuronide; AUC, area under the curve. * indicates statistical difference (p<0.05) between daily collection and 2 days per week collection frequency.

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Table 3.1: Reproductive steroid hormone metabolite parameters for the all cycles, eumenorrheic cycles only, and amenorrheic monitoring periods only analyses for all collection frequencies.

	Reduced Collection Frequency			
	Daily	5-d/wk	3-d/wk	2-d/wk
	mean±SE	mean±SE	mean±SE	mean±SE
All Cycles and Monitoring Periods (n=190)				
E1G cycle mean AUC (ng*day/mL)	856.0±36.3	844.0±35.3	829.0±35.1	789.0±34.2[*]
E1G cycle mean concentration (ng/mL)	29.5±1.2	29.6±1.2	27.4±1.2	29.6±1.2
PdG cycle mean AUC (μg*day/mL)	50.0±2.7	49.6±2.8	48.5±2.7	44.8±2.5[#]
PdG cycle mean concentration (μg/mL)	1.7±0.1	1.8±0.1	1.8±0.1	1.7±0.1
Eumenorrheic Cycles (n=94)				
E1G cycle mean AUC (ng*day/mL)	1037.5±50.7	1027.5±50.5	1014.7±50.7	972.5±49.2
E1G cycle mean concentration (ng/mL)	35.7±1.7	35.9±1.7	36.2±1.7	36.2±1.8
PdG cycle mean AUC (μg*day/mL)	68.4±4.2	68.4±4.2	67.0±4.1	62.2±4.0[#]
PdG cycle mean concentration (μg/mL)	2.4±0.1	2.4±0.2	2.4±0.2	2.4±0.2
Amenorrheic Periods (n=70)				
E1G cycle mean AUC (ng*day/mL)	539.2±27.7	532.6±27.3	515.5±26.5	488.6±27.5
E1G cycle mean concentration (ng/mL)	20.0±1.0	20.0±1.0	20.0±1.0	20.0±1.1
PdG cycle mean AUC (μg*day/mL)	25.6±1.6	25.2±1.6	24.2±1.5	22.7±1.5
PdG cycle mean concentration (μg/mL)	1.0±0.1	1.0±0.1	0.9±0.1	0.9±0.1

estrone-1-glucuonoide (E1G) and pregnanediol glucuronide (PdG) area under the curve (AUC) and mean concentration. d/wk, days per week.

* indicates significant differences ($p < 0.05$) between the reduced collection frequency and daily collection frequency.

# indicates a trend for a difference ($p < 0.1$) between the reduced collection frequency and daily collection frequency.

Figure 3.3: Bland Altman plots for all cycles 20-45 days in length and 28 day monitoring periods. The difference between daily and reduced collection frequencies are plotted against the mean of the daily and reduced collection frequency in the 190 paired measurements from the all cycles/monitoring periods analysis. The comparison of daily and 5-days/week collection frequency is in column 1, daily and 3-days/week collection frequency is in column 2, and daily and 2-days/week collection frequency is in column 3. Differences between daily and the reduced collection frequencies for E1G AUC (A-C) and PdG AUC (D-F) are demonstrated.

Table 3.2: Bland-Altman analysis for all menstrual cycle/monitoring period analyses of E1G AUC.

Method	Bland-Altman Analysis						
	Mean Difference		Lower Limit of Agreement		Upper Limit of Agreement		Proportional
	(ng*day/mL)	%	(ng*day/mL)	%	(ng*day/mL)	%	Bias
All Cycles and Monitoring Periods (n=190)							
Daily vs. 5-d/wk	12.0	1.4	-133.8	-15.7	157.9	18.6	0.0132
Daily vs. 3-d/wk	27.2	3.2	-141.8	-16.8	196.2	23.3	0.0140
Daily vs. 2-d/wk	67.0	8.2	-136.8	-16.6	270.8	32.9	0.0001
Eumenorrheic Cycles (n=94)							
Daily vs. 5-d/wk	10.0	1.0	-106.1	-10.3	126.1	12.2	0.6988
Daily vs. 3-d/wk	22.7	2.2	-122.1	-11.9	167.5	16.3	0.9908
Daily vs. 2-d/wk	65.0	6.5	-127.4	-12.7	257.3	25.6	0.1528
Amenorrheic Periods (n=70)							
Daily vs. 5-d/wk	6.6	1.2	-65.9	-12.3	79.1	14.8	0.4515
Daily vs. 3-d/wk	23.7	4.5	-60.4	-11.5	107.8	20.4	0.0405
Daily vs. 2-d/wk	50.6	9.8	-84.1	-16.4	185.2	36.0	0.8165

E1G, estrone-1-glucuronide; AUC, area under the curve; d/wk, days per week.

Table 3.3: Bland-Altman analysis for all menstrual cycle/monitoring period analyses of PdG AUC.

Method	Mean Difference		Lower Limit of Agreement		Upper Limit of Agreement		Proportional
	Bland-Altman Analysis						
	(µg*day/mL)	%	(µg*day/mL)	%	(µg*day/mL)	%	Bias
All Cycles and Monitoring Periods (n=190)							
Daily vs. 5-d/wk	0.3	0.6	-8.8	-17.7	9.5	19.0	0.1819
Daily vs. 3-d/wk	1.4	2.9	-12.7	-25.8	15.6	31.7	0.4675
Daily vs. 2-d/wk	5.1	10.8	-12.4	-26.3	22.7	47.9	0.0000
Eumenorrheic Cycles (n=94)-							
Daily vs. 5-d/wk	0.1	0.1	-12.1	-17.6	12.2	17.8	0.5247
Daily vs. 3-d/wk	1.4	2.0	-17.8	-26.3	20.5	30.3	0.3789
Daily vs. 2-d/wk	6.2	9.6	-15.8	-24.3	28.3	43.4	0.0541
Amenorrheic Periods (n=70)							
Daily vs. 5-d/wk	0.4	1.7	-3.5	-13.8	4.3	17.1	0.1814
Daily vs. 3-d/wk	1.4	5.6	-3.7	-15.0	6.5	26.3	0.0016
Daily vs. 2-d/wk	2.8	11.8	-4.4	-18.2	10.1	41.8	0.0314

PdG, pregnanediol glucuronide; AUC, area under the curve; d/wk, days per week.
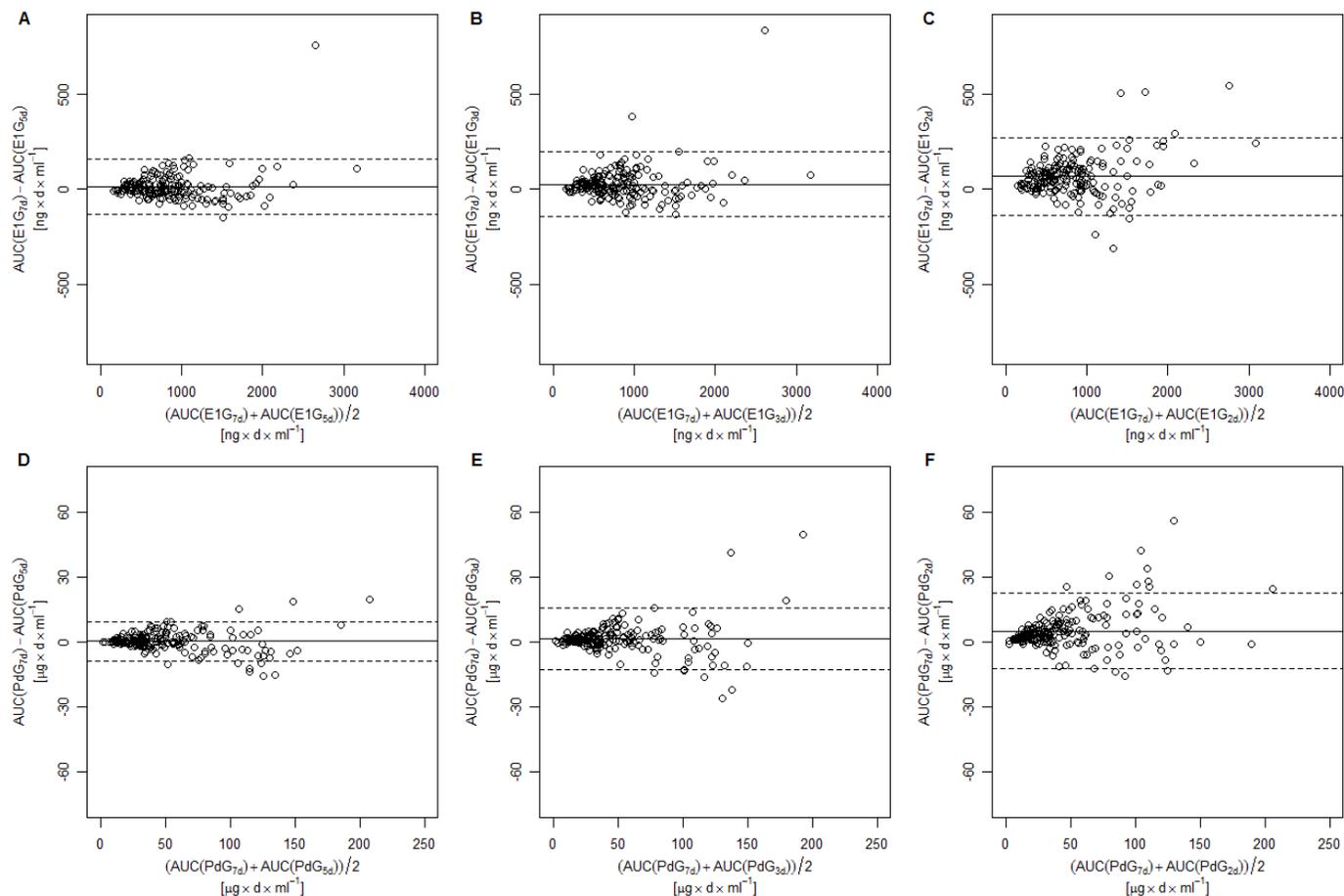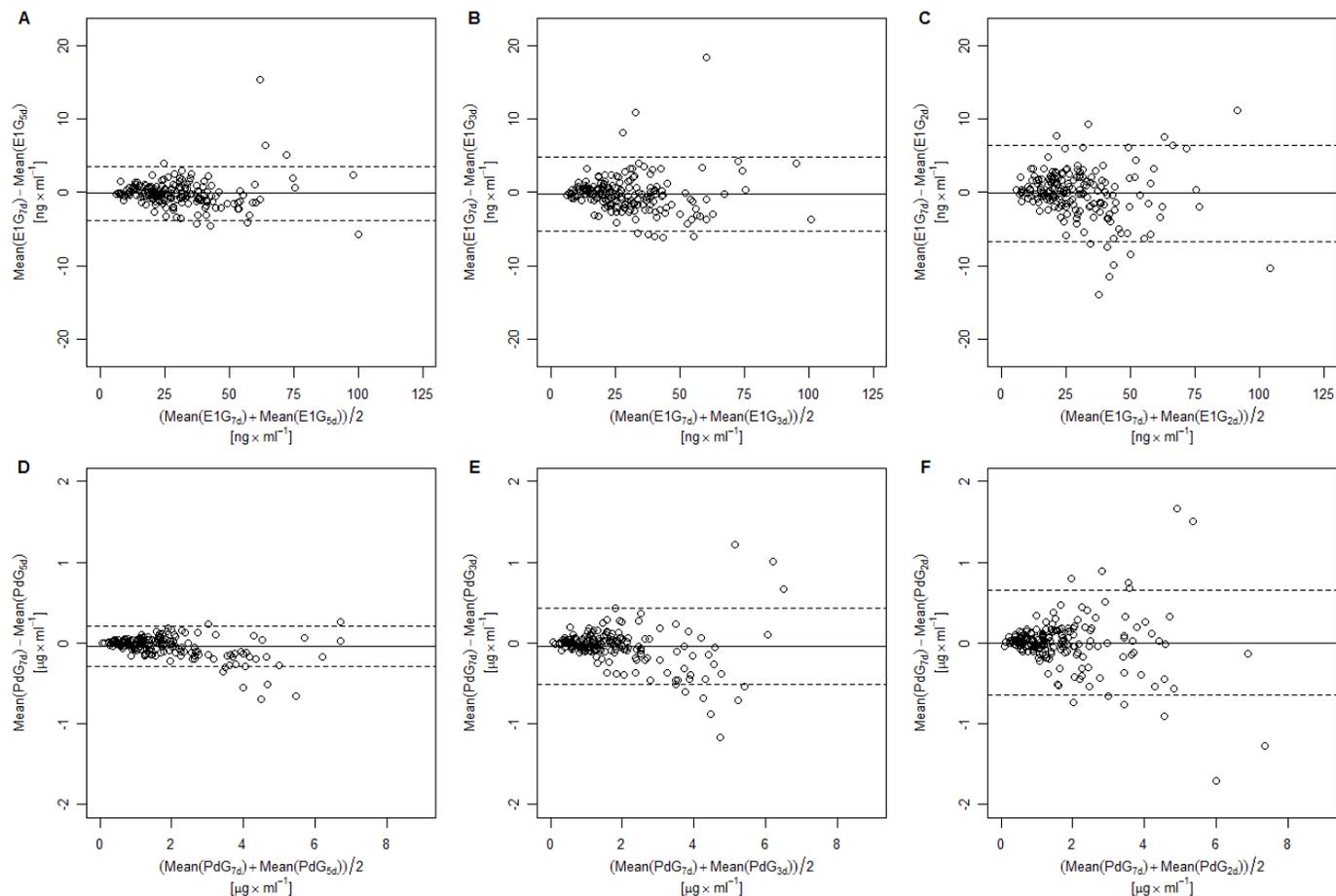
Figure 3.4: Bland Altman plots for all cycles 20-45 days in length and 28 day monitoring periods. The difference between daily and reduced collection frequencies are plotted against the mean of the daily and reduced collection frequency in the 190 paired measurements from the all cycles/monitoring periods analysis. The comparison of daily and 5-days/week collection frequency is in column 1, daily and 3-days/week collection frequency is in column 2, and daily and 2-days/week collection frequency is in column 3. Differences between daily and the reduced collection frequencies for E1G mean (A-C), and PdG mean (D-F) are demonstrated.

Bland Altman Analysis: The reduced collection frequencies for eumenorrheic cycles of 26-36 days in length, on average, under-estimate the daily E1G AUC as shown in **Table 3.2**. The E1G AUC for all educed collection frequencies demonstrate good agreement with daily urine collection as indicated by the 95% LOA including zero. The best agreement is observed with 5 day collection, indicated by the tighter range for the 95% LOA. A proportional bias was not observed in any of the reduced collection frequencies (p>0.20), as shown in **Table 3.2**. As shown in **Table 3.3**, the reduced collection frequencies for eumenorrheic cycles alone, on average, under-estimated the daily PdG AUC. The smallest range for the 95% LOA was observed in the 5-day collection frequency indicating the best agreement with daily sample collection. A proportional bias was not observed in any of the reduced collection frequencies (p>0.050) as shown in **Table 3.3**.

Reduction of the collection frequency for eumenorrheic cycles of 26-36 days in length, on average, over-estimated the E1G mean concentration as shown in **Table 3.4**. The 5-day collection frequency demonstrated the lowest degree of over-estimation of E1G cycle mean concentration, while the 2-day collection frequency demonstrated the greatest degree of over-estimation. A proportional bias was not observed in the 5-day and 3-day collection frequencies (p>0.10); however, a proportional bias was observed in the 2-day collection frequency (p=0.007) as shown in **Table 3.4**. On average, the daily PdG cycle mean concentration was over-estimated by the reduced collection frequencies for eumenorrheic cycles of 26-36 days in length as shown in **Table 3.5**. The best agreement was observed with 5-day collection frequency, indicated by the smaller range for the 95% LOA. A proportional bias was not observed in the 3-day collection frequency (p=0.20); however, a proportional bias was observed in the 5-day and 2-day collection frequencies (p<0.030) as shown in **Table 3.5**.

3. Sub-Analysis of Amenorrheic Monitoring Periods:

In a sub-analysis of amenorrheic monitoring periods of 28 days there were 19 participants and 70 monitoring periods with complete data. The participants were aged

Table 3.4: Bland-Altman analysis for all menstrual cycle/monitoring period analyses of E1G Mean.

Method	Bland-Altman Analysis						
	Mean Difference		Lower Limit of Agreement		Upper Limit of Agreement		Proportional
	(ng/mL)	%	(ng/mL)	%	(ng/mL)	%	Bias
All Cycles and Monitoring Periods (n=190)							
Daily vs. 5-d/wk	-0.1	-0.5	-3.8	-13.0	3.6	12.1	0.9569
Daily vs. 3-d/wk	-0.2	-0.8	-5.2	-17.7	4.8	16.1	0.5725
Daily vs. 2-d/wk	-0.1	-0.4	-6.7	-22.6	6.4	21.8	0.0352
Eumenorrheic Cycles (n=94)							
Daily vs. 5-d/wk	-0.3	-0.8	-3.8	-10.6	3.3	9.1	0.4931
Daily vs. 3-d/wk	-0.5	-1.4	-5.3	-14.7	4.2	11.8	0.1312
Daily vs. 2-d/wk	-0.5	-1.5	-7.1	-19.7	6.0	16.7	0.0067
Amenorrheic Periods (n=70)							
Daily vs. 5-d/wk	0.0	0.0	-1.8	-9.1	1.8	9.1	0.5498
Daily vs. 3-d/wk	0.0	-0.2	-2.9	-14.3	2.8	14.0	0.8204
Daily vs. 2-d/wk	0.0	-0.2	-5.7	-28.7	5.7	28.4	0.0066

E1G, estrone-1-glucuronide; d/wk, days per week.
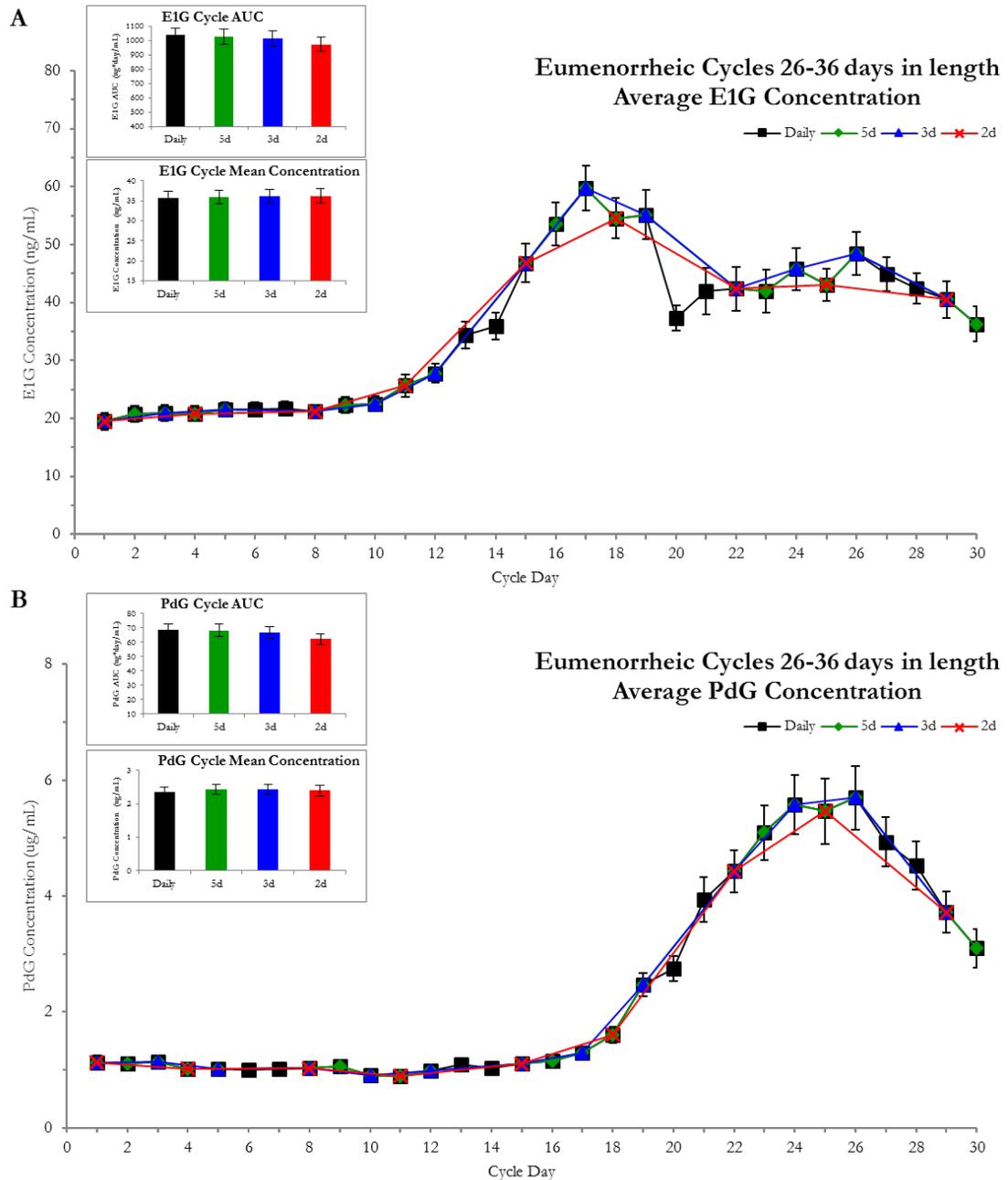
Figure 3.5: Composite graphs of urinary reproductive hormone excretion of E1G (A) and PdG (B) for eumenorrheic cycles of 26-36 days in length for daily, 5-day/week, 3-day/week, and 2-day/week urinary collection frequencies. The inset bar graphs depict the cycle AUC and mean concentration for daily, 5-day/week, 3-day/week, and 2-day/week collection frequencies. Cycle day one is the first day of menses. The data points for the central cycle/monitoring period days have an n=94. Cycle/monitoring period days at the beginning and end of the composite graph have a variable 'n' due to the varying lengths of the cycles included in the sub-sample. E1G, estrone-1-glucuronide; PdG, pregnanediol glucuronide; AUC, area under the curve.

21.2±3.5 years, weighed 57.3±7.8 kg, were 165.1±7.0 cm tall, and had a BMI of 21.1±2.4

kg/m^2. The average age at menarche was 14.1±1.4 years and the mean gynecologic age

was 7±3.9 years.

Mixed Model Analysis: Composite graphs of the average E1G and PdG concentrations,

respectively, across the entire monitoring period for daily, 5-day, 3-day, and 2-day collection

frequencies are shown in **Figures 3.6A and B**. The chronic suppression of E1G and PdG

that is characteristic of reproductive hormone concentrations among amenorrheic women is

clearly evident. Within the inset bar graphs and **Table 3.1**, the average AUC and cycle mean

concentrations are displayed for each frequency of urinary collection. There were no

significant differences detected between daily collection and each reduced collection

frequency (5-day, 3-day, or 2-day per week) with regard to E1G AUC ($p>0.12$) or mean

concentration ($p>0.99$). There were no significant differences detected between daily

collection and each reduced collection frequency (5-day, 3-day, or 2-day per week) with

regard to PdG AUC ($p>0.080$) or mean concentration ($p>0.99$).

Bland Altman Analysis: In amenorrheic 28-day monitoring periods daily sample collection

E1G AUC was, on average, under-estimated by the reduced collection frequencies (see

Table 3.2). The E1G AUC for all reduced collection frequencies demonstrate good

agreement with daily urine collection as indicated by the 95% LOA including zero. The best

agreement was observed with the 5-day collection frequency, indicated by the smaller range

for the 95% LOA. A proportional bias was not observed in the 5-day and 2-day collection

frequencies ($p>0.20$), but a proportional bias was observed in the 3-day collection frequency

($p=0.04$; see **Table 3.2**). Daily PdG AUC was, on average, under-estimated by the reduced

collection frequencies for 28-day monitoring periods (see **Table 3.3**). The smallest range for

the 95% LOA was observed in the 5-day collection frequency, indicating the best agreement

with daily sample collection. A proportional bias was not observed in the 5-day collection

frequency ($p>0.20$); however, a proportional bias was observed in the 3-day and 2-day

collection frequencies ($p<0.030$; see **Table 3.3**).

The 5-day collection frequency for 28-day monitoring periods, on average, estimated the daily E1G cycle mean concentration, while 3-day and 2-day collection frequencies over-estimated E1G cycle mean concentration (see **Table 3.4**). The calculated E1G cycle mean concentration for all 28-day monitoring periods and reduced collection frequencies demonstrate good agreement with daily urine collection as indicated by the 95% LOA including zero. A proportional bias was not observed in the 5-day and 3-day collection frequencies ($p>0.50$); whereas, a proportional bias was observed in the 2-day collection frequency ($p<0.007$; see **Table 3.4**). The 3-day and 2-day collection frequencies for 28-day monitoring periods under-estimate the daily PdG cycle mean concentration, on average, while the 5-day collection frequency over-estimated the PdG mean cycle concentration (see **Table 3.5**). The best agreement is observed with 5-day collection frequency, indicated by the smaller range for the 95% LOA. A proportional bias was not observed in any of the reduced collection frequencies ($p>0.20$; see **Table 3.5**).

Discussion

The present study was designed to assess the level of agreement between reduced urinary collection frequencies (5-days, 3-days, and 2-days per week) and the urinary gold standard of daily specimen collection in a sample of exercising eumenorrheic and amenorrheic women and in specific sub populations of eumenorrheic and amenorrheic exercising women. This report is the first to provide detailed information on the accuracy and precision of quantifying reproductive hormone exposure using a reduced sampling schedule. This report supports and builds upon the work of O'Connor et al. [40], which tested the presence and day of ovulation during reduced urinary collection frequencies. All comparisons between daily collection and each of the reduced collection frequencies (5-days, 3-days, and 2-days per week) for the average AUC and mean concentration for both E1G and PdG were not different when eumenorrheic cycles and amenorrheic monitoring

Table 3.5: Bland-Altman analysis for all menstrual cycle/monitoring period analyses of PdG Mean.

