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# Association of Dietary Factors with Menstrual Cycle Characteristics

Radhika Natarajan
*University of Massachusetts - Amherst*

# ASSOCIATION OF DIETARY FACTORS WITH MENSTRUAL CYCLE CHARACTERISTICS

A Dissertation Presented

by

RADHIKA BALAJI PRABHU

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# ASSOCIATION OF DIETARY FACTORS WITH MENSTRUAL CYCLE CHARACTERISTICS IN YOUNG WOMEN

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Approved as to style and content by:

_____
Alayne Ronnenberg, Chair

_____
Elizabeth Bertone-Johnson, Member

_____
Nancy Cohen, Member

_____
Carol Bigelow, Member

_____
Nancy Cohen, Department Head
Department of Nutrition

## DEDICATION

To my wonderful husband Prabhu, my beautiful children Sneha and Samir. Your constant love and support is the reason I got here. To my dearest mom, who I know would be so proud of me. To my dad, who has always believed in me through this journey.

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## ABSTRACT

## ASSOCIATION OF DIETARY FACTORS WITH MENSTRUAL CYCLE CHARACTERISTICS IN YOUNG WOMEN

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RADHIKA BALAJI PRABHU, B.S., SNDT UNIVERSITY

M.S., UNIVERSITY OF MASSACHUSETTS

Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Dr. Alayne Ronnenberg

Abnormal menstrual cycle length, pattern and bleed duration are associated with reduced fecundity and increased risk of miscarriage. The menstrual cycle is governed by the hypothalamus-pituitary-ovarian (HPO) axis, and nutritional factors may affect menstrual characteristics by influencing the HPO axis. Identifying these factors may lead to cost effective ways to improve reproductive outcomes.

In a cross-sectional analysis of 164 18- to 30-year-old women, we examined the association of adiposity, dietary fat intake and vitamin D status with menstrual cycle characteristics. Adiposity was measured using dual-energy X-ray absorptiometry (DXA); dietary intake was assessed using a food frequency questionnaire, and fasting venous samples were collected for measurement of vitamin D.

Most study participants were Caucasians (83%). Approximately 20% of women were overweight (BMI 25-29.9 kg/m$^2$); 6% were obese (BMI $\geq$ 30 kg/m$^2$). However, when using DXA measurements, 45% of women had a percent total body fat (%TBF) > 32, indicating obesity. The odds of having an irregular cycle pattern were three times higher among women with < 32% TBF compared to those with more adiposity [OR=3.1

95% CI (0.9 to 10.2) p=0.07]. When considering body fat distribution, the odds of an irregular cycle were 2.8 times higher in women with ≤ 41% gynoid fat [OR=2.8 95% CI(0.9, 8.6) p=0.07]; a one standard deviation increase in G/A (gynoid/android) fat mass was associated with a 50% decreased odds of an irregular cycle pattern [OR=0.45 95% CI (0.21, 1.1) p=0.07]. Among dietary factors, higher intake of n-6 PUFA was associated with irregular cycles (β= 0.16, p=0.05) and both short (β= 0.23, p<0.01) and long cycle length (β= 0.15, p=0.06). Furthermore, the odds of having sufficient vitamin D status (serum 25-hydroxyvitamin D > 50nmol/L) was 80% lower among women with irregular cycles compared to those with regular cycles (OR= 0.19; 95% CI: 0.04, 0.92). Our findings suggest that dietary factors are associated with menstrual cycle characteristics, particularly cycle regularity. If our findings are confirmed in larger prospective studies, they suggest that dietary manipulation may be one approach to improving menstrual cycle function.

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# CHAPTER 1

# INTRODUCTION

The onset of the menses signals the start of a woman's reproductive life. The menstrual cycle involves a complex interplay of steroid hormones regulated by the hypothalamus-pituitary-ovarian (HPO) axis (Mihm, Gangooly, & Muttukrishna, 2011; Owen, 1975; Speroff, 1999). Menstrual cycle characteristics of length and regularity vary considerably within women at various ages. Menstrual cycle length is long and irregular a few years after menarche, and similar irregularity and cycle length changes are seen a few years before menopause (Chiazze, Brayer, Macisco, Parker, & Duffy, 1968; Harlow & Ephross, 1995; Treloar, Boynton, Behn, & Brown, 1967; Vollman, 1977). Variations in cycle length, patterns and bleeding duration are lowest during the main reproductive years of 25 to 39 years of age (Chiazze et al., 1968; Harlow & Ephross, 1995; Treloar et al., 1967; Vollman, 1977). Much of the variability in menstrual cycle characteristics reflect underlying endocrine fluctuations particularly of estradiol (Landgren, Unden, & Diczfalusy, 1980; Sherman & Korenman, 1975). Epidemiologic studies have demonstrated that abnormal menstrual cycle length, pattern and bleed duration are associated with decreased fertility and fecundity, and increased risk of spontaneous abortion and gestational diabetes (Dishi et al., 2011; T. K. Jensen, Scheike, Keiding, Schaumburg, & Grandjean, 1999; Kolstad et al., 1999; Rowland et al., 2002; Small et al., 2010; Small et al., 2006; Steiner et al., 1999; Wise et al., 2011). Most of the variation in the cycle length is due to variations in the follicular phase length (Fehring, Schneider, & Raviele, 2006; Waller et al., 1998). Short cycle length is associated with a short follicular phase and high baseline and mean estrogen. The shortened follicular phase results in

early triggering of hormonal feedback mechanisms, resulting in shorter period of follicular development and poor oocyte quality (Fehring et al., 2006; Ouyang et al., 2007; Windham et al., 2002). Long cycle lengths, on the other hand, show lower baseline estrogen, delayed follicle recruitment and a prolonged estrogen peak, which also could affect hormonal feedback and oocyte quality (Fehring et al., 2006; Harlow, Baird, Weinberg, & Wilcox, 2000; Windham et al., 2002). Menstrual cycle length, pattern and bleed duration can be used as important determinants of a woman's reproductive capability. Identifying modifiable nutritional factors that affect menstrual cycle characteristics can be a cost effective way to improve a woman's fecundity and pregnancy outcomes.

The prevalence of obesity is rising among women of reproductive age (Morris, Picciano, Jacques, & Selhub, 2008; Morris, Sakakeeny, Jacques, Picciano, & Selhub, 2010). Obese women have decreased fertility and reproductive capability (Balen, Anderson, Policy, & Practice Committee of the, 2007; Brewer & Balen, 2010; Chang, Chen, Hsieh, & Chiu, 2009; Hartz, Barboriak, Wong, Katayama, & Rimm, 1979; T. K. Jensen et al., 1999; Pasquali, 2006; Pasquali, Pelusi, Genghini, Cacciari, & Gambineri, 2003; Rich-Edwards et al., 1994). Increased weight and BMI (>23.9 ) are associated with decreased cycle regularity and longer cycle lengths, presumably due to obesity-associated increases in peripheral estrogen production (Rich-Edwards et al., 1994). Body fat distribution plays an important role in menstrual function. Adiposity, particularly of the visceral type, is associated with subfecundity (Balen et al., 2007; Brewer & Balen, 2010; Pasquali, 2006; Pasquali et al., 2003). Menstrual characteristics and obesity in adolescent

and early adulthood are more predictive of subfecundity than is obesity later in reproductive life (Brewer & Balen, 2010; Pasquali, 2006; Pasquali et al., 2003; Rich-Edwards et al., 1994). Few studies have examined the association between various markers of visceral adiposity and menstrual cycle characteristics, and most conducted to date lacked accurate markers of adiposity and were conducted in a non-US population (Castillo-Martinez, Lopez-Alvarenga, Villa, & Gonzalez-Barranco, 2003; De Pergola et al., 2009).

A woman's dietary habits may affect her menstrual cycle characteristics (Baines, Powers, & Brown, 2007; Barr, 1999; Larsson, Kumlin, Ingelman-Sundberg, & Wolk, 2004; Nagata, Oba, & Shimizu, 2006; Pedersen et al., 1991). Epidemiologic studies demonstrate that following a vegetarian diet or a low-fat diet may be associated with longer menstrual cycle lengths and irregular patterns and other evidence from animal studies suggest that essential fatty acids such as omega-3 and omega-6 fats can indirectly affect estrogen production (Larsson et al., 2004; Nagata et al., 2006; Stocco, Wang, Jo, & Manna, 2005; Wathes, Abayasekara, & Aitken, 2007). However study results are inconclusive and this role has not been explored in younger premenopausal women. The role of various types of dietary fatty acids on estrogen and menstrual cycle characteristics is also unclear and has not been thoroughly explored.

There is some evidence that sunshine availability can affect menstrual cycle length and women have shorter cycles in summer as compared to winter (Danilenko, Sergeeva, & Verevkin, 2011; Lewis, Lucas, Halliday, & Ponsonby, 2010; Stumpf &

Denny, 1989). Vitamin D is made by body primarily from sunlight and rate of production varies with season and sunlight availability. Female rats with vitamin D deficiency had impaired fertility and these effects appear to mediated through reduced aromatase activity and estrogen levels (Halloran & DeLuca, 1980; Kinuta et al., 2000). There are no known studies that have directly examined the role of vitamin D status and menstrual cycle characteristics.

The current dissertation has been conducted using data from the UMass Campus Vitamin D Study (Bertone-Johnson, Chocano-Bedoya, Zagarins, Micka, & Ronnenberg, 2010). The UMass Vitamin D Status Study is a cross-sectional study of women aged 18 to 30 years living near the University of Massachusetts, Amherst. The women were recruited in the late luteal phase of their menstrual cycles and provided information on their diet, demographic, health and menstrual cycle characteristics and completed diet and health history questionnaires. Fasting venous blood samples were obtained, and dual energy x-ray absorptiometry (DXA) scans were performed. A total of 284 women were recruited between 2006 and 2011. The current dissertation proposes to study the association between adiposity, dietary fat intake and serum 25-hydroxyvitamin D concentration, a marker of vitamin D status on menstrual cycle length, pattern and bleed days. Identification of modifiable nutritional factors could lead to an inexpensive means of improving a woman's reproductive capability.

# CHAPTER

# 1. LITERATURE REVIEW

## 1.1 Menstrual Cycle

The onset of menstrual cycle is the beginning of the reproductive phase in a woman's lifetime. The menstrual cycle is the preparation of the woman's body towards a pregnancy and when it does not occur it leads to bleeding as a result of shedding of the uterine wall. It is characterized by the periodic bleeding approximately every 28 days. The menstrual cycle is divided into three stages: the follicular phase, ovulation and the luteal phase.

Follicular phase: It is also known as the proliferative phase. It starts from the first day of menses until ovulation occurs. It begins with the development and recruitment of a group of primordial follicles in the ovaries. These follicles develop through various stages to become primary follicles, and the dominant follicle prevails by the end of this phase and the rest of the follicles undergo atresia (Hawkins & Matzuk, 2008; Mihm et al., 2011; Owen, 1975; Speroff, 1999).

Ovulation: It is the rupture of the follicle and release of the ovum into the peritoneal cavity. The ovum travels into the fallopian tube after it is picked up by the fimbrae (Speroff, 1999).

Luteal phase: It is also known as the secretory phase. It usually lasts for 14 days, and the length of this phase does not vary much among women (Fehring et al., 2006). After the egg is released, the remaining granulosa cells of the ruptured follicle enlarge

and accumulate lutein. The luteinized granulosa cells forms the corpus luteum (Mihm et al., 2011; Owen, 1975; Speroff, 1999). The corpus luteum is an endocrine organ that secretes progesterone. Its main role is to prepare the endometrium for eventual implantation of the fertilized egg, which usually occurs 8 to 10 days after ovulation (Hawkins & Matzuk, 2008; Mihm et al., 2011; Owen, 1975; Speroff, 1999). If pregnancy does not occur, the corpus luteum undergoes luteolysis.

At the end of a menstrual cycle in the absence of pregnancy, hormone levels decline and the endometrium is shed along with blood. The average amount of blood loss is 30 milliliters (Harlow & Ephross, 1995; Hawkins & Matzuk, 2008; Mihm et al., 2011; Speroff, 1999).

### 1.1.1 Menstrual Cycle Regulation

The menstrual cycle is regulated by the hypothalamus-pituitary-ovarian (HPO) axis. The hypothalamus secretes the pulsatile gonadotropin releasing hormone (GnRH), which stimulates the anterior pituitary to secrete follicle stimulating hormone (FSH) and lutenizing hormone (LH) under certain conditions; these hormones, in turn, stimulate ovarian estrogen production and subsequent secretion of progesterone by the corpus luteum. These hormones are under several positive and negative feedback mechanisms during the various phases of the menstrual cycle (Hawkins & Matzuk, 2008; Mihm et al., 2011; Owen, 1975; Speroff, 1999).

**Regulation during the Follicular Phase:** The gonadotropin releasing hormone (GnRH) is released during the late luteal to follicular transition following the decline of

estrogen levels, which has negative feedback on pulsatile GnRH released by the hypothalamus. GnRH stimulates the pituitary to release follicle stimulating hormone (FSH). The rise in FSH starts the recruitment of a cohort of ovarian follicles (Mihm et al., 2011). The follicles grow and develop through various stages of preantral, antral and the dominant preovulatory follicle (Speroff, 1999). The preantral follicle is surrounded by many layers of granulosa cells and theca cells. In the granulosa cells, the rise in FSH leads to aromatization of androstenedione (androgen) to estrogen. FSH and estrogen together increase the number of FSH receptors in the follicle. The rise in FSH and estrogen triggers further development into antral follicles. The dominant follicle is selected around day 5 - 7 of the menstrual cycle, after which estrogen levels increase significantly (Mihm et al., 2011; Speroff, 1999). The follicle exerts a negative feedback on FSH, suppressing its release. The negative feedback of estrogen on FSH inhibits development of all but the dominant follicle. In the presence of estradiol, FSH leads to the formation of LH receptors in the granulosa cells (Mihm et al., 2011; Speroff, 1999). The mid-follicular estrogen levels rise, which in turn has a positive feedback on luteinizing hormone (LH) secretion. Following the rise in LH, androgen production in the theca cells increases, serving as a substrate for more estrogen production by the granulosa cells (Speroff, 1999). The high-estrogen environment in the dominant follicle has more FSH receptors and is, therefore, sensitive to even low levels of FSH. The dominant follicle develops into a preovulatory follicle from the antral stage. It is now larger in size with more lipid accumulation. LH levels start to rise following the positive feedback from estrogen in the mid follicular phase. The estrogen levels continue to rise and then peak, after which there is a surge in LH (Mihm et al., 2011; Speroff, 1999). At the end of the

follicular phase, one dominant preovulatory follicle prevails while the rest undergo atresia (Hawkins & Matzuk, 2008; Mihm et al., 2011; Owen, 1975; Speroff, 1999).

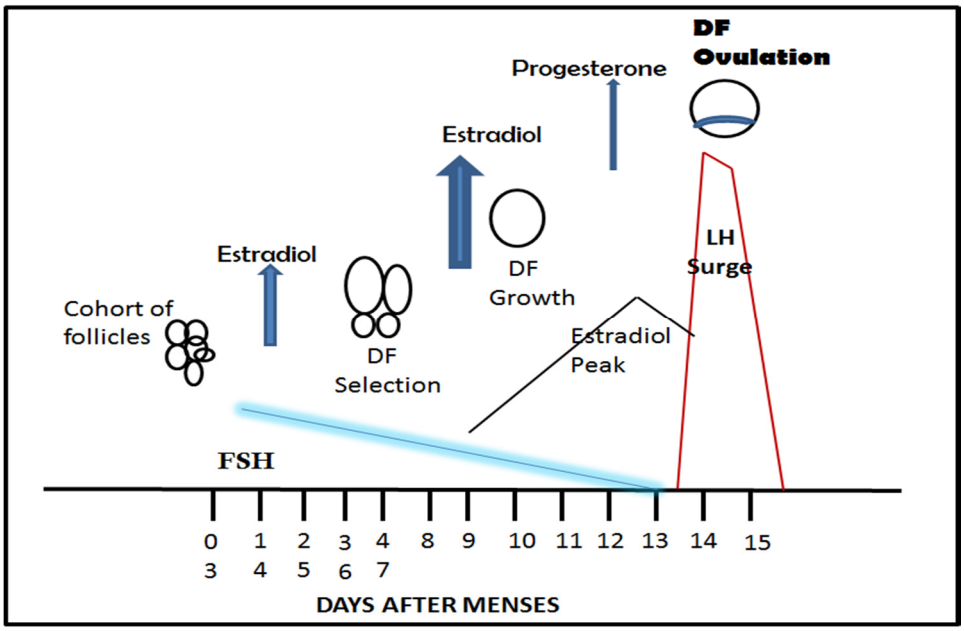


Figure 1 : Hormonal regulation during the follicular phase.

DF= dominant follicle. (Adapted from Mihm et al (2))

**Two-cell, two-gonadotropin regulation of estrogen synthesis:** LH receptors are located only on the theca cells, and under the influence of LH these cells synthesize androstenedione (androgen) from cholesterol. Aromatization of androgen to estradiol takes place only in the granulosa cells. FSH receptors are present in the granulosa cells. FSH induces aromatization by activating the P450arom gene (Speroff, 1999). The androstenedione produced in the theca cells diffuses to the granulosa cells where P450 enzymes aromatize it first to estrone and then subsequently to estradiol, which is then released in the blood. This is called the two-cell, two-gonadotropin regulation of estrogen synthesis (Speroff, 1999).

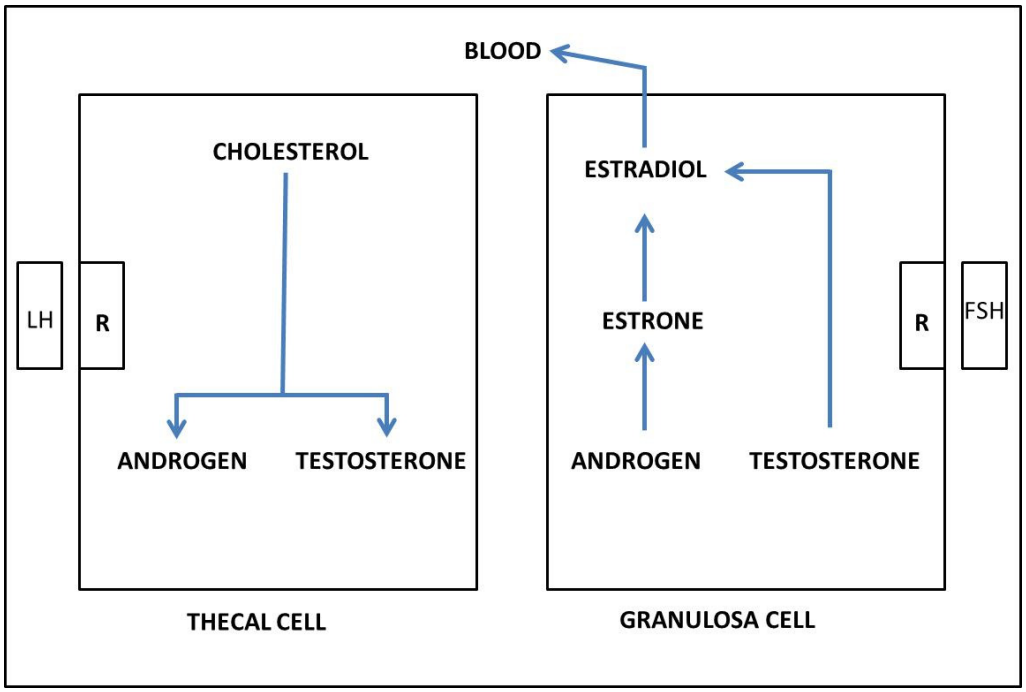


Figure 2 : Two-cell, two-gonadotropin regulation of estrogen synthesis.

LH-luteinizing hormone, FSH- follicle stimulating hormone, R- cell surface receptor.

**Ovulation:** Ovulation occurs 10 to 12 hours after the LH surge. The surge usually last for 48 to 50 hours in most women (Speroff, 1999). The LH surge causes luteinization of the granulosa cells and increases progesterone secretion. Estradiol levels fall immediately after the LH peak (Mihm et al., 2011; Owen, 1975; Speroff, 1999).