Method	Bland-Altman Analysis						Proportional Bias
	Mean Difference		Lower Limit of Agreement		Upper Limit of Agreement		
	(µg/mL)	%	(µg/mL)	%	(µg/mL)	%	
All Cycles and Monitoring Periods (n=190)							
Daily vs. 5-d/wk	0.0	-2.2	-0.3	-16.5	0.2	12.2	0.0000
Daily vs. 3-d/wk	0.0	-2.1	-0.5	-29.0	0.4	24.7	0.0115
Daily vs. 2-d/wk	0.0	0.2	-0.6	-37.3	0.7	37.7	0.0635
Eumenorrheic Cycles (n=94)							
Daily vs. 5-d/wk	-0.1	-2.9	-0.4	-16.3	0.3	10.6	0.0004
Daily vs. 3-d/wk	-0.1	-3.1	-0.7	-29.2	0.6	23.1	0.2167
Daily vs. 2-d/wk	0.0	-1.3	-0.9	-36.2	0.8	33.5	0.0296
Amenorrheic Periods (n=70)							
Daily vs. 5-d/wk	0.0	-0.6	-0.1	-11.8	0.1	10.5	0.2336
Daily vs. 3-d/wk	0.0	0.7	-0.2	-16.9	0.2	18.3	0.3317
Daily vs. 2-d/wk	0.0	1.6	-0.2	-26.3	0.3	29.4	0.4256

PdG, pregnanediol glucuronide; d/wk, days per week.
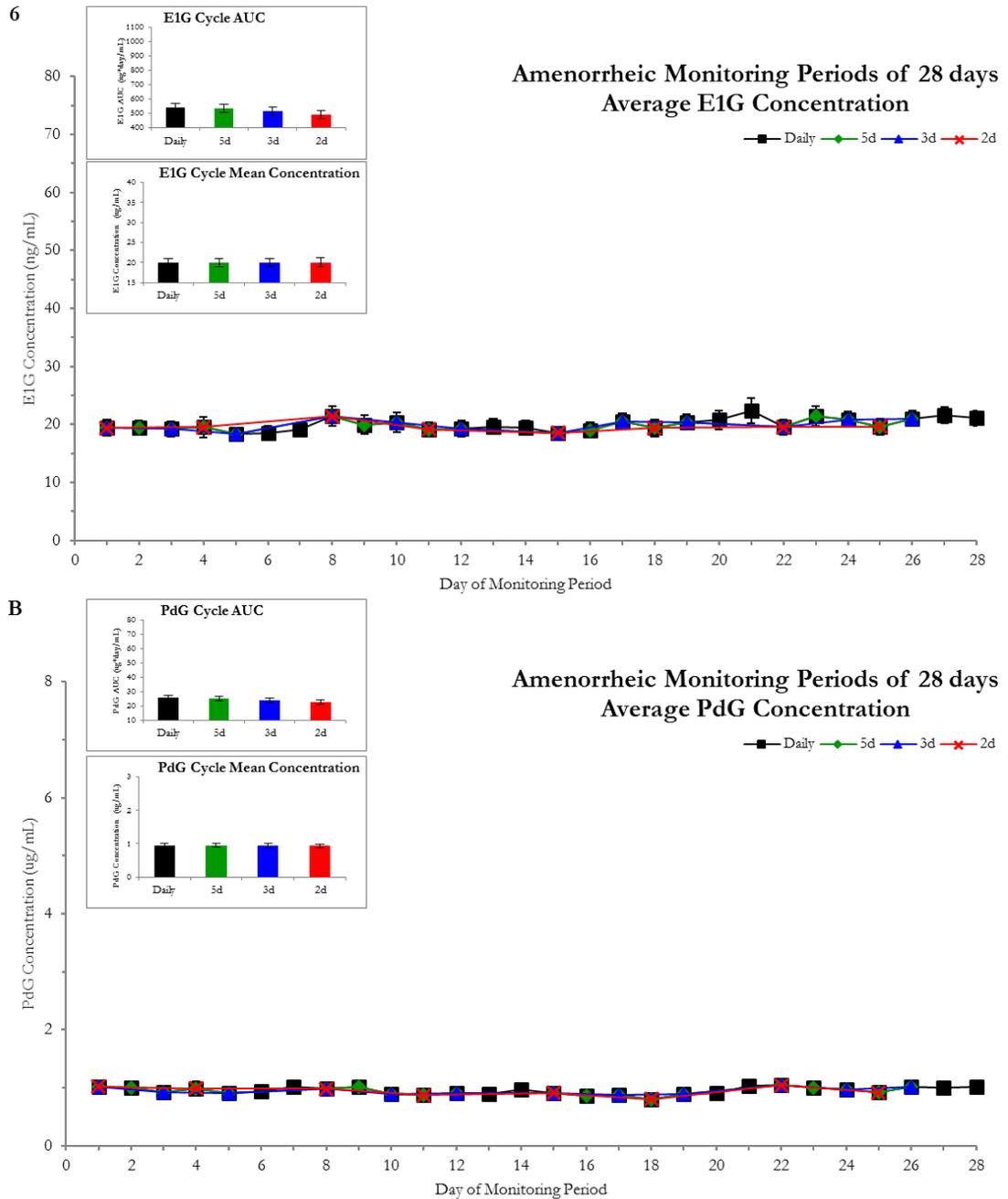
Figure 3.6: Composite graphs of urinary reproductive hormone excretion of E1G (A) and PdG (B) for 28 day amenorrheic monitoring periods for daily, 5-day/week, 3-day/week, and 2-day/week urinary collection frequencies. The inset bar graphs depict the monitoring period AUC and mean concentration for daily, 5-day/week, 3-day/week, and 2-day/week collection frequencies. Monitoring period day one is the first day of sample collection. The data points for the monitoring period days have an n=70. E1G, estrone-1-glucuronide; PdG, pregnanediol glucuronide; AUC, area under the curve.

periods were analyzed together and when analyzed separately; however, a difference was observed between daily collection and 2-days per week collection frequency with regard to the average E1G AUC in the complete sample analysis. In general the 5-day collection frequency demonstrated the best agreement with daily sample collection in all three analyses. The 3-day and 2-day collection frequencies also showed good agreement with daily sample collection in all three analyses of exercising women. The LOA for individual AUC and mean concentrations, however, appeared to increase with reduced sample collection frequencies. Depending on the required level of accuracy and precision, researches may choose to use varying sampling frequencies. The present analysis is the first to quantify mean error as well as individual agreement for urinary reproductive hormones.

The Bland Altman analysis was used to compare the daily and reduced collection frequencies [50]. This technique was used to assess the agreement between reduced collection frequencies (5-days, 3-days, and 2-days each week) and daily sample collection. If the differences between the reduced collection frequencies and daily collection is not large enough to change the interpretation of the research findings, daily collection could be replaced by the reduced collection frequencies or the collection frequencies could be used interchangeably. Though there were small over- or under-estimations of the AUC and mean concentrations observed in the Bland Altman analysis, good agreement among the reduced collection frequencies compared to daily collection frequency was observed according to the inclusion of zero in the Bland Altman LOA. The Bland Altman analysis indicated that the AUC for E1G and PdG from reduced urinary collection frequencies were within 9.8% and 11.8% of the daily measures, respectively, in the complete samples analysis and separate eumenorrheic and amenorrheic analyses. The AUC percent differences would have enabled O'Donnell et al. [51] and West et al. [25] to detect the observed 33%-98% differences in E1G AUC between sedentary and exercising eumenorrheic women and exercising amenorrheic women. The mean concentrations for E1G and PdG from reduced urinary

collection frequencies were within 1.5% and 3.1% of the daily measures, respectively, for all

three analyses. Differences in mean concentrations between ethnicities, which have been

reported to lie between 1.0 and 22.3% (E1G) and 3.0 and 31.7% (PdG) [52], would have

been observed using 2-days per week as collection frequency. However, observing subtle

differences of 1-5% in E1G and PdG AUC, as reported between sedentary and exercising

eumenorrheic women [22, 25] would require the urinary collection frequency to be no fewer

than 3-days per week (3.2% for E1G and 2.9% for PdG mean percent difference). Use of

reduced urinary collection frequencies as few as 2-days per week to assess group

differences in cycle AUC and mean concentration provides comparable values to daily

urinary collection. Thus, the use of reduced collection frequencies would still allow detection

of differences between experimental groups.

In a study by Mumford et al. [53] 8 serum samples were collected during biologically

relevant windows timed to a standardized 28-day menstrual cycle. The serum samples were

utilized to assess the exposure to reproductive hormones in women with short (<26 days),

normal (26-35 days), and long (>35 days) menstrual cycle lengths. Unfortunately, the

authors did not conduct a comparison of the 8 samples to daily sampling, but the AUC

values presented by Mumford et al. [53] demonstrated a high standard error indicating 8

samples per menstrual cycle to be too large a reduction in sample collection. The authors

commented that collecting only 8 serum samples throughout the menstrual cycle reduced

the generalizability of their findings in women with longer and more irregular cycles [3]. The

basic shape of the E1G and PdG curves across the menstrual cycles in our analysis indicate

that reducing the urinary collection frequency as low as 8 samples per cycle (2 days per

week in a 28-day menstrual cycle or monitoring period) increases the likelihood of missing

the peak hormonal concentrations, thus greatly altering the AUC and mean concentration

calculations. In our analysis a reduced collection frequency of less than 5 days per week

was associated with an increased range for the LOA due to the potential of missing peak

hormonal concentrations, which may also compromise the use of reduced collection

frequencies in studies evaluating individual subjects and clinical outcomes, such as day of ovulation.

The shape and timing of the peaks of E1G and PdG greatly affect the determination of exposure and cycle mean concentration. For example, the E1G peak is generally narrow and rises quickly to a peak, while the PdG peak is a broad curve. Hence, the narrow peak for E1G is more likely to be missed by a reduced sample collection frequency compared to the peak of a broad curve, like PdG. Differences in capture of the E1G and PdG peaks are shown in the composite graphs (**Figures 3.2, 3.5, and 3.6**). **Figure 3.5** shows that the broad PdG peak was not influenced by the reduced collection frequencies; however, the shape of the E1G peak is varied, though not visually different, between the reduced collection frequencies and daily collection hormone profile. Within a group analysis a proportion of the narrow peaks would be captured with the reduced collection frequencies, thus the increased LOA; however, when analyzing a cycle from an individual participant it is highly likely the E1G peak concentration would have occurred on a non-sample collection day. When evaluating individual cycles for a case report, as seen with Mallinson et al. [54], a reduction in the collection frequency as low as 2-days per week would not influence the characterization of the cycle via hormonal exposure in a participant who has had amenorrhea for a long period of time. In a participant who had amenorrhea for a short period of time a reduction in sample collection to 2 days a week would under-estimate the exposure of the participant to reproductive hormones due to higher variations in monitoring period peaks of E1G and PdG across a year [19].

Urinary samples are self-collectable, non-invasive, can be easily stored and transported, and have been shown to tolerate a wide variety of non-perfect experimental conditions in the field [11, 14]. Initially it was assumed that daily samples could be collected for prolonged periods of time with a high degree of compliance, however many recent studies have shown compliance to be highly variable depending on study length [10, 33, 36-39]. The level of compliance to sample collection may be due to personal or housemate

comfort level with storage of specimens or ability to store samples when away from the primary residential address. Participants in early studies which evaluated perceptions of urinary sample collection reported that the benefits of increased knowledge about their body outweighed the uncomfortable nature of urine collection [33, 55]. According to Wright et al. [56], urine collection was one of the least objectionable of the 8 methods used to assess reproductive function (transvaginal ultrasound, basal body temperature, salivary electrical resistance, blood sampling, salivary samples, vaginal mucus electrical resistance, and manual cervical mucus consistency). The study by Wright et al. [56] was one of the first to assess the attitudes of the general population, instead of nurses, to reproductive hormone collection methods. Anecdotal evidence from our laboratory has indicated that participant travel over weekends and for vacations lead to large gaps in sample collection and decreased compliance, thus the reduced collection frequencies evaluated in this paper provided the participants with weekends free from sample collection. In fact, for this analysis we excluded 67% of available cycles due to reduced compliance. In studies of long duration, it is unavoidable to have participants run into vacation time such as Thanksgiving, family summer holidays, or winter break, when reduced compliance to sample collection is more likely to occur.

One limitation of the present analysis was the restriction of the data set to only include subjects who were 100% compliant to the reduced collection frequencies. The presence of missed collection days within the reduced sample collection frequencies would decrease the accuracy of the urinary reproductive hormone metabolite profile across the menstrual cycle or monitoring period. Reduced collection frequency strategies still require the use of a valid menstrual cycle calendar in order to create an accurate presentation of the hormonal profile. Not all urinary collections for eumenorrheic cycles will begin on the first day of the menstrual cycle nor will the final collection always be on the last day of the menstrual cycle. Another limitation of the study was that we did not evaluate clinical reproductive outcomes in this analysis; however, such outcomes are integral in the

usefulness of reduced collection frequencies. Future studies should evaluate the validity of the reduced collection frequencies evaluated in this paper in detecting luteal sufficiency, and assessing this strategy in an independent sample.

The strengths of this analysis are that there were a large number of cycles included, individual participants provided multiple cycles, and the cycles used for reducing the collection frequency were based on menstrual cycles and 28-day monitoring periods that had all samples collected. In the data set, there are near equal numbers of short, normal, and long menstrual cycles in the eumenorrheic participant cycles and 28-day monitoring periods. Within our eumenorrheic cycles we intentionally included all cycle classifications (LPD, anovulatory, and ovulatory cycles) to show that even with cycles of highly variable hormone levels the reduced collection frequencies continue to have good agreement with daily urinary sample collection.

Though daily urinary collection is the most accurate reflection of reproductive hormone production when using urinary analysis, our results demonstrate that accurate E1G and PdG profiles of menstrual cycles of various lengths (20-45 days) and types (eumenorrheic and amenorrheic) can be measured with reduced urinary collection frequencies. This work supports and builds on the work of O'Connor et al. [40] demonstrating that a reduced sampling schedule can provide useful and accurate information in a manner that is comparable to that obtained from daily sampling of urine regarding ovarian hormone exposure and mean concentrations. The accuracy in quantifying exposure allows reduced collection frequency strategies to be utilized in under researched populations and in less developed regions around the world, where the capacity to store samples in a cold environment may be limited. The reduced collection frequencies produced composite E1G and PdG profiles for an entire cycle or monitoring period that were similar to the composite graphs of daily urinary collection. We have shown that daily ovarian steroid levels are not necessarily required to quantify AUC or mean values E1G or PdG across the entire menstrual cycle or monitoring period when conducting group examinations for the

assessment of disease risk, such as osteoporosis, endothelial dysfunction, ovarian cancer, and breast cancer, in large populations. Further research is needed to evaluate whether clinical outcomes, such as luteal sufficiency, are possible to determine through these specific reduced collection frequencies. We suggest reducing the urinary collection frequency in an effort to reduce the participant burden, increase compliance, and decrease project costs, depending on the accuracy and precision required to answer the reproductive questions of interest.

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Chapter 4: Study 2

Allaway HCM, John EM, Keegan TH, De Souza MJ. A pilot study on the utility of reduced urine collection frequency protocols for the assessment of reproductive hormones in adolescent girls. *In Review J Pediatr Endocrinol Metab.*

Abstract

To assess the feasibility of and compliance to collecting urine samples in pre- and postmenarcheal girls and determine if a reduced collection frequency was sufficient for assessing menstrual characteristics. Twenty-five postmenarcheal girls (11-17 years) collected samples using either a 2 or 3 samples/week protocol during one menstrual cycle. Exposure and mean estrone-1-glucuronide (E1G) and pregnanediol glucuronide (PdG) concentrations were calculated and evidence of luteal activity (ELA) was evaluated. Sixteen premenarcheal girls (8-11 years) collected one sample/month for 6 months. Samples were analyzed for E1G concentration. Participant compliance was calculated using dates on the urine samples and paper calendars. Participants collecting 3 samples/week were more compliant to collection than those collecting 2 samples/week ($83.6\pm2.6\%$ vs. $66.8\pm6.6\%$; $p=0.034$). There were no differences ($p>0.10$) between girls on the 2 vs. 3 samples/week protocols regarding paper calendar return ($81.8\pm12.2\%$ vs. $92.9\pm7.1\%$), recording menses ($55.6\pm17.6\%$ vs. $92.3\pm7.7\%$), or recording sample collection ($88.9\pm11.1\%$ vs. $84.6\pm10.4\%$). The average cycle length was 30.5 ± 1.3 days and 32% of cycles had ELA. The premenarcheal girls were 100% compliant to sample collection. Only 68.8% of participants returned the paper calendar and 81.8% of those participants recorded sample collection. The average E1G concentration was 15.9 ± 3.8 ng/mL. Use of a reduced collection frequency during a menstrual cycle in postmenarcheal, adolescent girls is feasible and provides informative data about menstrual characteristics. Collection of one sample/month in premenarcheal girls is feasible and detects the expected low E1G concentrations. Alternate strategies to the use of a paper calendar should be considered.

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Introduction

The gold standard of reproductive hormonal evaluation across the menstrual cycle requires frequent blood [1, 2], urine [3-6] or saliva [7, 8] samples. These assessments are generally limited by participant compliance to frequent sampling protocols and the cost of the assays. Daily sample collection presents a substantial study cost and high participant burden, contributing to high non-compliance and dropout rates [9], and may not be feasible for large, multi-center studies. Collection methods should yield good participant compliance to collection while providing reliable and sensitive menstrual cycle characteristics details [7]. In adolescent girls, it is especially difficult to design protocols for large, epidemiologic studies that yield good compliance and quantification of menstrual function characteristics, including estrogen exposure, which is important in many physiological systems [10-12].

Characterization of reproductive function in adolescent girls in the peri- and early postmenarcheal time period have utilized multiple methods, including menstrual calendars [2, 6, 8, 13-17], daily blood [2] or saliva [8] samples during a menstrual cycle [2, 8], serial blood sampling on one day [17-19], first morning urines once/week [14, 20], daily first morning urine [5, 6], 24-hour urine samples [15], and single time point samples [21-25]. Published reports using daily sampling methods are limited by small numbers and homogeneous racial/ethnic backgrounds [2, 5, 6, 8], thus reducing generalizability. Studies where less than daily sampling was utilized tended to have larger sample sizes; however, sample collection that is too infrequent will not provide sufficient data to characterize changes in reproductive function [14, 15, 20-25]. To adequately understand and evaluate the adolescent menstrual cycle, studies must include large numbers of girls of diverse racial/ethnic backgrounds and use protocols that are feasible.

There is scant information regarding compliance to reduced frequency biospecimen collection in adolescents, specifically longitudinal menstrual cycle data. In a study of 10 adolescent girls (11-13 years), 6 (60%) participants provided a minimum of 80% of all possible daily first morning urine samples and monthly menstrual calendars over two years

[6]. Compliance was high (94%) in a study of 174 girls ages 14-17 years that collected one early morning luteal phase urine sample in 2 non-consecutive menstrual cycles in a 6-month period correctly [13]. In another study, biweekly menstrual cycle calendars with adequate data (no or only 1 missing calendar) were returned by 168 (97%) participants [13].

We reported that a less frequent sample collection protocol provides adequate accuracy and precision for the purpose of describing menstrual cycle characteristics, such as cycle estrone-1-glucuronide (E1G) and pregnanediol glucuronide (PdG) exposure (area under the curve (AUC)) and mean integrated concentrations, in women ages 18-35 years [26]; however, compliance to reduced collection frequency has not been evaluated prospectively. The purpose of this study was to assess the feasibility and compliance of collecting urinary samples in pre- and postmenarcheal girls to determine if a reduced collection frequency (1 sample/month in premenarcheal and 2 or 3 samples/week in postmenarcheal) was sufficient for assessing characteristics of reproductive function (estrogen exposure and evidence of luteal activity).

Materials and Methods

Experimental Design: This study utilizes menstrual cycle data from girls recruited for two pilot studies conducted over a two-year period during 2013-2014 in the San Francisco Bay Area. Pilot Study 1 recruited 11 girls who were participants in the California site of the LEGACY Girls Study [27], and Pilot Study 2 included 30 girls recruited through community outreach. At the time of sample collection, participants of Pilot Study 1 were ages 13-17 years and all girls were postmenarcheal. Pilot Study 2 participants were ages 8-14 years and included 16 premenarcheal and 15 postmenarcheal girls. One Pilot Study 2 participant experienced menarche during the study and was included in both the premenarcheal and postmenarcheal analysis. One of the Pilot Study 2 postmenarcheal participants collected a single sample and did not return a paper calendar and was excluded from all analyses. Thus, the analysis for Pilot Study 2 is based on 14 postmenarcheal girls. Procedures for

both pilot studies followed the Declaration of Helsinki on ethical principles for medical research involving humans. The Institutional Review Board at the Cancer Prevention Institute of California (CPIC) approved both pilot studies. The participating mother or guardian provided written informed consent and participating girls signed an assent form prior to study participation.

Data Collection: Participating mothers/guardians completed online or mail questionnaires on demographic background, medical history, cancer family history, and early-life exposures. The Growth and Development questionnaire asked about the daughter's age at menarche, and Tanner stage of breast development using drawings [28]. Trained study staff conducted a home visit to take anthropometric measurements as previously described [27], including standing height (measured to the nearest 0.1cm), weight (measured to the nearest 0.1kg), waist and hip circumference (measured to the nearest 0.1cm), and body fat percentage. They also explained the urine sampling protocol and completion of the paper calendar. Study staff sent email or text message reminders weekly to postmenarcheal girls and monthly to premenarcheal girls.

Gynecologic age was calculated as current age minus age at menarche. The girls' body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). BMI percentiles by age were based on CDC growth charts and categorized as underweight (< 5th percentile), healthy weight (5th to < 85th percentile), and overweight/obese (≥ 85th percentile) [29]. Waist-to-hip ratio (WHR) was calculated as waist circumference (cm) divided by hip circumference (cm).

Urine Collection Procedures:

<u>Collection Frequencies:</u> Postmenarcheal participants were asked to collect first morning urine samples on the first Monday and Thursday (2 samples/week; Pilot study 1: n=11) or Monday, Wednesday, and Friday (3 samples/week; Pilot study 2: n=15) after the onset of menstrual bleeding [26]. Pilot Study 2 girls were asked to collect 3 samples/week for two consecutive cycles, beginning on the first Monday, Wednesday, or Friday after the onset of

menstrual bleeding [26]. Seven participants of Pilot Study 1 collected 2 samples/week for 2 weeks after the end of their menstrual cycle. Participants were provided a paper calendar to record days of menstrual bleeding and urine sample collection.

Premenarcheal participants (n=16) were asked to collect a first morning urine sample on the same day of the month for 6 consecutive months. Participants were provided with a paper calendar for recording urine sample collection days.

Urinary Sampling Procedure: Urine samples were collected by saturating a sponge and placing it in an airtight vial [30]. Samples were stored in the home freezer (-4°C) until returned to the laboratory at the CPIC. Vials were labeled with study ID and date of sample collection. If the participant was postmenarcheal, the menstrual cycle number was also recorded on the specimen label. Samples were kept cold by frozen ice packs during transport to the laboratory where samples were stored at -70°C before they were shipped on dry ice to the Women's Health and Exercise Laboratory at the Pennsylvania State University. Samples were thawed and centrifuged at 3000rpm for 20 min at 4°C to separate the urine from the sponge. Each sample was stored as 6 aliquots of 1800uL at -20°C until analysis. Specific gravity of all urine samples was measured using a hand refractometer (NSG Precision Cells, Inc., Farmingdale, NY).

Urinary Measurement of E1G and PdG: Microtiter plate competitive enzyme immunoassays (EIA) were used to measure E1G and PdG in postmenarcheal samples, as previously described [3, 26]. Premenarcheal samples were assayed for E1G. The secretion of E1G and PdG in the urine parallels serum concentrations of the parent hormones [4, 31]. Urinary concentrations of E1G and PdG were corrected for specific gravity to account for hydration status [32].

Compliance Calculations:

Urine Collection: Compliance was calculated as number of samples collected correctly divided by the total number of possible samples (determined by menses or first/last sample collection if no menstrual calendar was returned). For Pilot Study 2, we calculated the

compliance separately for each menstrual cycle and for both cycles combined. Additionally, we assessed the number of vials returned unlabeled or without biomaterial.

<u>Paper Calendars:</u> For postmenarcheal girls, we assessed the number who properly recorded the occurrence of menses and date of each urine collection on the calendar. For Pilot Study 2 participants, we assessed the calendar use separately for each menstrual cycle and for both cycles combined. For premenarcheal girls, we calculated the percentage of samples collected on the same day each month (correct collection) and the percentage of samples collected early or late.

Menstrual Cycle Assessment: We completed hormonal assessments of menstrual cycles only for participants who recorded the days of menstrual bleeding on their calendars in order to confirm the length of the menstrual cycle. We included one participant who did not complete the calendar, but was compliant to sample collection. Her hormone measures were utilized and calculated from the first to the final day of collection (monitoring period). E1G and PdG exposures across menstrual cycles were determined by calculating the AUC using Kaleidagraph Software (Synergy Software, Reading. PA, USA). We also calculated mean E1G and PdG concentrations across menstrual cycles. Evidence of luteal activity was determined based on maximum PdG measured in second half of menstrual cycle. One PdG concentrations above 2.5µg/mL in the second half of the menstrual cycle was considered as evidence of luteal activity [3, 33].

Statistical Analyses: All data are presented as mean ± standard error. Data were analyzed using SPSS for Windows (version 23.0, Chicago, IL). Differences in means were assessed using T-tests or Mann-Whitney U tests. Chi-square tests were used for categorical variables. Compliance was reported as a qualitative variable to summarize the feasibility and usefulness of the urine collection protocols.

Table 4.1: Descriptive characteristics of adolescent girls in Pilot Studies 1 and 2 (n=41).

	Postmenarcheal		Premenarcheal	P-value
	Pilot Study 1 (n=11)	Pilot Study 2 (n=14)	Pilot Study 2 (n=16)	(Postmenarcheal only)
Age (y)	**14.8 ± 0.4**	**12.4 ± 0.3**	10.1 ± 0.2	**< 0.001**
Age at Menarche (y)	11.8 ± 0.3	11.9 ± 0.2 *	N/A	1.000
Gynecologic Age (y)	**3.0 ± 0.5**	**0.6 ± 0.2 ***	N/A	**< 0.001**
Body Fat %	27.5 ± 3.2	24.0 ± 1.8	26.9 ± 1.7	0.330
Waist-to-Hip Ratio	0.8 ± 0.0	0.8 ± 0.0 [#]	0.8 ± 0.0	0.648
BMI Percentile for Age	64.9 ± 10.3	67.4 ± 5.8	50.7 ± 7.6	0.826
Weight Status [n (%)]				
Healthy Weight	6 (54.5)	11 (78.6)	13 (81.2)	
Overweight/Obese	5 (45.5)	3 (21.4)	3 (18.8)	
Race/Ethnicity [n (%)]				
Non-Hispanic White	3 (27.3)	6 (42.9)	7 (43.8)	
Asian American or Pacific Islander		3 (21.4)	7 (43.8)	
Asian Hispanic	2 (18.2)	1 (7.1)	1 (6.3)	
Hispanic	6 (54.5)	4 (28.6)	1 (6.3)	
Breast Tanner Stage [n (%)]				
Stage 1			7 (43.8)	
Stage 2			6 (37.5)	
Stage 3	3 (27.3)	6 (42.9)	3 (18.8)	
Stage 4	6 (54.5)	8 (57.1)		
Stage 5	2 (18.2)			

* n=13; One participant experienced menarche between survey collection in January and urine collection in August. Her gynecologic age is no more than 0.5 y.

[#] n=13; One participant did not have a waist circumference measurement.

Results

1. Postmenarcheal Participants:

        Participant characteristics are shown in **Table 4.1**. Pilot Study 1 participants were older than Pilot Study 2 participants (p<0.001), and they differed in gynecologic age (p<0.001). Participants did not differ (p>0.05) with respect to race/ethnicity, age at menarche, breast Tanner stage, BMI percentile, proportion in each BMI percentile, WHR, and percent body fat.

Feasibility and Compliance:

<u>Pilot Study 1:</u> Participants who used the 2 samples/week protocol (n=11) for one menstrual cycle were 66.8±6.6% (range 33-89%) compliant to collecting and storing urine samples. The number of possible samples for collection per participant ranged from 6-14 samples due to the varying menstrual cycle lengths (range 25-44 days). Seven participants collected additional samples beyond one menstrual cycle. Inclusion of these additional samples reduced the compliance to 63.2±5.1% (range 27-89%). The total number of possible samples for collection/participant ranged from 9-20 samples. The average menstrual cycle length was 31.5±2.2 days (range 25-44 days).

        Nine (81.8%) of 11 participants returned their paper calendar (**Figure 4.1A**). Of these, 5 (55.6%) participants recorded their menses on the calendar (**Figure 4.1C**). There was good concordance (89%) between the urine collections recorded on the calendar and the specimens returned. Of the 9 participants who returned paper calendars, 5 (55.6%) recorded on their calendars most (>80%) or all of the urine samples collected (**Figure 4.1B**). Only one participant did not record the date for the majority of her urine samples. Participating families found storage of the samples in the home freezer acceptable.

<u>Pilot Study 2:</u> Participants who collected 3 samples/week (n=14) for 2 consecutive menstrual cycles were 83.6±2.6% (range 71-100%) compliant to collecting and storing urine samples. The number of possible samples for collection/participant ranged from 20-34 samples. The average menstrual cycle length was 29.3±1.4 days (range 21-39 days).
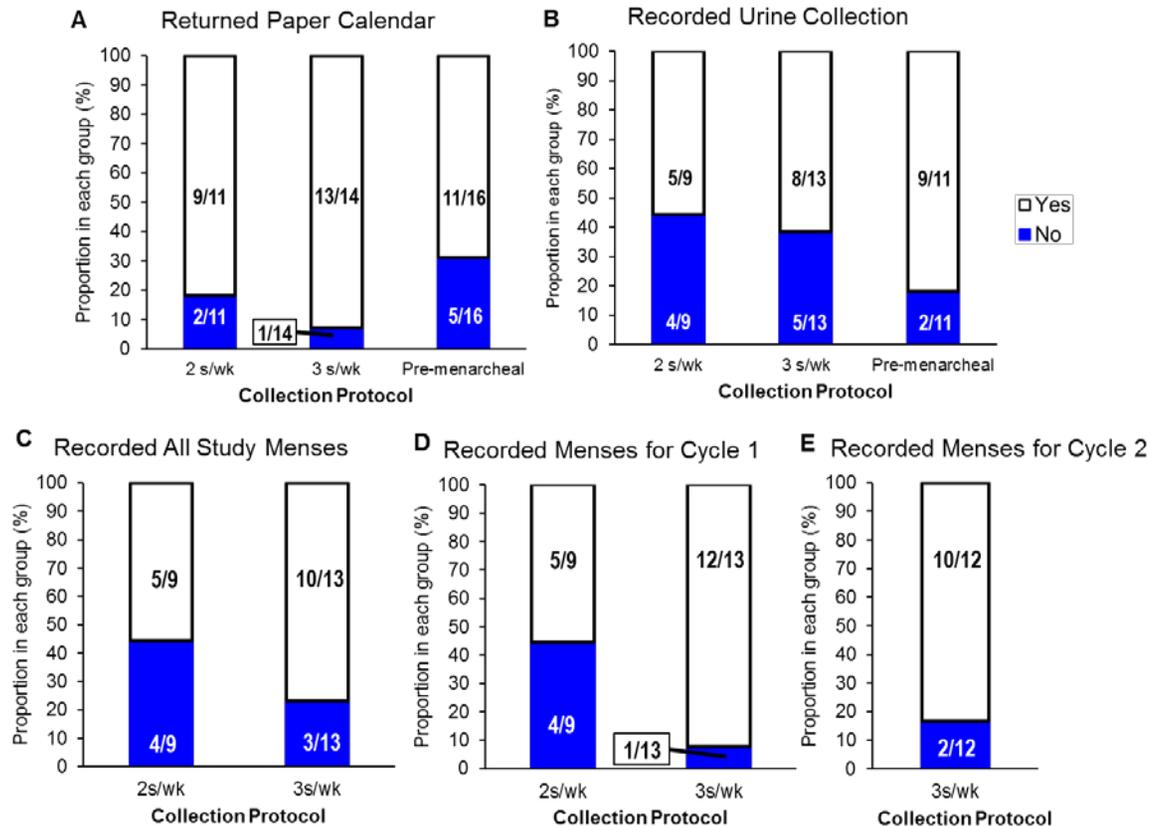
Figure 4.1: (A) Proportion of participants, postmenarcheal and premenarcheal, who returned their menstrual and urine collection calendar at the end of the pilot study. (B) Proportion of participants, postmenarcheal and premenarcheal, who returned collection calendars that recorded urine sample collection on the collection calendar. (C) Proportion of postmenarcheal participants who returned collection calendars and recorded all study menses on the collection calendar. (D) Proportion of postmenarcheal participants who returned collection calendars and recorded menses for determination of length of menstrual cycle 1. (E) Proportion of postmenarcheal participants of the 3 samples/week collection protocol pilot study who returned collection calendars and recorded menses for determination of length of menstrual cycle 2.
- s/wk indicates samples/week

Compliance to sample collection was 86.5±3.6% for cycle 1 and 79.7±5.5% for cycle 2 (p=0.403; **Figure 4.2C**). The average menstrual cycle length was 30.0±1.9 days (range 21-39 days) for cycle 1, and 28.5±2.08 days (range 21-39 days) for cycle 2.

Thirteen (92.9%) of 14 participants returned their paper calendar (**Figure 4.1A**). Of these, 10 (76.9%) recorded the days of menses (**Figure 4.1C**). There was no difference in the number of participants who returned calendars with adequate information to determine menstrual cycle length between the 2 menstrual cycles (92.3% vs. 83.3%, respectively; p=0.166; **Figures 4.1D and 4.1E**). There was a high concordance (95%) between the urine collections recorded on the paper calendars and the samples returned. Of the 13 participants who returned their calendars, 8 (61.5%) recorded most (>80%) or all of the urine samples collected (**Figure 4.1B**). Only one participant (7.1%) did not correctly date the majority of her samples. Temporary sample storage in the home freezer was acceptable to participants.

Pilot Study 1 vs. Menstrual Cycle 1 of Pilot Study 2: Pilot Study 2 participants (3 samples/week) were significantly more compliant to collecting urine samples over one menstrual cycle compared to Pilot Study 1 participants (86.5±3.6% vs. 66.8±6.6%, respectively; p=0.025; **Figure 4.2B**). When both cycle 1 and cycle 2 collections from Pilot Study 2 were included, compliance remained significantly different (83.6±2.6%; p=0.034, **Figure 4.2A**). Pilot Study 2 participants also had higher compliance to returning the paper calendar (92.9±7.1% vs. 81.8±12.2%, respectively; p=0.431) and recording their menses (92.3±7.7% vs. 55.6±17.6%, respectively; p=0.164), although the differences were not statistically significant. Compliance to recording urine collection dates was similar between the pilot studies (88.9±11.1% vs. 84.6±10.4%, respectively; p=0.896). Patient characteristics (age, gynecologic age, breast Tanner Stage, race/ethnicity) did not differ between participants with high (≥75%) and low (<75%) compliance between the two pilot studies.
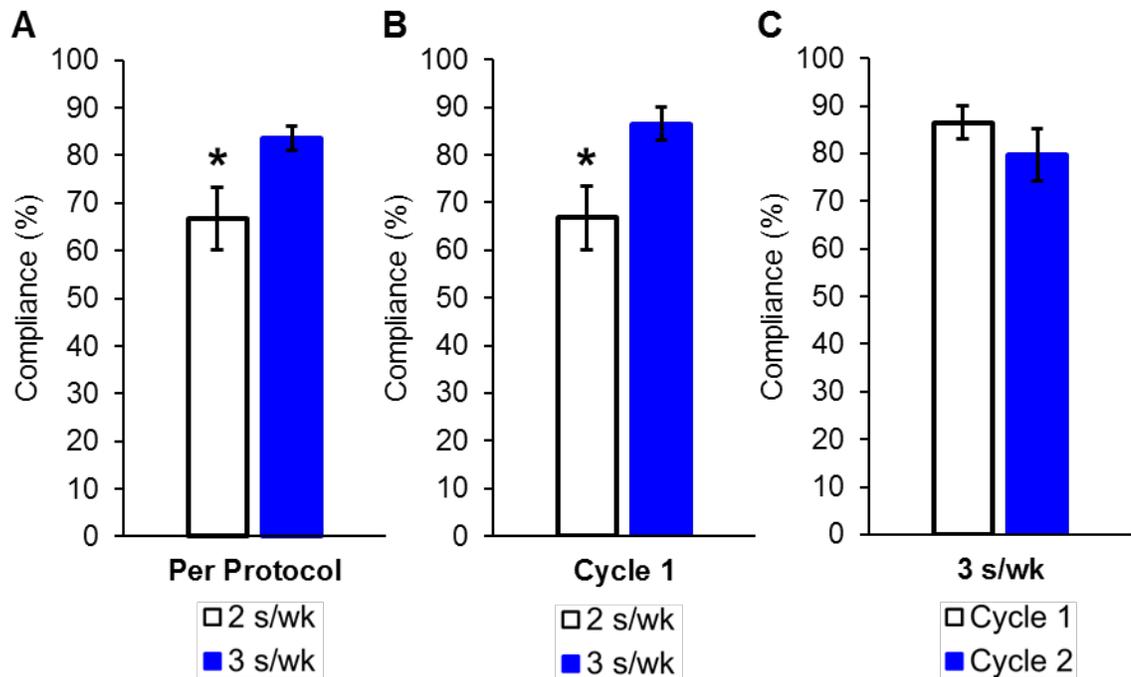
Figure 4.2: Urine sample collection compliance (A) per pilot study protocol for the 2 samples/week protocol compared to the 3 samples/week protocol participants (1 menstrual cycle for the 2 samples/week protocol and 2 consecutive menstrual cycles for the 3 samples/week protocol); (B) for menstrual cycle 1 only for the 2 samples/week protocol compared to the 3 samples/week protocol participants; and **(C)** for menstrual cycle 1 compared to menstrual cycle 2 for the 3 samples/week protocol participants. * indicates significant difference in compliance to collection (p<0.05).

Assessment of Menstrual Cycle Characteristics: We were unable to utilize the hormonal data from 17 of 42 cycles or monitoring periods due to either poor urine collection compliance (n=12; unlabeled samples, 7 or more consecutive missed samples) and/or inadequate recording of menses or failure to return the paper calendars (n=7). Of the excluded cycles, six were from Pilot Study 1, four were the additional collections by Pilot Study 1 participants, and seven were from Pilot Study 2. We included one monitoring period due to the participant's compliance to collection and the visualization of suppressed E1G and PdG concentrations across the entire 44-day monitoring period.

Representative menstrual cycles for Pilot Study 2 are presented in **Figures 4.3A-C** and for Pilot Study 1 in **Figures 4.3D-F**. Among the 25 cycles, the average cycle length was 30.5±1.3 days (range 21-44 days). The average cycle E1G and PdG AUCs were

1070.8±145.5 ng*d/mL and 33.4±5.3μg*d/mL, respectively. The mean concentrations of E1G and PdG were 42.5±5.2ng/mL and 1.3±0.2ug/mL, respectively. The average maximum PdG concentration in the second half of the cycle was 3.0±0.6μg/mL (range 0.22-12.83μg/mL). Evidence of luteal activity was observed in 32% of cycles (**Figures 4.3A, B, E, and F**). Using a maximum PdG concentration cutoff of 1.5μg/mL [6], 48% of cycles had evidence of luteal activity. The 6 monitoring periods excluded due to inadequate information on menses had an average PdG peak in the second half of the monitoring period of time of 2.02μg/mL. Two monitoring periods had PdG ≥2.5μg/mL and 4 monitoring periods had PdG ≥1.5μg/mL.

2. Premenarcheal Participants:

Mean age of premenarcheal participants was 10.1±0.2 years (range 8-11 years). They had a mean BMI percentile of 50.7±7.6, WHR of 0.8±0.0, and body fat percentage of 26.9±1.7%, and 18.8% were overweight/obese (**Table 4.1**). Participants' breast Tanner stages were 1 (43.8%), 2 (37.5%), or 3 (18.8%). Over half of the premenarcheal girls were racial/ethnic minorities.

Feasibility and Compliance: Of the 15 participants who remained premenarcheal during the pilot study, all collected the required one sample/month for 6 consecutive months (100% compliance). Most (99%) adequately labeled the samples and were 88% compliant to collecting samples within 3 days of the designated collection date. The remaining samples were collected 5-12 days earlier (3%) or 6-15 days later (9%) than scheduled. Eleven (68.8%) premenarcheal participants were compliant to returning the paper calendar (**Figure 4.1A**). Of these, 9 (81.8%) were compliant to recording the date and time of each urine collection on the calendar (**Figure 4.1B**). There was high concordance (93%) between the urine collections marked on the calendar and the urine specimens returned. Most (97%) samples contained sufficient biomaterial for analysis. Temporary sample storage in the home freezer acceptable to participants.
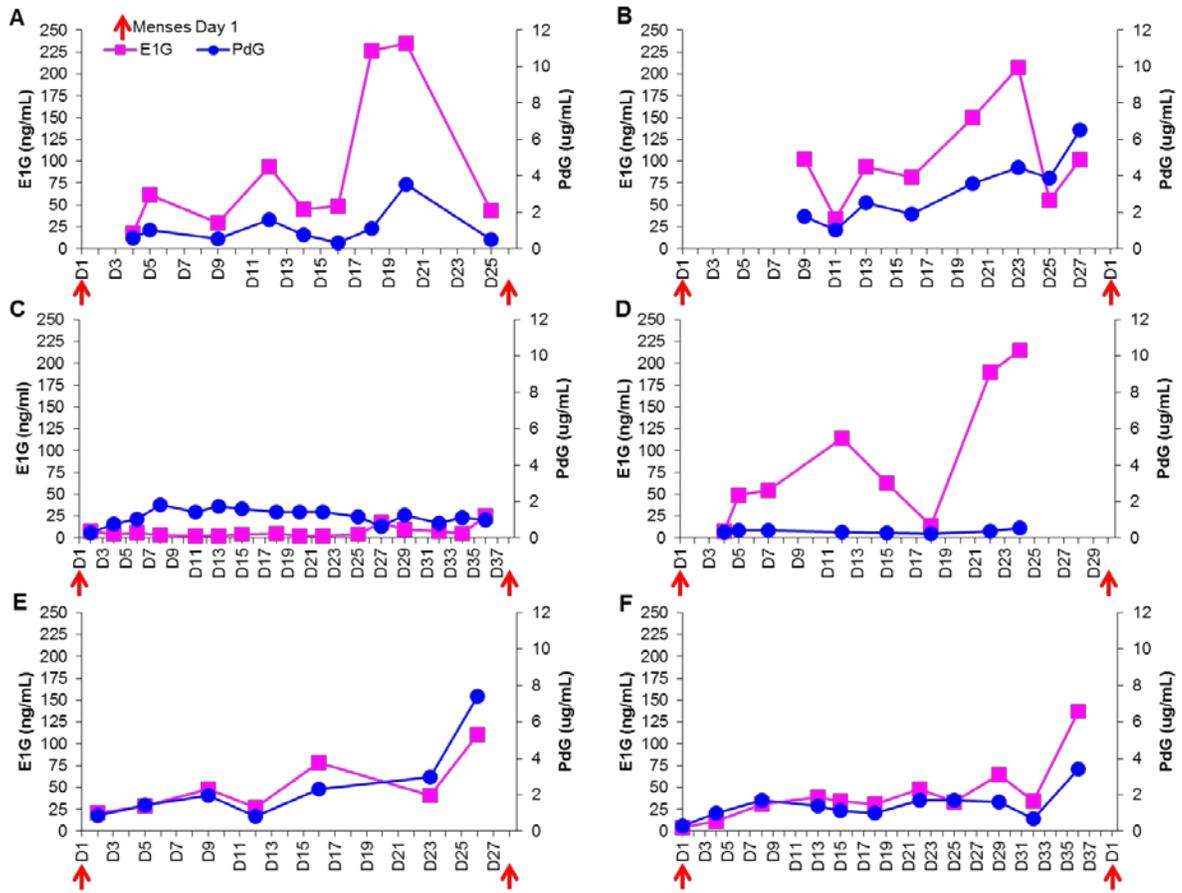
Figure 4.3: Representative estrone-1-gulcuronide (E1G) and pregnanediol-3-glucuronide (PdG) concentrations across menstrual cycles of the postmenarcheal participants. (A) A 11 year old (in 1st postmenarcheal year) 3 samples/week protocol participant with a 25 day cycle (estrogen activity and evidence of luteal activity). (B) A 12 year old (in 1st postmenarcheal year) 3 samples/week protocol participant with a 28 day cycle (estrogen activity and evidence of luteal activity). (C) A 13 year old (in 2nd postmenarcheal year) 3 samples/week protocol participant with a 37 day cycle (no estrogen activity and no evidence of luteal activity). (D) A 14 year old (in 2nd postmenarcheal year) 2 samples/week protocol participant with a 29 day cycle (estrogen activity and no evidence of luteal activity). (E) A 17 year old (in 4th postmenarcheal year) 2 samples/week protocol participant with a 27 day cycle (estrogen activity and evidence of luteal activity). (F) A 14 year old (in 3rd postmenarcheal year) 2 samples/week protocol participant with a 38 day cycle (late in the menstrual cycle estrogen activity and no evidence of luteal activity).

Hormonal Characteristics: Average E1G concentration over the 6-month collection period was 15.9±3.8ng/mL (range 1.4-60.0ng/mL). The average E1G for collections 1-5 were 15.6±4.0, 10.2±2.1, 11.8±2.4, 15.6±3.9, 12.5±2.9, and 12.9±2.7ng/mL, respectively (**Figure 4.4A**). The E1G and PdG concentrations observed in the participant who experienced menarche during the study are shown in **Figure 4.4B**. This participant collected a single premenarcheal sample with elevated E1G and low PdG. During her menstrual cycle, a slow rise in E1G concentrations was observed with fluctuations in PdG that did not significantly rise above 2.0µg/mL.

Discussion

Our pilot studies are the first to examine prospective compliance to reduced urine collection frequencies. We found that the collection of 2 or 3 samples/week over 1-2 menstrual cycles in postmenarcheal girls and one monthly sample over 6 consecutive months in premenarcheal girls is feasible, that girls had good compliance, and that the data generated from these reduced sampling protocols are useful for assessing general characteristics of reproductive hormones in adolescent girls. This study builds upon previous work [26, 34] demonstrating that researchers can utilize a reduced sampling protocol and obtain useful information regarding ovarian hormone exposure, when accompanied by an appropriately completed menstrual calendar, in studies of adolescent girls.

Overall, participants using the 3 samples/week protocol were more compliant to sample collection than the participants using the 2 samples/week protocol. Compliance to urine collection did not significantly differ between the 2 consecutive menstrual cycles for the participants using the 3 samples/week protocol. Anthropometric characteristics did not differ between participants with high and low compliance. Moreover, the participants in the 2 samples/week protocol were participating in the LEGACY Girls Study [27], which required completion of other study components that could have impacted compliance to this pilot study. Premenarcheal girls were highly compliant to collection of urine samples over the course of a 6-month time period.
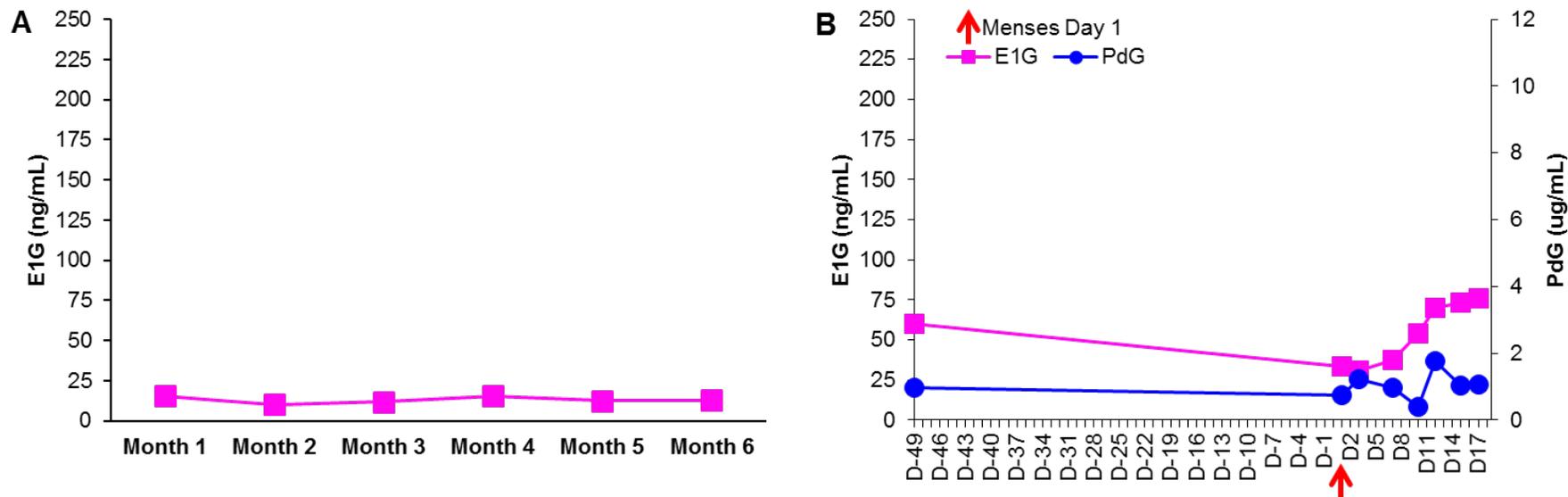
Figure 4.4: (A) Average estrone-1-gulcuronide (E1G) concentrations over the 6 month collection period for premenarcheal pilot study participants. (B) E1G and pregnanediol-3-glucuronide (PdG) concentrations observed in an 11 year old participant whose transition through menarche was observed during the study urine collection timeframe. The urinary sample labeled as D-49 was collected under the premenarcheal protocol. The red arrow indicates day 1 of menses and the subsequently collected urine samples were collected following the 3 samples/week protocol for a partial menstrual cycle.

Urine was chosen due to its non-invasiveness and self-collectability, compared to blood, over long periods of time, while still providing useful information regarding menstrual cycle function. Our results demonstrate that prospective collection of a reduced frequency protocol may have compliance issues related to the combination of weekdays utilized (i.e., a Monday/Tuesday being a less intuitive combination than Monday/Wednesday/Friday). The missed collection days within the reduced sample collection protocol prevented accurate analyses of the metabolite profile. Reduced collection frequencies still require completion of a valid menstrual calendar to create an accurate presentation of the hormone profile.

Compliance to completing the paper calendar was lower than expected [13] for both the pre- and postmenarcheal participants. Adolescent participants may have been poor recorders and collectors during the study due to their inexperience with menstrual cycling and terminology surrounding menstrual cycles. This was evident in two cases in particular, who only collected urine while menstruating. Some young girls may not have felt comfortable in talking to their parents about their menstrual cycle, thus decreasing compliance to the calendars even with high compliance to collecting urine on the correct days. As a result, alternate modes for completing menstrual calendars should be considered. With the widespread use of mobile technologies among adolescents, electronic menstrual cycle tracking applications may be useful tools to clinicians and researchers for educating young girls about normal menstrual cycles and monitoring cycle length and bleeding patterns [35, 36]. Thus, the development of menstrual cycle applications specifically for education and research in adolescent girls during the early years after menarche is important to consider.

Initial estrogenic activity results in thelarche and menarche is reported to occur 2.3±1.0 years after thelarche [35, 37, 38]. The median age at thelarche has been decreasing since the 1970's, whereas the age at menarche has remained relatively stable in Caucasian females [38, 39]. Even with the younger age at initiation of puberty, a propensity for young girls to experience long and inconsistent menstrual cycles remains due to the immaturity of

the hypothalamic-pituitary-ovarian axis and a large proportion of cycles are thought to be anovulatory [35, 40]. Thus, there is a need to understand hormonal variation across menstrual cycles in conjunction with observable external signals in adolescent girls as they mature gynecologically. In this study, we have demonstrated that reduced frequency protocols were sufficient to determine overall exposure to E1G and PdG during an adolescent menstrual cycle, when provided adequate information on menses (inadequate data for 40.5% of cycles). Although we obtained limited details regarding cycle quality (i.e., day of ovulation and follicular and luteal phase length), we were able to determine evidence of luteal activity. Future studies enrolling girls into pubertal cohorts to disentangle the impact of pubertal timing, growth rate in height, onset of first menses, and hormonal aspects of gynecologic maturation on disease risk later in life should consider utilizing urine collection with a reduced collection protocol. In the premenarcheal participants, we were able to measure low concentrations of E1G in the once/month samples over a 6-month sampling period. Researchers should consider utilizing urine sample collection to evaluate changes in ovarian steroid hormone exposure over time in future cohorts of pre- and postmenarcheal adolescent girls.