**Luteal phase regulation**: After ovulation, the corpus luteum, forms from the remaining granulosa cells, acts as an endocrine organ and mainly secretes progesterone. The corpus luteum requires LH support (Owen, 1975; Speroff, 1999). Around eight or nine days after ovulation, progesterone and estradiol levels peak. This is the time when implantation of the fertilized ovum takes place (Mihm et al., 2011; Owen, 1975; Speroff, 1999). Estrogen levels therefore rise twice in the menstrual cycle, once on the mid-follicular phase and once in the mid-luteal phase. Estradiol is responsible for the

proliferative changes of the endometrium during the follicular phase, and progesterone triggers differentiation of the cells of the endometrium during the luteal phase (Mihm et al., 2011). Both estradiol and progesterone decline at the end of the cycle which leads to the shedding of the endometrium (Mihm et al., 2011; Owen, 1975).

**Other Follicular and Luteal secretions**: Other peptides are secreted during the follicular and luteal phases, which are also involved in menstrual cycle regulation. Inhibins and activins are a family of peptides secreted by the granulosa cells that regulate FSH release (Mihm et al., 2011; Speroff, 1999). Estradiol and inhibin inhibit FSH release, whereas activin stimulates the release of FSH (Mihm et al., 2011; Speroff, 1999).

Two types of inhibins, inhibin A and inhibin B have been identified. Inhibin A secretion is correlated with follicular estradiol secretion. The levels rise after the dominant follicle selection and peak with the mid-cycle estradiol peak; the levels fall after ovulation. After ovulation inhibin A is secreted by the corpus luteum. Inhibin B levels rise steadily in the luteal-follicular transition and peak on day 5 of menses, reflecting the emergence of the cohort of growing follicles and an initial decline in FSH levels. The levels decline in the late follicular phase (Mihm et al., 2011; Speroff, 1999).

Activins are peptides involved in stimulating FSH secretion. Secretion of activin A rises in the mid-luteal phase followed by the decline in levels of inhibin A, estradiol and progesterone (Mihm et al., 2011). This is reflective of the rise in FSH during the luteal-follicular transition (Speroff, 1999).

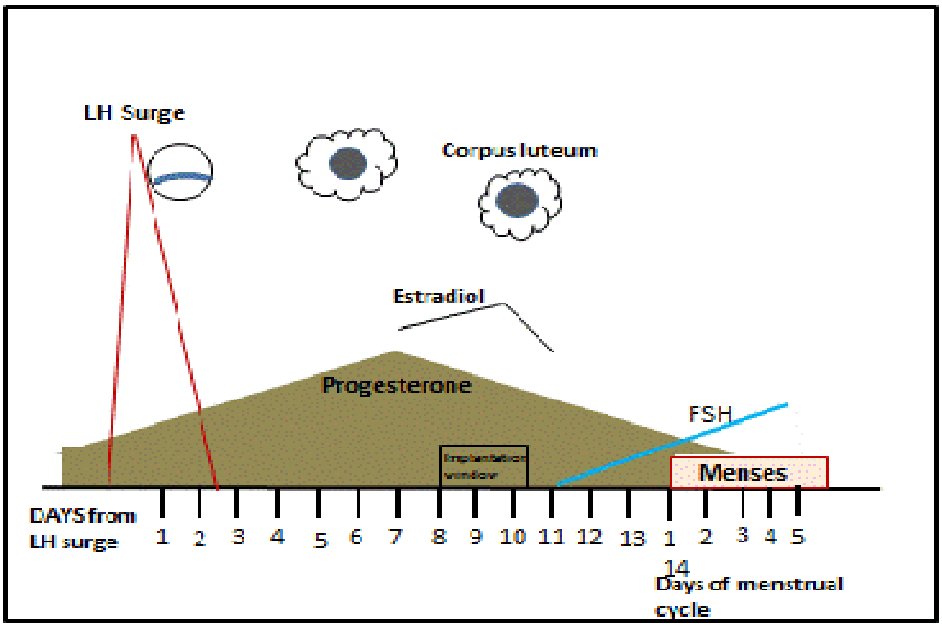


Figure 3 : Hormonal regulation during the luteal phase.

LH= luteinizing hormone, FSH = follicle stimulating hormone. (Adapted from Mihm et al. (Mihm et al., 2011))

### 1.1.2 Steroid Synthesis and Metabolism

The ovary produces three types of sex steroids: estrogens, progestins and androgens. The starting molecule in steroid synthesis is cholesterol. Cholesterol can be produced in situ from the 2-acetate molecule; however, ovarian production is not adequate to meet the demand ("Hormone Biosynthesis, Metabolism, and Mechanism of Action," 1999; Miller & Auchus, 2011). The organs depend on cholesterol from the blood stream from LDL for steroid synthesis. The gonadotropic hormones from the anterior pituitary bind to the cell surface receptor of a G-protein to activate the second messenger system through activation of adenylate cyclase. Cyclic AMP (cAMP) activity increases, which in turn

stimulates production of enzymes and protein required for steroid synthesis ("Hormone Biosynthesis, Metabolism, and Mechanism of Action," 1999).

Cholesterol synthesis occurs in the mitochondria following free cholesterol transport into the mitochondria. The first rate limiting step in steroid synthesis is the transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane. Several proteins have been identified to be involved in this step, including sterol carrier protein (SCP2), steroidogenic activator protein (SAP) and steroidogenic acute regulator protein (STAR). The STAR protein is the most favored protein for this step ("Hormone Biosynthesis, Metabolism, and Mechanism of Action," 1999). STAR increases steroid production by mediating transfer of cholesterol into the mitochondria of the adrenals and the gonads. A mutation in the STAR gene leads to failure of steroid synthesis in the adrenals and gonads ("Hormone Biosynthesis, Metabolism, and Mechanism of Action," 1999; Miller & Auchus, 2011). Once in the mitochondria, the cholesterol is converted to pregnenolone by the P450scc enzyme. Pregnenolone can be converted to progesterone, androstenedione or estrogen, based on the binding tropic hormone and the menstrual phase. Pregnenolone is converted to progesterone in a two-step reaction. Androstenedione is formed by conversion of pregnenolone to various intermediates by the enzyme P450c17 (Abetew et al., 2011; "Hormone Biosynthesis, Metabolism, and Mechanism of Action," 1999; Miller, 2008). Androstenedione is further converted to estrone (E1) by P450arom enzyme, and estrone can be converted to estradiol. Alternatively, androstenedione can also be converted to testosterone, which is converted to estradiol by the enzyme P450arom (Miller, 2008; Miller & Auchus, 2011). P450arom is expressed in several tissues. It is highly regulated in the ovary but non-

regulated in the adipose and placenta ("Hormone Biosynthesis, Metabolism, and Mechanism of Action," 1999). Estradiol is produced mainly by androstenedione via estrone. Estrone is secreted in significant amounts daily. Both estradiol and testosterone circulate bound primarily to the carrier protein, sex hormone binding globulin (SHBG). Around 10-30% of estradiol is bound to albumin and only 1% is available in free, unbound form. Androstenedione is primarily bound to albumin with only 8% bound to SHBG ("Hormone Biosynthesis, Metabolism, and Mechanism of Action," 1999).

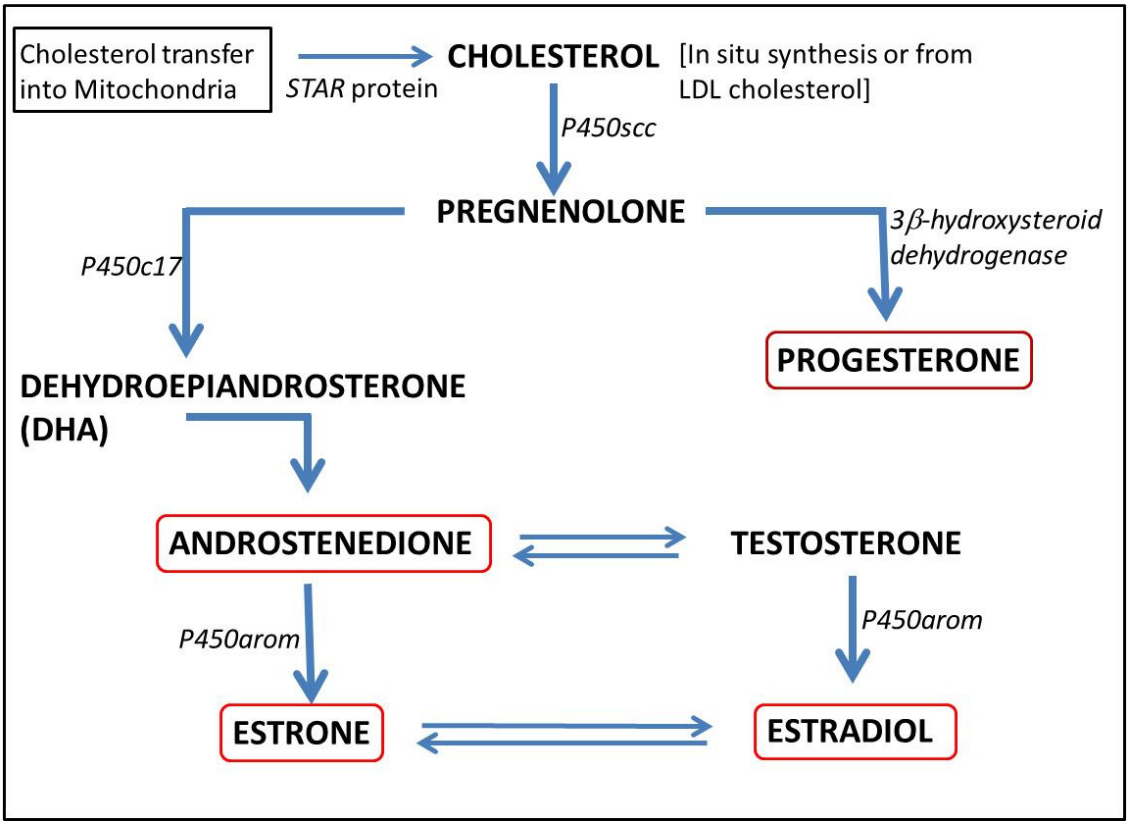


Figure 4 : Steroid Synthesis from Cholesterol in the Mitochondria.

**Estrogen Metabolism and Excretion:** Estrone (E1), estradiol (E2) and estriol (E3) are estrogens. Estradiol is the most active metabolite; estrone, which has lower metabolic activity, can be interconverted to estradiol. Estriol, is a relatively inactive metabolite of estrone and estradiol. Androgens are the main precursor of estradiol

("Hormone Biosynthesis, Metabolism, and Mechanism of Action," 1999). They are converted to estradiol by the aromatase enzyme P450arom (Miller, 2008; Miller & Auchus, 2011). Estrogen can be produced from androgens in the ovaries, and adrenals and in peripheral tissue such as adipose tissue, skin and endometrium. The total circulating amount of E2 is the sum of ovarian, adrenal and peripheral conversion of androgen to estrogen ("Hormone Biosynthesis, Metabolism, and Mechanism of Action," 1999). Estradiol and other active steroids are deactivated by conversion into sulfate and glucuronide metabolites. These metabolites are water soluble and are excreted through the urine or through the intestinal tract through bile ("Hormone Biosynthesis, Metabolism, and Mechanism of Action," 1999). Some of the estrogen metabolites can be reactivated by deconjugation through certain intestinal enzymes and introduced back into the circulation (Goldin & Gorbach, 1988; "Hormone Biosynthesis, Metabolism, and Mechanism of Action," 1999).

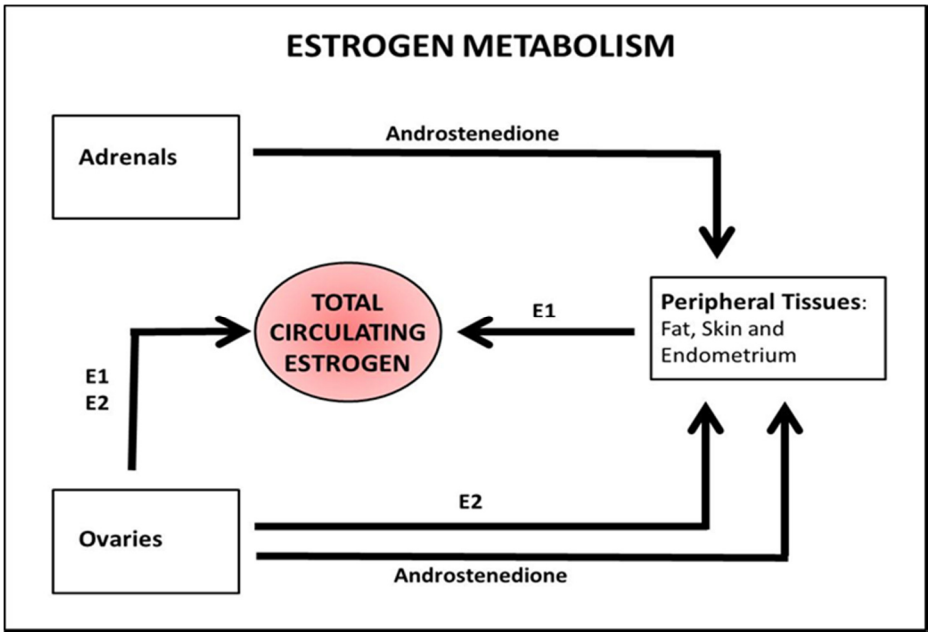


*Figure 5 : Estrogen* Metabolism.

E1-estrone, E2-estradiol.

### 1.1.3 Menstrual Cycle Characteristics and their Role in Fecundity and Spontaneous Abortion.

Menstrual cycle length is defined as the number of days starting from the first day of bleeding to the day before the start of the next period. Much of the information on menstrual cycle length and variability comes from 3 classic studies using menstrual diary records. Treolar et al., conducted a study of 2700 U.S. women aged 10 to 56 years for nearly 30 years. The women maintained annual menstrual cards and reported all and any medical or surgical histories throughout their follow-up (Treloar et al., 1967). Chiazze et al., followed cycle length and patterns of 2316 U.S. and Canadian women 15 to 44 years of age for 2 years (Chiazze et al., 1968). Vollman et al., followed 691 Swiss women 11 to 58 years of age for 1 to 39 years (Vollman, 1977). These studies showed high variability in cycle length, with long cycles immediately after menarche and similar variability and

changes in cycle length a few years before menopause (Chiazze et al., 1968; Treloar et al., 1967). All 3 studies reported mean cycle length between 28 to 31 days at the ages of 20 to 35 years. Their analyses also showed lowest variability in cycle length during these years (Chiazze et al., 1968; Treloar et al., 1967). A decrease in cycle length by one day was observed after the age of 35 (Treloar et al., 1967; Vollman, 1977). Similar variability in pattern and changes in bleed duration are also seen a few years after menarche and before menopause (Harlow & Ephross, 1995). Most cycle lengths fall between 26 to 31 days in the main reproductive years of ages 20 to 35 (Chiazze et al., 1968; Treloar et al., 1967; Vollman, 1977).

Abnormal menstrual cycle length, pattern and bleed duration have been used as proxy measures to assess a woman's fecundity and time to pregnancy. Both short and long cycles are associated with reduced fecundity and increased time to pregnancy (T. K. Jensen et al., 1999; Kolstad et al., 1999; Small et al., 2010; Small et al., 2006; Wise et al., 2011). A recent prospective study by Wise et al. of 2,653 Danish women 18 to 40 years old showed that women with shorter cycle length (<25 days) had the lowest fecundity (Wise et al., 2011). The study showed an inverse "u-shaped" association between cycle length and fecundity. Adjusted fecundability ratio, defined as the probability of conception within a cycle, when compared with average cycle lengths of 27-29 days were 0.64 (95%CI 0.49-0.84) for <25 days, 0.94 (95%CI 0.77-1.13) for 25-26 days, 1.10 (95% CI 0.97-1.25) for 30-31 days, 1.35 (96%CI 1.06-1.73) for 32-33 days and 1.17 (95% CI 0.91-1.49). The association for short cycle length (<25 days) was most significant. This study excluded women with irregular cycles and it is unclear how the study defined the criteria for irregular cycles. Also unclear is whether women with polycystic ovarian

syndrome (PCOS) were excluded from the sample. Exclusion of women with irregular cycles without PCOS is likely to cause bias because women with irregular cycles will also tend to have longer cycle length, which would lead to an overestimation of the fecundability ratios. However, the results of this study are consistent with other studies in a similar population (Kolstad et al., 1999). The bleed duration and intensity, age of menarche and time to menstrual regularization were not associated with fecundity in this study (Wise et al., 2011).

A study by Small et al., conducted in 470 women examined associations between cycle length, bleed duration and outcomes of fecundity and spontaneous abortion. The probability of conception was lower following a shorter cycle length (<30 days), combined fecundity ratio FR = 0.60 (95% CI 0.4 -1) (Small et al., 2006). Fecundity was also lower following longer (>31 days) cycles FR= 0.69 (95% CI 0.42-1.12) and 0.63 (95% CI 0.38-1.03) in a model using all pregnancies and in models using clinical pregnancies respectively (Small et al., 2006). They found that compared to 30 to 31 day cycle lengths, embryos conceived during both shorter and longer cycles were more likely to end in spontaneous abortion (SAB). The association of bleed days with fecundity and SAB demonstrated that women with bleed duration of 5 days was associated with highest fecundity, whereas bleed durations of less than 4 days (FR=0.6 95% CI 0.3-0.9) or up to 4 days (FR=0.5 95% CI 0.3-0.8) had significantly lower fecundity ratios. The study showed an association between cycle length and SAB [OR=3.0 (95% CI 0.9-9.6)] for short cycles and [OR=3.0 (95% CI 0.9 -10.6)]. This association was not statistically significant. Women with menstrual bleed duration of greater than 5 days had a low likelihood of spontaneous abortion (OR=0.4 95% CI 0.1 to 1.1) when compared to 5 day

bleed days (Small et al., 2006). This was the first study that used previous menstrual cycle characteristics as the exposure as it is most indicative of the woman's fecundity. One of the limitations of this study is that it did not adjust for physical activity which can confound the results (Small et al., 2006). However, the results are similar to other studies (Kolstad et al., 1999). Quinby et al., 1993 demonstrated that women with long cycle length (>34 days) was associated with recurrent miscarriage (Quenby & Farquharson, 1993). A separate analysis of the hormonal profile of 20 women 10 with regular cycle length (27 to 31 days) and 10 with long cycle length (>34 days) was conducted by the same study. The women with long cycle lengths had significantly lower estradiol in the luteal phase as compared to women with normal cycle lengths. The study hypothesized that low estradiol levels during the luteal phase may be associated with recurrent pregnancy loss due to poor implantation and an underdeveloped endometrium (Quenby & Farquharson, 1993). However this study was conducted in 203 women attending a Miscarriage Clinic and caution must be used when generalizing the results to the normal population.

Another study by Small et al., examined the association of menstrual cycle variability (cycle pattern) with fecundity and spontaneous abortion (Small et al., 2010). Menstrual cycle variation was assessed using the standard deviation calculated from means of menstrual cycle lengths reported in diaries. The probability of conception was significantly lower among women with greater menstrual cycle variability FR=0.49 (95%CI 0.31-0.77) than among women with lower cycle variability. The association was independent of the mean cycle length and age. This study stratified their analysis based on timing of intercourse and the results did not vary. They also found that 66% of

subclinical pregnancy losses were in women with highly variable cycles. The study strengths include the use of menstrual diaries, prospective nature of the study and urine analysis to assess early pregnancy loss and a pregnancy. However the study population had women with generally lower fecundity, were white and well educated. These factors may limit the generalizability of the study (Small et al., 2010).

Two other studies used self-reported cycle pattern to assess variability (T. K. Jensen et al., 1999; Steiner et al., 1999). Their results were similar to Small et al., suggesting that irregular cycles had lower fecundity ratio (Small et al., 2010). Oral contraceptive use in Jensen et al., found that women with cycle length >35 days had a lower fecundity odds ratio of 0.74 (95% CI 0.63-0.67) (T. K. Jensen et al., 1999). These studies may be subject to recall bias as those who have difficulty becoming pregnant are likely to differentially recall their cycle pattern (Steiner et al., 1999). Another study by Kolstad et al., 1999 of 295 couples used the difference between usual cycle length and an observed cycle length to assess cycle pattern variation (Kolstad et al., 1999). They too found that women with >10 day variation from their usual cycle length have a fecundity that is 25% that of a woman with no variation (OR 0.25; 95%CI 0.09-0.68) (Kolstad et al., 1999).