We have demonstrated that in postmenarcheal adolescent girls, a 3 samples/week urine collection protocol provided high compliance and is useful for assessing menstrual characteristics. The low concentration of E1G expected in premenarcheal adolescent girls can be measured. Use of electronic menstrual cycle applications should be considered over paper calendars in this population.

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Chapter 5: Study 3

Allaway HCM, Weaver CM, Misra M, Williams NI, Mallinson RJ, Petkus DL, Stone MS, Southmayd EA, Martin BR, De Souza MJ. Oral contraceptives decrease recombinant human GH-stimulated hepatic IGF-1 secretion more than non-oral contraceptives in healthy young women.

Abstract

Combined hormonal contraceptive (CHC) therapy may be associated with negative effects on BMD that are likely route dependent and involve the hepatic growth hormone (GH)/insulin-like growth factor-1 (IGF-1) axis. The purpose of this study was to assess the effects of combined oral contraceptive (COC) and contraceptive vaginal ring (CVR) therapy on hepatic IGF-1 production compared to a non-therapy Control group in response to recombinant human growth hormone (rhGH) (IGF-1 Generation Test). Healthy, premenopausal women chose to participate in the Control group (n=8) or to be randomly assigned to receive COC (n=8) or CVR (n=8) for two contraceptive cycles. All participants completed a modified IGF-1 Generation Test beginning between days 2-5 of the menstrual cycle prior to the initiation of CHC therapy (baseline). The IGF-1 Generation Test was repeated beginning between days 15-17 of the second CHC intervention cycle in the COC and CVR groups or between days 2-5 of the subsequent menstrual cycle in the Control group (post-therapy). During the IGF-1 Generation Tests, fasting blood samples were obtained on days 1, 2, 4, 6, and 8 and rhGH injections were administered on days 2, 3, 4, and 5. Day 1 and 2 (basal) average IGF-1, peak increment IGF-1 (peak minus basal) concentration, and area under the curve (AUC) of the increment IGF-1 concentrations were calculated for both IGF-1 Generation Tests. Multiple t-tests with a Bonferroni correction, univariate ANOVA, and repeated measures ANOVA were utilized to compare baseline and post-therapy measurements within and among groups. Participants in the COC, CVR, and Control groups were similar in age (23.0±0.7y), gynecologic age (10.0±0.8y), BMI (22.5±0.6), and VO_{2peak} (44.8±1.5mL/kg/min). Basal IGF-1 concentrations were significantly decreased in the COC group compared to the Control group (p=0.013). There were

interaction and period (p<0.02) effects on peak IGF-1 concentrations; however, there was no group (p=0.27) effect. Peak IGF-1 concentration was suppressed post-therapy compared to baseline in the COC group (p<0.01), but did not differ in either the CVR or Control groups (p>0.12). There were interaction and period (p<0.03) effects but no group (p=0.41) effect on IGF-1 Generation Test AUC. The IGF-1 AUC was suppressed post-therapy compared to baseline in the COC group (p<0.01). These data suggest that rhGH-stimulated hepatic synthesis of IGF-1 is affected by route of CHC administration, such that greater suppression occurs during short-term COC use. Therefore, use of COC therapy may potentially suppress bone formation.

Introduction

Since the approval of the first oral contraceptive pill in the 1960's, combined hormonal contraceptives (CHCs) have been used by millions of women worldwide [1, 2]. The most popular CHCs include combined oral contraception (COC), the transdermal contraceptive patch (TDC), and the contraceptive vaginal ring (CVR) [3]. Between 2011 and 2013 approximately 62% of women age 15-44 in the United States were currently using some form of hormonal or non-hormonal contraception [4], of which approximately 16% were using a COC [4].

Multiple research teams have reported on the association between COC use and bone mineral density (BMD). Effects of COC use on bone health have recently been summarized in detail [5]. Briefly, investigators evaluating past and/or current COC use in cross-sectional analyses have demonstrated mixed results with some reports of protection against low BMD [6, 7], some reports of decreased BMD (0.5-1.5%) [8, 9], and other reports of no differences in BMD [10-15]. Additionally, prior COC use that exceeded 24 months resulted in greater loss of lumbar spine BMD [15]. Compared to non-users, larger losses of BMD were observed at the hip and smaller gains at the total body in users of COCs with 30-35µg and <30µg ethinyl estradiol (EE) after 12 months of discontinuation compared to non-users [15]. Other investigators have observed that lumbar spine BMD did not change among adolescents taking COCs while non-adolescent users experienced a 2% increase in BMD [16], indicating that COC use may suppress bone accrual. Based on these conflicting findings, further efforts to understand the effects of COC use on bone are critically important, particularly if these effects have the potential to be negative.

Use of CHC therapy may be associated with negative effects on BMD that are likely route-dependent and involve the hepatic growth hormone (GH)/insulin-like growth factor-1 (IGF-1) axis. The COC pathway of absorption results in the "first pass" effect, where the EE dose is metabolized in the liver. This process is known to cause decreased hepatic hormone and growth factor synthesis and increased hepatic binding protein synthesis [17, 18].

Systemic IGF-1 is predominantly synthesized in the liver; its secretion is GH dependent [19-21] and provides an important stimulus for bone growth [22]. COCs may decrease IGF-1 synthesis in young women [23, 24] due to the first pass effect; however, results of such studies have not been consistent [6, 23, 25, 26]. The direct systemic absorption of EE with TDC and CVR use (non-oral CHCs), circumvents the hepatic portal circulation and may exert fewer negative effects on liver IGF-1 synthesis [27-29] and thus may be less detrimental to bone. To date, there have been three published studies exploring the impact of 12-24 months of non-oral CHC use on BMD that demonstrate ambiguous results, including: 1) an attenuation of BMD at the hip (0.8 vs 2.5%) and lumbar spine (1 vs 2.8%) for TDC users compared to non-users [28], 2) no difference in lumbar spine BMD between TDC users, CVR users, and Controls [30], and 3) no difference in lumbar spine or hip BMD z-scores between CVR user and non-users [31].

A second mechanism possibly contributing to the observed suppression of IGF-1 concentrations is a reduced hepatic response to GH. A novel test, the IGF-1 Generation Test, is used to test hepatic responsiveness to GH stimulation [32-35] and has the potential to amplify subtle differences that are not otherwise detectable with fasting basal concentrations of IGF-1. In postmenopausal women, oral estrogen therapy reduced rhGH stimulated peak IGF-1 production by 20% [34, 35], indicating the potential for a reduction in liver responsiveness to GH secondary to oral estrogen therapy. In premenopausal women, liver responsiveness to GH decreases with increasing endogenous estradiol concentration [36] and peak IGF-1 production is reduced by 36% in COC users (mono and triphasic 20-35μg EE) compared to non-users, demonstrated via an extended rhGH stimulation study [37]. The IGF-1 Generation Test, however, has never been performed prospectively in premenopausal women following CHC initiation.

To date there are no direct comparisons of COC and CVR (or other non-oral) therapies that explore the influence of CHC therapy on the GH/IGF-1 axis in healthy young women. The purpose of this study was to assess the effects of COC and CVR therapy on

hepatic IGF-1 production compared to a non-therapy control group. We hypothesized that post-therapy basal IGF-1 concentration would be reduced in the COC group compared to the CVR and Control groups with no difference between the CVR and Control groups. We also hypothesized that the response to the IGF-1 Generation Test (peak and area under the curve) following CHC therapy will be attenuated in the COC group compared to the CVR and Control groups.

Methods

Experimental Design: This study was a prospective, open label, randomized control study examining the impact of CHC use on hepatic production of IGF-1 in young women aged 18 to 30 years. The study was completed at two sites, the Pennsylvania State University (PSU; n=17) and Purdue University (n=9). Assessments were completed during a baseline natural menstrual cycle and following 1.5 contraceptive cycles of either COC or CVR (42 days) or one natural menstrual cycles (21-35 days) in a Control group. The impact of COC versus CVR use on IGF-1 production was assessed using basal concentrations and a modified IGF-1 Generation Test. The Institutional Review Boards of PSU and Purdue University approved the study protocol. All participants signed informed consent prior to initiating screening procedures.

Participants: Women not currently using hormonal contraceptives were recruited. Eligibility included: 1) age 18 to 30 years, 2) BMI 18 to 29kg/m^2, 3) non-smoking, 4) naïve to hormonal contraceptives or not using hormonal contraceptives for at least six months prior to study entry, 5) not lactating, pregnant or intending to become pregnant in the next six months, 6) no apparent metabolic, endocrine, musculoskeletal, or severe psychiatric disease, 7) if physically active the primary mode was required to be weight bearing, 8) able to maintain current exercise training and diet, and remain weight stable (±2kg), and 9) having at least 9 menses in the past 12 months. Participants were excluded if they: 1) regularly consumed large amounts of soy products or grapefruit, 2) had a diagnosis of liver or renal disease, 3)

had malabsorption or skeletal disorder, 4) had uncontrolled thyroid abnormalities, 5)

chronically used non-steroidal anti-inflammatory drugs, 6) used medications known to have

interactions with hormonal contraception, 7) have any contraindication for hormonal

contraception use as proposed by the World Health Organization [38], or 8) were a Division

1 athlete on or off season (PSU site).

Screening: Young women desiring and not desiring hormonal contraception were screened

for this study. Anthropometrics, to include height (to the nearest 0.5 cm) and weight (to the

nearest 0.01 kg), were measured. Participants completed questionnaires to assess medical,

menstrual, and exercise history, eating behaviors, and psychological health. A blood sample

was taken and a physical exam was performed to determine overall health. Participants age

21 and over were required to provide evidence of a normal PAP smear in the past 18

months.

Study Phases: The study had three study periods: baseline, intervention, and post-therapy

(**Figure 5.1**). *Baseline* occurred during the first natural menstrual cycle and testing began

between days 2-7 of the cycle. Each participant completed an IGF-1 Generation Test,

started taking calcium (up to 1000mg/d) and vitamin D (800IU/d) supplements based on

estimated daily calcium intake (Brief Calcium Assessment Tool), completed an aerobic

fitness assessment (VO_{2peak}), a dual x-ray absorptiometry (DXA) scan to assess body

composition, weekly body weight measurements, a menstrual calendar, and a seven-day

exercise training log. During week three of baseline, participants desiring contraception were

randomized to either COC or CVR treatment groups. The *intervention* period began on the

first day of the second or third natural menstrual cycle following the baseline menstrual cycle

(due to scheduling the post-therapy testing). The intervention period was completed during

one natural menstrual cycle for the Control group or during 1.5 contraception cycles if

randomized to either COC or CVR groups. The duration of 1.5 contraception cycles was

necessary to 1) ensure a minimal yet adequate treatment period, 2) allow ample time to

schedule all follow-up testing, and 3) ensure post-therapy testing occurred while COC or

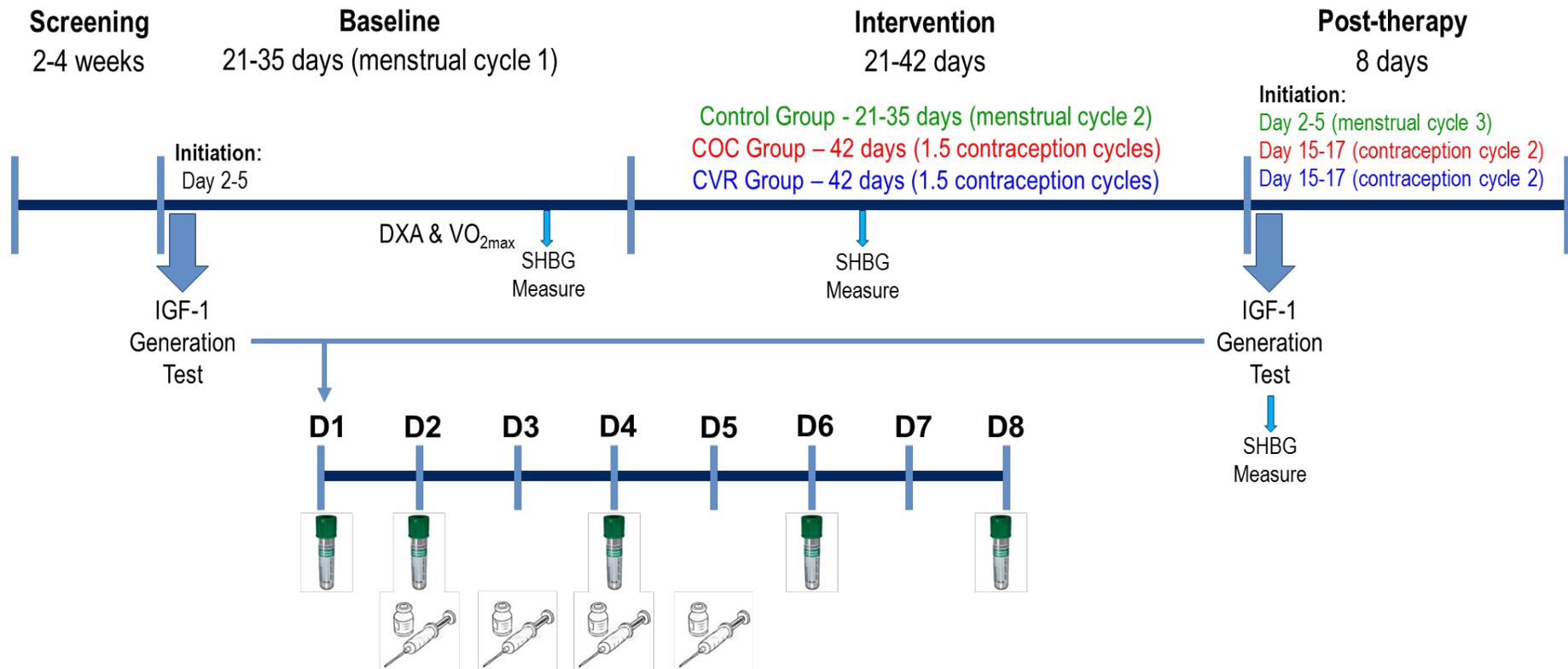**Figure 5.1:** Schematic of the study design. The study was comprised of a screening period followed by a Baseline period (1 menstrual cycle), Intervention period (1 menstrual cycle or 1.5 contraception cycles), and a Post-therapy period (8 days). Inset is a schematic of the 8 day protocol for the IGF-1 Generation Test, which occurred at the beginning of the Baseline period and during the Post-therapy period.

CVR therapy was not interrupted with a hormone-free interval. During the intervention period, body weight was measured weekly as participants continued taking calcium and vitamin D supplements, completed a seven-day exercise training log, and completed a menstrual/contraceptive therapy calendar. *Post-therapy* began between days 2-7 of the menstrual cycle following the intervention period menstrual cycle for the Control group or between days 15-17 of the second contraception cycle for the CHC groups. During post-therapy, participants completed an IGF-1 Generation Test, continued taking calcium and vitamin D supplements, continued taking CHC therapy (COC or CVR), completed a 7-day exercise log, and completed a menstrual/contraceptive therapy calendar.

Participant Grouping Categories: Participants not desiring hormonal contraception self-selected into the Control group (n=8). Participants desiring hormonal contraception were randomized to use either a monophasic COC (Reclipsen Actavis plc, Parsippany-Troy Hills, NJ; n=8) or CVR (NuvaRing[TM], Merck, Kenilworth, NJ; n=8) for two contraceptive cycles. The COC contained 30µg EE and 150µg desogestrel and the CVR contained 15µg EE and 120µg etonogestrel. The first contraception cycle of the intervention period for both COC and CVR participants was used as marketed (21 days of active hormones and a 7-day hormone-free interval) and was initiated on day 1 of the menstrual cycle. The second contraception cycle was initiated on the day immediately following the hormone-free interval. For the second contraception cycle participants did not have the hormone-free interval to ensure uninterrupted hormone exposure during the post-therapy testing. Participants were provided with additional COC pills or a CVR to allow for continued use of the COC or CVR until the end of the post-therapy IGF-1 Generation Test.

Insulin like Growth Factor Generation Test: Participants underwent a baseline and post-therapy modified IGF-1 Generation Test (inset **Figure 5.1**), with testing occurring before 0930 h for both tests. The protocol for the modified IGF-1 Generation Test was 8 days in duration, with the rhGH injections beginning on day 2 of the protocol. Four rhGH injections were given subcutaneously into the abdomen at a dose of 0.033mg/kg/day [39] using the

weight taken the morning of the first injection by Clinical Research Center personnel. The

baseline IGF-1 Generation Test was initiated between days 2-7 of the baseline menstrual

cycle. The post-therapy IGF-1 Generation Test was initiated between days 2-7 of the

menstrual cycle following the intervention period for the Control group or between days 15-

17 of the second contraception cycle of the COC and CVR groups. Participants were asked

to refrain from resistance exercise for the duration (8 days) of the IGF-1 Generation Test

and to not exercise in the morning before any blood draws.

On day 1 of the IGF-1 Generation Test participants had a fasting blood draw. On day

2, participants had a urine pregnancy test performed and body weight was measured to the

nearest 0.01kg. If the urine pregnancy test was negative, participants had a fasting blood

draw followed by rhGH injection 1 of 4 (Omnitrope Sandoz, Holzkirchen, Germany). On day

3, participants had rhGH injection 2 of 4. On day 4 participants had a fasting blood draw and

rhGH injection 3 of 4. On day 5 participants had rhGH injection 4 of 4. On days 6 and 8

participants had a fasting blood draws. No testing occurred on day 7. All blood draws were

antecubital for the purposes of evaluating IGF-1 concentrations.

Dual X-ray Absorptiometry: Participants had a DXA scan of the total body to assess body

composition at baseline. Measurements at the PSU site were performed using a GE Lunar

iDXA (enCORE 2008 software version 12.10.113). Measurements at the Purdue University

site were performed using a GE Lunar iDXA (enCORE version 15 SP1). The precision for

the PSU site iDXA body composition is 0.6% and 1.1% for lean and fat mass variables,

respectively.

Exercise Testing: Measurement of VO_{2peak} was performed during the study on a treadmill

using breath-by-breath indirect calorimetry [SensorMedics Vmax metabolic cart, Yorba

Linda, Ca (PSU) and (Parvomedics TrueOne metabolic cart, Sandy, UT (Purdue

University)]. Participants acclimated to the indirect calorimetry equipment for one minute

standing still, followed by two minutes at a walk (2.5mph), and two minutes at a jog (4-

5mph). The test then began at a self-selected treadmill speed until volitional exhaustion

(rating of perceived exertion ≥ 18). During the exercise test, the incline was increased 2.0% every 2 minutes until 6.0% followed by 1.0% every minute until volitional exhaustion. Peak VO_2 criteria included achieving a perceived exertion ≥ 18, age-predicted maximal heart rate (208-(0.7*age)), respiratory exchange ratio ≥ 1.1, and an observed plateau in oxygen consumption despite an increase in exercise workload [40].

Compliance: Participant compliance was monitored via assessment of daily menstrual calendars on which the participant monitored menstrual symptoms, side effects of the COC or CVR therapy (if randomized to CHC use), as well as time of COC pill ingestion or day of CVR insertion. Compliance was also monitored by way of returned COC and CVR packaging and a fasting blood samples taken to measures sex hormone binding globulin (SHBG), which is known to rise by a minimum of 40% from baseline with COC therapies not containing levonorgestrel [41, 42]. Samples to assess compliance were obtained between day 10-22 of the baseline period menstrual cycle, between days 13-21 of the intervention period menstrual cycle/contraception cycle, and between days 13-21 of the post-therapy contraception cycle. No post-therapy sample was taken in the Control group due to the post-therapy testing occurring during the follicular phase, not the luteal phase, for this study.

Hormone Assessment: All blood samples were allowed to clot for 45 minutes at room temperature (20-24ºC) and then centrifuged at 3000 rpm for 15 min at 4ºC. Serum was aliquoted into 2mL polyethylene storage tubes and stored frozen at -80ºC until analysis. Aliquots stored at Purdue University were shipped overnight on dry ice to PSU and stored frozen at -80ºC until analysis. Total IGF-1 was measured in duplicate using chemiluminescent immunometric assay (IDS-iSYS; Immunodiagnostic Systems Limited, Gaithersburg, MD). Analytical sensitivity for the total IGF-1 assay was 8.8ng/mL and the assay limits were 10-1200ng/mL. The intra-assay and inter-assay coefficients of variation were 0.97% and 0.8%, respectively. SHBG was analyzed using a chemiluminescence immunoassay analyzer (Immulite, Diagnostic Products Corporation, Los Angeles, CA) through competitive immune assay. Analytical sensitivity for the SHBG assay was 0.2nmol/L

and the assay upper limit was 180nmol/L. Samples measured above the assay upper limit were diluted and assayed again, with the reported concentration being the product of the dilution factor. The intra-assay coefficient of variation was 1.47%.

Statistical Analysis: Analyses were performed with SAS (version 9.4, Cary NC). Data were assessed for normality and outliers prior to analysis. No differences (P>0.190) in body composition variables were observed between study sites, therefore, body composition variables by study group were analyzed as both sites combined. Differences between study groups were assessed with Proc Mixed (age, gynecologic age, baseline and post-therapy IGF-1 Generation Test weight, average daily and total rhGH dose, VO_{2peak}, and body composition variables) or Proc NPar1Way (BMI). Basal IGF-1 concentrations for the baseline and post-therapy IGF-1 Generation Tests are the average of the day 1 and day 2 IGF-1 concentrations from each IGF-1 Generation Test. The IGF-1 response to rhGH stimulus during the IGF-1 Generation Test was calculated by subtracting basal (day 1 and 2 average) IGF-1 concentration from measured (day 4, 6, and 8) IGF-1 concentration. These values are referred to as "increment IGF-1." The increment IGF-1 concentrations were used to assess IGF-1 peak and AUC. The AUC was calculated for the baseline and post-therapy IGF-1 Generation tests using basal, increment day 4, increment day 6, and increment day 8 with Kaleidagraph Software (Synergy Software, Reading, PA, USA). Data with multiple observations per participant (basal IGF-1, peak increment IGF-1, IGF-1 AUC) were analyzed as repeated measures (study period) using Proc Mixed with Tukey-Kramer post hoc analyses. The model included the fixed effects of group, study period, and group*study period interaction. Repeated measures over time were modeled with unstructured covariance model. Denominator degrees of freedom were estimated with user of the Kenward-Rogers method. Change in basal IGF-1, peak increment IGF-1, and AUC of the increment IGF-1 were calculated by subtracting the baseline values from the post-therapy values. Change data were analyzed using Proc Mixed with Tukey-Kramer post hoc analysis or multiple student t-tests with Bonferroni adjustment. Compliance was assessed for each

study period (baseline, intervention, and post-therapy) independently utilizing Proc Mixed

with Tukey-Kramer post hoc analysis or Proc Npar1way. Data are presented as mean ±

standard error. Using sensitivity analysis, with 3 groups, 23 participants, 2 measurements

(baseline and post-therapy), an alpha=0.05, and adequate power (1-β=0.80) we are able to

detect an effect size of 0.35.

Results

Sixty participants signed informed consent and entered screening (**Figure 5.2**).

Thirty-four participants withdrew (n=22) or were found to meet study exclusion criteria during

screening (n=12). Twenty-six participants entered and completed baseline testing. Eight

Control participants entered the intervention period and 18 participants were randomized to

COC (n=9) or CVR (n=9). Three CVR participants experienced break-through bleeding, of

which one withdrew from the study and two determined the side effect was mild enough to

continue study participation. Eight Control and 8 CVR participants completed post-therapy

testing. Eight COC participants completed the post-therapy testing; one participant was lost

to follow-up on day 2 of the post-therapy testing. Analyses were performed on participants

who completed both baseline and post-therapy testing (COC=8, CVR=8, Control=8).

Participant demographics are shown in **Table 5.1**. Participants in the COC, CVR,

and Control groups did not differ in age or gynecologic age (p>0.120). BMI and weight

measured for the baseline and post-therapy IGF-1 Generation Tests were not different

among the COC, CVR, and Control groups (p>0.604). Participants in the COC, CVR, and

Control groups had similar VO_{2peak} values (p=0.682). Participants in the COC, CVR, and

Control groups had similar body fat percentage, lean mass, android/gynoid percent fat ratio,

trunk/leg percent fat ratio, fat mass index, lean mass index (p>0.506).