Several plausible biologic mechanisms can be involved in this association of cycle length and fecundity. Much of the variability in cycle length is attributed to the follicular phase length (Fehring et al., 2006). Short cycle lengths reflect shorter follicular phase lengths as 84% of cycle length variability is a result of variation in follicular phase (Danilenko et al., 2011; Fehring et al., 2006; Waller et al., 1998). In 20 to 39 years old women shortened follicular phase is associated with higher follicular phase estrogen

(Small et al., 2006; Waller et al., 1998; Windham et al., 2002). Short cycle length is associated with a short follicular phase and high baseline and mean estrogen. The shortened follicular phase results in early triggering of hormonal feedback mechanisms, resulting in a short period of follicular development and poor oocyte quality (Fehring et al., 2006; Ouyang et al., 2007; Windham et al., 2002). In older women changes in ovarian hormones also include increased follicular phase estrogen and these women also have decreased fertility (Small et al., 2006). A recent study by Mumford et al., 2012 demonstrated that women with short cycles have higher late luteal and early follicular phase FSH, early estrogen peaks in the follicular phase, and decreased progesterone levels during the luteal phase (Mumford et al., 2012). These hormonal variations are similar to those seen in reproductive aging, a period in which there is decreased fertility (Sherman & Korenman, 1975; Small et al., 2006; Windham et al., 2002). Short cycle lengths may also be associated with poor oocyte quality due to immature follicular development (Small et al., 2006; Windham et al., 2002). Long cycle lengths, on the other hand, show lower baseline estrogen, delayed follicle recruitment and a prolonged estrogen peak, which also could affect the hormonal feedback mechanisms and oocyte quality (Fehring et al., 2006; Harlow et al., 2000; Windham et al., 2002). Low follicular phase estrogen is associated with decreased fecundity (Windham et al., 2002). Mumford et al., 2012 demonstrated that women with long cycles (>35 days) had a delayed FSH and estradiol peaks. This can affect the oocyte quality (Mumford et al., 2012). Short and long cycle lengths are also more likely to be associated with anovulation and hence lower fecundity (Harlow et al., 2000; Harlow & Ephross, 1995; Mumford et al., 2012; Small et al., 2006).

Bleed duration is also associated with both fecundity and spontaneous abortion and may be used as a fertility indicator (Small et al., 2006). Small et al., 2006 postulate that the decrease in bleed days may be caused from a quick drop in estrogen from the prior cycle as a result of follicles deficient in estrogen production (Small et al., 2006). Endocrine hormones fluctuations may be associated with bleed duration. These hormones play a major role in endometrium development (Mihm et al., 2011; Vitzthum, 2009; Vitzthum, Spielvogel, Caceres, & Miller, 2001). Short bleeds are associated with inadequate endometrium buildup. This could lead to unsuccessful implantation and higher risk to spontaneous abortion (Quenby & Farquharson, 1993; Small et al., 2006). Growth of the endometrium starts during the follicular phase and is estrogen dependent (Mihm et al., 2011; Speroff, 1999).

Irregular cycles may be indicative of an underlying endocrine disturbance in the hypothalamic-pituitary-ovarian feedback system. High variability in cycles is seen during the perimenopausal period when estrogen levels are affected. This period is also marked by lower fertility (Harlow & Ephross, 1995; Sherman & Korenman, 1975; Small et al., 2010; Small et al., 2006). Decreased estrogen levels have been associated with lower possibility of conception (Small et al., 2010; Venners et al., 2006).

The current literature available on menstrual cycle characteristics including cycle length, pattern and bleed days shows that these characteristics are important determinants of a woman's fertility and fecundity, influencing a woman's likelihood of successfully getting pregnant and sustaining the pregnancy (T. K. Jensen et al., 1999; Kolstad et al., 1999; Rowland et al., 2002; Small et al., 2010; Small et al., 2006; Wise et al., 2011). Menstrual cycle characteristics can be used as a non-invasive, inexpensive way

to assess a woman's reproductive health. Identifying modifiable nutritional factors that affect menstrual cycle characteristics can be a cost effective way to improve a woman's reproductive capability and pregnancy outcomes.

### 1.2 Obesity

Obesity is defined as body mass index (BMI) $\geq$ 30 kg/m$^2$. The number of people in the obese and morbidly obese (BMI>40) category has seen a dramatic increase since 1990 (Flegal, Carroll, Kit, & Ogden, 2012; Jeffery & Utter, 2003; Roth, Qiang, Marban, Redelt, & Lowell, 2004). In the 1990s, the Behavioral Risk Factor Surveillance System (BRFSS) data showed 10 states with <10% obesity and no state having >15% prevalence in obesity. In comparison, data from 2006 show only 4 states with <20% obesity and 22 states with a prevalence of $\geq$25% obesity; in two of these states the prevalence of obesity was >30% ("Behavioral Risk Factor Surveillance System," 2013). According to the NHANES study, in 2007-08 age-adjusted prevalence of obesity was higher in women as compared to men. In women of reproductive age (20 to 39y), 34 % are obese as compared to 27.5% of men in the same age range. The prevalence of obesity in women of reproductive age has increased in the past 9 years from 28.4% in 1999 to 34% in 2008 (Flegal et al., 2012; Ogden, 2012).

In Massachusetts, the prevalence of obesity among both genders has increased in the past 12 years from 14.3% in 1998 to 23.6% in 2010 (Ogden, 2012). The prevalence has doubled in women from 11.1% to 21.1% from 1998 to 2010. The 25 to 34 age group has had a 10% increase in the prevalence of obesity in the same time period and this increase was more pronounced in females. All education levels, including college

graduates and those with some post-high school education, showed an increase in obesity by at least 10% (Ogden, 2012).

Although BMI is an easy and inexpensive measure of obesity, it has several limitations, including it does not measure body fat or body fat distribution (Kennedy, Shea, & Sun, 2009; Novotny, 2012; Shuster, Patlas, Pinthus, & Mourtzakis, 2012). Percent body fat at a BMI level varies based on gender, age and adiposity status and BMI does not capture this. BMI can fail to capture changes in body composition as a result of exercise or weight loss (Kennedy et al., 2009; Novotny, 2012; Shah & Braverman, 2012). Measurement of percent body fat is a more useful method for quantifying adiposity. Body fat is metabolically responsible for disease risk. Several methods are available for estimating percent body fat including dual energy x-ray absorptiometry (DEXA), computed tomography, and underwater weighing (Lee & Gallagher, 2008; Shuster et al., 2012). In females, percent body fat cut-points are as follows;

Table 1.1 : Percent body fat cut points in females (Pi-Sunyer, 2000).

| **Categories** | **Body fat Percent** |
|---|---|
| Too low | <= 10% |
| Acceptable (low) | 9 – 23% |
| Acceptable (high) | 24 – 31% |
| Too High | >= 32% |
| Optimal | 18 – 25% |

In males the percent body fat cut-points are lower and are as follows;

Table 1.2 : Percent body fat cut points in males (Pi-Sunver, 2000).

| **Categories** | **Body fat Percent** |
|---|---|
| Too low | <= 5% |
| Acceptable (low) | 6–15% |
| Acceptable (high) | 16 –24% |
| Too High | >= 25% |
| Optimal | 12 –18% |

Body fat distribution is also a more sensitive risk factor to chronic disease in comparison to total body fat alone (Shuster et al., 2012). Body fat distribution is either visceral/abdominal and peripheral body fat. Based on the type of body fat distribution it is either android (apple-shape) obesity where the fat is located around the upper body and abdomen, or gynoid (pear-shape) obesity where the fat is distributed around the hips and thighs. Women tend to have gynoid type of fat distribution and men tend to have android, however visceral adiposity is also seen among women (Novotny, 2012).

Obesity also increases an individual's rate of mortality from acute or chronic disease. Many factors contribute to obesity, including high energy intake, decreased physical activity, sedentary lifestyle, increase in fast food etc. (Jeffery & Utter, 2003). Obesity increases an individual's risk of chronic disease like type 2 diabetes, coronary artery disease, hypertension, stroke, gall stones, osteoarthritis, and certain cancers including endometrial, breast, uterine, cervical and prostate, (Mutsaerts et al., 2012; Roth et al., 2004). The current epidemic of obesity has seen an increase in obesity among women of reproductive age, which influences reproductive outcomes (Flegal et al., 2012;

Ogden, 2012). Obese women are more likely to experience menstrual irregularities, anovulation, polycystic ovarian syndrome (PCOS), decreased fertility and fecundity. In pregnant women, obesity increases risk of numerous complications, including gestational diabetes, preeclampsia, spontaneous abortions and caesarian type deliveries (Balen et al., 2007; Mutsaerts et al., 2012). Given the sentinel nature of menstrual cycle characteristics, understanding the influence of body fat and body fat distribution on these characteristics could help identify mechanisms through which obesity influences reproductive success.

### 1.2.1 Role of Adiposity and Menstrual Cycle Characteristics.

Obesity is associated with menstrual abnormalities, decreased reproductive capability and infertility (Balen et al., 2007; Brewer & Balen, 2010; Chang et al., 2009; Hartz et al., 1979; T. K. Jensen et al., 1999; Pasquali, 2006; Pasquali et al., 2003; Rich-Edwards et al., 1994). The association between obesity and menstrual function has been explored in several studies. Hartz et al (Hartz et al., 1979; Hartz, Rupley, Kalkhoff, & Rimm, 1983) conducted two studies to explore this association in two large cohorts of women (n=26,638 and n=11,791). They found that women with irregular cycles and long cycle lengths were significantly heavier than those with normal cycles irrespective of parity and age. They also found that women with higher waist-to-hip ratios were more likely to have irregular menstrual cycle (OR =1.56; $p<0.001$). These studies had statistically significant findings, but had several limitations in terms of not adjusting for several known confounding variables, such as smoking, parity or oral contraceptive use. Rich-Edwards et al., 1994 used BMI at 18 years of age as a measure of obesity and found a J-shape relation between BMI and menstrual regularity in the Nurses' Health Study II (Rich-Edwards et al., 1994). They concluded that a BMI >23.9kg/$m^2$ was associated with

decreased menstrual cycle regularity. Two other studies also found similar relationship with BMI and menstrual cycle regularity. However both these studies had very vague definitions of "menstrual cycle regularity" and menstrual function was a secondary outcome (Brown, Mishra, Kenardy, & Dobson, 2000; Lake, Power, & Cole, 1997).

BMI is easy to measure and a useful tool for assessing adiposity in research studies; however, BMI only estimates adiposity. Metabolically, menstrual cycle function is influenced by the actual amount of body fat (Brewer & Balen, 2010). In this respect, BMI is not the most accurate measure of adiposity since it is based on body weight, rather than on body composition. Although DXA measurements have been used increasingly in research to estimate percent body fat, Douchi et al., 2002 is the only known study to use DXA measurements of body fat to study the association with menstrual cycle characteristics (Douchi et al., 2002). They conducted a case-control study in Japanese women 20 to 39 years of age where cases (n= 39) were defined as women with menstrual disorders who either had oligomennorhea (six or fewer menstrual cycle per year) or women with secondary amenorrhea. The controls (n= 44) were women with regular menstrual cycles with a cycle length between 25 to 35 days. Adiposity was estimated using BMI, total body fat percentage, and body fat at various body sites, such as trunk fat or trunk- to-leg fat. Studies have used trunk-to-leg fat ratio as a measure of upper or lower body fat distribution (Brufani et al., 2012; Douchi et al., 2002; Zillikens et al., 2010). They found that the trunk-to-leg fat ratio was significantly higher in cases than in controls [cases 1.48 +/-0.29 and in controls was 1.25 +/-0.38 ($P<0.01$)] (Douchi et al., 2002). However, this study had a very small sample size (n=83), used no statistical measures of association, used only cycle regularity as its sole outcome, and was

conducted in a non U.S population, which have ethnic and lifestyle differences that may limit the generalizability of their findings.

In addition to the quantity of body fat, the distribution of body fat also plays an important role in reproductive health. Visceral fat is metabolically more active than subcutaneous fat, and it plays a greater role in obesity-related chronic disease (Brewer & Balen, 2010; Wronska & Kmiec, 2012). Menstrual cycle characteristics can be affected by a higher amount of visceral fat. De Pergola et al., 2009 examined the association of visceral fat with oligomennorhea, defined as menstrual cycle length of greater than 35 days, in 266 Finnish women aged 18 to 40 years (De Pergola et al., 2009). They found that waist circumference was significantly associated with oligomennorhea (defined as cycle length >35 days) after adjusting for variables like insulin resistance and hyperinsulinemia (OR: 1.45 95% CI 1.04-2.04) BMI alone was not significantly associated with menstrual cycle length. Women with long menstrual cycle length had a higher BMI, waist circumference and higher fasting insulin than did women with normal menstrual cycle length (De Pergola et al., 2009). This study had several limitations, including the relatively high mean age of the women (33 years), since menstrual cycle characteristics at that age are not predictive of reproductive capability (Sherman & Korenman, 1975; Small et al., 2006; Windham et al., 2002). In addition, women in the study were either obese or overweight-- no normal-weight women served as a control group. Furthermore, visceral fat was estimated from waist circumference, rather than from DXA measures for body fat measurement, and waist circumference does not distinguish between central fat that is subcutaneous and visceral central fat.

Castillo-Martinez et al., 2003 conducted a cross sectional study in 120 women aged 18 to 40y from an obesity clinic (Castillo-Martinez et al., 2003). They assessed central adiposity using waist circumference and waist-to-hip ratio, and they examined the association between visceral adiposity and menstrual cycle length. They found that both waist circumference and waist-to-hip ratio were higher among women with longer menstrual cycle lengths (>35 days). However, this study was conducted in obese or overweight subjects, did not use DXA measures of visceral adiposity and both mean age (30y) and BMI (37 kg/m2) were relatively high (Castillo-Martinez et al., 2003). Moreover, other cycle characteristics, such as regularity and bleed duration, were not assessed. Two other large studies that evaluated BMI and menstrual cycle length and regularity found that BMI was significantly associated with menstrual cycle length and cycle variability (Chang et al., 2009; Rowland et al., 2002). However, neither study used DXA estimates of body fat, nor associations with body fat distribution were not evaluated.

Because adipose tissue is an important site for extra-gonadal steroid synthesis—particularly estradiol (E2) and estrone (E1) production through conversion of androgens by aromatase enzyme—sex hormone secretion and bioavailability are altered in obese persons. In general, women with higher body fat have increased aromatase activity, leading to higher estrogen levels (Turner, 2011). This increased estrogen production is associated with decreased levels of transport proteins, such as sex hormone binding globulin (SHBG), which delivers estrogen to its target organs (Brewer & Balen, 2010; Flegal et al., 2012). Changes in the steroid pool can, in turn, affect menstrual cycle

characteristics. Because adipose tissue serves as a site of peripheral estrogen secretion, increased steroidogenesis occurs in obese women.

**Visceral Fat:** Distribution of the body fat may play a bigger role in regulation of the steroid production and distribution. Visceral adiposity is associated with increased insulin levels, higher androgen production which, in turn, lead to decreased hepatic synthesis of SHBG (Brewer & Balen, 2010). This regulatory disturbance leads to an inflated pool of free steroid hormones and further affects the delivery of these to the target organs (Brewer & Balen, 2010). Some evidence suggests that higher visceral fat is associated with menstrual cycle irregularity (Douchi et al., 2002). On the other hand, gynoid/peripheral type obesity (body fat in the hips and thigh region) may also be associated with menstrual cycle function, perhaps because fat depots in the buttocks and thighs have higher aromatase expression than do abdominal fat depots and are major sites for extragonadal estrogen production (Simpson, 2000). Estrogens increase SHBG and insulin and androgens decrease its levels (Balen et al., 2007). SHBG levels are less affected in gynoid obesity and it is not accompanied by the inflated free steroid pool seen in visceral adiposity. In the absence of visceral obesity it may be likely that peripheral body fat may help regularize menstrual function by contributing to the total estrogen pool (Brewer & Balen, 2010; Goumenou, Matalliotakis, Koumantakis, & Panidis, 2003). Thus, it appears that the pattern of body fat distribution may play a greater role in menstrual function than total body fat alone, especially in obesity.

**Role of leptin:** Abnormal menstrual cycle function in obese individuals may be mediated through other biological mechanism, including leptin secretion by adipocytes (Brewer & Balen, 2010). Leptin is a regulatory protein secreted by adipose tissue that helps to control food intake and metabolism of fat and glucose. Leptin levels are proportional to body fat stores, with lower levels observed when fat stores are low and higher levels observed with increased fat stores (Gosman, Katcher, & Legro, 2006). In the recent years, leptin has been studied for its role in reproductive functions (Brewer & Balen, 2010; Castillo-Martinez et al., 2003). The genetically leptin-deficient obese Ob/Ob mice are infertile and have hypogonadism (Brewer & Balen, 2010; Goumenou et al., 2003). The possible effects of leptin on the menstrual cycle in obese women could be through dysregulation of GNRH secretion, altered ovarian steroidogenesis and dyregulation of folliculogenesis (Brewer & Balen, 2010). A recent study by Asimakopoulos et al., 2009 conducted in 19- to 30-year-old women during the course of one menstrual cycle shows significant variations in leptin levels (Asimakopoulos et al., 2009). Mean leptin concentrations rise slowly during the menstrual cycle, with higher values during the luteal phase and the lowest during the follicular phase (Asimakopoulos et al., 2009). Similar results were seen in other studies (Brannian & Hansen, 2002). With normal serum levels, leptin affects reproductive function through stimulatory effects on the HPO axis, but elevated levels may cause dyregulation of this axis (Brewer & Balen, 2010). The hypothalamus and pituitary both have leptin receptors, and leptin may be involved in the control of GnRH secretion. In obese women, leptin secretion may lead to dysregulation of GnRH Leptin receptors are also present in the ovaries, and mRNA expression of leptin has been seen in human theca and granulosa cells of the ovary as well as oocytes and

endometrial cells (Brewer & Balen, 2010). In cultured granulocytes, leptin exerts dose-dependent inhibition of estradiol synthesis in response to FSH and IGF1 stimulation, possibly suggesting a paracrine role for leptin (Agarwal, Vogel, Weitsman, & Magoffin, 1999). In another in vitro study, high concentration of leptin inhibited follicular development and increased follicular steroidogenesis in cultured mouse follicles (Swain, Dunn, McConnell, Gonzalez-Martinez, & Smith, 2004). Thus, excess leptin, as seen in obese women, may affect menstrual function; however, studies on a direct association between leptin and menstrual cycle regularity in obese or overweight women are scarce.

**Summary:** Energy stores as body fat play an important role in human reproduction. From an evolutionary perspective, it makes sense that in times of reduced food availability and lower body fat stores, the body uses existing reserves to sustain maternal life while reducing the chances of pregnancy and the extra demands involved in a long period of gestation. This is seen during such times by a reduction in menstrual regularity and decreased menstrual function, which are important determinants of a woman's reproductive capability. Similarly, in times of excess, energy stored as body fat as seen in obesity can also affect menstrual regularity or function and in turn influence a woman's reproductive potential (Douchi et al., 2002; Hartz et al., 1979; Rich-Edwards et al., 1994). Apart from being an energy reserve, body fat plays an important role in regulation of menstrual cycle regularity, possibly through its role in the regulation of steroidogenesis (Brewer & Balen, 2010; Brown et al., 2000; Simpson, 2000). Despite the importance of adiposity, few studies conducted in the US have used DXA measures of body fat to assess its association with menstrual cycle length and pattern. Most studies have used

BMI as a measure of obesity, but BMI does not accurately estimate body fat especially in young women. Distribution of body fat is important as well, and the association of android and gynoid obesity with menstrual cycle characteristics has not been adequately explored, and existing studies were conducted in older women 35y and older, and age can affect menstrual cycle regularity, length and bleed duration (Chiazze et al., 1968; Harlow & Ephross, 1995; Treloar et al., 1967; Vollman, 1977). Menstrual cycle characteristics and obesity in younger women are more predictive of subfecundity than is obesity later in life (Pasquali, 2006; Pasquali et al., 2003; Rich-Edwards et al., 1994). Most studies have focused on how obesity affects menstrual cycle regularity alone; to our knowledge, no studies have explored the association of markers of adiposity with menstrual cycle length and bleed duration. Both of these menstrual cycle characteristics are also determinants of the hormonal environment, and they can affect a woman's reproductive capability. Because several research gaps exist in the current literature, this study aims to evaluate the association of total body fat as well as body fat distribution with menstrual cycle characteristics in young premenopausal women.

### 1.3 Vitamin D

Vitamin D, a seco-steroid hormone, is well-known for its classic role in calcium and phosphorus homeostasis and bone mineralization (Holick, 2011). Deficiency of this fat soluble vitamin causes bone deformities in children, known as rickets, and in adults causes osteomalacia. Unlike typical vitamins, which must be provided through diet or supplements, vitamin D can be synthesized endogenously upon exposure of skin to

sunlight of certain wavelengths, and few dietary sources of vitamin D are generally available.

**Cutaneous Synthesis and Activation of Vitamin D:** Vitamin D can be synthesized in the body upon exposure to sunlight. UVB rays from sunlight with wavelengths between 290nm and 315nm act on 7-dehydrocholesterol present on the skin to make previtamin D. Previtamin D3 is then converted to Vitamin D3 by thermal transformation. Prolonged exposure to sunlight can lead to conversion of previtamin D to inactive catabolic compounds called lumisterol and tachysterol and can also cause vitamin D to degrade to suprasterol I, suprasterol II and 5,6-trans-vitamin D (Bender, 2001).

The newly formed vitamin D enters the circulation and binds to a specific vitamin D binding protein called $\alpha_1$-globulin.Vitamin D bound to $\alpha_1$-globulin travels to the liver where it undergoes hydroxylation at carbon 25 by 25-hydroxylase (CYP27A) to form 25-hydroxyvitamin D (25-D). This is the main circulating form of the vitamin, which has a half-life of 3 weeks. The normal range for 25-D in blood is between 20-87 nmol/L (Bender, 2001). The 25-hydroxyvitamin D undergoes further hydroxylation at the 1 position by renal and extra renal enzyme 1$\alpha$-hydroxylase (CYP27B1), producing 1,25-dihydroxyvitamin D (1,25-D). This is the active form of vitamin D and is capable of producing the functional effects of vitamin D. The ubiquitous enzyme 24-hydroxylase (CYP24) can inactive both vitamin D metabolites, converting 25-D to 24,25-dihydroxyvitamin D and 1,25-D to 1,24,25 $(OH)_3$ (calcitetrol). These metabolites are further degraded and excreted as calcitroic acid. CYP24 expression is regulated by 1,25-D levels (Sutton & MacDonald, 2003).

Vitamin D from dietary sources is taken up by enterocytes and incorporated into chylomicrons. These chylomicrons are released into the lymphatic system, and then drained into the blood stream (Bender, 2001). The chylomicron remnants reaches the liver where the vitamin D is hydroxylated to 25-D by 25-hydroxylase and enters the circulation bound to vitamin D binding protein (Norman, 2001). It can undergo further hydroxylation in the kidney.

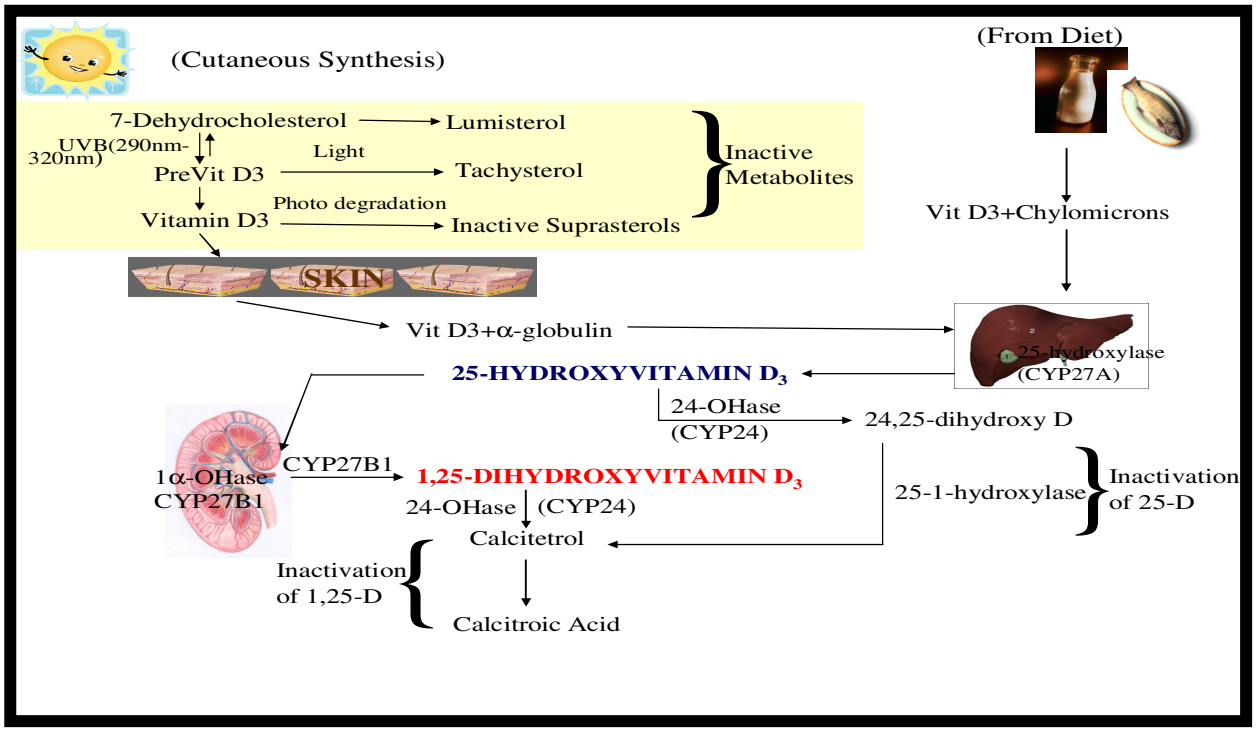


Figure 6: Cutaneous Synthesis and Activation of Vitamin D.

This figure shows the various vitamin D metabolites derived through cutaneous synthesis and diet to the formation of the 25-hydroxyvitamin $D_3$ (circulating form) and its conversion to 1,25-dihydroxyvitamin $D_3$ (active form) and their respective inactivation to calcitroic acid. Also shown are the major enzymes involved in the activation and inactivation of these metabolites.

**Dietary sources and Dietary Reference Intakes (DRI) for Vitamin D:**

Fortified milk, eggs from hens fed vitamin D-fortified feed, fatty fish, fish oils, and animal liver are the main dietary sources of vitamin D (Bender, 2001). Vitamin D has an established Recommended Dietary Allowance (RDA) that represents a daily intake sufficient to maintain bone health and normal calcium metabolism in healthy people ("Vitamin D: Fact Sheet for Health Professionals," 2011). The RDA for vitamin D for the various age groups and gender are as follows:

Table 1.3 : Recommended Dietary Allowances (RDAs) for Vitamin D ("Vitamin D: Fact Sheet for Health Professionals," 2011)

| AGE | MALE | FEMALE |
|---|---|---|
| **0 – 12 months** | 400 IU (10 mcg) | 400 IU (10mcg) |
| **1 – 13 years** | 600 IU (15 mcg) | 600 IU (15 mcg) |
| **14 – 18 years**<br>**Pregnancy/Lactation** | 600 IU (15 mcg)<br>600 IU | 600 IU (15 mcg)<br>600 IU |
| **19 - 50 years**<br>**Pregnancy/Lactation** | 600 IU (15mcg)<br>600 IU (15 mcg) | 600 IU (15 mcg)<br>600 IU (15 mcg) |
| **51 – 70 years** | 600 IU (15 mcg) | 600 IU (15 mcg) |
| **> 70 years** | 800 IU (20 mcg) | 800 IU (20 mcg) |

**Factors Affecting Vitamin D Synthesis**

Vitamin D synthesis from sunlight is affected by various factors. Some of the factors affecting its synthesis from sunlight are as follows:

(i) Clothing: Women who wear long robes and head covering or those who are covered from head to toe are likely to block cutaneous synthesis of vitamin D. Clothing absorbs

the ultraviolet radiation required for vitamin synthesis from sunlight (Bender, 2001; "Vitamin D: Fact Sheet for Health Professionals," 2011).

(ii) Latitude: During the months from November through February people in northern latitudes do not make vitamin D from sun exposure. This is because the angle of the sun rays blocks the wavelengths required for conversion of 7-dehydrocholesterol to previtamin D. Hence no cutaneous synthesis of vitamin D occurs (Bender, 2001; Dawson-Hughes, 2001; "Vitamin D: Fact Sheet for Health Professionals," 2011).

(iii) Skin tone: The skin pigment melanin acts as a natural sunscreen, hence people with darker skin require much more sun exposure than light skinned people for cutaneous vitamin D synthesis. Studies have reported lower serum 25-D levels among individuals of African American descent compared to individuals of Caucasian descent (Bender, 2001; Dawson-Hughes, 2001; "Vitamin D: Fact Sheet for Health Professionals," 2011).

(iv) Sunscreen: Sunscreens with SPF of 8 or greater blocks UV rays required for cutaneous synthesis of vitamin D ("Vitamin D: Fact Sheet for Health Professionals," 2011).

(v) Age: Aging causes a decrease in vitamin D synthesis, absorption and metabolism. Aging can cause a decrease in synthesis of vitamin D by skin due to decreased 7-dehydrocholesterol levels. Hence there is a decrease in serum levels of 25-D. Older adults are more likely to spend time indoor and have limited sun exposure which would further contribute to their decreased serum levels of 25-D (Bender, 2001; Dawson-Hughes, 2001; "Vitamin D: Fact Sheet for Health Professionals," 2011).

**Vitamin D Metabolism**

The main function of vitamin D is maintaining the calcium and phosphorus levels in the blood. Vitamin D level is regulated by PTH, 1,25-dihydroxyvitamin D (1,25-D) and serum calcium and phosphorus levels. Parathyroid hormone (PTH) is secreted by the parathyroid glands located in the neck around the thyroid gland in response to low levels of calcium in the blood. The parathyroid glands have cell surface calcium sensors that can detect low levels of calcium in the blood. PTH is released in response to hypocalcemia. The increase in PTH levels is sensed by PTH sensors in the kidney (Bender, 2001; Holick, 2000). Once PTH is released it acts on the kidneys by increasing the activity of 1$\alpha$-hydroxylase, which causes increased production of 1,25-D from 25-D. 1,25-D brings about the effects of increasing serum calcium levels through the intestine and bones. In the intestine, 1,25-D directly increases intestinal absorption of calcium by increasing the synthesis of proteins like calbindin, which is responsible for the transport of calcium across the intestinal mucosa (Holick, 2000). It also increases activity of other proteins, such as alkaline phosphatase, calmodulin, low-affinity calcium-dependent ATPases, and brush border actin involved in calcium regulation and bone formation (Norman, 2001).

In the bone, 1,25-D can indirectly affect osteoclast activity and increase serum calcium levels through bone resorption. 1,25-D interacts with vitamin D receptors (VDR) present on osteoblasts and increase the expression of RANKL (receptor activator of NFkB ligand) (Holick, 2011). The receptor RANK present on preosteoclasts interacts with RANKL resulting in the formation of multinucleated osteoclasts (Holick, 2000, 2011). These osteoclasts secrete HCL that dissolve bone matrix causing release of

calcium into the blood. 1,25-D mobilizes bone to release calcium through its action on osteoblasts. In turn, 1,25-D levels can negatively regulate PTH. Low levels of 1,25-D increase PTH secretion whereas high levels inhibit PTH secretion (Bender, 2001; Holick, 2011). Further, high 1,25-D levels down-regulate the activity of renal 1α-hydroxylase and up-regulate 24-hydroxylase activity (Sutton & MacDonald, 2003). This brings about the catabolic breakdown of 1,25-D to the inactive metabolite 1,24,25-D (calcitetrol).

1α-hydroxylase is the renal enzyme involved in the activation of 25-D to 1,25D. Disruption of the 1 α-hydroxylase gene in null mutant mice results in hypocalcemia, hyperparathyroidism, growth retardation, osteomalacia and reproductive dysfunction (Sutton & MacDonald, 2003). Recent studies have demonstrated the presence of 1α-hydroxylase in extra-renal tissues such as immune cells, breast, pancreas and the prostate (Martini & Wood, 2006). 1,25-D regulates cell differentiation function in certain tissues and the presence of 1α-hydroxylase in these tissues could play an important role in the paracrine regulation of 1,25-D (Lips, 2006). In the extra renal tissues, 1α-hydroxylase is influenced by cytokines (Lips, 2006; Peterlik & Cross, 2005).

**Mechanism of Action of Vitamin D:** Vitamin D exerts its metabolic activity through both non-genomic and genomic mechanisms of action. The genomic responses, also called the less rapid or genome-initiated responses, can take minutes to hours or days. The genomic responses of 1,25-D are mediated through the nuclear vitamin D receptor (VDR) (Holick, 2000). 1,25-D binds to VDR in the nucleus of the cell, which brings about a conformational change in VDR and facilitates the formation of a heterodimer with retinoid X receptor (RXR) (Holick, 2000). This further causes phosphorylation to

activate binding to the VDRE (vitamin D response element) in the promoter region of target genes and causes the up-regulation of vitamin D-responsive genes, thereby causing the tissue-specific responses (Holick, 2000). Some of the genes upregulated by 1,25-D are calbindin D, osteocalcin, osteopontin, plasma membrane calcium pump, interleukin-1, and interleukin-6 (Bender, 2001; Holick, 2000). These genes are involved in calcium metabolism and bone health (Bender, 2001; Holick, 2000).

VDR is a 50 kDa nuclear receptor which has a high affinity to 1,25-D. It is a member of the nuclear receptor family of ligand activated transcription factors (Sutton & MacDonald, 2003). It was first found in the intestinal mucosa, but was subsequently identified in other tissues, including the kidneys, bone, parathyroid glands, β-cells of the pancreas, placenta, hypothalamus, pituitary, ovary, uterus, mammary glands, skin, thymus, monocytes, macrophages and T lymphocytes (Bender, 2001; Lerchbaum & Obermayer-Pietsch, 2012; Parikh et al., 2010). The primary amino acid sequence of VDR has 5 functional domains which include regions for nuclear localization, DNA binding, heterodimerization, ligand binding and transcriptional activation (Norman, 2001). Regulation of gene transcription is dependent on the ligand binding to the receptor (Norman, 2001). An unoccupied VDR does not bring about the gene transcription as that of a VDR bound to its ligand 1,25-D. Shape of the ligand is also an important aspect for the up- or down-regulation of the genomic responses. VDR bound to the hat icon shape of 1,25-D leads to the appropriate gene responses (Norman, 2001).

Non-Genomic or Rapid responses - Non-genomic responses take a few seconds or minutes for the tissue-specific responses, which are mediated by a cell membrane receptor for vitamin D called MARRS (membrane activated rapid response steroid

binding protein) (Rohe, Safford, Nemere, & Farach-Carson, 2005). The binding of the 1,25-D to the cell membrane receptor triggers a number of signal transduction pathways, which leads to the corresponding physiological response (Holick, 2000). In enterocytes, ligand binding to the receptor causes G-protein activation leading to phospholipase C-linked hydrolysis of phosphatidylinositol bisphosphate (PIP2). This causes release of diacylglycerol and inositol triphosphate ($IP_3$). IP3 leads to the opening of calcium transport channels in the ER leading to the release of Ca+ ions, which activates calcium-dependent proteins like calmodulin. This brings about the appropriate physiological responses. Diacylglycerol leads to activation of protein kinase C and mitogen activated (MAP) kinases, which in turn also causes the physiological responses. These physiological responses include transcaltachia, activation of voltage-gated calcium channels, increase in intracellular calcium and induction of phospholipids and sphingolipid turnover (Bender, 2001).

### 1.3.1 Vitamin D, The Menstrual cycle and Fertility.

Seasonal changes in light can influence menstrual cycle length and variability (Danilenko et al., 2011; Stumpf & Denny, 1989; Sundararajan, Subbaraj, Chandrashekaran, & Shunmugasundaram, 1978). As early as 1894, observations made in Eskimo women during the 4-month period of arctic night showed suppressed menstrual function, whereas menstrual cycle length returned to normal during the light period (Stumpf & Denny, 1989). Several studies have focused on the effects of melatonin, which is secreted in the absence of light, but none has studied the effects of vitamin D as a

possible explanation for this association. Vitamin D levels vary considerably in humans and are affected by seasonal variation of sunlight availability (Holick, 2011).

Sundarajan et al, 1977 investigated the seasonal variation of the menstrual cycle in a retrospective cohort of women ages 20 to 30y from the University of Minnesota during 1930 to 1970. Menstrual intervals had a sinusoidal pattern with longer cycles in fall and shorter cycles in spring and summer. The data showed a solar movement in the menstrual cycle length with shorter cycles in warmer days and longer cycles in colder days (Sundararajan et al., 1978). A recent study by Danilenko et al., 2010 examined the effects of season on ovarian-menstrual function. In summer versus winter, menstrual cycle length was shorter (by 0.9 days), there was a higher frequency of ovulation, and increased FSH secretion was seen. The study also concluded that higher sunshine was significantly associated with shorter cycle length (Danilenko et al., 2011). Similar seasonal trends were seen in conception rates in northern countries. Conception rates were low in dark long winters and peaked during summer months (Lerchbaum & Obermayer-Pietsch, 2012).

**Animal Studies:** These effects can possibly be explained by seasonal variation of vitamin D levels, although there are no known studies that have evaluated this hypothesis in humans. Several animal studies have demonstrated a role for vitamin D in fertility (Halloran & DeLuca, 1980; Kwiecinksi, Petrie, & DeLuca, 1989). Halloran et al., 1980 studied weanling rats consuming vitamin D-replete or -deficient diets until maturity. They found that the vitamin D deficient rats had 75% reduced fertility and this was associated with decreased probability of getting pregnant and increased likelihood of complications during pregnancy. They also found that the vitamin D-deficient rats that

did not get pregnant and were not cycling in their normal 4 day pattern (Halloran & DeLuca, 1980). Kwiecinski et al., 1989 similarly saw reduced fertility and mating success in vitamin D deficient female rats. The reduced fertility was not corrected by normalizing the hypocalcemia, but required 1,25-D suggesting that 1,25-D had a direct role (Kwiecinksi et al., 1989).

Studies in rats have shown the presence of vitamin D receptor (VDR) in the ovary, uterus, endometrium and the hypothalamus, suggesting an active role for vitamin D in these tissues (Lerchbaum & Obermayer-Pietsch, 2012). A recent study in humans demonstrated the presence of VDR mRNA in ovaries including mixed ovarian cells and purified granulosa cells (Parikh et al., 2010). Granulosa cells of the ovary are the site for aromatase conversion of androgens to estrogen, and the presence of the VDR mRNA in this tissue suggests a role of vitamin D in steroidogenesis of sex hormones (Parikh et al., 2010). Kinuta et al., 2000 showed that female VDR null mice had impaired folliculogenesis, uterine hypoplasia and ovarian aromatase activity that were 24% of that in wild-type mice. The VDR null mice had decreased P450 aromatase enzyme activity and decreased expression of the CYP19 gene that encodes the P450 enzyme. P450 aromatase is a key enzyme required in estrogen synthesis from androgens (Kinuta et al., 2000). Calcium supplementation for the hypocalcemia seen in the VDR null mice increased aromatase activity to 60% of the wild type mice and also increased the CYP19 gene expression higher than that seen in the VDR null mice (Kinuta et al., 2000). The results from this study indicate that the action of vitamin D on estrogen biosynthesis is only partially explained through calcium homeostasis, but a direct regulation on aromatase expression cannot be ruled out (Kinuta et al., 2000). A more recent study by

Parikh et al., 2010 demonstrated the presence of VDR in animal and human ovarian tissues. Cultured human ovarian tissues when treated with 1,25-D stimulated estradiol production by 9% ($p<0.02$), estrone production by 21% ($p<0.002$) and progesterone production by 13% ($p<0.001$). When insulin was also added, then insulin and 1,25-D synergistically increased estradiol production by 60% ($p<0.005$). This increase in estrogen production by 1,25-D may be a result of increase in aromatase activity caused by 1,25-D in the human ovary (Parikh et al., 2010). Although most of these studies have been conducted in animal or cell culture models, they suggest a possible role of vitamin D in steroidogenesis.