Daily IGF-1 concentrations during the IGF-1 Generation Tests were graphed as the

difference from basal IGF-1 concentrations for each group (Control, COC, and CVR) and

study period (baseline and post-therapy) (**Figure 5.3 A-C**). The average daily dose of rhGH
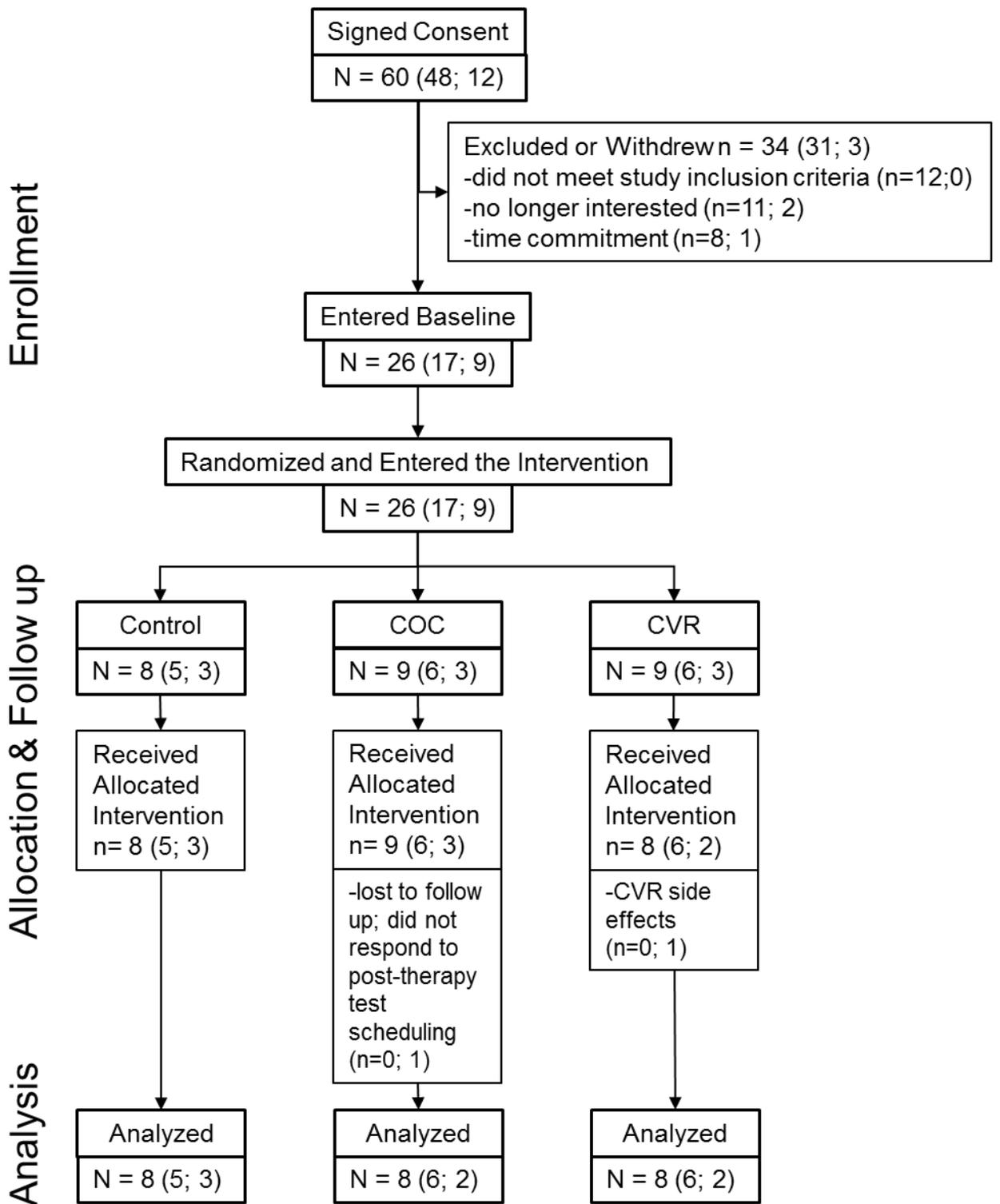
Figure 5.2: Progression of study participants through the pilot study.

Table 5.1: Participant demographic and body composition characteristics by study group.

	Control n=8	COC n=7	CVR n=8	P-value
Demographics				
Age (y)	23.6 ± 1.02	22.3 ± 1.28	23.1 ± 1.36	0.879
Gynecologic Age (y)	9.5 ± 1.20	9.9 ± 1.64	10.6 ± 1.63	0.812
BMI (kg/m^2)	21.9 ± 1.24	23.2 ± 1.26	622.5 ± 0.56	0.252
Baseline IGT Weight (kg)	58.73 ± 4.40	60.30 ± 2.32	59.53 ± 2.16	0.919
Post IGT Weight (kg)	58.92 ± 4.74	61.65 ± 2.38	59.46 ± 1.96	0.901
VO$_{2peak}$ (mL/kg/min)	44.55 ± 2.63	43.50 ± 3.11	46.28 ± 2.46	0.873
Body Composition				
Body Fat %	29.38 ± 2.89	32.34 ± 2.02	30.14 ± 1.43	0.472
Lean Mass (kg)	38.49 ± 2.03	37.87 ± 1.66	39.27 ± 1.35	0.824
Android/Gynoid % Fat Ratio	0.72 ± 0.08	0.81 ± 0.04	0.71 ± 0.07	0.563
Trunk/Leg % Fat Ratio	0.78 ± 0.08	0.85 ± 0.03	0.79 ± 0.06	0.589
Fat Mass Index (kg/m^2)	6.54 ± 1.01	7.52 ± 0.85	6.73 ± 0.44	0.471
Lean Mass Index (kg/m^2)	14.47 ± 0.40	14.50 ± 0.53	14.89 ± 0.30	0.779
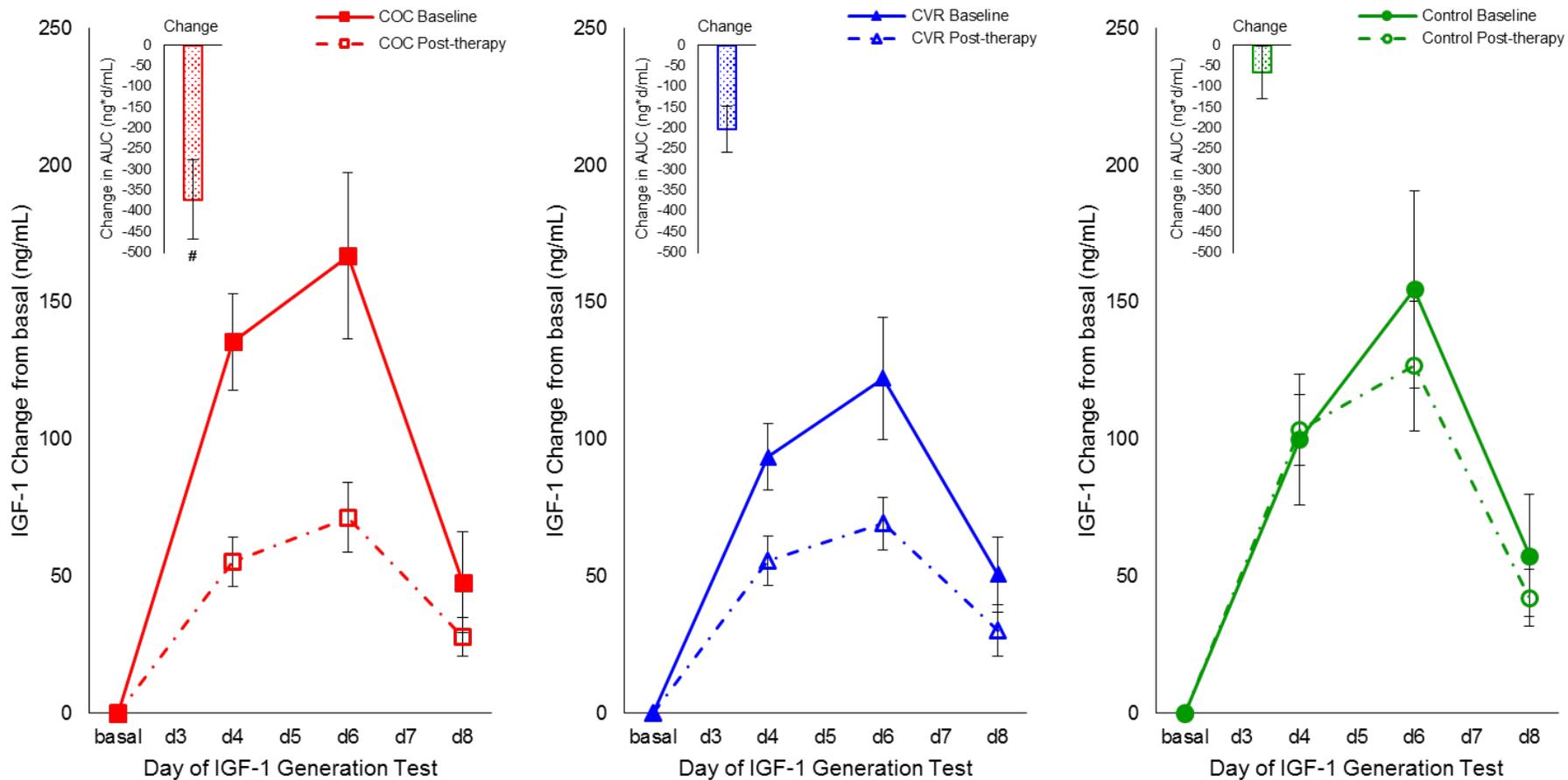
Figure 5.3: Daily concentrations of IGF-1 for baseline (solid lines and filled symbols) and post-therapy (dashed lines and open symbols) period IGF-1 Generation Tests. (A) COC group (squares); (B) CVR group (triangles); (C) Control group (circles). Concentrations for days 4, 6, and 8 were subtracted from the basal IGF-1 concentration (average of IGF-1 Generation Tests days 1 and 2) for each group and study period (baseline and post-therapy). Inset is area under the curve (AUC) for the change in IGF-1. [#] indicates difference from Control group $P<0.05$.

did not differ among the COC, CVR, and Control groups during the baseline (2.0±0.2 vs 2.0±0.1 vs 1.9±0.1mg/day, respectively; p=0.929) or post-therapy IGF-1 Generation Tests (2.0±0.2 vs 2.0±0.1 vs 1.9±0.1mg/day, respectively; p=0.904). The total rhGH dose for all participants during the IGF-1 Generation Test was 7.9±0.2mg (range: 5.9-11.2mg) at baseline and 7.8±0.2mg (range: 5.9-11.4mg) post-therapy.

There was no interaction (group*study period) effect (p=0.106) observed for basal IGF-1 concentrations (**Figure 5.4A**). Additionally, there was no group (COC vs. CVR vs. Control) effect (p=0.099) observed for basal IGF-1 concentrations; however, there was a study period (baseline vs. post-therapy) effect (p=0.014), such that basal IGF-1 concentrations were suppressed at post-therapy compared to baseline, regardless of study group. Multiple Students t-test comparisons (with Bonferroni correction for multiple comparisons) on the change scores from baseline to post-therapy revealed that basal IGF-1 concentration decreased significantly in the COC group compared to the Control group (**Figure 5.4A**; p=0.039); however, there was no difference between the CVR and Control groups (p=0.921). These findings indicate that basal IGF-1 concentration was decreased significantly post-therapy in the COC therapy group only.

There was an interaction (group*study period) effect (p=0.012) observed for peak increment IGF-1 concentration (i.e. difference between basal (day 1 and 2 average) and measured maximum IGF-1 concentration) during the IGF-1 Generation Tests (**Figure 5.4B**). There was a period (baseline vs. post-therapy) main effect (p<0.001) but no group (COC vs. CVR vs. Control) main effect (p=0.267) observed for peak increment IGF-1 concentrations. Post hoc analysis indicated peak increment IGF-1 concentrations were greater during the baseline compared to the post-therapy IGF-1 Generation Test for the COC group (p<0.001). There were no significant differences between baseline and post-therapy peak increment IGF-1 concentrations for the CVR or Control groups (p>0.080). No significant differences were observed among any of the groups at baseline (p>0.703) or post-therapy (p>0.118).
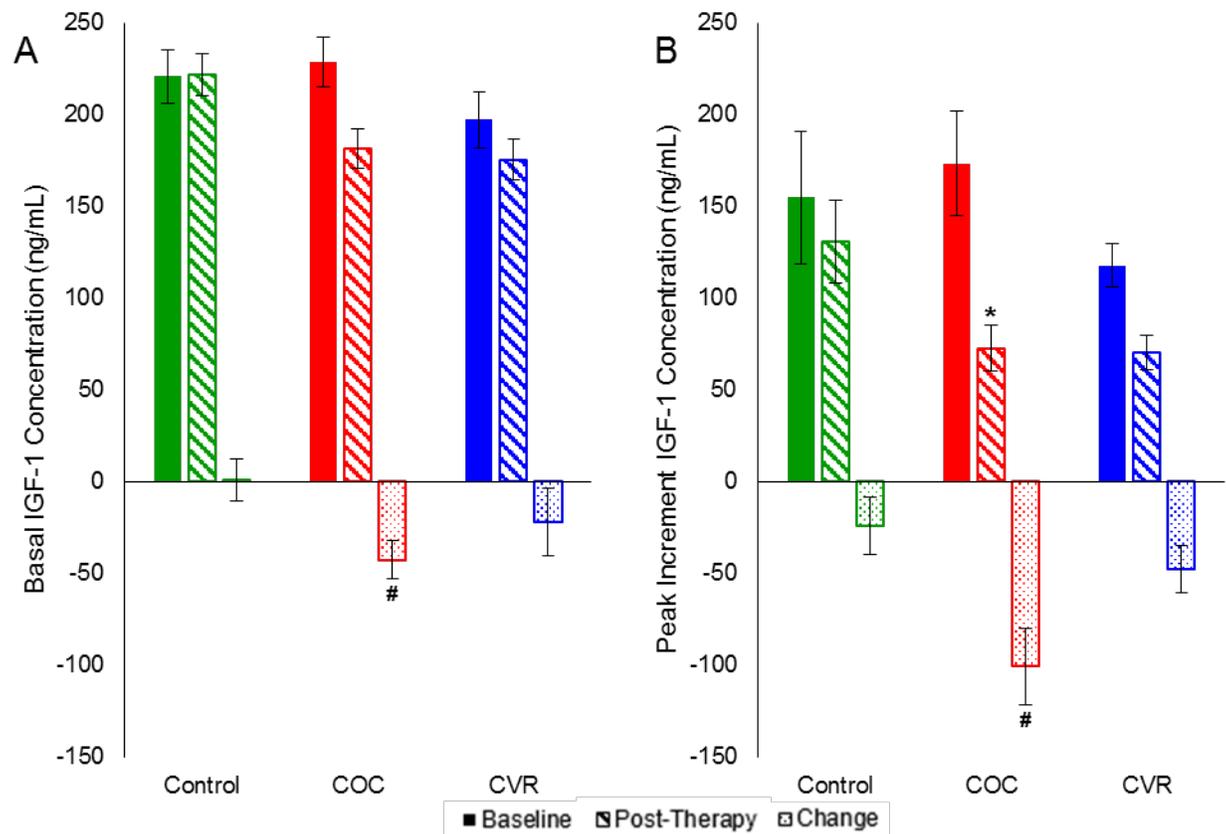
Figure 5.4: (A) Basal IGF-1 concentrations and (B) Peak increment IGF-1 concentrations for baseline (solid) and post-therapy (hashed) IGF-1 Generation Tests and absolute change (dotted) for the Control (green), COC (red), and CVR (blue) groups. Data presented as mean ± SEM.
* indicates difference within group baseline to post-therapy P<0.05.
# indicates difference from Control group P<0.05.

Change in peak increment IGF-1 concentrations were different among groups (**Figure 5.4B**; COC vs. CVR vs. Control; p=0.024). Post hoc analyses of the change scores (baseline to post-therapy) indicated that peak increment IGF-1 concentrations for the COC group were significantly decreased compared to the Control group (p=0.011), indicating that peak IGF-1 concentration was decreased post-therapy in the COC group. No significant decrease in peak increment IGF-1 was observed in the CVR group compared to the Control group (p=0.589).

There was an interaction (group*study period) effect (p=0.026) observed for IGF-1 AUC during the IGF-1 Generation Tests (**Figure 5.3 A-C**). There was also a main effect of study period (p<0.001) but no treatment group effect (p=0.408) observed for IGF-1 AUC.

Post hoc analyses indicated the AUC was reduced during the post-therapy test compared to the baseline test for the COC group (p=0.001). There were no significant differences in AUC between baseline and post-therapy IGF-1 Generation Tests for the CVR or Control groups (p>0.107). No significant differences were observed at baseline between each paired combination of the groups (p>0.869) or post-therapy between each paired combination of the groups (p>0.129). Change between baseline and post-therapy AUCs were different among groups (**Figure 5.3 A-C insets**; COC vs CVR vs Control; p=0.045). Post hoc analyses of the change scores (baseline to post-therapy) indicated the IGF-1 AUC for the COC group was significantly decreased compared to the Control group (p=0.020), indicating that rhGH-stimulated IGF-1 production post-therapy was reduced in the COC group. There were no significant differences between the CVR group and the Control group (p=0.399).

Figure 5.5 displays the compliance to the study protocol, based on SHBG concentrations. Baseline SHBG concentrations did not differ among the COC, CVR, and Control groups (p=0.949). During the intervention, SHBG concentrations were significantly different among the COC, CVR, and Control groups (p<0.001). Post hoc analyses indicated the Control group had significantly lower SHBG than the COC and CVR groups (p<0.001), while SHBG did not differ between the COC and CVR groups (p=0.714). At post-testing, the COC and CVR groups did not differ in SHBG concentrations (p=0.725).

Discussion

The present study provides valuable information about the impact of exogenous EE in CHCs, taken via oral and non-oral routes, on the GH/IGF-1 axis, since IGF-1 is anabolic to bone [22, 43] and has downstream effects on bone metabolism [44-46]. This is the first study to date that prospectively compared the influence of COC versus CVR on the GH/IGF-1 axis in healthy, young women using an IGF-1 Generation Test, resulting in two major and novel findings. Firstly, COC, not CVR, therapy reduced basal hepatic IGF-1 production. Secondly, the IGF-1 response to an exogenous rhGH stimulus, evaluated as AUC and peak

increment IGF-1 concentration, was suppressed following COC use but not following CVR use. While it is noteworthy that a simple examination of basal IGF-1 concentrations at baseline and post-therapy was sufficient to expose differing effects of COC vs CVR therapy, a more complete understanding about the shifts in the GH/IGF-1 axis dynamics following CHC therapy were highlighted through utilization of the endocrine probe of an IGF-1 Generation Test. These findings suggest that rhGH-stimulated hepatic synthesis of IGF-1 is affected by route of CHC administration, such that the oral route of administration (i.e. COC) resulted in a greater suppression of IGF-1 than did the vaginal route (i.e. CVR).
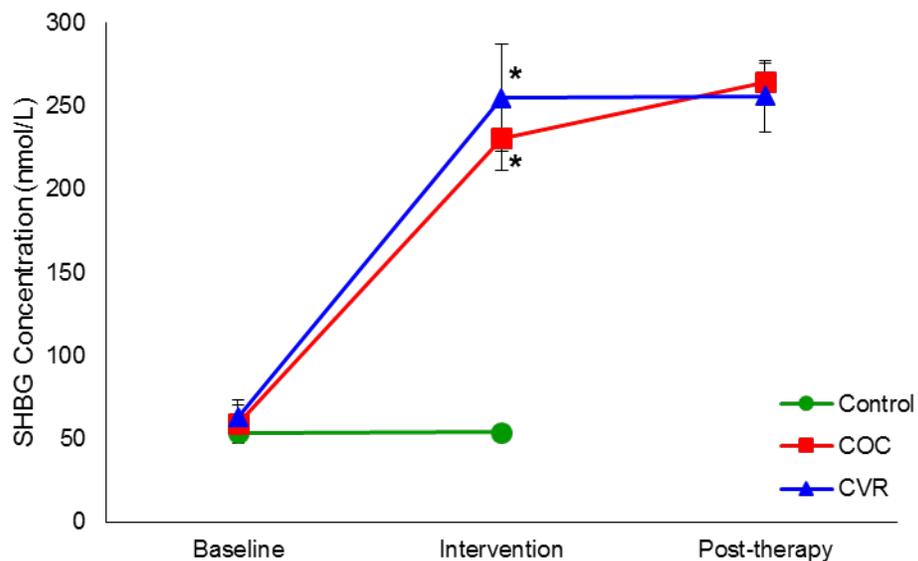
Figure 5.5: Compliance to study period and study group. SHBG concentrations for the control (green circle), COC (red square), and CVR (blue triangle) groups for baseline, intervention, and post-therapy compliance assessments.
* indicates difference from control group $p < 0.05$.

Our major finding that absolute reduction in basal IGF-1 concentration from baseline to post-therapy was significant only in the COC group while no changes were observed following non-oral therapy (CVR) are consistent with other prospective and cross-sectional reports [23, 24, 28, 47, 48]. In cross-sectional analyses of COC users, reductions of 11-30%

in basal IGF-1 concentrations were reported in current users of various doses of COCs (20-35µg EE) [23, 24, 47, 48]. Notably, a clear dose-response was observed with EE doses, such that the higher the EE dose (20-35µg), the lower the basal IGF-1 concentration (300-250ng/mL) that was observed [24], suggesting more profound detriments to bone health may be observed with increased EE dose. Conversely, in two cross-sectional studies, Elkazaz et al. [6] and Massa et al. [26] reported no difference in basal IGF-1 concentrations between current COC users and non-users. Conflicting results have also been demonstrated, in older (31-40 years old) women who initiated COC use after age 25, in whom IGF-1 concentrations were 28% higher than never-users [23]. On the other hand, Harel et al. [28] demonstrated no change in basal IGF-1 concentration in young women (12-21 years old) after 6 and 12 month of non-oral TDC therapy, which is consistent with our findings that the non-oral route of CHC fails to suppress basal IGF-1 concentrations.

An important and novel finding of this study is the observation of suppressed hepatic IGF-1 response to rhGH stimulation during a modified IGF-1 Generation Test following COC therapy, but not CVR therapy. Our finding of a 57% suppression of peak IGF-1 concentration in the COC group is similar to responses to COC therapy reported following an extended rhGH stimulation study (2 weeks at 1-3U/m^2 body surface) in premenopausal women. In non-COC users, peak IGF-1 concentrations following rhGH stimulation were 52% above basal IGF-1 concentrations, but only 16% above basal concentrations in women taking COCs (mono and triphasic; 20-35µgEE) [37]. Reports in postmenopausal women on oral estrogen therapy [34, 35] reveal additional parallels. For example, Lieberman et al. [34] first demonstrated a 58% suppression of peak IGF-1 response to a single rhGH injection (0.1mg/kg dose) in a cross-sectional study of postmenopausal women using and not using oral estrogen therapy. Similarly, in a 6 week crossover study of three different estrogen formulations (oral estradiol valerate (1mg/12hr), low-dose transdermal estradiol (50µg/d), and high-dose transdermal estradiol (200µg/d)), Lissett and Shalet [35] demonstrated peak IGF-1 concentrations following rhGH stimulation (7mg single bolus) were suppressed by

approximately 21% during the oral estrogen treatment period but only by 13% with the high-dose transdermal estrogen treatment. The peak IGF-1 concentration in the low-dose transdermal estrogen period did not differ from the pre-therapy test [35]. Our observations, in concert with reports in pre- and postmenopausal women, indicate that the use of oral estrogen therapies lead to reductions in hepatic capacity to respond to GH stimulation, a response that may be obviated by alternative route of estrogen administration.

Our findings of a reduced hepatic responsiveness to rhGH stimulation following short-term COC use likely has downstream implications for bone turnover, which could influence bone health in the long-term with continued COC use. Indeed, receptors for IGF-1 have been localized on multiple components of skeletal tissue, including fibroblasts [45] and osteoblasts [44], and IGF-1 has been demonstrated to directly stimulate collagen synthesis [46]. Thus, IGF-1 is widely regarded as bone anabolic and strong positive correlations have been reported between IGF-1 and biochemical markers of bone formation, including procollagen type I N-terminal propeptide (P1NP), osteocalcin, and bone specific alkaline phosphatase (BSAP) [49-51]. As such, we hypothesize that COC use may indirectly impact bone turnover through suppression of IGF-1 concentrations. Supporting this notion, decreases in osteocalcin and BSAP have been reported in COC users (20-35µg EE) compared to non-users [37, 52, 53], and this is in contrast to no observed change in osteocalcin following 12 months of TDC and CVR use compared to non-users [30].

Bone resorption must also be considered, as estrogen inhibits bone resorption [54] and exogenous estrogen administration through CHC alters the estrogenic, and thus the resorptive, environment. Several investigators have examined markers of bone formation (P1NP and osteocalcin) and markers of bone resorption (N- and C-terminal cross-linked telopeptides of collagen I (nTX and cTX)) in cross-sectional studies of users and nonusers of COCs. Consistently, suppression of markers of formation *and* resorption have been reported with COC use [52, 55-57]. Longitudinal studies strengthen these findings, demonstrating that initiation of COC therapy results in reduction of bone formation and

resorption markers [58, 59]. Notably, Polatti et al. [59] found that reductions in markers of bone resorption after 60 months of COC coincided with no changes in BMD, while nonusers experienced increased BMD over the 60-month study. Indeed, BMD is the clinical outcome typically of interest when assessing bone health, yet the links between CHC use, the GH/IGF axis, and BMD are not well elucidated. Elkazaz and Salama [6] examined a large cohort of women with varying histories of COC use and reported that, compared to past COC users, women who were current COC users had significantly lower circulating IGF-1 concentrations in concert with significantly lower hip, forearm, and spine BMD, findings which remained significant after adjusting for age and BMI. Interestingly, in past-users and never-users, IGF-1 concentration was correlated with BMD, but this correlation was not significant in current COC users [6], suggesting a dysregulation of the GH/IGF-1 axis and bone turnover with oral exogenous estrogen administration. Overall, these findings reveal an overall dampening of bone turnover with COC use, due to effects on both formation (presumably through the GH/IGF-1 axis) and bone resorption (through altered estrogen exposure), that may have detrimental consequences for BMD. Whether these detriments can be avoided with non-oral routes of CHC administration remains to be evaluated, but the findings of the present study are promising, as CVR therapy altered the GH/IGF-1 axis less than COC therapy.

These results provide data from which to develop additional studies to elucidate the impact of CHC use on various aspects of bone health and the mechanisms by which CHC use augments bone health in young women, a demographic where initiation of CHC use is common practice. Additionally, these results provide the data to explore the role of route, EE dose, and generation of progestin on the GH/IGF-1 axis. With respect to progestin, Balogh et al. [47] demonstrated a 30% reduction in basal IGF-1 following 21 day use of a 4th generation COC (30µg EE and 2000µg dienogest) compared to a 12% reduction with a 2nd generation COC (30µg EE and 125µg levonorgestrel). On the other hand, Harel et al. [28] did not observe differences in basal IGF-1 between TDC users (20µg EE and 150µg

norelgestromin, 3rd generation progestin) and non-users at 6 or 12 months of follow-up. Clearly, the complex interaction of route of administration, EE dose, and generation of progestin requires further inquiry, especially in light of the discontinuation of the TDC Ortho-Evra^{TM} (Janssen Pharmaceuticals, Raritan, NJ) in the United States, the FDA approval of a generic transdermal patch (Xulane^{TM}, Mylan Pharmaceuticals, Canonsburg, PA) in 2014, and with the current development of AG200-15 [60], which utilizes levonorgestrel and a lower EE dose compared to previously approved TDC options. The constant evolution of CHC therapy on the market requires a continued, vested interest in the impacts of new therapies on bone physiology and overall skeletal health.