**Epidemiologic Evidence:** The only epidemiologic study to investigate the association between vitamin D and estrogen and progesterone was conducted by Knight et al., 2010 on young women ages 18 to 30 years. 101 non-contraceptive users were recruited during their luteal phase and provided blood samples and completed demographic and lifestyle questionnaires. After adjusting for age, BMI, ethnicity, season, alcohol use, smoking and physical activity, each 10nmol/L increase in 25-D was associated with a 10% decrease in progesterone (CI 5-14%) and a 3% decrease in estradiol (CI 0-6%) (Knight, Wong, Blackmore, Raboud, & Vieth, 2010). The reduction in estradiol was marginally significant. However this study had limitations in the timing of hormone evaluation as the study subjects provided blood samples during the luteal phase, which may not be significant in terms of fertility since the follicular phase estrogen levels is more relevant in terms of folliculogenesis, ovulation and oocyte quality (Mihm et al., 2011; Sherman & Korenman, 1975; Speroff, 1999).

Menstrual cycles reflect changes in ovarian steroid hormone production, particularly estrogen, which can affect menstrual cycle function (Landgren et al., 1980; Sherman & Korenman, 1975). Studies in humans have shown 1,25-D, the active metabolite of vitamin D, elevated during the menstrual cycle suggesting a possible role for vitamin D during the menstrual cycle (Thys-Jacobs, McMahon, & Bilezikian, 2007). Thys-Jacobs et al., 2007 observed in premenopausal women that 1,25-D levels progressively increased from the follicular phase with mid-cycle levels almost doubling compared to early follicular phase. Similar results were also reported by others and the elevations in 1,25-D corresponded with the estrogen peaks during the menstrual cycle (Thys-Jacobs et al., 2007). It is unclear whether these observations are because estrogen modulates 1,25-D or if 1,25-D has some role to play in the menstrual cycle. Regardless of vitamin D's role, however, controversy still exists concerning the variability of calcium regulating hormones, particularly 1,25-D, during the menstrual cycle.

**Summary:** In summary, the role of vitamin D in fertility and menstrual cycle function is still an evolving subject. Menstrual cycle characteristics of cycle length, pattern and bleed duration are determinants of a woman's reproductive capability (Dishi et al., 2011; T. K. Jensen et al., 1999; Kolstad et al., 1999; Rowland et al., 2002; Small et al., 2010; Small et al., 2006; Steiner et al., 1999; Wise et al., 2011). Menstrual cycle function is a surrogate measure of a woman's fertility, her ability to get pregnant and sustain her pregnancy (T. K. Jensen et al., 1999; Kolstad et al., 1999; Rowland et al., 2002; Small et al., 2010; Small et al., 2006; Wise et al., 2011). There is no known study that directly looked at the role of vitamin D and menstrual cycle function. Menstrual cycle characteristics of length and pattern have seasonal variation and vitamin D primarily synthesized through sunlight

also fluctuates with seasonal changes (Danilenko et al., 2011; Dawson-Hughes, 2001; Norman, 2001; Stumpf & Denny, 1989; Sundararajan et al., 1978). The existence of VDR in the ovaries and the hypothalamus is suggestive of a possible role for vitamin D in these tissues. Furthermore vitamin D can affect aromatase activity an important enzyme in estrogen biosynthesis and expression of CYP19 the gene encoding it (Kinuta et al., 2000). Based on the current evidence it seems that vitamin D may play an important role in endocrine function in premenopausal women. Identifying modifiable nutritional factors that affect menstrual cycle characteristics can be a cost effective way to improve a woman's fecundity and pregnancy outcomes. It is well known that suboptimal vitamin D levels are high in women of nubile age (Yetley, 2008). Improving a woman's vitamin D status is safe and may be effective in improving a woman's reproductive capability. Studies on association of serum vitamin D levels and menstrual cycle characteristics are scarce, and more research is needed to investigate this role further in young premenopausal women. Hence, the current study aims to evaluate the association between serum vitamin D and menstrual cycle characteristics including cycle length, pattern and bleed duration, all of which are important determinants of a woman's reproductive capability in young premenopausal women.

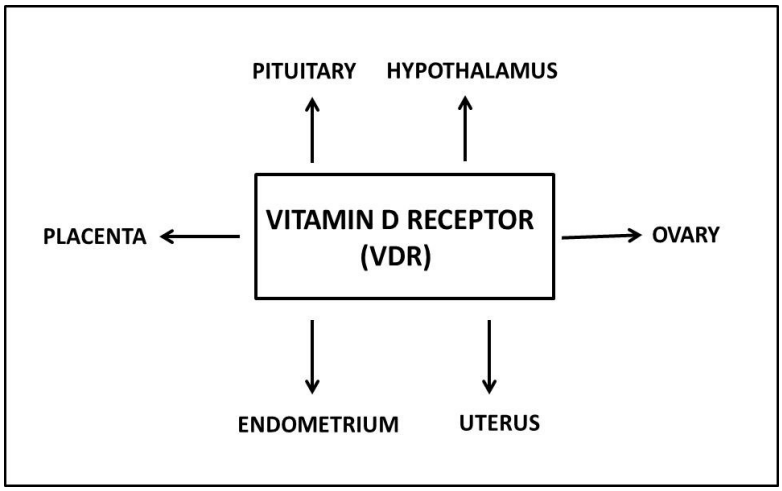


Figure 7 : Vitamin D Receptor (VDR) expressed in various tissues involved with reproduction.

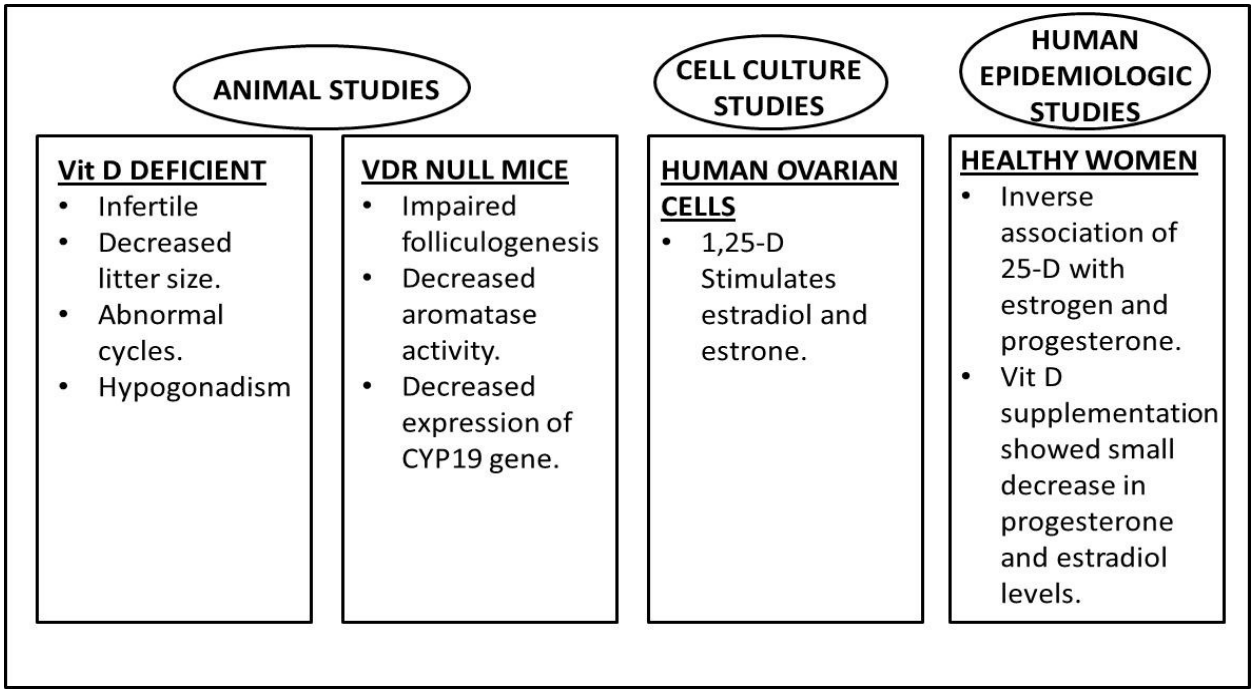


Figure 8: Summary of study results on Vitamin D and reproduction and steroid synthesis.

## 1.4 Dietary Fats

Dietary fats are energy-yielding macronutrients that comprises 33 % of caloric intake in the average US diet (Wright, Wang, Kennedy-Stephenson, & Ervin, 2003). Fats provide 9-kilocalorie energy per gram. Certain dietary fats are essential nutrients required to perform many important functions in the body. Dietary fat can be stored in adipose tissue and can be used as an energy source during malnutrition and starvation. It forms structural fat which surrounds important organs and protects it from injury. Dietary fat stored subcutaneously serves as insulation (Spector, 2000; Sul, 2000). Fats are also required for digestion, absorption and transport of fat soluble vitamins and phytochemicals like lycopene and carotenoids. They form the structural component of the cell membrane which forms functional barriers between the cell and the extracellular surface. Lipids are required for synthesis of hormones including the sex steroid hormones (derived from cholesterol) and eicosanoids, including prostaglandins and leukotrienes derived from polyunsaturated fatty acids (Spector, 2000; Sul, 2000).

95% of the lipids in our diet are from triglycerides also known as fats and oils. The remaining fats from our diet are phospholipids which form 2 % of our diet and a small percentage from sterols. Triglycerides have a glycerol backbone with a fatty acid attached to each of its three glycerol carbons ("Lipids," 2011). The fatty acids are the key building blocks and determine the characteristic of a fat. The fatty acids can vary based on chain length and degree of saturation (Brenna, 2000). The degree of saturation influences the firmness and stability of these fats. Fatty acids are categorized as follows based on degree of saturation:

**Saturated fats** – when there is no double bond between any of the carbon atoms it is called saturated fatty acid. Dietary sources include solid fats like butter, lard, animal fats, coconut oil, and palm kernel oil (Brenna, 2000). The American Dietary Guidelines 2010 recommends saturated fat intake of <10% of total calories. The average American consumes 11% of their total calories from saturated fat (Services, 2010; Wright et al., 2003).

**Unsaturated Fats** – This term describes fatty acids that have one or more double bonds between the carbon atoms. They are further divided based on the number and position of their double bond. Monounsaturated fats (MUFA) contain one carbon-carbon double bond. Dietary sources of MUFA include olive oil and canola oil. Polyunsaturated fats (PUFA) contain two or more carbon-carbon double bonds (Brenna, 2000; Services, 2010). Based on the location of the carbon-carbon double bond from the terminal methyl group, PUFA is further classified as omega-3 fatty acids or omega-6 fatty acids (Brenna, 2000; Spector, 2000). Dietary sources of omega-6 fats include safflower oil, sunflower oil, corn and soybean oil. Dietary sources of omega-3 fatty acids include fish like sardine, salmon, tuna, atlantic mackerel, and plant sources like canola oil, flaxseed oil and walnuts (Services, 2010). Some omega-3 and omega-6 fatty acids are essential fatty acids as the body cannot synthesize them. Humans lack the desaturase enzyme required to synthesize the omega-3 and omega-6 fatty acid and hence they need to be provided from our diet (Spector, 2000). Humans are unable to synthesize the 18- carbon omega-3 fatty acid or the omega-6 fatty acid but they can convert these to the longer chain 20 and 22 carbon fatty acids (Spector, 2000).

**Essential Fatty acids**: In 1929 two scientists demonstrated that omega-6 fatty acid (linoleic acid) deficiency in rats caused cessation of growth, dermatitis, loss of water through skin, fatty liver and loss of reproductive capacity in male and female rats (Spector, 2000). It was later also demonstrated that this fatty acid was essential in humans. The omega-6 (linoleic acid) and the omega-3 (alpha-linolenic acid) polyunsaturated fatty acids are required for 2 major functions. They are required for synthesis of lipid biomediators, such as eicosanoids, and for production of membrane phospholipids with optimal structural properties and signal transduction properties (Spector, 2000).

Omega-6 PUFAs are required for production of eicosanoids. Arachidonic acid (20:4), formed from linoleic acid, is the primary substrate for eicosanoid synthesis (Brenna, 2000; Spector, 2000). Arachidonic acid (AA) is stored in membrane phospholipids, and activation of phospholipase A2 leads to the liberation of arachidonic acid. AA can be converted to eicosanoids by the cycloxygenase, lipoxygenase or the cytochrome P450 pathways present in the cells. The cyclooxygenase (COX) pathway produces prostaglandins (PGs) and thromboxanes (TXs), and the lipoxygenase pathway produces leukotrienes (LTs) (Brenna, 2000). These compounds are lipid biomediators involved in activating intracellular signaling pathways or initiating transcriptional mechanisms. The eicosanoids formed from the COX pathway are involved in reproduction, modulating cardiovascular function, blood coagulation and inflammation (Brenna, 2000; Spector, 2000). The omega-6 fatty acids and the eicosanoids are ligands for peroxisome proliferator activated receptor (PPAR) and they can affect gene expression in lipid metabolism (Spector, 2000). Recent evidence from animal studies has suggested a

possible role for arachidonic acid and its eicosanoid metabolites, such as prostaglandins (PG series), in sex steroid synthesis through various mechanisms (Larsson et al., 2004; Stocco et al., 2005; Wathes et al., 2007).

Omega-3 (alpha-linolenic acid) fatty acids are required for the formation of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are also available from dietary sources. The EPA is also a substrate for eicosanoids; however it is a weak substrate. It competes with arachidonic acid for eicosanoid synthesis (Spector, 2000). Recent studies highlight the role of essential fatty acids in women's reproductive health (C. L. Jensen, 2006; McKeegan & Sturmey, 2011; Wathes et al., 2007). Both essential fatty acids can affect reproductive function through a variety of mechanisms. They may influence sex steroid synthesis through the action of eicosanoids, they may be involved in oocyte development, and they may decrease preterm delivery and reduce risk to preeclampsia (C. L. Jensen, 2006; McKeegan & Sturmey, 2011; Mozurkewich & Klemens, 2012; Wathes et al., 2007).

### 1.4.1 Dietary Fats, Menstrual Cycle Characteristics and Fertility

Diet pattern has been associated with menstrual abnormalities (Baines et al., 2007; Barr, 1999; Griffith & Omar, 2003; Pedersen et al., 1991). For instance, vegetarians are more likely to have irregular menstrual cycles compared to non-vegetarians (Baines et al., 2007; Barr, 1999; Griffith & Omar, 2003; Pedersen et al., 1991). A recent study by Baines et al., 2006 assessed the health status of 9113 vegetarian and semi-vegetarian Australian women ages 22 to 27 years. They found that vegetarians reported higher menstrual irregularities compared to semi-vegetarians (Baines et al.,

2007). In another study by Pederson et al., of 41 non-vegetarian and 34 vegetarian premenopausal Caucasian women, the incidence of menstrual irregularities was 4.9% in non-vegetarians but 26.5% in vegetarian women.

The menstrual abnormalities seen in vegetarians may be attributed to inadequate energy availability, body fat composition or, more importantly, dietary components (Barr, 1999; Pedersen et al., 1991). Vegetarians differ from non-vegetarians in terms of dietary components, particularly in quantity and type of dietary fat (Barr, 1999). Vegetarians also differ in their intake of types of fat particularly of saturated, omega-3 fats and omega-6 fats (Griffith & Omar, 2003; Pedersen et al., 1991).

**Dietary fat and Menstrual Cycle Length and Pattern:** To date, five studies have explored the association between dietary fat and menstrual cycle length and pattern. Three of those studies are intervention studies, one is a prospective cohort study and one is a cross sectional study. These studies used cycle length as a proxy measure of lifetime estrogen exposure, which has been studied as a potential risk factor for breast cancer (Aubertin-Leheudre et al., 2008; Nagata et al., 2006; Wu, Pike, & Stram, 1999). Nagata et al., 2006 examined the role of dietary fat and menstrual cycle length among 341 Japanese women 18 to 29 years of age followed for an average of 3 menstrual cycles. Total fat intake was not associated with menstrual cycle length but PUFA intake was significantly inversely associated with menstrual cycle length. After controlling for covariates such as age, energy and duration of the study period, women in the highest quintile of PUFA intake had shorter cycle length compared to women in the lowest quintile of PUFA intake (31d vs. 33d; p for trend= 0.03). However the study did not

adjust for fiber or soy intake although both have been associated with menstrual cycle length (Nagata, Takatsuka, Kawakami, & Shimizu, 2000). Since the study population was Japanese women, their soy intake would likely be greater than that of non-Asian populations.

Kato et al., 1999 studied epidemiologic correlates with menstrual cycle length in 4900 premenopausal women under age 45 years from the New York University Women's Health Study. They found that after adjusting for age, BMI, and number of cigarettes smoked, risk of irregular cycles was marginally associated with total dietary fat intake (OR-1.14; $p < 0.05$). However, the study population consisted of mainly middle aged women (mean age was 40y) who attended a mammographic screening (Kato et al., 1999). The age of the women would limit the generalizability of this study as these women were self-selected and may already have ongoing hormonal changes that may affect their menstrual cycle characteristics.

Bagga et al., 1995 followed 12 Caucasian women average age of 33yrs for a 3-month intervention study (Bagga et al., 1995). The women had a high-fat diet with 30% energy from dietary fat with 15 to 25 g fiber for the first month, followed by a low-fat diet of 10% energy from fat and 25 to 35g fiber for the next 2 months. They measured the change in menstrual cycle length as well as the change in serum estrogen levels by collecting blood samples during the diet intervention period. They found that at the end of the low-fat diet period cycle length decreased from baseline by 3 days and estrogen levels (E1 and E2) decreased significantly in both the follicular and luteal phases. Furthermore, the change in the cycle length was reflected in the follicular phase length; the luteal phase length remained the same. However, this study had a small sample size

with only 12 women (Bagga et al., 1995). Jones et al., 1987 reported a significant increase in cycle length at the end of 2 months of a low fat diet (Jones, Judd, Taylor, Campbell, & Nair, 1987). Reichman et al., 1992 conducted an intervention study on 31 healthy women ages 20 to 40 years for nine menstrual cycles. They assessed the effect of dietary fat on the length of the follicular phase of the menstrual cycle. It is known that variations in cycle length are reflected by changes in follicular phase length and luteal phase remains the same (Reichman et al., 1992). The women were put on a high fat diet (40% energy from fat) for 4 menstrual cycles after a one month baseline period of free-living followed by 4 menstrual cycles of low fat diet (20% energy from fat). The women were randomized into one of 2 groups of polyunsaturated fat/saturated fat ratio of either 1 or 0.3. There was a significant increase in length of the follicular phase when women consumed the low-fat diet as compared to when they consumed a high fat diet ($p<0.006$). The average increase in length of follicular phase was 1.9 days. There was no difference in average follicular phase length seen between the two P/S groups (Reichman et al., 1992).

The existing studies of dietary fat intake and menstrual cycle length or regularity have several research gaps in terms of sample size, generalizability, and age, and the results are inconclusive. The studies conducted so far have focused on menstrual cycle length as a proxy measure of lifetime estrogen exposure which is a risk factor to breast cancer. Hence the women in these studies have a higher age group and are not representative of the reproductive age group. Variations in cycle pattern and cycle length reflect underlying endocrine fluctuations of the hypothalamic-pituitary-ovarian (HPO)

axis, particularly related to estrogens (Landgren et al., 1980; Sherman & Korenman, 1975).

**Dietary fats and Estrogen:** Several studies that have explored the association between dietary fats and circulating estrogens suggest that a low-fat diet can decrease circulating serum estrogens (Turner, 2011). Wu et al., 1999 conducted a meta-analysis of ten dietary fat intervention studies in premenopausal women. A pooled estimate of change following ten dietary interventions in estradiol levels was -6.7% (95%CI -11.1% to -2.1%) in premenopausal women. The percent decrease in estradiol concentrations was greatest in the study in which dietary fat intake was as low as 12%. The change in estradiol was independent of participant's body weight as their weight did not change during the study period. The intervention studies with low fat diet also had participants with a higher fiber intake (Wu et al., 1999). Fiber intake is inversely associated with serum estradiol levels due to increased intestinal excretion of estrogen metabolites. Fiber can bind with estrogen increasing its fecal excretion and reducing the reabsorption of estrogen metabolites back into the enterohepatic circulation (Das, 2008; Forman, 2007). However, estradiol reductions were seen in studies having low (<20g), medium (21 to 29g), or high (>30g) fiber intake in this meta-analysis study, suggesting that fat may play a more important role than fiber alone. There were only 3 studies in the meta-analysis that had a very high fiber intake of 40g the others were <28g fiber.

Furthermore, Goldin et al., 1988 evaluated separate effects of fat and fiber and they found that fat reduction was associated with higher free estradiol and lower estrone; fiber intake was associated only with reduced total estradiol (Goldin & Gorbach, 1988). The reductions in estrogen by fiber may be attributed to a specific type of fiber,

particularly insoluble fibers found in whole grains such as wheat, and bran. It is likely that some of the reductions seen in estradiol could be attributed to the fiber intake. An important limitation was the timing of collection of blood for estrogen measurement. Some studies timed their blood collection during the follicular phase while others did not. This is important as estrogen levels vary based on the phase of the menstrual cycle (Mihm et al., 2011; Speroff, 1999). This could lead to misclassification of the outcome causing a bias toward the null.

A recent study by Aubertin-Leheudre et al., 2008 showed that total dietary fat, saturated fat and MUFA were significantly correlated with plasma estrogens (estradiol and estrone) as well as urinary estrogens after adjusting for dietary fiber in 50 premenopausal women. Pearson's correlation coefficient for dietary fat and plasma E1 (estrone) was 0.447, E2 (estradiol) was 0.432, E1+E2 was 0.432 and total urinary estrogens were 0.563 after adjusting for total fiber intake (Aubertin-Leheudre et al., 2008).

Another study by Nagata et al., 2000 found a significant positive association between total fat, MUFA and plasma estrone levels in the follicular phase in premenopausal women (Nagata et al., 2006). Although several studies showed positive associations between dietary fats and estrogen, other studies have found no association. A recent cross-sectional study by Cui et al., 2010 followed 595 premenopausal women (mean age 43y) to investigate the association of dietary fat, carbohydrates and fiber on endogenous hormone levels. No significant association was observed between total fat, saturated, monounsaturated or animal fat, fiber or carbohydrates and estrogen, androgen or SHBG (Cui, Rosner, Willett, & Hankinson, 2010). However, this study also had

women in the older age group (43 years) who would have underlying endocrine hormonal fluctuations seen with age and are not representative of the ideal reproductive age.

Several research gaps exist in the studies focusing on dietary fat and menstrual cycle length as well as in those focusing on fat and estrogen levels. These studies were conducted to assess breast cancer risk and the women were older (>35 years of age) and not representative of women of reproductive age. Age can affect estrogen levels as well as a woman's menstrual cycle characteristics (Chiazze et al., 1968; Harlow & Ephross, 1995; Treloar et al., 1967; Vollman, 1977). Further, there are generalizability issues, the results are still inconclusive and studies have not adequately explored roles of the various types of fat, particularly omega-3 fats and omega-6 fats and their ratio. The results could be confounded by fiber intake as most of these studies were low fat high fiber intervention studies. Furthermore menstrual cycle length was the only cycle characteristic studied; menstrual cycle pattern and bleed duration have not be adequately explored.

**Biological mechanism of action of fatty acids on sex steroid synthesis:** High fat diet is associated with altered beta-glucuronidase activity, decreased excretion of estrogen metabolites and increased reuse of estrogen metabolites. This also results in increased circulation of total estrogen (Goldin & Gorbach, 1988). However, animal studies have proposed other mechanisms by which certain types of fat can affect menstrual function by influencing steroid synthesis (Wathes et al., 2007). There is evidence highlighting mechanisms by which PUFAs, particularly omega-3 and omega-6 fatty acids, and their metabolites can directly or indirectly influence pathways involved in steroidogenesis (Das, 2008; "Hormone Biosynthesis, Metabolism, and Mechanism of Action," 1999;

Larsson et al., 2004; Spector, 2000; Stocco et al., 2005; Wathes et al., 2007). Some of the proposed mechanisms include regulation of genes involved in sex steroid synthesis (Stocco et al., 2005; Wathes et al., 2007).

Arachidonic acid (AA) derived from dietary omega-6 fatty acids and its metabolites can influence steroidogenesis directly through the steroid acute regulator protein (STAR) (Stocco et al., 2005; Wathes et al., 2007). STAR is a protein involved in the rate limiting step in acute steroid synthesis ("Hormone Biosynthesis, Metabolism, and Mechanism of Action," 1999). Cholesterol is the starting molecule in steroidogenesis and STAR increases steroid production by mediating cholesterol transfer into the mitochondria of adrenal glands and the gonads (Speroff, 1999; Stocco et al., 2005). The tropic hormone-based release of arachidonic acid and its metabolites are involved in the expression of STAR protein and steroid synthesis (Stocco et al., 2005; Wathes et al., 2007).

Alterations in the omega-6 to omega-3 ratios of PUFA in the diet can affect the cell membrane phospholipid composition. Arachidonic acid a metabolite of omega-6 and eicosapentaenoic acid (EPA) is a metabolite of omega-3 fatty acid are stored in cell membranes and are substrates for prostaglandin synthesis. EPA is also available from dietary sources. The proportion of these will affect the type of prostaglandins synthesized as they compete for the enzymes involved in their metabolism (Larsson et al., 2004; Spector, 2000; Wathes et al., 2007). This can affect steroid synthesis (Larsson et al., 2004; Stocco et al., 2005; Wathes et al., 2007). Dietary intake of PUFAs can also affect prostaglandin production by affecting the expression and cellular concentrations of the various enzymes involved (Wathes et al., 2007).

The PGE3 series of prostaglandins derived from arachidonic acid has been shown to stimulate aromatase P450 activity. However the PGE2 series of prostaglandins derived from EPA does not stimulate aromatase P450 activity (Larsson et al., 2004; Stocco et al., 2005). Alterations in the ratio of omega-6 to omega-3 fatty acid may play a role in regulating estrogen production through AA or EPA and its respective prostaglandin series (Larsson et al., 2004). However, no known studies have directly explored this association in humans.

Studies conducted in rats have shown that omega-3 fatty acids can decrease the concentration and activity of HMGCoA reductase, the enzyme that catalyzes the synthesis of mevalonate, which is the rate-limiting step for cholesterol biosynthesis (Das, 2008; Larsson et al., 2004). HMGCoA reductase inhibitors, such as the medications, statin are used to treat hypercholesterolemia. However, supplementation with omega-3 fatty acid is not known to decrease cholesterol (Larsson et al., 2004; Spector, 2000). PUFAs can also affect other transcription factors such as peroxisome proliferator-activated receptors (PPARs). PPARs are activated by PUFAs, which are endogenous ligands for it. PPARs can affect gonadal steroid synthesis (Wathes et al., 2007).

**Summary:** Estrogen is of critical importance in menstrual function, fecundity and reproductive function. Some evidence suggests dietary fats can affect estrogen levels as well as menstrual cycle length and pattern (Baines et al., 2007; Barr, 1999; Kato et al., 1999; Nagata et al., 2006; Pedersen et al., 1991; Wu et al., 1999). However study results are inconclusive and this role has not been explored in younger premenopausal women. The role of different type of fats on estrogen and menstrual cycle characteristics is also unclear. Some studies suggest dietary fats can have different effects based on the

reproductive stage of a woman (Hilakivi-Clarke et al., 2002; Ronco, De Stefani, & Stoll, 2010; Wronska & Kmiec, 2012). It is important to study the role of dietary fats during the reproductive age. Existing literature from animal studies has highlighted a biological role for PUFAs, particularly omega-6 and omega-3 fatty acids, in steroid synthesis through various pathways (Das, 2008; Larsson et al., 2004; Stocco et al., 2005; Wathes et al., 2007). However, no human studies have adequately explored this role. Menstrual cycle length, pattern and bleed duration can be used as important determinants of a woman's reproductive capability. Variation in menstrual cycle characteristics reflects underlying endocrine fluctuations, particularly of estrogen (Landgren et al., 1980; Sherman & Korenman, 1975). Identifying modifiable nutritional factors that affect menstrual cycle characteristics can be a cost effective way to improve a woman's reproductive success and pregnancy outcomes. The current study will assess the role of various types of dietary fat (total, saturated, omega-3 and omega-6 fatty acids) and their association with menstrual cycle characteristics including cycle length, pattern and bleed duration in young premenopausal women.

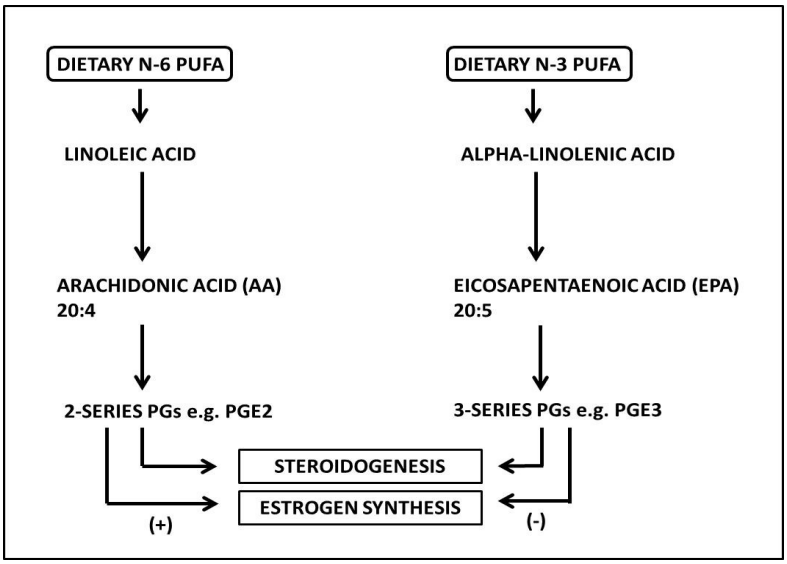


Figure 9 : Dietary PUFA and Steroidogenesis.

PGs – prostaglandins.

## 1.5 References


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# CHAPTER 2

# MENSTRUAL CYCLE CHARACTERISTICS ARE ASSOCIATED WITH ADIPOSITY IN YOUNG WOMEN

## 2.1 ABSTRACT

**Background**: Excess body fat, particularly regional fat, influences sex steroid levels and has been adversely associated with menstrual cycle characteristics. Abnormal menstrual cycle characteristics are associated with reduced fecundity. Previous studies examining these associations have assessed adiposity using proxy measures, such as body mass index (BMI), which may not accurately estimate body fat. The current cross-sectional study evaluates the association of three menstrual cycle characteristics (cycle pattern, cycle length and bleed days) with estimates of body fat and body fat distribution obtained through duel-energy x-ray absorptiometry (DXA) in young women.

**Methods**: The study subjects were a subset of 164 18- to 30-year-old women participants in the UMass Vitamin D Status Study who reported no use of hormonal contraceptives. Total body fat (TBF) and regional body fat, including gynoid fat (GF) and android fat (AF), were measured using DXA; information on menstrual cycle characteristics (cycle pattern, length and bleed days) was obtained through a self-reported questionnaire.

**Results**: In multivariable logistic regression analyses, the odds of having an irregular cycle pattern were three time higher in women with ≤ 32% TBF [OR=3.1 95% CI (0.9 to 10.2) p=0.07] compared to those with higher levels of body fat. When DXA estimates of

body fat distribution were considered, the odds of an irregular cycle pattern were 2.8 times higher in women with ≤ 41% gynoid fat [OR=2.8 95% CI(0.9, 8.6) p=0.07]. In addition, a one standard deviation increase in G/A (gynoid/android) fat mass tended to be associated with a 50% decreased odds of having an irregular cycle pattern [OR=0.45 95% CI (0.21, 1.1) p=0.07]. BMI and WC were not associated with menstrual cycle pattern, and cycle length was not associated with any measures of adiposity.

**Conclusion:** Our findings suggest that a certain level of overall body fatness—and particularly lower body adiposity (GF% and G/A)—are associated with optimal menstrual function.

## 2.2 INTRODUCTION

Nearly two-thirds of U.S. women of reproductive age are overweight or obese (Flegal et al., 2012; Ogden, 2012). Excess body fat influences sex steroid levels and has been adversely associated with menstrual cycle length and pattern (Brewer & Balen, 2010; Douchi et al., 2002; Hartz et al., 1979; Rich-Edwards et al., 1994). Evidence from epidemiologic studies suggests abnormal menstrual cycle characteristics are associated with reduced fecundity and increased risk of miscarriage and gestational diabetes, possibly due to altered endocrine function (Dishi et al., 2011; T. K. Jensen et al., 1999; Kolstad et al., 1999; Rowland et al., 2002; Small et al., 2010; Small et al., 2006; Steiner et al., 1999; Wise et al., 2011). These observations suggest that one mechanism through which excess adiposity may influence reproductive success is by altering menstrual characteristics.

The menstrual cycle involves a complex interplay of steroid hormones regulated by the hypothalamus-pituitary-ovarian (HPO) axis (Mihm et al., 2011; Owen, 1975; Speroff, 1999). Menstrual cycle characteristics, including length and regularity, vary considerably within women at various ages. Much of the variability in menstrual cycle characteristics reflects underlying endocrine fluctuations, particularly of estrogen synthesis (Landgren et al., 1980; Sherman & Korenman, 1975).

Some evidence indicates that the pattern of body fat distribution may play an important role in menstrual function. Adiposity, particularly central—or visceral—adiposity, is associated with abnormal menstrual function and an adverse hormonal profile (Balen et al., 2007; Brewer & Balen, 2010; Pasquali, 2006; Pasquali et al., 2003). Visceral adiposity, which has also been termed "android" fat because it predominates in male obesity, contrasts with "gynoid" fat, which is more typical of female obesity and refers to concentration of fat tissue in the hips and upper thighs (Karastergiou, Smith, Greenberg, & Fried, 2012; Novotny, 2012). Visceral fat can lead to regulatory disturbances that increase the pool of sex steroid hormones, decrease sex-hormone binding globulin (SHBG) levels and interfere with hormone delivery to target organs (Brewer & Balen, 2010).

Previous studies assessing the role of body fat and menstrual cycle characteristics have assessed adiposity using proxy measures, such as body mass index (BMI), waist circumference or waist-to-hip ratio (Chang et al., 2009; Hartz et al., 1979). Because these measures may fail to accurately estimate body fat (Flegal et al., 2009), studies relying on these estimates may mis-characterize the association of body fat with menstrual cycle characteristics. To overcome this shortcoming, we assessed adiposity

using dual energy x-ray absorptiometry (DXA), which is an accurate, validated measure of total percentage body fat (Bolanowski & Nilsson, 2001; Haarbo, Gotfredsen, Hassager, & Christiansen, 1991). Recently, algorithms have been developed that allow estimation of regional fat depots (gynoid and android) using data from DXA scans (Zhang et al., 2007). Use of DXA measurements to estimate total body fat and the relative proportion of android and gynoid fat tissue improves our estimates of adiposity and thus helps to clarify its relationship with menstrual cycle characteristics.

The current study evaluates the association of three menstrual cycle characteristics (cycle pattern, cycle length and bleed days) with DXA estimates of body fat and body fat distribution in young women participating in the UMass Vitamin D Status Study (Bertone-Johnson et al., 2010).

## 2.3 METHODS

### 2.3.1 Study Design, Study Setting and Population

This study uses data collected as part of the UMass Vitamin D Status Study, a cross-sectional study designed to assess vitamin D status in young women and to identify its dietary, environmental and lifestyle determinants. The current analysis examines the association between body fat and menstrual cycle length, menstrual pattern regularity and number of bleeding days.

The UMass Vitamin D Status Study is based at the University of Massachusetts, Amherst. The study subjects consisted of 288 female students of all ethnicities between 18 to 30 years of age. Women on campus were recruited through brochures distributed at various classes and displayed on bulletin boards in various departments in the university

and through references from women who participated in the study. Information about women was collected at one study visit during the mid-to-late luteal phase of their menstrual cycle. During the study visit, the participant completed a study questionnaire that collected information on demographics, health history, and menstrual symptoms. A trained research assistant measured anthropometric information, and after completing appropriate training, completed DXA scans in the DXA facility at the university health services.

Women were ineligible for the study if they were currently pregnant or not menstruating, currently reported experiencing depression, had high blood pressure, kidney or liver disease, bone diseases, rheumatologic diseases, multiple sclerosis, cancer, diabetes, hyperparathyroidism, hyperlipidemia, polycystic ovaries, or chronic digestive disorders, or if they were taking medications such as corticosteroids (e.g. prednisone; anabolic steroids), anticonvulsants (e.g. Depakote), Tagamet or propranolol. Women with the above conditions were excluded from participating in the study as each of the conditions or medications could potentially influence vitamin D status. In addition, women using oral contraceptives (OC) have regular cycles due to the hormonal content of the pill, which is not indicative of the real nature of their menstrual cycle before OC use. For these analyses, therefore, we excluded women using oral or other hormonal contraceptives (n=124).

### 2.3.2 Adiposity

Three measures of adiposity, including BMI, percent total body fat (%TBF) and waist circumference (WC), were considered exposure variables. Because this is a cross-

sectional study, we cannot establish a temporal relationship. However, underlying biology suggests that menstrual cycles are affected by adiposity and not the reverse. Anthropometric measures were collected during a single clinic study visit. Weight was measured using a calibrated scale, and height was measured using a stadiometer. BMI was calculated as weight in kilograms divided by the square of height in meters. Waist circumference was measured at the umbilicus using a standard tape. Percent total body fat (%TBF) was measured by DXA using the whole body scan mode on a narrow angle fan GE Lunar Prodigy scanner (GE Lunar Corp., Madison, WI). The scan was performed with the subject lying in a supine position with her arms and legs positioned as per the manufacturer's specifications. The same technician completed all DXA measurements. The machine was calibrated daily using the standard calibration method provided by the manufacturer. The coefficient of variation for repeated scans of the manufacturer's phantom is less than 1% (Zagarins, Ronnenberg, Gehlbach, Lin, & Bertone-Johnson, 2012). For DXA regional fat analysis, the software automatically demarcates the boundaries for the android and gynoid region of interest (ROI). The android ROI, which is an estimate of visceral adiposity, was defined as the area between the ribs and the pelvis. The gynoid ROI includes the hips and upper thigh (Zhang et al., 2007). Body fat distribution was measured as android fat percentage (AF%) and gynoid fat percentage (GF%) using DXA scan measurements. The fat percentage was defined as the ratio of fat mass in the specified region divided by the tissue mass (fat mass+lean mass) of the specified region.

### 2.3.3 Menstrual Cycle Characteristics

In statistical models, menstrual cycle length, pattern and bleed duration were modeled as the independent outcome variables, as explained previously. Information on menstrual cycle characteristics was collected through a self-reported study questionnaire with questions similar to the Nurses' Health Study 2. Menstrual cycle length categories were: <21 days, 21 to 25 days, 26 to 31 days, 32 to 39 days, 40 to 50 days, >50 days, too irregular to count. To ensure adequate sample sizes for modeling, we collapsed these categories to ≤25 days (short), 26 to 31 days (typical) and ≥ 32 days (long). Menstrual cycle variability information was collected through a cycle pattern question with the following categories: extremely regular (no more than 1 to 2 days before or after expected), very regular (within 3-4 days), regular (within 5-7 days), usually irregular and always irregular. Cycle pattern was collapsed to regular (for ≤7 day variation) and irregular (for > 7 day variation). Bleed duration information was collected under the following four categories: < =3 days, 4-5 days, 6-7 days, ≥ 8 days. For statistical analyses, we collapsed bleed duration into three categories: ≤ 3 days (short), 4 to 5 days (regular), > 5 days (long). For these analyses, we excluded women using oral or other hormonal contraceptives (n=124). In our cohort of women, OC users differed significantly from non- users (n=164) only with respect to their menstrual cycle characteristics. The current analysis includes 164 women.

### 2.3.4 Covariate Assessment

Information on covariates was collected through the clinic study questionnaire and through anthropometric measures assessed during the study visit. Information concerning smoking, parity, and age at menarche was collected through self-reported

entry in the study questionnaire (Liu, Gold, Lasley, & Johnson, 2004). Physical activity can affect menstrual cycle function (Orio et al., 2013) and information on participant's physical activity was assessed in the study questionnaire. Participants reported their average weekly physical activity using a modified version of the Nurses' Health Study II questionnaire (Wolf et al., 1994; Zagarins et al., 2012). Activities included walking, jogging, running, bicycling, aerobics/dance/rowing machine, tennis/racket sports, swimming, yoga/pilates, and weight training/resistance exercises. The responses ranged from 0 to 11 or more hours per week. Metabolic equivalents (MET) scores were assigned to each activity using the method used by Ainsworth et al., 2000 (Ainsworth et al., 2000).