In summary, our study provided insight into the effects of oral versus non-oral CHC therapy on the GH/IGF-1 axis using an IGF-1 Generation Test. Specifically, results demonstrated that basal hepatic IGF-1 production and the response to an rhGH stimulus, evaluated as AUC or peak increment IGF-1, was suppressed with COC use and not CVR use compared to the Control group. These findings likely have downstream consequences for bone turnover and, with long-term use, could influence BMD.

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CHAPTER 6: STUDY 4

Allaway HCM, Williams NI, Mallinson RJ, Wagstaff DA, Scheid JL, De Souza MJ. The impact of non-traditional DXA measures of body composition distribution on menstrual recovery of women with functional hypothalamic amenorrhea.

Abstract

In exercising women with functional hypothalamic amenorrhea (FHA) nutritional interventions aimed at increasing body weight should be the primary strategy for treatment to achieve resumption of menses (ROM). However, it is unclear how different components of body weight as well as the distribution of body fat influence ROM. Recently, novel measures of body composition and fat distribution that go beyond the traditional measures have been obtained using dual x-ray absorptiometry (DXA). The purpose of this study was to assess the extent to which changes in traditional and non-traditional measures of body composition predict ROM in exercising women with FHA. Exercising women between 18-35 years were recruited for a nutritional intervention. 31 of the recruited women presented with FHA and are included in the present analysis. Participants were grouped based on ROM, which was defined as occurrence of the first menstrual cycle of the parent intervention being less than 90 days in duration. 11 women were categorized as FHA-R and 20 women were categorized as FHA-NR. DXA assessments were conducted at baseline and across the parent intervention. The DXA assessment closest to the resumption menstrual cycle for participants who experienced ROM (FHA-R) and the final DXA of the parent study for participants who did not experience ROM (FHA-NR). Traditional [lean body mass (LBM), body fat percentage (BF%)] and non-traditional [fat mass index (FMI), lean mass index, trunk/leg percent fat ratio (T/L% ratio), trunk percent fat (T% fat), leg percent fat (L% fat)] were obtained from DXA scans, in addition to the traditional anthropometric measures of body weight and BMI, at baseline and post-study. The duration of time between baseline and post-study was 1-37 weeks for FHA-R women and 1-49 weeks for FHA-NR women. Independent Student t-tests were used to assess differences between FHA-R and FHA-NR

groups. Forward stepwise logistic regression was used to determine predictors of ROM (dichotomous variable). FHA-R and FHA-NR women did not differ in age, gynecologic age, height, weight, BMI, exercise volume, or nutrient intake at baseline ($p>0.080$). FHA-R women had greater BF%, FMI, T% fat and L% fat compared to FHA-NR women at baseline and post-study ($p<0.050$). Baseline and post-study BF%, FMI, T% fat and L% fat were positively correlated with ROM ($p<0.050$). In a logistic regression model of baseline and post-study BF%, FMI, T% fat, and L% fat measures, post-study BF% was the only significant predictor of ROM ($p=0.016$, $R^2=0.233$). In a logistic regression model of baseline and post-study non-traditional measures (FMI, LMI, T/L% ratio, T% fat, L% fat), post-study L% fat was the only significant predictor of ROM ($p=0.020$, $R^2=0.221$). We demonstrate that FHA-R and FHA-NR women did not differ with respect to weight, BMI, LBM (traditional measures) at baseline or post-study. FHA-R and FHA-NR women differed with respect to FMI, T% fat, and L% fat (non-traditional measures). Post-study BF% was the strongest predictor of ROM.

Introduction

Both pediatric and adult psychiatric guidelines highlight the importance of weight restoration and resumption of menses (ROM) in the treatment recommendations for female patients with anorexia nervosa and functional hypothalamic amenorrhea (FHA) [1, 2] because the ROM is an important indicator of recovery from anorexia nervosa [3-5]. The Female Athlete Triad Coalition Consensus Statement also notes that ROM (or menstrual recovery) is a top priority in the treatment of exercising women with FHA [6, 7]. Commonly used indicators of weight restoration include targeted ranges of expected or ideal body weight, body mass index (BMI) or BMI percentile, and body fat percentage.

Weight restoration, defined through measures of body weight and BMI, is the current anthropometric and clinically relevant measurement that best predicts functional recovery of the hypothalamic-pituitary-ovarian axis [8-18]. In a case-control study, Dueck et al. [13] reported that a 2.7kg increase in body weight in one athlete with FHA was accompanied by an increase in morning LH pulsatility such that it matched that of three ovulatory women. Some researchers have reported percent weight gain [8] or attainment of 90-95% of expected body weight to be associated with ROM [12, 15, 17, 19] in exercising women and anorexia nervosa patients with FHA. Additionally, BMI and BMI-percentiles are used as markers of weight restoration. In female anorexia nervosa patients, ROM has been associated with the attainment of a BMI greater than 18 kg/m^2 [16, 20-23] or between the 25th and 50th percentiles [11]. Falsetti et al. [24] reported a 24.6% increased probability of menstrual recovery in anorexia nervosa patients with FHA for each 1 kg/m2 increase in BMI. Similarly, Dempfle et al. [12] reported that the odds of persistent amenorrhea doubled for every 1kg/m^2 decrease in BMI. Though increases in BMI have been associated with menstrual recovery in adolescent and adult females, some investigators have reported no differences in BMI increases between those who do resume and who do not resume normal menstrual function [9, 10, 12, 14].

In a similar fashion body fat percentage has been found to be a significant predictor of menstrual recovery [14, 17, 25] compared to fat mass or lean body mass [25]. El Ghoch et al. [14] demonstrated that body fat percentage at inpatient discharge was the best independent predictor of ROM at 1 year of follow up, such that one unit change in body fat percentage increased the odds of ROM occurring by 14%. A relative sparing of body fat percentage has been associated with the preservation of menstrual function in extremely low weight women [26]. However, Golden et al. [15] and Arimura et al. [9] reported no significant difference in mean body fat percentage between anorexia nervosa patients who resumed and who did not resume menses, indicating a need for further exploration of the role of body composition in ROM.

Unfortunately, approximately 15% of patients who increase their body weight and maintain weight stability do not achieve menstrual recovery [27-29], which may indicate individual susceptibility to menstrual dysfunction, resistance to resumption [11, 15, 30], or may reflect a lack of complete resolution of body composition abnormalities (i.e., lower fat mass despite weight gain) [29]. An understanding of the distribution (upper or lower body) of weight gain may play a critical role in determining whether the ROM will occur with weight gain.

In 2013 the International Society for Clinical Densitometry (ISCD) published a position paper that discussed how researcher should use and report body composition variables [31]. Specifically, the ISCD recommended that researchers utilize the unique measures of body composition assessed by dual x-ray absorptiometry (DXA) in addition to the traditionally reported fat mass, body fat percentage, and lean body mass. The non-traditional variables that the ISCD proposed include android to gynoid fat ratio (A/G ratio), trunk to leg fat ratio (T/L ratio), fat mass index (FMI), and lean mass index (LMI) [31]. The non-traditional measures are based on the prevailing idea that the pattern of adipose distribution is more clinically relevant than is the total quantity of adipose tissue [32]. FMI and LMI are measures similar to BMI and are total body fat or lean mass in kg divided by

height in m^2. To date, no research group has published a study that used the newly recommended ISCD variables of body composition and menstrual recovery [31], further exploration of the pattern of body composition distribution among women with FHA who achieve ROM vs. those who do not achieve ROM may provide clinically relevant knowledge about key contributors to menstrual recovery.

Additionally, few researchers have published studies that address the question, Does the distribution of the weight gain, based on DXA measures of body composition (trunk fat mass), influence or predict menstrual recovery? Some investigators have reported trunk fat mass is associated with ROM [13] or preservation of menstrual function [26]. However, results have been inconsistent [14], indicating the need for further research. In adolescents and adults with anorexia nervosa increased trunk not extremity fat was associated with ROM [33, 34], and individuals with higher baseline cortisol gained greater trunk fat with weight gain [33-35]. Though ROM was correlated with increased trunk fat, greater abdominal fat has also been correlated with increased risk for metabolic and cardiovascular diseases [36-39] and reduced reproductive hormone exposure [40]. In To date, no researchers have published studies that address the impact of a change in body composition with weight gain or the distribution of body composition on the ROM in exercising women with FHA.

In conducting the present study, our objective was to assess the extent to which temporal changes in traditional and non-traditional measures of anthropometry and body composition predict ROM in exercising women with FHA. Additionally, we wanted to determine if traditional or non-traditional measures are better predictors of menstrual recovery in exercising women with FHA. The four *traditional* measures evaluated were weight, BMI, body fat percentage and LBM. The *non-traditional* measures evaluated were FMI, LMI, T/L ratio, trunk percent fat, and leg percent fat. We propose three hypotheses. First, at baseline, compared to women with FHA who do not experience menstrual recovery, women with FHA who experience menstrual recovery will not differ with respect to *traditional* or *non-traditional* measures of anthropometry and body composition. Second, at post-study,

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compared to women with FHA who do not experience menstrual recovery, women with FHA who experience menstrual recovery will have greater weight, BMI, body fat percentage, FMI, trunk percent fat, and leg percent fat and lower LBM, LMI, and T/L ratio. Third, measures of lower body fat (T/L ratio and leg percent fat) and total body fat (BMI, body fat percent, and FMI) will be stronger predictors of ROM than will other measures of anthropometry and body composition (weight, LBM, LMI, trunk percent fat).

Methods:

Experimental Design: A secondary analysis of a prospective, repeated measures parent study was used to determine the effect of changes in body composition (body weight, BMI, body fat percentage, lean body mass, T/L ratio, FMI, LMI) on resumption of menstrual function in exercising, young women who suffer from FHA. Specifically, the present analysis used a one-between, one-within-subject design: body composition of women with FHA who had ROM were compared women with FHA who did not have ROM.

The present analysis includes data from a randomized controlled trial (RCT), REFUEL, that was designed to assess the effects of 12 months of increased energy intake (20-40% above baseline energy expenditure requirements) on indices of menstrual status and bone health in women with exercise-associated menstrual disturbances (EAMD) that included FHA and oligomenorrhea (long and inconsistent menstrual cycles of 36-90 days). For the REFUEL study, the EAMD participants were randomized to one of two intervention groups: 1) an EAMD group that increased energy intake (EAMD+Cal), 2) an EAMD group that maintained their baseline energy intake (EAMD Control). The EAMD+Cal and EAMD Control participants were compared to exercising women assigned to an exercising control group who presented with current ovulatory menstrual cycles (Ov Control). Participants were recruited on a rolling basis over eight years and observed for 13 months (1 month baseline and 12 months intervention). All participants were "exercising", which was defined as participation in purposeful physical activity for a minimum of two hours/week. The present

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analysis includes 31 women who had a current menstrual status of FHA when they entered the screening phase of the parent study, which was confirmed by baseline reproductive hormone measurements. REFUEL was conducted at two sites, the University of Toronto (UT) and the Pennsylvania State University (PSU) from 2006 to 2014. The study protocol was approved by the Research Ethics Board at UT and the Institutional Review Board at PSU. All participants signed informed consent prior to initiating screening procedures.

The Parent Study "REFUEL"

Participants: Participants were young, adult women. Eligibility criteria for this study were: 1) aged 18 to 35 years, 2) good health as determined by a medical exam, 3) body mass index (BMI) 16 to 25 kg/m2, 4) no chronic illness (including hyperprolactinemia and thyroid disease), 5) currently participating in a minimum of two hours/week of purposeful exercise, 6) non-smoker, 7) not currently dieting, 8) not taking any hormonal therapy for at least six months, 9) no current clinical diagnosis of eating or psychiatric disorders, 10) not pregnant, lactating or planning to become pregnant, 11) no medication use that would alter metabolic or reproductive hormone concentrations, and 12) no other contraindications that would preclude participation in the study. Women who reported regular menstrual cycles during the six month period prior to enrollment and demonstrated an ovulatory menstrual cycle during the baseline phase of the parent study were eligible to participate in the OV Control group. Women who reported no menses in the three months prior to study enrollment or who reported six or fewer cycles in the 12 months prior to study enrollment, confirmed by an amenorrheic monitoring period or oligomenorrheic menstrual cycle during baseline, were eligible for the EAMD groups. Advertisements place on university campus bulletin boards and in community newspapers, in association with television and radio advertisements were used for the parent study recruitment.

Figure 6.1 displays the progression of participants through the parent study, the "REFUEL" RCT. Two hundred women signed an informed consent and were assessed for

eligibility of the parent study *screening period.* Fifty-eight participants were excluded or withdrew during screening; 142 participants remained and entered the *baseline period*. One hundred eighteen participants remained and entered the *intervention phase* after 24 participants did not complete the baseline period. Seventy-eight participants were randomized to one of two EAMD groups and 40 participants were assigned to the OV Control group; 36 participants were randomized to the EAMD Control group and 42 participants were randomized to the EAMD+Cal group. Seventy-one participants completed the first six months of the study (26 Ov Control, 19 EAMD Control, 26 EAMD+Cal); and 55 participants completed 9 to 13 months of the study (20 Ov Control, 16 EAMD Control, 19 EAMD+Cal). *The present analysis* used the data provided by 31 participants (12 EAMD Control, 19 EAMD+Cal) who entered the screening phase and reported no menses in the three months prior to study enrollment. The absence of menses was confirmed during a baseline amenorrheic monitoring period. Eighteen participants included in the present analysis completed a minimum of 6 months of the intervention.

Screening Procedures: Trained study staff assessed EAMD and OV Control participant demographic information during the screening period.

i. Anthropometric Assessment and Physical Exam: Total body weight was measured to the nearest 0.1 kg and height was measured to the nearest 1.0 cm. BMI was calculated as the body weight divided by height squared (kg/m^2). A physical exam was performed to determine health status and to rule out any physical signs or symptoms of polycystic ovarian syndrome (i.e., acne, hirsutism) or eating disorders.

ii. Fasting Serum Metabolic Panel - To rule out endocrine and metabolic disease, a fasting blood sample was obtained and analyzed for complete blood count, basic chemistry panel, and an endocrine panel (follicle stimulating hormone, luteinizing hormone, estradiol, prolactin, thyroid stimulating hormone, thyroxine, total and free testosterone, and dehydroepiandrosterone sulfate). Women with a high free androgen index (FAI), as

determined by a ratio of total testosterone to sex hormone binding globulin greater than 6.6, which indicated the upper cutoff of the 95% confidence interval for this sample of women, were excluded.

iii. Questionnaires – Participants completed questionnaires on medical and menstrual history, eating attitudes and behaviors [41, 42], exercise participation, bone health, and psychological health [43-46].

iv. Diet and Exercise Assessment - Participants completed a 3-day diet log for dietary energy intake assessment and a 7-day exercise log for purposeful exercise energy expenditure assessment. A registered dietician performed a structured interview with all participants to assess eating patterns, relationship with food, and food preferences in an effort to determine participants' willingness to comply with the study protocol. Additionally, usual dietary intake was used to determine if participants consumed the adequate intake of 1000-1300 mg/day of calcium and 400 IU/day of Vitamin D [47, 48].

v. Psychological Assessment - A research psychologist, with expertise in clinical eating disorders, conducted a semi-structured interview with each participant to rule out current clinical eating disorders and other psychiatric disorders.

vi. Dual X-Ray Absorptiometry - A dual-energy x-ray absorptiometry (DXA) scan was performed to assess body composition [body fat percentage (%BF), fat mass (FM, kg), lean body mass (LBM, kg), trunk, and leg percent fat] and bone mineral density (BMD) at the total body, lumbar spine, and dual femur.

Baseline Procedures: Baseline was initiated on a random day for EAMD participants and on day 1 of the menstrual cycle following completion of the screening phase. Baseline for EAMD participants was a 28-day monitoring period and for OV control participants was a single menstrual cycle. On day 1 of baseline all participants initiated calcium and vitamin D supplements, which were provided as necessary to attain the current recommendation for
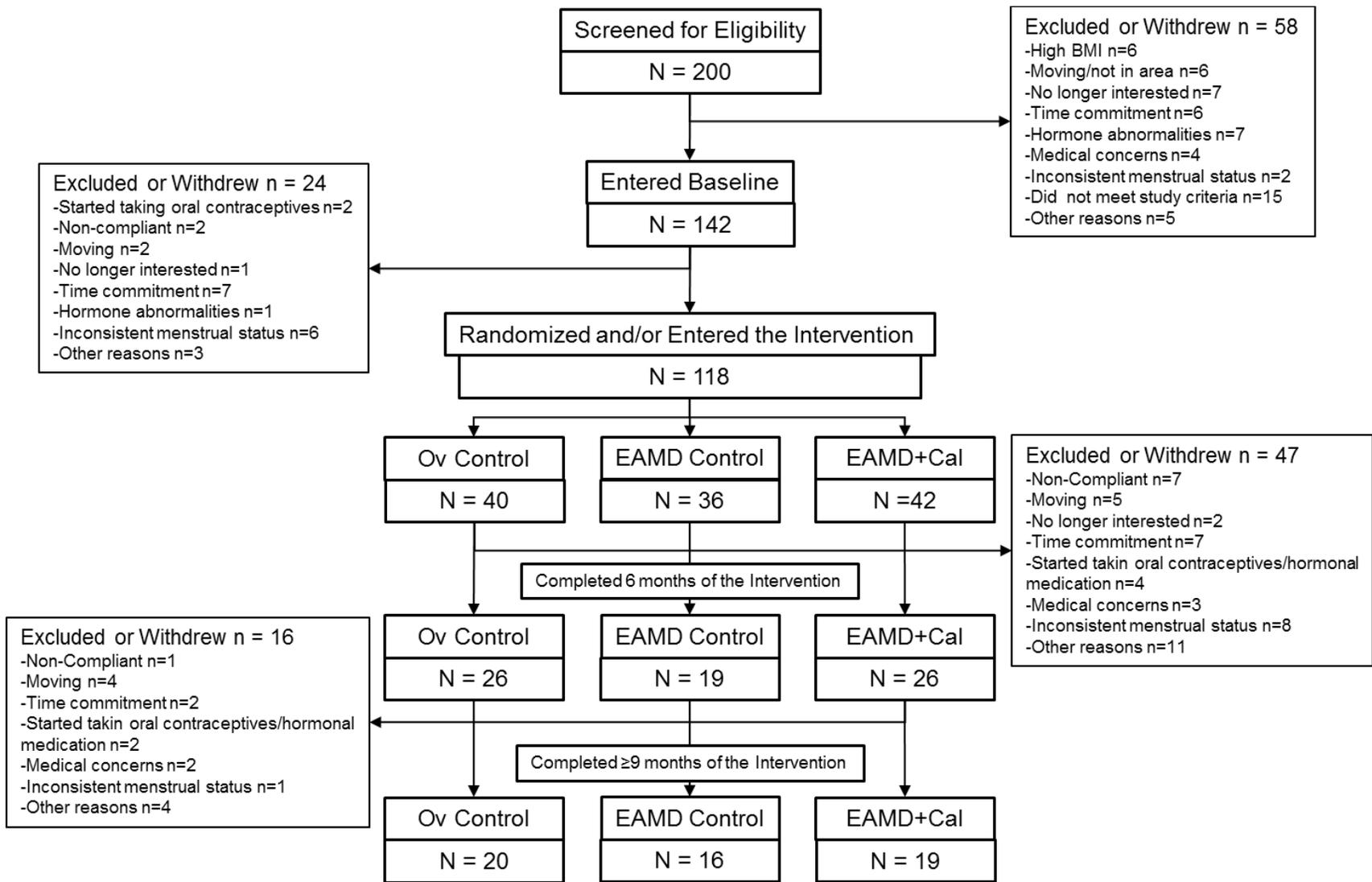
Figure 6.1: Progression of study participants through the parent randomized control trial.

adequate intake for calcium and vitamin D3 intake [47, 48]. Calcium and vitamin D3 were used as control measures similar to other studies of bone health [49-52].

i. Anthropometric Assessment – Total body weight was measured to the nearest 0.1 kg each week during baseline. Baseline values for weight and BMI were reported as the average of all baseline and screening measurements.

ii. Resting Energy Expenditure – REE was determined by indirect calorimetry using a ventilated hood system (SensorMedics Vmax Series, Yorba Linda, CA, USA) once during week 3 of baseline. Volunteers were instructed not to exercise or ingest caffeine within 24 hours, refrain from ingesting food and alcohol within 12 hours prior to testing, and arrive at the lab within 90 minutes of waking. Before conducting the REE analysis, weight (kg) was measured and recorded with the height and age recorded during screening. REE measurements were performed between 0630 and 1000 h in a lit room at a comfortable temperature setting (20-24C). After volunteers lay quietly for 45 minutes, a transparent canopy was placed over their head. Volunteers were instructed to lie flat on their back ad remain awake during the 30 minute measurement period. Oxygen consumption (VO2; mL/min) and carbon dioxide production (VCO2; mL/min) were measured every 20 seconds. Following the REE test the VO2 and VCO2 measures were assessed for steady state, which was achieve when the volume of expired air (VO2) and respiratory quotient values were not varying by more than 10% or 5%, respectively. Only steady state data was used to calculate with the Weir equation [53]: REE (kcal/day)=[3.94(VO2)+1.11(VCO2)]*1.44. REE was used to calculate baseline energy expenditure needs as explained below.

iii. Dietary Energy Intake – Energy intake (kcal/day) was assessed using three-day diet logs recorded for two week days and one weekend day during week 3 of baseline. On-site registered dietitians met with the participants to instruct them on how to accurately record energy intake on the 3-day diet log. Participants were specifically instructed to measure (using standard measuring cups/tools) and record all food and beverages consumed in detail. The nutrient data from the 3-day diet logs were coded and analyzed for total

kilocalories using the Nutrition Data System for Research (NDSR 2008 Version; University of Minnesota; Minneapolis, MN, USA). Daily kilocalories consumed over the 3-day recording period were averaged. Baseline values for energy intake are reported as the average of baseline and screening measurements. Three-day diet logs recording energy intake have been shown to provide comparable data to 7-day logs in women who may under-report their energy intake, including lean women [54]. Additionally, 3-day diet logs have been shown to reduce participant burden and improve compliance [55]. Participants with dietary habits that did not comply with the study protocol were excluded.

iv. Purposeful Exercise Energy Expenditure – Participants kept logs of the purposeful exercise each week of baseline and provided a measurement of exercise volume over a 7-day period (min/wk) [56]. Purposeful exercise energy expenditure (EEE) was measured at baseline using heart rate data. Energy expended during purposeful exercise sessions was measured using the OwnCal feature of the Polar S610 or RS400 heart rate monitors (Polar Electro Oy, Kempele, Finland), which has been validated for estimating EEE from heart rate [57]. The OwnCal feature includes rest in the estimation of energy expenditure, thus measured REE (kcal/min) was subtracted from the heart rate monitor readout to obtain EEE. For purposeful exercise session during which participants did not heat rate monitors, the Ainsworth et al. [58, 59] compendiums of physical activities were used to determine the appropriate metabolic equivalent (MET) level for the exercise performed [60]. To calculate EEE, the MET level was multiplied by the duration (min) of the session and the measured REE (kcal/min), and measured REE (kcal/min) was subtracted from this value to adjust for the fact that MET data includes REE.

v. Aerobic Exercise Capacity - VO_2peak (mL/kg/min) was measured during a progressive treadmill test to volitional exhaustion [61] using breath-by-breath indirect calorimetry (SensorMedics Vmax metabolic cart, Yorba Linda, Ca) on a single occasion during the baseline period. After a 3-5 minute warm-up, the VO_2peak test was initiated at a participant selected comfortable running speed at a 0.0% grade. Every 2 minutes the grade was

increased by 2.0% until reaching 6.0%, thereafter the incline was increased 1.0% every minute until volitional exhaustion. Peak VO_2 criteria included achieving a perceived exertion ≥ 18, age predicted maximal heart rate (208-(0.7*age)), respiratory exchange ratio ≥ 1.1, and an observed plateau in oxygen consumption despite an increase in exercise workload [62].

vi. Menstrual Status Assessment – Participants in the REFUEL study collected daily, first morning void urine samples for one 28-day monitoring period, which were assayed for estrone-1-glucuronide (E1G), pregnanediol glucuronide (PdG), and luteinizing hormone (LH). Participants recorded urine collection and/or menstrual symptoms daily on menstrual calendars. Initial classification of menstrual status prior to the intervention was based on self-reported menstrual history for the 12 months prior to study entry and urinary E1G, PdG, and LH profiles during the baseline monitoring period. Menstrual status for EAMD participants with FHA was defined as no reported menses for the past 3 months and evidence of suppressed concentrations of E1G and PdG at baseline. E1G and PdG exposures across the monitoring period were determined by calculating the area under the curve (AUC) using Kaleidagraph Software (Synergy Software, Reading. PA, USA). Mean E1G and PdG concentrations across the cycle were also calculated.

vii. Urinary Reproductive Hormone Measurement – Microtiter plate competitive enzyme immunoassays (EIA) were used to measure E1G and PdG, as previously described [63, 64]. The secretion of these metabolites of estrogen and progesterone in the urine parallels serum concentrations of the parent hormones [65]. Urinary concentrations of E1G and PdG were corrected for specific gravity, determined using a hand refractometer (NSG Precision Cells, Inc., Farmingdale, NY), to account for hydration status [66-68]. The inter-assay coefficients of variation for high and low internal controls for the E1G assay are 12.2% and 14.0% respectively. The PdG intra- and inter-assay variability for the PdG assay are 13.6% and 18.7% [63, 69]. For samples below or above the sensitivity of the assay, the low and

high sensitivity values were used to estimate E1G (3.9 and 250 ng/mL) and PdG (97.5 and 25,000 ng/mL) concentrations.