### 2.3.5 Statistical Analysis

All statistical analyses were conducted using IBM SPSS version 22. The characteristics of the study population were reported using means, standard deviations, and ranges for continuous variables (physical activity score, BMI, body fat percentages, waist circumference and years since menarche) and counts and percentages for categorical variables (menstrual cycle length, cycle pattern, bleed duration, age at menarche, oral contraceptive use, smoking status, BMI, body fat percent and waist circumference). We examined the distribution of continuous outcome variables to assess their normality; all were reasonably approximately normally distributed.

Bivariate analyses were conducted to evaluate potential confounders by cross tabulating covariates against menstrual cycle characteristics and measures of adiposity. Differences in means were assessed using t-tests and ANOVA, and differences in

proportions were assessed using Chi square tests and Fisher's exact test for cells with small frequencies.

Univariable linear regression analyses were conducted to provide unadjusted associations between the various menstrual cycle characteristics and DXA-derived body fat variables, including % total body fat (%TBF), gynoid fat percentage (GF%), android fat percentage (AF%) and gynoid/android ratio (G/A ratio). Variables that were significantly associated with the measures of adiposity were evaluated for inclusion in the multivariable models. Multivariable linear regression analysis was used to study the association of the menstrual cycle pattern, bleed duration and cycle length and markers of body fat and body fat distribution, controlling for those covariates that caused a 10% change in the coefficient for body fat. Age and smoking were included in the model a priori. We calculated beta coefficients and 95% confidence intervals. We considered a $p < 0.05$ to be statistically significant.

We conducted logistic regression and estimated odds ratios and 95% confidence intervals to study the association of body fat estimates with menstrual cycle pattern. We tested several models with various covariates, including physical activity, age at menarche, years since menarche; our fully adjusted final model included covariates that caused a 10% change in the estimates for menstrual cycle pattern. We considered $p< 0.05$ to be statistically significant.

## 2.4 RESULTS

### *Subject Characteristics*

Descriptive characteristics of the 164 eligible women from the UMass Vitamin D Study are shown in **Table 2.1**. The mean (± SD) age of this young adult group was 21.5 (± 3.1) years. The average BMI was 23.1 (± 3.3) kg/m$^2$, which is within the recommended normal BMI range (18.5-24.9 kg/m$^2$), as was the mean waist circumference (30.7 ± 3.6 inches). Overall, the mean level of physical activity of the group (51.5 ± 46.1 METs/week), calculated from subject estimates of type and duration of daily activities, was considered moderate by government guidelines ("Physical activity guidelines for Americans," 2008).

According to standard BMI cut-off values (Flegal et al., 2012), approximately 20% of women were considered overweight (BMI 25-29.9 kg/m$^2$) and 6% were obese (BMI ≥ 30 kg/m$^2$). However, total body fat—as measured by DXA—indicated that the mean %TBF in this group was 32.1% (±8.1%), which is higher than the recommended level of < 32%, and 45% of women were classified as obese based on %TBF > 32 (Pi-Sunyer, 2000). Estimates of the average relative distribution of body fat between gynoid (hips and thighs) and android (mid-trunk) fat depots in these subjects was calculated from DXA measurements as 40.8% gynoid (GF) and 34.7% android (AF), and the ratio of gynoid to android fat mass (G/A) was 3.4 (±1.1)g [**Table 2.1**]. Irregular menstrual cycle pattern, cycle length and number of bleed days were reported by 11.6%, 40.2% and 38.6% of the women, respectively (**Table 2.1**).

In bivariate analyses, greater relative body fat and a more gynoid fat distribution pattern were associated with a more regular menstrual cycle. Both %TBF and GF% were lower in women with an irregular menstrual cycle pattern than in those with a regular pattern (**Table 2.2**). Women whose %TBF was less than 32—the cut-off for obesity—

were nearly three times as likely to report irregular cycles (16.3% vs. 5.6%; p<0.03) than women with a %TBF of 32 or above. No other measures of adiposity (BMI, WC, AF%, G/A) were significantly associated with menstrual cycle pattern. No adiposity measures were associated with menstrual cycle length (**Table 2.2a**). Mean BMI and WC were lower in women with shorter or longer bleed days (**Table 2.2a**).

In addition to percent body fat measures, other subject characteristics were associated with menstrual cycle pattern. Women with irregular cycle patterns were twice as likely to have started menstruating after age 12 (16.7% vs. 7.0%; p<0.05) and reported fewer years since menarche (YSM: 7.1y vs. 9.8y; p< 0.01). Moreover, women who reported a later age at menarche also had a lower mean %TBF (30.6 vs. 33.3 %TBF; *p< 0.04);* GF % (39.6% vs. 41.7% GF; *p< 0.04)* and a tendency to lower AF% (32.9 vs. 36.2 % AF; *p< 0.06)*. Menstrual cycle pattern was not associated with age, height, physical activity or smoking status. Among covariates, only height differed significantly across categories of menstrual cycle length (Table 2.2c).

**Menstrual Cycle Characteristics**

***Menstrual Cycle Pattern:***

To evaluate the impact of potential covariates on the association of adiposity measures with menstrual cycle pattern, we developed unadjusted and adjusted linear regression models (Table 2.3a). In both the unadjusted models and model 1 (adjusted for age and smoking), %TBF and GF% were inversely associated with irregular cycle pattern, although these associations failed to reach statistical significance (p < 0.07). These associations were attenuated, however, in the fully adjusted model (Model 2,

adjusting for physical activity, age at menarche, years since menarche and smoking status) and were no longer statistically significant. However, although the G/A ratio was not associated with cycle pattern in the unadjusted model, it was inversely associated with cycle pattern in both adjusted models. AF% was not associated with menstrual cycle pattern (or any other menstrual cycle characteristics) in unadjusted or adjusted linear regression models.

In logistic regression analyses, the odds of having an irregular cycle pattern were more than three times higher in women with ≤ 32% TBF compared to women with higher levels of adiposity, although this association was no longer statistically significant in model 2 [OR=3.1 95% CI (0.9 to 10.2) p=0.07]. When DXA estimates of body fat distribution were considered, the risk of irregular cycle pattern tended to be 2.8 times higher in women with ≤ 41% gynoid fat. Additionally, one standard deviation increase in G/A fat mass ratio was marginally associated with a 50% lower odds of having irregular cycle pattern in model 1 and fully adjusted model 2. BMI, WC and %AF were not associated with irregular cycle pattern (table 2.3c).

***Menstrual Cycle Length:*** Menstrual cycle length is the interval from the first day of one menses to the start of the next menses. A short or long cycle may indicate an abnormal hormonal feedback mechanism, which may affect menstrual phase lengths and, in turn, ovulation and the quality of the oocyte (Fehring, Schneider, and Raviele, 2006; Ouyang et al., 2007; Windham et al., 2002). Although %TBF tended to be associated with menstrual cycle length in the unadjusted model and in Model 1, additional adjustments for physical activity, age at menarche, years since menarche and smoking status (Model 2) weakened the association further (Table 2.3a).

***Menstrual Cycle Bleed Days:*** Bleed days indicate the duration of menstrual bleeding and reflect endometrial development, which is mediated by the ovarian hormones (Vitzthum, 2009). In all unadjusted and adjusted linear regression models, both BMI and WC were significantly associated with both short and long number of bleed days (Table 2.2b). The number of menstrual cycle bleed days was not significantly associated with %TBF, GF%, AF%, or G/A fat mass ratio (Table 2.3a).

## 2.5 DISCUSSION

In the current analysis, measures of body fatness and fat distribution were associated with menstrual cycle pattern and the number of menstrual bleed days. Irregular cycle pattern was associated with low %TBF. When DXA estimates of body fat distribution were considered, low GF% and low G/A fat mass ratio were associated with irregular cycle pattern. However, BMI or WC measures were not associated with irregular cycle pattern. The observed association of cycle pattern and gynoid fat appear to be biologically plausible because adipose tissue is an active endocrine organ involved in sex hormone synthesis, particularly estrogen synthesis, and is a site for aromatase activity, which converts androgen to estrogen (Brewer & Balen, 2010). In particular, fat depots of gynoid type have higher aromatase activity, which may play a role in regulating menstrual function (Simpson, 2000). Our findings suggest that lower estimates of body fat and gynoid fat (%TBF, GF%, and G/A ratio) tended to be associated with increased odds of irregular cycle pattern, but proxy measures of body fat, such as BMI and WC was not. These findings indicate that a certain level of overall body fatness may be needed

for ovarian function and that the level of gynoid fat may be particularly important for cycle regularity.

Our study population was a sample of young (18-30 years of age), healthy, mostly nonsmoking women. When BMI was used to classify adiposity, few (4%) in our cohort were considered obese (BMI $\geq$ 30 kg/m$^2$), but when obesity was defined as total percent body fat $\geq$ 32% TBF, 42.8% of participants were classified as obese. This discrepancy between BMI and %TBF classifications highlights the importance of selecting an accurate measure of adiposity. Findings from previous studies have highlighted the inadequacy of BMI to estimate adiposity. In a study by Kennedy et al., one-third of the women were misclassified by BMI criteria compared to %TBF from DXA. The rate of misclassification was higher in females under 40 years of age, leading the researchers to conclude that BMI inaccurately predicted true adiposity, particularly in normal weight and obese women (Kennedy et al., 2009).

Although we found that irregular cycle pattern was associated with low %TBF, it was not associated with BMI or WC. This disparity underscores the inadequacy of BMI as a proxy measure of fat mass, which may underestimate the fat mass in this cohort of young premenopausal women. Most existing studies that have investigated these associations have used BMI or WC as a measure of adiposity (Chang et al., 2009; Rich-Edwards et al., 1994). Although they found abnormal cycle pattern to be associated with higher BMI, in our study population, BMI did not adequately identify women at risk of irregular menstrual patterns when compared to DXA measures of body fat. Our study is one of the few that have used DXA estimates of body fat, which is considered a more accurate measure of fat mass than BMI (Flegal et al., 2009).

Our DXA measures of body fat distribution indicated that higher GF%, which refers to fat percentage in the hips and thigh region, was associated with more regular cycle patterns. In contrast, we found no significant association with AF%, a DXA estimate for abdominal fat. To our knowledge, only one other study used DXA measures to evaluate the association of body fat with menstrual cycle pattern (Douchi et al., 2002). The women in the Douchi et al. study had a BMI > 25kg/$m^2$ and were divided into two groups: women with menstrual disorders (included oligomenorrhea and amenorrhea) and controls without menstrual disorders. They found that women who had menstrual disorders had significantly higher trunk fat mass and trunk-leg fat ratio. They found no association with percent body fat or leg fat mass (Douchi et al., 2002). Although these findings suggest that upper body adiposity is associated with menstrual disorders, the use of trunk fat mass is not considered an accurate measure of abdominal fat as it estimates fat mass from the chest, abdomen and pelvic region and also estimates the subcutaneous adipose tissue from the chest, back and breasts (Zillikens et al., 2010). Unlike Douchi et al., we used a more focused DXA estimate for abdominal fat (Zillikens et al., 2010) that did not include chest fat, and we did not find any significant association with AF% (a measure of "android" or abdominal body fat). The reason for these different observations is unknown. However, our cohort was a younger group of women who had a narrow range of AF%, which may explain our different findings.

One well-known factor associated with menstrual dysfunction is low body fat. This association has been studied in the context of menstrual dysfunction and eating disorders (Poyastro Pinheiro et al., 2007; Vyver, Steinegger, & Katzman, 2008). For example, Pinheiro et al., found that women with amenorrhea and oligomenorrhea

(infrequent cycles) had a significantly lower lifetime BMI than those with regular menstrual function (Poyastro Pinheiro et al., 2007). Brown et al., also reported that women with BMI <18.5 kg/$m^2$ were more likely to have irregular cycles (Brown et al., 2000). We also found that irregular cycle pattern was associated with low body fat, and our study was unique in finding that a low gynoid body fat distribution pattern was associated with irregular cycles.

Adipose tissue is an extragonadal site for estrogen secretion and accounts for around one-third of circulating estrogen in premenopausal women (Frisch, 1996). Estrogen plays an important role in menstrual function and hence women with low body fat may experience menstrual cycle pattern abnormalities. Furthermore, a minimum fat mass is required for the onset of menarche. Studies suggest a threshold of 17% body fat is required for the onset of menarche and a minimum weight representing 22 % body fat is required for maintenance of menses in women over 16 years (Frisch, 1996; Vyver et al., 2008). It is likely that in order to maintain regular cycle characteristics women may require an optimal or threshold body fat percent and distribution. The amount of body fat can determine estrogen metabolism. Lean women have higher amounts of the 2-hydroxylated form of estrogen, which is a weak form of estrogen with little affinity for the estrogen receptor. In contrast, obese women have higher levels of the more potent 16-hydroxylated form of estrogen, which has higher affinity for the estrogen receptor and has potent estrogenic activity (Frisch, 1996).

Gynoid fat depots—those in the buttocks and thigh—have higher aromatase activity and may play a role in regulating menstrual function by affecting estrogen production (Simpson, 2000). We found that irregular menstrual cycles were more

common in women with a lower percentage of gynoid fat, which may be related to the endocrine function of these depots. In order to further understand the role of gynoid and android patterns of body fat distribution, we used a gynoid/android (G/A) fat mass ratio to assess upper and lower body fat distribution. Several metabolic studies have used similar ratios such as A/G fat ratio (android/gynoid), trunk-to-leg fat ratio or leg/trunk fat mass ratio as indicators of upper or lower body fat distribution (Aasen, Fagertun, & Halse, 2008; Douchi et al., 2002; Shao et al., 2014; Tremollieres, Pouilles, & Ribot, 1996). In our study, irregular cycle pattern was significantly associated with a low G/A fat mass ratio. Body fat distribution expressed as the G/A ratio better explained the inverse association with irregular cycle pattern than the GF%.

Our study is one of the first to highlight the role of DXA estimates of body fat distribution with menstrual cycle characteristics in young healthy premenopausal women. Our results agree in part with Douchi et al., the only other study that used DXA estimates of body fat. Using a case-control study, they found that obese women with menstrual disorders had a higher trunk-to-leg fat ratio (as a marker of upper or lower body fat distribution) compared to control obese women with no menstrual disorders ($1.48 \pm 0.29$ Vs.$1.25 \pm 0.38$, $p<0.01$). They concluded that greater upper body obesity is associated with menstrual disorders. The authors noted that upper body obesity contributes to an androgenic environment, low SHBG and an abnormal hormonal profile that may partially contribute to the menstrual disorders in these women (Douchi et al., 2002). This study was conducted in older obese Japanese women and may not be generalizable to the U.S. population. Further, we used the ratio of gynoid to android fat based on DXA estimates

of fat mass in these regions. This ratio may better capture the proportion of fat in that area than the trunk to leg fat ratio.

Several mechanisms may explain our observed association between irregular cycle pattern and low G/A fat mass. Menopause is associated with a change in body fat distribution with a shift toward increased abdominal fat, a phenomenon that has been attributed to the decline in estrogen and an increase in androgen levels (Cao, Zhang, Zou, & Xia, 2013; Toth, Tchernof, Sites, & Poehlman, 2000; Tremollieres et al., 1996). Irregular cycle is also associated with hormonal variations that may be influenced by body fat distribution. In addition, women with upper-body adiposity have lower sex hormone binding globulin (SHBG), with higher levels of free androgen and testosterone compared to women with lower-body adiposity (Kirschner et al., 1990; Wei, Schmidt, Dwyer, Norman, & Venn, 2009). The study by Kirschner et al., reported that obese women with lower-body adiposity had higher SHBG and did not have the elevated levels of free estrogen or free androgens seen in women with upper-body adiposity and irregular cycles. This difference in the hormonal milieu could affect menstrual cycle characteristics. It is likely that having a higher ratio of G/A fat mass, which indicates greater lower-body adiposity, may benefit menstrual function. However, due to the cross-sectional nature of the study design, we cannot determine if the body fat distribution causes irregular cycle pattern or vice versa. We did not find a significant association of irregular cycle pattern with AF%.

We found that short and long bleed duration was significantly associated with low BMI and WC, but was not associated with any DXA estimates of body fat. Bleed days indicate the duration of menstrual bleeding and reflect endometrial development, which is

mediated by the ovarian hormones (Vitzhum et al., 2009). We are unsure of the basis for our findings and further studies will be needed to understand these associations.

One of the strengths of this study is the use of DXA estimates of body fat and body fat distribution, a tool that has been validated to measure adiposity (Haarbo et al., 1991; Kaul et al., 2012). However, very few studies have used this measure, and ours is the first to use DXA estimates of body fat distribution in the context of menstrual cycle characteristics. We found that DXA estimates of body fat provide more information on adiposity and its distribution than does BMI. Our study is the first to highlight the association between irregular cycle pattern and low GF% and low G/A fat mass ratio, suggesting that lower body adiposity may likely play an important role in regulating menstrual cycle pattern.

Our study had some potential limitations that need to be considered when interpreting our results. We used self-report to assess menstrual cycle characteristics, which can cause measurement errors that would bias the results toward the null. However, participants were enrolled and information was collected in the late luteal phase of their menstrual cycle so they are more likely to recall their cycle characteristics accurately and may be less prone to memory errors. Although we measured and adjusted for many known confounders, we did not adjust for other potentially important variables, such as stress levels. Since our study population was college-aged women, the stress levels would likely be similar among study participants. Also, the study population was fairly homogenous since all participants were 18 to 30 year-old women and 83% of them were Caucasians, which may limit the generalizability of our results.

In summary, we found that low %TBF but not BMI and WC was associated with irregular cycle pattern and with low GF% and low G/A ratio, which are indicators of lower-body or peripheral adiposity. Thus, our findings suggest that regional body fat distribution may be involved in regulating menstrual function. However, these findings need to be replicated by larger studies. Future studies that assess hormonal levels in addition to cycle characteristics prospectively are required to better understand the biological basis for these associations. Evidence shows that menstrual cycle dysfunction can affect reproductive health and women with abnormal cycle patterns are more likely to have miscarriages and have reduced fecundity (Kolstad et al., 1999; Small et al., 2010; Small et al., 2006; Wise et al., 2011). Menstrual cycle characteristics are easy to assess and provide a non-invasive and inexpensive method to assess a woman's reproductive health. Identifying modifiable nutritional risk factors like body fat is a cost effective way to improve a woman's reproductive capability.