Intervention Procedures: Prior to the start of the intervention, EAMD participants were randomly assigned to either a treatment group (EAMD+Cal) or a control group (EAMD Control). Additionally, an OV Control group was followed for the same duration of time.

i. Energy Prescription – The EAMD+Cal participants were provided an energy prescription of increased energy intake 20-40% above their baseline energy requirements and asked to maintain their usual exercise training regimen for the intervention phase of the study. Baseline energy requirements were operationally defined as the sum of the laboratory measured REE and purposeful EEE. Participants in the EAMD+Cal group were requested to increase their energy intake through the use of nutritional and sports energy supplements or with foods they typically eat. Energy bars (primarily PowerBars and Clif Bars) that contained approximately 220-300 calories were provided to EAMD+Cal participants by the research staff and used as a strategy to increase energy intake throughout the day. Participants in the EAMD Control and OV control groups were asked to maintain their baseline physical activity level and energy intake.

ii. Nutritional Intake Monitoring – The EAMD+Cal participants met with a registered dietitian at the end of baseline and bi-weekly for the first 3 months of the study followed by monthly for the remainder of the study. The dietitian monitored the participants for compliance to the energy prescription through review of monthly 3-day diet logs (two week days and one weekend day) and in person interviews to monitor changes in nutritional and eating behavior characteristics. The dietitian also provided strategies to participants to achieve the prescribed energy intake. EAMD Control and Ov Control participants met with the registered dietitian monthly throughout the study.

iii. Psychological Status and Behavior Monitoring – The EAMD+Cal participants met with a research psychologist bi-weekly for the first three months and then monthly for the

remainder of the study to monitor general psychological and eating behavior status. The psychologist also provided assistance in implementing the energy prescription and other lifestyle changes to ensure compliance to the intervention. The participants in the EAMD Control and OV Control groups met with the research psychologist monthly throughout the study.

iv. Anthropometric Assessment – Participants body mass was assessed at the beginning of every 4 week interval during the intervention. BMI was calculated from the prospective body weights and the screening height measurement.

v. Dual X-Ray Absorptiometry – A dual-energy x-ray absorptiometry (DXA) scan was performed to assess body composition [percent body fat (%BF), fat mass (FM, kg), lean mass (LBM, kg), trunk, and leg percent fat] at intervention months 2, 3, 6, 9, and 12.

vi. Menstrual Status Assessment – Participants in this analysis collected daily, first morning void urine samples for consecutive blocks of 28-day monitoring periods for the entire intervention. Assessment of urinary reproductive hormones are described in baseline procedures. The menstrual calendars were used to monitor the occurrence of menstrual bleeding and symptoms throughout the study.

Analyses and Statistics

i. Resumption of Menses: Successful ROM for the 31 FHA participants included in the present analysis was defined as the occurrence of the first menstrual cycle of the parent intervention being less than 90 days in duration. Participants with FHA who had ROM (FHA-R) were compared women with FHA who did not have ROM (FHA-NR), irrespective of parent study randomized treatment group. Menstrual recovery was recorded as weeks into the intervention. When menstrual recovery occurred the data from the 28-day monitoring periods of daily urine samples the participants collected were aligned to the menstrual cycle based on self-reported menses after E1G and PdG assays were completed.

ii. Anthropometry and Body Composition Variables: Traditional anthropometry variables of interest are weight and BMI. Traditional body composition variables of interest are body fat percentage and lean body mass. Non-traditional body composition variables of interest are FMI, LMI, the T/L ratio, and trunk and leg fat percentage. The T/L ratio will be calculated as trunk percent fat/leg percent fat. The FMI and LMI variables will be calculated as fat mass in kg/height in m^2 and lean body mass in kg/height in m^2. Baseline variables are the anthropometric and body composition measures taken prior to randomization to the parent study intervention. Post-study variables for EAMD-NR are the anthropometric and body composition measures taken at the participants final study visit. Post-study variables for the EAMD-R participants are the study time point closest to observed ROM.

Participants from the University of Toronto cohort were scanned using a GE Lunar Prodigy (enCORE 2002 software version 6.50.069; n=8), early participants in The Pennsylvania State University cohort were scanned using a Hologic QDR45000W DXA scanner (Hologic Inc. Bedford MA; n=5), and later participants in the Pennsylvania State University cohort were scanned using a GE Lunar iDXA (enCORE 2008 software version 12.10.113; n=19). A cross calibration study was performed to remove systematic bias between the systems, consistent with the ISCD guidelines, and all systems were calibrated to the iDXA. Equations were derived using simple linear regression to remove biases.

iii. Statistical Analyses: Preliminary analyses were used to assess continuous demographic and body composition variables for normality and for specific indications of non-normality (i.e., the presence of outliers). We used Student's t-test to determine if the FHA-R women differed from the FHA-NR women with respect to the following baseline demographic variables: gynecologic age, height, BMI, fat mass, body fat percent, LBM, and VO_{2peak}. Wilcoxon Signed-Rank test was used to compare the two groups baseline demographic characteristics that exhibited marked skew: age, menarcheal age, weight, duration of amenorrhea, energy intake, and exercise volume. Group differences with respect to six continuous reproductive hormone characteristics were analyzed with independent and with

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paired Student t-tests with a Bonferroni adjustment. The latter was used to address the issue of multiple comparisons with a procedure that controls the familywise Type I error rate. The type I error rate for the individual comparisons was set at p=0.025 for cycle E1G AUC and mean, day 2-12 E1G AUC and mean, and cycle PdG AUC and mean (**Table 6.2**).

Change and percent change in nine anthropometric and body composition variables were calculated as change to resumption for FHA-R (i.e., the observed measurement at resumption minus the observed baseline measurement) and as change to post-study for FHA-NR (i.e., the observed measurement at post-study minus the observed baseline measurement). Student t-tests were used to assess differences in traditional and non-traditional anthropometry and body composition between FHA-R and FHA-NR at baseline, post-study, change, and percent change. Correlation analyses were performed to determine the associations among baseline, post-study, and among the change in continuous anthropometry measures with ROM (bivariate yes, no variable). Forward stepwise logistic regression (entry p=0.05, exit p=0.10) was used to determine predictors of ROM and odds ratios calculated from parameters of covariates. Predictors were chosen based on the literature as well as those that had significant correlation to ROM in our sample. Predictors were entered into three models: 1) all baseline and post-study anthropometric and body composition variables significantly correlated with ROM in our data set, 2) all baseline and post-study *traditional* anthropometric and body composition variables, and 3) all baseline and post-study *non-traditional* body composition variables. Last observation carried forward (LOCF) was used to address the missing post-study data of 5 participants (1 FHA-R, 4 FHA-NR women). Analyses were performed with SPSS for windows (version 23, Chicago, IL).

Results:

Demographics

We report findings based on 31 young, otherwise healthy women who completed the screening and baseline phase of the parent study characterized as having FHA. Resumption

of menses was observed in 11 of the participants (1 EAMD Control and 10 EAMD+Cal participants). **Table 6.1** presents the baseline demographic characteristics of the 31 participants. Age, age at menarche, gynecologic age, and duration of amenorrhea at study entry did not differ between FHA-R (n=11) and FHA-NR (n=20) participants (p>0.200). Moreover, the FHA-R women did not differ from the FHA-NR women with respect to height, weight, BMI, fat mass, or LBM (p>0.080). Baseline body fat percentage was significantly lower in FHA-NR participants compared to FHA-R participants (p=0.035). Finally, aerobic fitness (VO_{2peak}), exercise volume (min/week), and energy intake (kcal/day) at study entry were similar between FHA-R women and FHA-NR women (p>0.090).

Reproductive Hormone Measures

Figure 6.2 displays the baseline and post-study composite monitoring periods or menstrual cycle data for FHA-R and FHA-NR participants. **Table 6.2** presents summary statistics for participants' urinary reproductive hormone measures. The 11 FHA-R women did not differ from the 20 FHA-NR women with respect to cycle E1G (AUC and mean; p > 0.690), days 2-12 E1G (AUC and mean; p>0.880), or cycle PdG (AUC and mean; p>0.690) at baseline. The FHA-NR women had similar cycle E1G (AUC and mean) and day 2-12 E1G (AUC and mean) at baseline and post-study (p>0.230), but a decrease in cycle PdG (AUC and mean; p<0.020) from baseline to post-study monitoring period was observed. Resumption of menses occurred on average by week 13 (range: week 1 to 37). The average length of the first menstrual cycle that was experienced by the 11 FHA-R participants was 32 days (range: 16 to 48 days). Seven women experienced a eumenorrheic first menstrual cycle (resumption menstrual cycle; <36 days in length), and three women experienced an oligomenorrheic first menstrual cycle (36 to <90 days in length). Two women had an ovulatory first menstrual cycle, and eight participants had an anovulatory first menstrual cycle. The resumption menstrual cycle did not differ from the baseline monitoring period of FHA-R participants for cycle PdG (AUC and mean; p>0.320), cycle E1G (AUC and mean; p>0.041), and day 2-12 E1G (AUC and mean; p>0.300), after Bonferroni adjustment

Table 6.1: Baseline demographic characteristics of FHA participants who resumed and did not resume menstrual function.

	FHA-NR	FHA-R	
	n = 20	n = 11	**P-value**
Age	20.8±0.6	21.8±0.9	0.451
Age at Menarche	13.4±0.3	13.3±0.3	0.951
Gynecologic Age	7.4±0.7	8.5±0.9	0.332
Duration of Amenorrhea (days)	475±101	256±20	0.204
Height (cm)	167.0±1.3	166.9±1.9	0.977
Weight (kg)	54.86±1.89	57.02±2.21	0.502
BMI (kg/m^2)	19.6±0.6	20.4±0.5	0.382
Fat Mass (kg)	11.2±1.0	13.9±0.9	0.088
Body Fat %	20.2±1.3	24.5±1.1	**0.035**
Lean Body Mass (kg)	41.3±1.3	40.4±1.4	0.672
VO$_{2peak}$	52.8±2.5	46.2±2.9	0.102
Exercise Volume (min/week)	406±60	318±118	0.091
Energy Intake (kcal/d)	2124±284	1937±134	0.699

to control the familywise Type I error. The resumption menstrual cycle of FHA-R women differed in cycle E1G AUC (p=0.007) and cycle PdG AUC (p=0.023) from the post-study monitoring period of the FHA-NR participants. The resumption menstrual cycle of FHA-R women did not differ from the post-study monitoring period of FHA-NR participants for cycle E1G mean (p=0.032), day 2-12 E1G (AUC and mean; p>0.850), or cycle PdG mean (p=0.054), after Bonferroni adjustment to control the familywise Type I error.

Anthropometric and Body Composition Measures

Table 6.3 displays participant traditional anthropometric and body composition measures. There were no differences between the 11 FHA-R women and the 20 FHA-NR with respect to weight, BMI, or LBM at baseline (p>0.380), post-study (p>0.220), change (p>0.350) or percent change (p>0.500) from baseline to post-study. Although, the FHA-R women had a greater body fat percentage at baseline (p=0.035) and at post-study (p=0.024) than the FHA-NR women, the two groups did not differ with respect to change or percent change in any anthropometric or body composition variable from baseline to post-study (p>0.830).

A. FHA-NR Baseline Monitoring Period
n=20
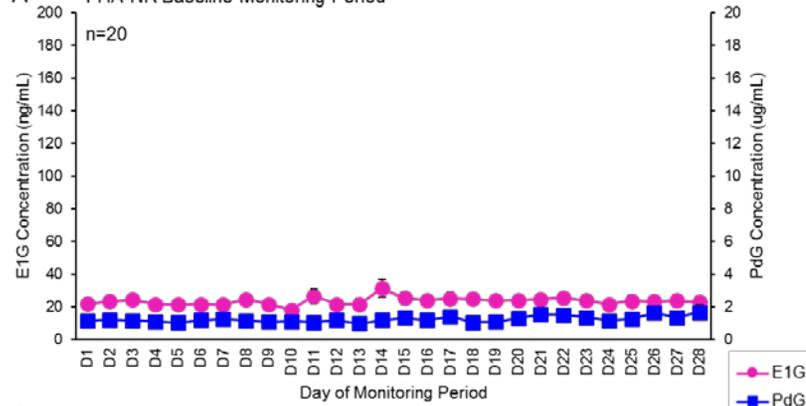
B. FHA-R Baseline Monitoring Period
n=11
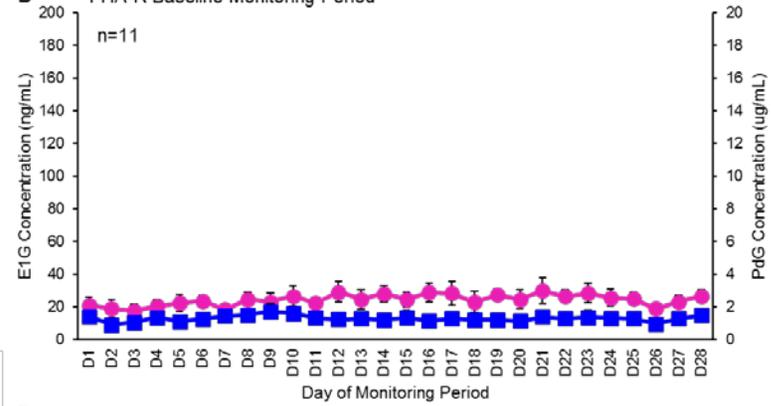
C. FHA-NR Post-Study Monitoring Period
n=20

D. FHA-R Resumption Menstrual Cycle
n=11

E1G
PdG
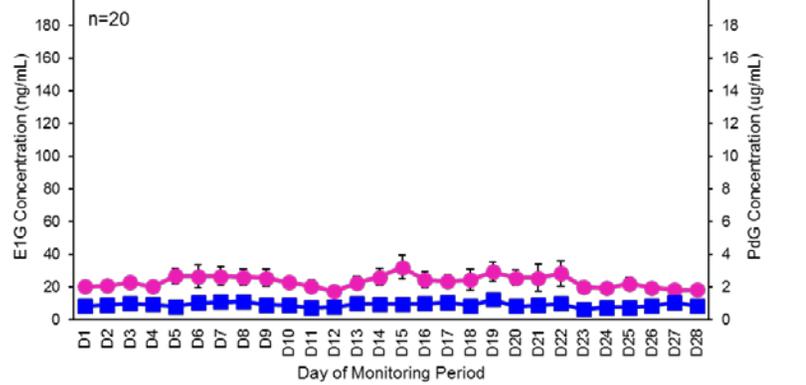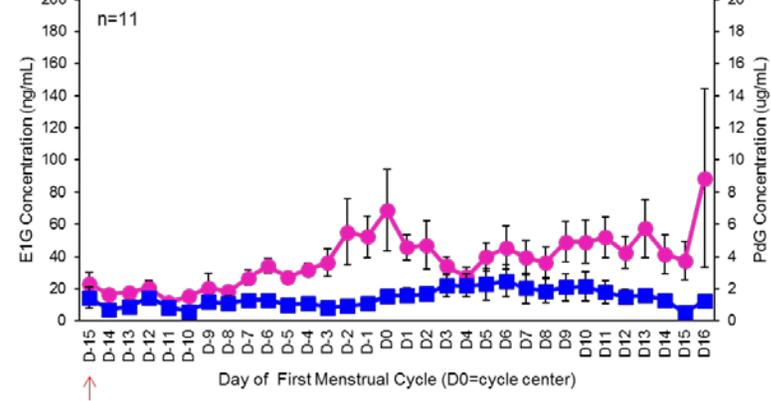
Figure 6.2: Composite monitoring period and menstrual cycle graphs of reproductive hormones, estrone-1-glucuronide (E1G) and pregnanediol glucuronide (PdG), in functional hypothalamic amenorrheic women who experienced resumption of menses (FHA-R) and did not experience resumption of menses (FHA-NR). (A) Composite baseline 28-day monitoring period graph featuring the reproductive hormone profile of the FHA-NR women. (B) Composite baseline 28-day monitoring period graph featuring the reproductive hormone profile of the FHA-R women. (C) Composite post-study 28-day monitoring period graph featuring the reproductive hormone profile of the FHA-NR women. (D) Composite menstrual graph featuring the reproductive hormone profiles of the resumption menstrual cycle of the FHA-R women. The chronic suppression of E1G and PdG are classic characteristics of the hormonal status in FHA.

Table 6.2: Reproductive hormone characteristics of FHA participants who resumed and did not resume menstrual function

	FHA-NR Baseline Monitoring Period n=20	FHA-NR Post-Study Monitoring Period n=20	FHA-R Baseline Monitoring Period n=11	FHA-R Resumption Menstrual Cycle n=11	P-value *	P-value **	P-value #	P-value ##
E1G AUC	623.84±60.67	652.74±95.43	664.31±83.69	1038.80±134.83	0.696	**0.007**	0.232	0.041
E1G Integrated Mean	23.07±2.24	24.87±3.73	24.59±3.10	34.66±3.67	0.693	0.032	0.313	0.110
E1G Day 2-12 AUC	222.43±21.47	232.77±33.83	225.86±27.25	185.66±33.34	0.923	0.366	0.809	0.330
E1G Day 2-12 Integrated Mean	22.26±2.12	23.10±3.23	22.74±2.59	18.82±3.12	0.890	0.389	0.852	0.302
PdG AUC	32.69±4.20	22.46±2.15	35.35±4.97	43.48±8.79	0.696	**0.023**	**0.003**	0.328
PdG Integrated Mean	1.22±0.16	0.93±0.10	1.32±0.19	1.38±0.22	0.697	0.054	**0.014**	0.722

Familywise Type I error rate adjusted for multiple t-tests by Bonferroni adjustment **p=0.025**

* Comparison between FHA-NR and FHA-R Baseline Monitoring Period

** Comparison between FHA-NR Post-study Monitoring Period and FHA-R Resumption Menstrual Cycle

Comparison between FHA-NR Baseline and Post-study Monitoring Periods.

Comparison between FHA-R Baseline Monitoring Period and Resumption Menstrual Cycle

Estrone-1-Glucuronide (E1G); Pregnanediol Glucuronide (PdG); Area Under the Curve (AUC)

Table 6.3: Differences in traditional anthropometrics and body composition between women with FHA who resumed menses versus those who did not resume menses.

	FHA-NR	FHA-R	
	n = 20	n = 11	P-value
Weight (kg)			
Baseline	54.86±1.89	57.02±2.21	0.502
Post-Study	55.88±1.85	58.61±1.97	0.352
Change	1.02±0.40	1.60±0.56	0.400
Percent Change	2.04±0.72	2.93±1.25	0.508
BMI (kg/m^2)			
Baseline	19.64±0.58	20.42±0.54	0.382
Post-Study	20.00±0.55	21.01±0.48	0.230
Change	0.36±0.14	0.59±0.19	0.353
Percent Change	2.06±0.71	2.94±1.25	0.513
Body Fat %			
Baseline	20.15±1.33	24.55±1.12	**0.035**
Post-Study	21.43±1.27	25.91±1.02	**0.024**
Change	1.27±0.62	1.36±0.52	0.921
Percent Change	6.76±2.45	7.80±4.85	0.832
Lean Body Mass (kg)			
Baseline	41.30±1.26	40.44±1.45	0.672
Post-Study	41.59±1.27	41.11±1.48	0.815
Change	0.29±0.34	0.67±0.23	0.445
Percent Change	0.91±0.83	1.42±0.52	0.668

Table 6.4 presents summary statistics for participant non-traditional body composition measures. There were no differences between FHA-R and FHA-NR participants for LMI or T/L ratio at baseline (p>0.220), post-study (p>0.090), change (p>0.440) or percent change (p>0.660) from baseline to post-study. Greater baseline and post-study FMI (p<0.040), trunk % fat (p<0.045), and leg % fat (p<0.045) were observed in FHA-R participants compared to FHA-NR participants. There were no differences in change or percent change from baseline to post-study between FHA-R and FHA-NR participants for FMI (p>0.480), trunk % fat (p>0.780), or leg % fat (p>0.840).

Table 6.5 displays the Pearson product-moment and Spearman rank-order correlations between ROM, measured as a dichotomous variable (yes/no), and four *traditional* and five *non-traditional* anthropometric and body composition variables. Resumption of menses was not correlated with baseline (p>0.020) or post-study (p>0.090)

weight, BMI, LBM, LMI, or T/L ratio. Moreover, change (p>0.325) and percent change (p>0.250) of weight, BMI, %BF, LBM, FMI, LMI, T/L ratio, trunk % fat, or leg % fat were not correlated with ROM. Resumption of menses was positively correlated with baseline body fat percent (r=0.381, p=0.035), FMI (r=0.430, p=0.016), trunk % fat (r=0.482, p=0.006), and leg % fat (r=0.366, p=0.043). Resumption of menses was also positively correlated with post-study body fat percent (r=0.404, p=0.024), FMI (r=0.381, p=0.035), trunk % fat (r=0.482, p=0.040), and leg % fat (r=0.383, p=0.033).

Table 6.4: Differences in non-traditional body composition between women with FHA who resumed menses versus those who did not resume menses.

	FHA-NR	FHA-R	
	n = 20	n = 11	**P-value**
Fat Mass Index (kg/m^2)			
Baseline	4.02±0.37	5.02±0.34	**0.018**
Post-Study	4.31±0.34	5.45±0.32	**0.035**
Change	0.29±0.13	0.44±0.14	0.488
Percent Change	9.07±3.02	11.82±6.11	0.654
Lean Mass Index (kg/m^2)			
Baseline	14.79±0.37	14.47±0.26	0.561
Post-Study	14.89±0.38	14.71±0.29	0.744
Change	0.12±0.12	0.24±0.08	0.442
Percent Change	0.90±0.83	1.42±0.52	0.667
Trunk/Leg % Fat Ratio			
Baseline	0.67±0.04	0.74±0.02	0.231
Post-Study	0.70±0.03	0.76±0.02	0.095
Change	0.03±0.02	0.02±0.01	0.841
Percent Change	4.98±1.88	5.14±5.07	0.972
Trunk % Fat			
Baseline	16.79±1.60	21.35±1.13	**0.007**
Post-Study	18.28±1.51	23.00±1.05	**0.040**
Change	1.49±0.76	1.64±0.68	0.893
Percent Change	10.57±3.47	12.72±8.47	0.784
Leg % Fat			
Baseline	24.52±1.39	28.96±1.26	**0.043**
Post-Study	25.70±1.33	30.16±1.16	**0.033**
Change	1.18±0.55	1.20±0.49	0.890
Percent Change	5.09±2.19	5.82±3.11	0.845

Table 6.5: Pearson product-moment and Spearman rank-order correlations between resumption of menses and traditional or non-traditional anthropometric and body composition variables at baseline, post-study, change and percent change.

	Resumption of Menses			Resumption of Menses	
	Rho	P-value		Rho	P-value
Traditional Variables			*Non-Traditional Variables*		
Body weight (kg)			Fat Mass Index (kg/m^2)		
Baseline**	0.128	0.492	Baseline**	**0.430**	**0.016**
Post-study	0.173	0.352	Post-study	**0.381**	**0.035**
Change	0.157	0.400	Change	0.157	0.399
Percent Change	0.204	0.271	Percent Change	0.053	0.778
BMI (kg/m^2)			Lean Mass Index (kg/m^2)		
Baseline	0.163	0.382	Baseline	-0.108	0.561
Post-study	0.222	0.230	Post-study	-0.061	0.744
Change	0.173	0.329	Change	0.088	0.640
Percent Change	0.211	0.253	Percent Change	0.080	0.667
Body Fat %			Trunk/Leg % Fat Ratio		
Baseline	**0.381**	**0.035**	Baseline	0.221	0.231
Post-study	**0.404**	**0.024**	Post-study**	0.309	0.090
Change	0.061	0.745	Change	0.044	0.814
Percent Change	0.040	0.832	Percent Change	0.007	0.972
Lean Body Mass (kg)			Trunk % Fat		
Baseline	-0.079	0.672	Baseline**	**0.482**	**0.006**
Post-study	-0.044	0.815	Post-study	**0.371**	**0.040**
Change	0.093	0.618	Change	0.080	0.670
Percent Change	0.080	0.668	Percent Change	0.051	0.784
			Leg % Fat		
			Baseline	**0.366**	**0.043**
			Post-study	**0.383**	**0.033**
			Change	0.053	0.775
			Percent Change	0.008	0.968

** Spearman rank-order Correlation

To determine whether traditional or non-traditional body composition measures collected at baseline and post study predicted ROM, a logistic regression model was fit to data from all 31 FHA women included in the present analysis. Of the variables that demonstrated a significant correlation with ROM (baseline and post-study body fat %, FMI, trunk % fat, and leg % fat), post-study body fat percentage was the only significant predictor of ROM (p=0.016, R^2=0.233, OR=1.226, 95%CI=1.00-1.49). Similarly, when all baseline and post-study *traditional* anthropometric and body composition measures (weight, BMI, body fat

%, LBM) were entered into a logistic regression model, post-study body fat percentage (p=0.016, R^2=0.233, OR=1.23, 95%CI=1.00-1.49) was the only significant predictor of ROM. Inclusion of baseline and post-study *non-traditional* body composition measured (FMI, LMI, T/L ratio, trunk % fat, leg % fat) for baseline and post-study in a logistic regression analysis, only post-study leg % fat was a significant predictor of ROM (p=0.020, R^2=0.221, OR=1.22, 95%CI=1.00-1.49). Change and percent change of traditional and non-traditional anthropometric and body composition variables did not significantly predict ROM.

Discussion:

Among exercising women with FHA, an increase in energy intake, without change in exercise volume, was well received and holds the potential to be an effective treatment strategy to improve menstrual dysfunction secondary to an energy deficiency. Relatively few studies about fat distribution in exercising women with FHA have been published. Additionally, the effects of weight gain on regional body composition and the subsequent impact on ROM have yet to be fully explored in this population. FHA-R participants entered the study with and maintained a greater body fat percentage, FMI, trunk percent fat, and leg percent fat than the FHA-NR participants. Though only 35% of our FHA participants experienced ROM during the intervention, the change in all anthropometric and body composition variables were the same for FHA-R and FHA-NR participants. As such, more time and more women are likely required to observe differences in women with subtle energy deficits and baseline body composition within the normal range. Among the factors assessed in this study, post-study body fat percentage was the strongest predictor of ROM; additionally, non-traditional variables of body composition (FMI, trunk % fat, and leg % fat) were positively correlated with menstrual resumption.

We hypothesized that FHA-R participants would not differ from FHA-NR participants with regard to anthropometric and body composition variables at baseline. Though we did not observe differences in weight, BMI, LBM, LMI, or T/L ratio between FHA-R and FHA-NR

participants, we did observe greater baseline body fat percentage, FMI, trunk percent fat, and leg percent fat in FHA-R participants compared to FHA-NR participants. This is partially consistent with the published literature of ROM in exercising women and female anorexia nervosa patients. Misra et al. [35] reported that baseline BMI, fat mass, body fat percentage, and trunk percent fat did not differ between adolescent anorexia nervosa patients who experienced ROM compared to patients who did not experience ROM while undergoing inpatient therapy. Arimura et al. [9] reported that in young adult women with anorexia nervosa who experienced ROM did not differ in weight, BMI, or body fat percentage at baseline from participants who did not experience ROM. However, this is in contrast to reports of significant differences in BMI [25] and body fat percentage [17, 25] in adolescent and young adult women with anorexia nervosa who experience ROM compared to those who do not experience ROM with treatment. Similar to our results Lagowska et al. [70] reported no baseline differences in weight or BMI in exercising women with EAMD who experienced ROM and those who did not experience ROM. Additionally, Lagowska et al. [70] observed that EAMD participants who experienced ROM entered the study with a higher body fat percentage than participants who did not experience ROM, which is similar to our observations in exercising women with FHA who experienced ROM.

Contrary to our hypothesis, we did not observe differences at post-study with respect to weight, BMI, LBM, LMI, or T/L ratio. We did observe that FHA-R participants continued to have greater body fat percentage, FMI, trunk percent fat and leg percent fat compared to FHA-NR participants. Our results support the findings of Lagowska et al. [70] who observed parallel increases in weight, BMI and body fat percentage in their study population, such that weight and BMI continued to be similar between groups and body fat percentage continued to be greater in participants who experienced ROM compared to those who did not experience ROM. Our results indicate that the FHA-NR participants may have been in a greater energy deficit than FHA-R. Indeed, 55% of the FHA-NR participants had been randomized to the EAMD Control group of the parent study and were asked to maintain

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baseline exercise and energy intake. The nine participants in the FHA-NR group who had been randomized to the EAMD+Cals group of the parent study likely needed a longer duration of time in the intervention to attain increases in body fat percentage, FMI, trunk percent fat, and leg percent fat comparable to the point at which FHA-R participants began baseline. The differences between baseline and post-study data observed in participants who experience ROM and those that do not experience ROM, in both the anorexia nervosa literature [9, 35] and the literature in exercising women with the female athlete triad [71], indicate that the severity of the energy deficit may play a role in changes in body composition.

Additionally, we did not observe any differences in change or percent change for any anthropometric or body composition variables between FHA-R and FHA-NR participants. This is in contrast to reports in adolescent anorexia nervosa patients, where patients who experience ROM have a significantly greater percent increases in BMI, LBM, fat mass, trunk percent fat, and trunk to extremity fat ratio [34, 35]. Similarly, weight and BMI change was different at 5-year follow up in amenorrheic college athletes who had ROM and those that did not have ROM [8]. Contrary to our hypotheses, we did not observe lower LBM or LMI in FHA-R participants compared to FHA-NR participants. Measures of LBM may more significantly predict improvements in energetic status than estrogen status. Indeed, LMI has been demonstrated to be an indicator of undernutrition in other populations [72-74], thus may be a better predictor of recovery of energetic status than as a predictor of recovery of menstrual function. Alterations in LBM and LMI should be evaluated in concert with measurements of energy balance, resting energy expenditure, and metabolic hormones triiodothyronine and insulin-like growth factor-1, which have been reported to be reflective of energetic status in exercising women with FHA and anorexia nervosa patients [75-78].

Though body fat percentage, FMI, trunk percent fat, and leg percent fat at baseline and post-study were positively correlated with ROM, within our logistic model with those specific variables, post-study body fat percentage was observed to be the only predictor of

ROM. Additionally, within a model of only non-traditional body composition variables, post-study leg percent fat was the only significant predictor of ROM. These findings are partially in support of Frisch and McArthur's [79] hypothesis that 22% body fat is necessary to restore and maintain menstrual cycles in women of ages 16 years and older. In the present analyses, the FHA-R women began the study with an average body fat percentage of 24%, while the FHA-NR women began the study with 20% body fat. Similarly, Lagowska et al. [70] demonstrated that exercising women with FHA who went on to achieve menstrual recovery initiated the study at a body fat percentage of 22% compared to 19% in those women who did not achieve menstrual recovery. Misra et al. [35] demonstrated in female adolescent anorexia nervosa patients that had between 18 and 24% body fat, some girls would achieve menstrual recovery while others would not, indicating that a body fat percentage value to initiate menstrual recovery is *not* a population-based threshold and is rather individual-dependent.

The etiology of FHA is multifactorial, thus the recovery from FHA will indeed be multifactorial, such that body composition, recovery from energy deficiency, and endocrine markers of energy stores and energetic status (leptin, insulin-like growth factor-1, triiodothyronine) will provide insights into ROM. However, further research will be necessary to determine which variables should be monitored clinically to predict ROM, as Misra et al. [35] demonstrated increases in body fat but not leptin predicted ROM in adolescent anorexia nervosa patients even with differences in serum leptin between those who resumed menses and those that did not resume menses. Additionally, Corr et al. [80] demonstrated no differences in circulating leptin between eumenorrheic and amenorrheic exercising, young women after adjusting for body fat percentage.

One limitation of this study is the liberal definition of menstrual resumption. Had we used a more strict definition of menstrual resumption such as 3 consecutive cycles of <36 days [35, 70], we may have observed differences in additional measures at baseline and post-study, and some differences in change or percent change in anthropometric and body

composition measures. Another limitation is the small sample size, which is why the liberal definition of menstrual resumption was chosen. Similarly, we utilized T/L ratio as discussed in the ISCD position paper [31] due to the reflection of fat distribution to the upper and lower body; however, literature in ROM in anorexia nervosa patients has used a trunk to extremity percent fat ratio [33, 34]. The extremity percent fat includes leg and arm fat. A further exploration of ROM in exercising women with FHA should explore the use of the trunk to extremity percent fat ratio in addition to T/L ratio, enabling comparison to anorexia nervosa literature, where the energy deficit causing the FHA is greater. One strength of this study is the use of daily urinary reproductive hormone assessment to evaluate reproductive status in conjunction with self-reported ROM. Self-report of menses can be used to categorize menstrual cycle length status; however, this method does not allow for the identification of subtle menstrual disturbances. In our sample, of the women whose first menstrual cycle was less than 90 days in length, seven had eumenorrheic cycles and three had oligomenorrheic cycles, which could be determined with self-report. What would not have been observed with self-report is that 80% of these cycles were anovulatory, indicating the need for further evaluation of these individuals to observe continued recovery.

In summary, the present analysis provides insight into the role of body composition in menstrual recovery in young, exercising women with FHA. Specifically, results demonstrated post-study body fat percentage was the strongest body composition predictor of ROM. Additionally, of the non-traditional body composition variables, post-study leg percent fat was the strongest predictor of ROM, while the T/L ratio was not correlated with ROM. These findings demonstrate the multifactorial etiology of FHA and the need to understand the complex interaction of neuroendocrine and localized metabolic signals.

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Chapter 7

General Discussion

The overarching purpose of this dissertation was to examine how endocrinologic and reproductive health in premenopausal women is influenced by combined hormonal contraceptive (CHC) use and changes in body composition. Additionally, methods of obtaining accurate information about reproductive hormone secretions were explored in female populations of varying age to improve assessments of reproductive health. Menses is the external sign of reproductive cyclicity in humans. From menarche to menopause the occurrence of menses at regular intervals is a critical vital sign [1] and is important in maintaining the endocrine environment for skeletal [2-4] and cardiovascular [5-7] health and reducing the risk of ovarian [8] and breast cancer [9, 10]. Young girls and women are a demographic in whom changes in body composition occur, which can coincide with alterations in lifestyle including the initiation of fitness or sport programs and CHC use. Involvement in regular physical activity is important for general health; however, many women who are physically active are at risk for developing menstrual dysfunction [11, 12]. The specific goals of this dissertation were to: (1) assess the average and individual agreement of daily and reduced sample collection frequencies (5, 3, or 2 samples/week) of estrone-1-glucuronide (E1G) and pregnanediol glucuronide (PdG) exposure (Study 1), (2) assess the compliance of young girls to prospectively collecting 2 or 3 urine samples/week for a menstrual cycle (Study 2), (3) understand the impact of CHC administration on hepatic insulin-like growth factor-1 (IGF-1) production (Study 3), and (4) determine if non-traditional dual x-ray absorptiometry (DXA) measures of body composition predict resumption of menses (ROM) in amenorrheic exercising women (Study 4).

Daily sample collection is the most accurate reflection of reproductive hormone exposure, especially when using urine analysis; however, balancing the accuracy of data with participant compliance, burden, and project costs plays a large role in determining study design. To date, there is little information [13] regarding the accuracy and precision of

calculated hormone exposure when less than daily sample collection frequency is used. We are the first to report that with perfect compliance to sample collection, reproductive hormone exposure, based on E1G and PdG area under the curve (AUC) and mean, is accurately quantified using reduced sample collection frequencies. We demonstrated that 5 samples/week (weekdays) had the best agreement with daily collection, while 3 (Monday, Wednesday, and Friday) and 2 (Monday and Thursday) samples/week collection frequencies had good agreement with daily sample collection. While our retrospective assessment of reduced sample collection demonstrated accurate quantification of hormone exposure, prospective data was unavailable to determine if compliance to the reduced sample collection frequencies would be better than compliance to daily collection.

Our second study is the first to prospectively assess compliance to reduced sample collection at frequencies that were previously demonstrated to provide adequate data on reproductive hormone status, while also assessing reproductive hormone exposure. We observed a reduced compliance to collection of 2 samples/week compared to collecting 3 samples/week. Additionally, we observe evidence of luteal activity in 32% of the prospectively collected cycles. Therefore, we were able to conclude that in the absence of perfect sample collection the frequency of 2 samples/week is not enough to provide adequate information regarding reproductive hormone metabolites to determine exposure or luteal activity. Collection of 3 sample /week occurs at time intervals that are seemingly more intuitive (Monday, Wednesday, Friday), which may have aided in the increased compliance observed.

In addition to there being little prospective information in the literature regarding reduced sample collection strategies, there is also conflicting or no information regarding the influence of route of CHC administration on hepatic IGF-1 production in young women [14-18]. We assessed total IGF-1 in the early follicular phase of a natural menstrual cycle and again following 1.5 cycles of combined oral contraceptive (COC) or contraceptive vaginal ring (CVR) use. Route of CHC administration influenced IGF-1 production such that basal

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IGF-1 concentrations and the response to exogenous growth hormone (GH) were suppressed following COC use but not following CVR use. The overall implications of our findings likely have downstream effects on bone turnover. With long-term COC use, a continued suppression of IGF-1 could influence bone health, since adequate IGF-1 concentrations are necessary for anabolic effects on bone during adolescence and young adulthood [19].

Though it is unclear if all types of exogenous estrogen administration can negatively impact bone, the effect of suppressed endogenous estrogen on bone has been clearly demonstrated [3, 4, 20-22]. Menstrual recovery by women with functional hypothalamic amenorrhea (FHA), whether secondary to anorexia nervosa [23, 24] or the Female Athlete Triad [25], is important to the overall recovery of the individual. Clinically, weight restoration has been positively associated with resumption of menses (ROM) in FHA populations [26-32]; however, approximately 15% of individuals who restore weight do not resume menses [28, 32-36]. This indicates that weight gain alone may not be enough for ROM, and perhaps the distribution of weight gain may be more important in assessing if menstrual recovery will occur. To this end, we investigated whether non-traditional measures of anthropometry based on DXA body composition scans [37] were better predictors of menstrual recovery than traditionally used measures of anthropometry. A greater number of non-traditional measures (fat mass index, trunk % fat, and leg % fat) were significantly positively correlated with ROM than traditional measures (body fat %) at baseline and post study; however, ROM was not significantly correlated with change or percent change of any one anthropometric or body composition variable. Of all baseline and post-study variables evaluated, post-study body fat percentage was the strongest significant predictor of ROM, while a separate regression of only non-traditional variables demonstrated leg percent fat to be a significant predictor of menstrual recovery.

Conclusions

Proper endocrine and reproductive health is of vital importance to young women, with perturbations in the endogenous hormonal profile leading to adverse long-term clinical outcomes (CVD, osteoporosis, cancer, etc.). Because of this, we sought to examine the effect of specific lifestyle considerations on the hormonal profiles of premenopausal women. The administration of CHCs and changes in body composition, which may or may not coincide with each other, are common occurrences in young women and are each known to have an impact on endocrine and reproductive health. While exogenous estrogen use can serve multiple purposes in this population, the route of administration should be carefully considered taking into account the newfound knowledge that COCs can pose a greater risk to future bone health due to their suppression of IGF-1, which is required for bone anabolic processes [19, 38]. With respect to body composition, weight gain is positively associated with ROM [27-29, 32, 39, 40], but not all those who gain weight will also resume menses [34, 35, 41] and achieve the optimal reproductive hormonal profile associated with eumenorrheic, ovulatory cycles. We found that, as opposed to total changes in weight, measurements regarding the composition and pattern of weight gain may be of greater utility when predicting ROM and these non-traditional measures of regional body composition require further investigation.

Additionally, in an attempt to balance the demands of accurate data collection with the logistical concerns associated with subject participation and study design we utilized both retrospective and prospective studies to determine the most appropriate sample collection frequency for the assessment of reproductive hormone exposure. Daily urine collection allows for the most accurate measurement of reproductive hormone exposure [42] but this frequency is not always appropriate or feasible as part of a research study. The finding that a sampling collection frequency of 3 samples/week (Monday, Wednesday, Friday) provides good tradeoff between accuracy and compliance is of great utility for future research in this area. Together, all of these findings contribute to a greater understanding of

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the details regarding alterations and assessment of the intricate endogenous endocrine profile of premenopausal women.

Future Directions

Although this dissertation has contributed novel findings regarding the endocrine and reproductive health of premenopausal women, this work should serve as a foundation for future research in the field. This work is the first to have identified individual differences in prospectively collected urine samples at a more than once weekly, reduced collection frequency. With simulated perfect compliance to reduced collection, 5 samples, 3 samples, and 2 samples per week collection frequencies provide accurate E1G and PdG profiles. From our retrospective analysis we demonstrated that 382 menstrual cycles/monitoring periods from 61 participants needed to be excluded from the analyses due to noncompliance to daily collection. In our prospective evaluation of 3 samples and 2 samples per week collection frequencies, adolescent participants collecting 3 samples per week were more compliant than those collecting 2 samples per week. Additionally, collection of urine with a frequency of 3 or 2 samples per week were able to provide overall details regarding cycle quality through evidence of luteal activity in 32% of cycles evaluated. Future studies are needed to prospectively evaluate the compliance to 5 samples per week collection, as well as to further evaluate the compliance to 3 samples per week collection frequency for longer study intervals and in additional population age ranges. Within studies to evaluate compliance to 5 and 3 samples per week, the clinical outcomes of luteal sufficiency and hormone exposure should be evaluated. Though there is the potential for increased compliance to collection by participants, if clinical outcomes are not observable, these collection patterns will not be useful as research strategies.

A subcomponent of our prospective evaluation of sample collection frequencies was the collection of monthly samples by premenarcheal girls. We demonstrated with this analysis that our E1G assay is sensitive enough to observe the low concentrations observed

in premenarcheal adolescents. This information is imperative in the development of further studies to evaluate the impact of changes in the timing of the pubertal transition on the maturation of the hypothalamic-pituitary-ovarian axis [1, 43]. Adolescence is a period of growth and maturation, where endocrine system maturation and puberty are occurring, which have significant impact on bone growth, body composition, psychological factors, and exercise status. Evaluating the changes in the timing of these events will require the recruitment of young premenarcheal girls into cohorts and following the cohorts through the first year of menarche. The methodology of a reduced sampling frequency would decrease costs and potentially increase compliance for a large longitudinal study aimed at understanding the menarcheal transition in the midst of changing global nutritional and general health conditions [44-48]. Reduced collection frequencies would be a great methodology to employ to evaluate resumption of menstrual function in longitudinal cohorts of young women with FHA since it may take 12 or more months in a non-pharmacologic intervention to achieve resumption of menses [27, 49].

In our adolescent study we also observed a lower than expected compliance to use of the paper menstrual calendar. The reduced compliance to the paper calendar methodology suggests the need for alternate modes of calendars to be considered. With continued technological development and increased technological literacy, the need for a change in menstrual cycle and sample collection monitoring with adolescent and young adult research populations may be necessary. It has been proposed that electronic menstrual cycle tracking applications may be useful tools for clinicians and researchers to educate young girls about what is normal for a menstrual cycle and when to seek medical advice for menstrual cycle abnormalities [1, 50]. Current availability of applications to track menstrual cycle length, menses duration, and symptoms are not focused on education of young girls and women about normal menstrual function, especially associated with the menarcheal transition, when multiple physiologic changes are occurring. Development of a research specific menstrual cycle tracker application would allow for multiple ways to

increase compliance including 1) automatic prompting for sample collection, 2) notifications to update tracker with menses and symptoms, and 3) ability for the research team to download the data without transcription errors. Development of a research focused menstrual cycle tracker app would allow for education, increased compliance, and reduced missing data in research that requires self-reported menstrual data as an endpoint.

In the current study, evaluating the impact of short term CHC use on markers of bone turnover is an important next step. Strong positive correlations have been reported between IGF-1 and biochemical markers of bone formation, including procollagen type I N-terminal and C-terminal propeptide (P1NP and P1CP), osteocalcin, and bone specific alkaline phosphatase (BSAP) [51-53], while an IGF-1 global knockout model suggests that IGF-1 is required for normal osteoclast differentiation [54]. COC use, therefore, may indirectly impact bone turnover through suppression of IGF-1 concentrations. Decreased serum (osteocalcin, BSAP, and P1CP) markers of bone formation have been reported in COC users (20-35μg EE) compared to non-users [14, 55-57]. Conflicting results have been reported with longer duration evaluation, such that no change in osteocalcin was observed over 9-12 months of COC use (15-30μg EE) [58, 59]. Similarly, longer term use of TDC and CVR demonstrated no differences in osteocalcin, while decreasing concentrations of urinary bone resorption markers [pyridinoline (PYD) and deoxypyridinoline (DPYR)] [16, 60]. Use of transdermal estradiol by adolescent anorexia nervosa patients with FHA for 12 months resulted in decreased c-terminal cross-linked telopeptide (CTx) and no change in P1NP [61]. Grinspoon et al. [55] reported short-term COC use decreased urinary markers [n-terminal cross-linked telopeptide (NTx) and DPYR] of bone resorption and increased osteoprotegrin, indicating reduced osteoclast function. Thus, alterations in bone turnover by CHC use may be affected by age, reproductive status, length of use, and route of administration. One of the most crucial steps for extending the work of this dissertation is exploring the impact of CHC use on bone health in adolescents and young adults through biomarker assessments and longitudinal (12-24 months) bone density and strength measurements.

Longitudinal data is important in skeletal health as it is a tissue known for a slow rate of change which varies depending on the proportion of trabecular and cortical bone at the site [62]. To have a full understanding of the association between CHC use and skeletal health the cohorts evaluated must be of varying BMI, reproductive, and exercise statuses. Misra et al. [61] demonstrated in adolescent anorexia nervosa patients with amenorrhea that 18 months of transdermal 17β estradiol use, with oral medroxyprogesterone for 10 days each month, lead to no differences in basal IGF-1 or P1NP, but observed a decrease in CTx, a marker of bone resorption, compared to controls. Spine and hip z-scores increased over 18 months in the transdermal estradiol treated group compared to the control group, even after controlling for baseline age and weight [61]. Though the estrogen component used by Misra et al. [61] was different than the conventional CHC estrogen, the impact on IGF-1 was not different than observed by Harel et al. [16], who utilized a transdermal contraceptive (TDC) with 20μg EE and 150μg norelgestromin. The complex interaction with route of administration is not only limited to the dose of the estrogen component, but also to the generation of the progestin which may play an additional role in the impact of CHC use on skeletal health. A 30% reduction in basal IGF-1 concentrations were observed following 21 days of use of a COC with a 4th generation progestin (dienogest) compared to a 12% reduction observed following use of a COC with a 2nd generation progestin (levonorgestrel) [63]. The complex interplay of route of administration, estrogen, and progestin are complicated by the development of a new TDC by Agile pharmaceuticals [64] that utilizes a 2nd generation progestin, in contrast to the 3rd generation progestin used in the generic TDC currently on the market and the currently approved CVR. Beyond route of administration and hormone dose, the interaction of exercise with skeletal health with CHC use is an important area to explore. This is in light of the observed decrease in spinal and femoral neck BMD following COC use and the initiation of resistance exercise combined with jump rope training [65]. Exercise is important since the variability of sport loading modality (including a

sedentary lifestyle) influences bone strength and geometry irrespective of CHC use and reproductive status [66, 67].

Longitudinal studies of CHC use are difficult to conduct for multiple reasons. First, randomization to CHC use or abstention, is associated with high levels of noncompliance and cross over between the study groups. Additionally, randomization to one CHC route, EE dose, or progestin for long periods of time may lead to noncompliance based on known side effects, such as break through bleeding and bloating, that may be a nuisance to some but not all participants. Similarly, participant desire to use CHC and comfort with route of administration may change with age altering compliance and study retention.

The definition of ROM is varied within the anorexia nervosa and Female Athlete Triad literature, such that a single spontaneous menses up to regular menstrual cycles for 6 months have been reported [26, 27, 29, 31, 49, 68]; however, oftentimes the requirement for menstrual recovery is not defined [28, 39, 69-73]. Whether the definition of ROM is strict or liberal the impact of nutritional interventions to resolve the energy deficit leading to the first menses will likely follow a similar trajectory in women with FHA. The etiology of ROM is likely multifactorial requiring alterations in body composition, recovery from an energy deficiency, and restoration of endocrine markers that were suppressed (leptin, IGF-1, triiodothyronine) or elevated (cortisol, ghrelin, peptide YY) with FHA. Four important endocrine markers of energy deficiency that should be evaluated in association with ROM are leptin, IGF-1, triiodothyronine (T_3), and cortisol to determine which endocrine markers should be monitored clinically to predict ROM. Though differences in serum leptin exist between anorexia nervosa patients who experience ROM and those who do not experience ROM, Misra et al. [72] has demonstrated that increases in body fat, not leptin, predict ROM in adolescent anorexia nervosa patients. Additionally, circulating leptin levels were not able to distinguish between eumenorrheic and amenorrheic exercising, young women after adjustment for body fat percentage [74]. In a case-study of recovery from short and long term amenorrhea, Mallinson et al. [68] documented increases in leptin, T_3, and resting

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energy expenditure, while ghrelin decreased. Interestingly, ROM in anorexia nervosa adolescents and adults is associated with increased trunk fat not extremity fat [75, 76], and individuals with higher baseline cortisol gained greater trunk fat with weight gain [72, 75, 76]. Determining the best combination of clinical measures to track in patients with FHA to predict complex ROM is necessary as interplay between body composition and energetic signals is complex.

An important extension of ROM is the understanding of recovery of ovarian function. In an anorexia nervosa population weight gain is a prerequisite for the initiation and restabilization of menses frequency; however, the precise amount, tissue type, and distribution of weight gain is still unclear. Additionally, the temporal association between increased energy intake, weight gain, and ROM remains unclear. The utilization of daily urine collection for reproductive hormone evaluation does provide some information regarding the temporal relationship between increased energy intake, weight gain, and ROM [68] but the hormonal measure is limited and does not provide detailed information regarding follicular development, corpus luteum function, or endometrial function. There is a need to follow nutritional intervention patients prospectively using endovaginal ultrasound to understand the temporal link between increased energy intake and changes in follicle development, endometrial development, and corpus luteum function in young adult women. Endovaginal ultrasound is a highly sensitive clinical and research tool that can show small antral follicles, follicle growth [77], endometrial development [78], and corpus luteum formation (if ovulation was to occur) [79]. Pelvic ultrasound has been used to assess ovarian and uterine maturity, through volume measures, in adolescent anorexia nervosa patients [80-82]. These reports in adolescents, where pelvic not endovaginal ultrasound is the standard of care, were single time point scans or pre- and post-intervention scans and not serial scanning across an intervention. Additionally, with the advancement in ultrasound technology and better imaging quality obtained with endovaginal ultrasound, a more comprehensive understanding of the relationship between energy intake and ovarian

function in young adult women would be developed. The association between changes in circulating leptin and IGF-1 observed with increased energy intake will be highly correlated with follicle growth due to the presence of leptin and IGF-1 receptors on the ovary [83-86]. Development of a further understanding of the coordinated response of the hypothalamic-pituitary-ovarian axis to metabolic fuel availability will enable the more complete understanding of whether the distribution of body composition gain impacts ROM.

Expanding upon our observed results of body fat associations with ROM, measures of LBM may be better predictors of recovery of energetic status rather than as predictors of ROM. Specifically, low LMI is an indicator of undernutrition in other populations [87-89], thus increases in LMI may be a better predictor of recovery of energetic status. To that end, alterations in LBM and LMI should be evaluated in association with measures of energy balance, resting energy expenditure, and measures of hormones suppressed with energy deficiency, such as T_3 and IGF-1 [90-93]. Gains in LBM or LMI will likely reflect an energy replete status and will be associated with increases in T_3 and IGF-1. Additionally, LBM and IGF-1 are strongly correlated with bone mass and geometry [19, 20, 94-97], thus recovery of both lean mass and fat mass with ROM is imperative for overall health.

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Selected Peer-reviewed Publications (out of 8)

Koehler K, Williams NI, Mallinson RJ, Southmayd E, **Allaway HCM**, De Souza MJ. Low resting metabolic rate in exercise-associated amenorrhea is not due to a reduced proportion of energetically expensive tissue compartments. *American Journal of Physiology Endocrinology and Metabolism.* 2016, 311(2): E480-487.

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