Table 2.1, Descriptive characteristics of study participants (n=164)

| Characteristics | n | Mean ± SD[a] |
|---|---|---|
| **Age (years)** | 164 | 21.5 ± 3.1 |
| **Height (cm)** | 164 | 165.9 ± 6.4 |
| **Weight (kg)** | 164 | 63.7 ± 10.6 |
| **BMI (kg/m$^2$)** | 164 | 23.1 ± 3.3 |
| **Waist Circumference (inches)** | 164 | 30.7 ± 3.6 |
| **% Total Body Fat (TBF)** | 157 | 32.1 ± 8.1 |
| Missing | 7 | |
| **Gynoid Fat % (GF)** | 157 | 40.8 ± 6.4 |
| **Android Fat % (AF)** | 157 | 34.7 ± 10.6 |
| **Gynoid:Android (G/A) fat mass (g)** | 157 | 3.4 ± 1.1 |
| **Age at menarche (years)** | 164 | 12.5 ± 1.5 |
| **Years since menarche (YSM)** | 164 | 8.9 ± 3.5 |
| **Physical Activity (METs/week)** | 164 | 51.5 ± 46.1 |

[a]SD - Standard deviation;

Table 2.1(cont'd) Descriptive characteristics of study participants (n=164)

| Characteristics | | n (%) |
|---|---|---|
| **BMI (kg/m$^2$):** | < 18.5 | 9 (5.5) |
| | 18.5 - 24.9 | 116 (70.7) |
| | 25 - 29.9 | 33 (20.1) |
| | > 30 | 6 (3.7) |
| **%TBF:** | < 32 | 86 (54.8) |
| | ≥ 32 | 71 (45.2) |
| **WC (inches):** | ≥ 35 | 23 (14) |
| | < 35 | 141 (86) |
| **Age at Menarche:** | ≤ 12 y | 86 (52.4) |
| | >12 y | 78 (47.6) |
| **Current smokers:** | Yes | 9 (5.5) |
| | No | 154 (94.5) |
| | Missing | 1 |
| **Cycle Pattern** | Regular (≤ 7 days) | 145 (88.4) |
| | Irregular (> 7 days) | 19 (11.6) |
| **Cycle Length** | Short (≤ 25 days) | 32 (19.5) |
| | Typical (26 to 31 days) | 98 (59.8) |
| | Long (> 32 days) | 34 (20.7) |
| **Bleed Days** | Short (≤ 3 days) | 25 (15.3) |
| | Regular (4 to 5 days) | 100 (61.3) |
| | Long (> 5 days) | 38 (23.3) |

BMI-body mass index, WC-waist circumference, TBF- total body fat;

Table 2.2a Association of adiposity measures with menstrual cycle characteristics (n=164)

| Body Fat Estimates | Menstrual Cycle Pattern | | | Menstrual Cycle Length | | | | Menstrual Bleed Days | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Regular n=145 | Irregular n=19 | | Short n=32 | Typical n=98 | Long n=34 | | Short n=25 | Regular n=100 | Long n=38 | |
| | | | P[b] | Mean ± SD | | | P[c] | | | | P[c] |
| **%TBF** | 32.5 ± 7.8 | 27.8 ± 9.3 | 0.06 | 31.9 ± 7.4 | 33.0 ± 8.0 | 30.0 ± 8.9 | 0.22 | 29.6 ± 6.8 | 33 ± 8.7 | 31.3 ± 7.5 | 0.14 |
| **%GF** | 41.0 ± 6.2 | 37.9 ± 7.7 | **0.04** | 40.9 ± 6.2 | 41.3 ± 6.2 | 39.2 ± 7.1 | 0.27 | 39.3 ± 5.2 | 41.3 ± 7.0 | 40.5 ± 5.8 | 0.37 |
| **%AF** | 35.0 ± 10.6 | 31.5 ± 11 | 0.15 | 35.0 ± 10.0 | 35.0 ± 10.8 | 32.6 ± 11 | 0.44 | 32.1 ± 9.8 | 36.1±11.1 | 32.8 ± 9.7 | 0.13 |
| **G/A fat mass ratio** | 3.4 ± 1.1 | 3.4 ± 0.9 | 0.84 | 3.2 ± 0.8 | 3.4 ± 1.2 | 3.5 ± 1.3 | 0.61 | 3.6 ± 1.2 | 3.3 ± 1.0 | 3.6 ± 1.2 | 0.13 |
| **BMI** | 23.2 ± 3.3 | 22.0 ± 2.8 | 0.13 | 23.0 ± 2.8 | 23.3 ± 3.4 | 22.5 ± 3.5 | 0.47 | 22.0 ± 2.5 | 23.6 ± 3.6 | 22 ± 2.6 | **0.03** |
| **WC** | 30.8 ± 3.6 | 30.0 ± 3.2 | 0.48 | 30.0 ± 3.2 | 31 ± 3.7 | 30.5 ± 3.5 | 0.40 | 29.6 ± 3.0 | 31 ± 3.8 | 30 ± 3.2 | **0.04** |
| | | | P[a] | n (%) | | | P[a] | | | | P[a] |
| **%TBF** | | | | | | | | | | | |
| < 32 % | 72 (83.7) | 14 (16.3) | **0.03** | 15 (17.4) | 50 (58.1) | 21 (24.4) | 0.50 | 16 (18.6) | 48 (55.8) | 22 (25.6) | 0.30 |
| ≥ 32 % | 67 (94.4) | 4 (5.6) | | 14 (19.7) | 45 (63.4) | 12 (16.9) | | 8 (11.4) | 47 (67.1) | 15 (21.4) | |
| **WC** | | | | | | | | | | | |
| < 35 inches | 125 (88.7) | 16 (11.6) | 0.52 | 30 (21.3) | 83 (58.9) | 28 (19.9) | 0.35 | 23 (16.4) | 82 (58.6) | 35 (25) | 0.19 |
| ≥35 inches | 20 (87) | 3 (13) | | 2 (8.7) | 15 (65.2) | 6 (26.1) | | 2 (8.7) | 18 (78.3) | 3 (13) | |

[a]p-values- chi square test of association; [b]p-values - two sample t-test; [c]p-value - one way analysis of variance F test
[d]SD-standard deviation; TBF-total body fat;
GF- gynoid fat; AF- android fat; WC- waist circumference; BMI- Body mass index

Table 2.2b Association of covariates with menstrual cycle characteristics (n=164)

| Covariates | Menstrual Cycle Pattern | | | Menstrual Cycle Length | | | | Menstrual Bleed Days | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Regular | Irregular | | Short | Typical | Long | | Short | Regular | Long | |
| | n=145 | n=19 | | n=32 | n=98 | n=34 | | n=25 | n=100 | n=38 | |
| | | | P[b] | (Mean+SD[d]) | | | P[c] | | | | P[c] |
| **Age (years)** | 21.7 ± 3.0 | 20.6 ± 2.0 | 0.14 | 21.3 ± 2.9 | 21.8 ± 3.4 | 21.1 ± 2.0 | 0.42 | 21.7 ± 3.1 | 21.4 ± 2.9 | 21.7 ± 3.3 | 0.70 |
| **Physical activity** | 50.5 ± 46.3 | 59.3 ± 45.2 | 0.43 | 49.8 ± 44.6 | 50.2 ± 48.4 | 56.9 ± 41.7 | 0.74 | 65.9 ± 54.9 | 49.8 ± 43.7 | 47.4 ± 45.9 | 0.24 |
| **Height (cm)** | 165.9 ± 7.0 | 166.8 ± 6.0 | 0.54 | 163.0 ± 7.1 | 167.0 ± 6.1 | 166.0 ± 5.8 | **0.01** | 166.0 ± 7.3 | 166.0 ± 6.1 | 165.0 ± 6.6 | 0.62 |
| **YSM** | 9.8 ± 3.6 | 7.1 ± 2.6 | **0.01** | 8.8 ± 2.8 | 9.3 ± 4.0 | 8.2 ± 2.6 | 0.24 | 9.3 ± 3.5 | 8.8 ± 3.4 | 9.2 ± 3.7 | 0.87 |
| | | | P[a] | N (%) | | | P[a] | | | | P[a] |
| **Age at menarche** | | | | | | | | | | | |
| ≤12 years | 80 (93.0) | 6 (7.0) | 0.05 | 16 (18.6) | 54 (62.8) | 16 (18.6) | 0.70 | 14 (16.5) | 50 (58.8) | 21 (24.7) | 0.80 |
| >12 years | 65 (83.3) | 13 (16.7) | | 16 (20.5) | 44 (56.4) | 18 (23.1) | | 11 (14.1) | 50 (64.1) | 17 (21.8) | |
| **Current smokers** | | | | | | | | | | | |
| Yes (n=9) | 9 (100) | 0 (0) | 0.60 | 2 (22.2) | 5 (55.6) | 2 (22.2) | 0.95 | 2 (22.2) | 6 (66.7) | 1 (11.1) | 0.60 |
| No (n=154) | 135 (87.7) | 19 (12.3) | | 29 (18.9) | 93 (60.4) | 32 (20.8) | | 23 (15.0) | 93 (60.8) | 37 (24.2) | |

YSM-years since menarche; physical activity is expressed as metabolic equivalents (METS);*p-value<0.05; [d]SD-standard deviation

[a]p-values- chi square test of association; [b]p-values - two sample t-test; [c]p-value - one way analysis of variance F test

Table 2.2c Distribution of DXA-derived body fat estimates by covariates (n=164)

| Covariates | BMI | P[a,b] | WC | P[a,b] | %TBF | P [a,b] | GF% | P[a,b] | AF% | P[a,b] | G/A | P[a,b] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean±SD[d] | | | | | | | | | | | |
| **YSM** | | | | | | | | | | | | |
| ≤ 6 years | 22.9 ± 3.3 | 0.70 | 31.0 ± 3.9 | 0.70 | 31.7 ± 8.3 | 0.80 | 40.4 ± 6.5 | 0.70 | 35.2 ± 11.0 | 0.80 | 3.3 ± 0.8 | 0.63 |
| >6 years | 23.0 ± 3.3 | | 30.6 ± 3.5 | | 32.0 ± 8.1 | | 40.9 ± 6.4 | | 34.6 ± 10.6 | | 3.4 ± 1.2 | |
| **Age at Menarche** | | | | | | | | | | | | |
| ≤12 years | 23.2 ± 3.6 | 0.45 | 30.8 ± 3.7 | 0.50 | 33.3 ± 8.3 | **0.04** | 41.7 ± 6.3 | **0.04** | 36.2 ± 10.4 | 0.06 | 3.3 ± 1.1 | 0.12 |
| > 12 years | 22.8 ± 2.9 | | 30.5 ± 3.5 | | 30.6 ± 7.7 | | 39.6 ± 6.5 | | 32.9 ± 10.8 | | 3.5 ± 1.1 | |
| **Physical Activity** | | | | | | | | | | | | |
| 1st tertile | 23.8 ± 3.7 | 0.13 | 31.7 ± 3.7 | **0.03** | 34.6 ± 8.7 | **0.01** | 42.6 ± 6.7 | **0.01** | 38 ± 10.8 | **0.01** | 3.1 ± 1.1 | 0.08 |
| 2nd tertile | 22.6 ± 3.3 | | 30.2 ± 3.8 | | 32.0 ± 7.4 | | 41.1 ± 5.7 | | 34 ± 10.4 | | 3.5 ± 1.1 | |
| 3rd tertile | 22.8 ± 2.8 | | 30.1 ± 3.0 | | 29.6 ± 7.5 | | 38.7 ± 6.3 | | 32.1 ± 10 | | 3.2 ± 1.1 | |
| **Current Smokers** | | | | | | | | | | | | |
| Smokers | 23.3 ± 1.5 | 0.50 | 30 ± 1.8 | 0.60 | 30.3 ± 5.8 | 0.50 | 38.5 ± 5.2 | 0.30 | 32.0 ± 7.7 | 0.40 | 3.3 ± 0.8 | 0.80 |
| Non-smokers | 23.1 ± 3.4 | | 30.7 ± 3.8 | | 32.2 ± 8.2 | | 40.9 ± 6.5 | | 34.9 ± 10.9 | | 3.4 ± 1.1 | |

*p-value≤0.05; [b] p-value - one way analysis of variance F test; [d]SD-standard deviation; TBF- total body fat; GF-gynoid fat; AF – android fat; WC- waist circumference

[a] p-value - two sample t-test;

YSM-years since menarche;

Table 2.3a Association of DXA-derived body fat estimates and menstrual cycle characteristics using unadjusted and adjusted multivariable linear regression (n=164)

| | Menstrual Cycle Pattern | | | Menstrual Cycle Length | | | | | Menstrual Bleed Days | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Regular | Irregular | | Short | | Typical | Long | | Short | | Regular | Long | |
| **DXA- BF Estimates** | | Beta (95% CI) | P | Beta (95%CI) | P | | Beta | P | Beta | P | | Beta | P |
| **%TBF** | | | | | | | | | | | | | |
| Crude | Ref | -0.15 (-7.7, -0.2) | 0.07 | -0.05 (-4.4, 2.4) | 0.57 | Ref | -0.15 (-2.0, 0.1) | 0.09 | -0.15 (-7.0, 0.2) | 0.06 | Ref | -0.10 (-4.8, 1.3) | 0.26 |
| Model 1¥ | Ref | -0.16 (-8.1, -0.1) | **0.04** | -0.04 (-4.3, 2.6) | 0.60 | Ref | -0.15 (-2.0, 0.1) | 0.07 | -0.15 (-7.0, 0.3) | 0.07 | Ref | -0.09 (-5.0, 1.4) | 0.26 |
| Model 2† | Ref | -0.13 (-7.3, 0.8) | 0.10 | -0.04 (-4.2, 2.6) | 0.60 | Ref | -0.13 (-1.9, 0.2) | 0.13 | -0.12 (-6.4, 0.8) | 0.14 | Ref | -0.09 (-4.8, 1.4) | 0.28 |
| **GF%** | | | | | | | | | | | | | |
| Crude | Ref | -0.16 (-6.4, -0.1) | **0.04** | -0.02 (-3.1, 2.3) | 0.80 | Ref | -0.13 (-1.6, 0.2) | 0.11 | -0.11 (-4.9, 0.8) | 0.17 | Ref | -0.06 (-3.3, 1.6) | 0.50 |
| Model 1¥ | Ref | -0.18 (-6.8, -0.5) | **0.03** | -0.02 (-3.0, 2.5) | 0.90 | Ref | -0.14 (-1.6, 0.1) | 0.10 | -0.11 (-4.8, 0.9) | 0.19 | Ref | -0.06 (-3.4,1.6) | 0.50 |
| Model 2† | Ref | -0.15 (-6.1, 0.3) | 0.07 | -0.01 (-2.9, 2.5) | 0.90 | Ref | -0.11 (-1.4, 0.3) | 0.18 | -0.08 (-4.2, 1.5) | 0.36 | Ref | -0.06 (-3.3, 1.6) | 0.48 |
| **AF%** | | | | | | | | | | | | | |
| Crude | Ref | -0.11 (-8.9, 1.6) | 0.17 | -0.01 (-4.8, 4.2) | 0.89 | Ref | -0.11 (-2.3, 0.5) | 0.20 | -0.14 (-8.7, 0.8) | 0.10 | Ref | -0.13 (-7.3, 0.8) | 0.11 |
| Model 1¥ | Ref | -0.13 (-9.6, 1.1) | 0.12 | -0.01 (-4.8, 4.3) | 0.90 | Ref | -0.11 (-2.4, 0.4) | 0.18 | -0.13 (-8.6, 0.9) | 0.12 | Ref | -0.13 (-7.4, 0.8) | 0.12 |
| Model 2† | Ref | -0.09 (-8.6, 2.2) | 0.25 | -0.01 (-4.7, 4.3) | 0.90 | Ref | -0.10 (-2.2, 0.6) | 0.27 | -0.11 (-8.0, 1.7) | 0.20 | Ref | -0.13 (-7.2, 0.8) | 0.12 |
| **G/A ratio** | | | | | | | | | | | | | |
| Crude | Ref | -0.02 (-0.6, 0.5) | 0.90 | -0.06 (-0.6, 0.3) | 0.44 | Ref | 0.04 (-0.1, 0.2) | 0.67 | 0.13 (-0.1, 0.9) | 0.13 | Ref | 0.14 (-0.1, 0.8) | 0.10 |
| Model 1¶ | Ref | -0.12 (-0.8, -0.03) | **0.04** | -0.09 (-0.6, 0.1) | 0.15 | Ref | -0.06 (-0.2, 0.1) | 0.33 | 0.02 (-0.3, 0.4) | 0.81 | Ref | 0.06 (-0.2, 0.4) | 0.31 |
| Model 2¶ | Ref | -0.13 (-0.9, -0.05) | **0.03** | -0.08 (-0.6, 0.1) | 0.16 | Ref | -0.06 (-0.2, 0.1) | 0.33 | 0.03 (-0.3, 0.4) | 0.68 | Ref | 0.06 (-0.2, 0.5) | 0.35 |

Outcomes - %TBF (percent total body fat); GF% (gynoid fat); AF% (android fat); G/A ratio (gynoid fat mass/android fat mass)

¥Model 1- adjusted for age and smoking status; † Model 2 - adjusted for physical activity, age at menarche, years since menarche, smoking status

¶-model also adjusted for %TBF

Table 2.3b Association of BMI and WC and menstrual cycle characteristics using unadjusted and adjusted multivariable linear regression (n=164)

| | **Menstrual Cycle Pattern** | | | **Menstrual Cycle Length** | | | | | **Menstrual Bleed Days** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Regular** | **Irregular** | | **Short** | | **Typical** | **Long** | | **Short** | | **Regular** | **Long** | |
| | | Beta (95%CI) | P | Beta (95%CI) | P | | Beta (95% CI) | P | Beta (95% CI) | P | | Beta (95% CI) | P |
| **BMI** (kg/m$^2$) | | | | | | | | | | | | | |
| Crude | Ref | -0.12 (-2.8, 0.4) | 0.13 | -0.04 (-1.6, 1) | 0.60 | Ref | -0.10 (-0.7, 0.2) | 0.23 | -0.16 (-2.8, 0) | 0.05 | Ref | -0.18 (-2.6, -0.2) | **0.03** |
| Model 1¥ | Ref | -0.12 (-2.8,0.3) | 0.12 | -0.04 (-1.6, 1) | 0.67 | Ref | -0.09 (-0.7, 0.2) | 0.25 | -0.16 (-2.8, 0) | 0.05 | Ref | -0.19 (-2.7, -0.2) | **0.02** |
| Model 2† | Ref | -0.10 (-2.7,0.6) | 0.20 | -0.04 (-1.6,1.1) | 0.67 | Ref | -0.08 (-0.7, 0.2) | 0.30 | -0.16 (-2.9, 0.3) | 0.06 | Ref | -0.19 (-2.6, -0.2) | **0.02** |
| **WC** (in) | | | | | | | | | | | | | |
| Crude | Ref | -0.06 (-2.3, 1.1) | 0.48 | -0.11 (-2.3, 0.5) | 0.20 | Ref | -0.05 (-0.6, 0.3) | 0.50 | -0.17 (-3.2, -0.1) | **0.04** | Ref | -0.16 (-2.7, 0) | 0.05 |
| Model 1¥ | Ref | -0.06 (-2.4, 1) | 0.45 | -0.09 (-2.2, 0.7) | 0.30 | Ref | -0.05 (-0.6, 0.3) | 0.50 | -0.17 (-3.3, -0.2) | **0.03** | Ref | -0.17 (-2.8, -0.1) | **0.04** |
| Model 2† | Ref | -0.04 (-2.2, 1.3) | 0.59 | -0.09 (-2.2, 0.7) | 0.30 | Ref | -0.04 (-0.6, 0.4) | 0.63 | -0.16 (-3.2, -0.5) | **0.04** | Ref | -0.17 (-2.8, -0.1) | **0.04** |

Outcomes -BMI (body mass index) and WC (waist circumference) in inches

¥Model 1- adjusted for age and smoking status; † Model 2 - adjusted for physical activity, age at menarche, years since menarche, smoking status

Table 2.3c Relative odds of irregular menstrual cycle pattern by adiposity measures (n=164)

| | Menstrual Cycle Pattern | | | | | |
|---|---|---|---|---|---|---|
| | Odds Ratio (95% CI) | | | | | |
| | **Crude** | **p-value** | **Model 1** | **p-value** | **Model 2** | **p-value** |
| **BMI** | 0.88 (0.74 - 1.04) | 0.13 | 0.88 (0.75 - 1.04) | 0.13 | 0.87 (0.73 - 1.04) | 0.13 |
| **WC** | 0.95 (0.83 - 1.10) | 0.48 | 0.94 (0.82 - 1.10) | 0.41 | 0.95 (0.82 - 1.10) | 0.48 |
| **% TBF (1SD)** | 0.91 (0.88 - 1.01) | 0.07 | 0.93 (0.87 - 0.99) | 0.04 | 0.94 (0.87 - 1.01) | 0.08 |
| **% TBF** | | | | | | |
| ≤ 32 | 3.3 (1.01-10.39) | 0.05 | 3.5 (1.01 - 11.41) | 0.04 | 3.1 (0.9 - 10.20) | 0.07 |
| >32 | Referent | | Referent | | Referent | |
| **Gynoid fat % (1SD)** | 0.92 (0.85 -0.99) | 0.05 | 0.91 (0.84 - 0.98) | 0.03 | 0.92 (0.85 - 1.01) | 0.06 |
| **GF % categories** | | | | | | |
| ≤ Median GF% | 2.8 (0.95 - 8.30) | 0.06 | 3.1 (1.04 - 9.40) | 0.04 | 2.8 (0.93 - 8.58) | 0.07 |
| > Median GF% | Referent | | Referent | | Referent | |
| **Android fat %(1SD)** | 0.98 (0.92 - 1.02) | 0.17 | 0.96 (0.91 - 1.01) | 0.11 | 0.97 (0.92 - 1.02) | 0.18 |
| **AF % categories** | | | | | | |
| ≤ Median AF% | 1.6 ( 0.6 - 4.47) | 0.33 | 1.8 (0.64 - 4.90) | 0.27 | 1.6 (0.58 - 4.59) | 0.35 |
| > Median AF% | Referent | | Referent | | Referent | |
| **G/A fat ratio‡ (1SD)** | 0.96 (0.61 - 1.52) | 0.84 | 0.47 (0.22 - 1.04) | 0.06 | 0.47 (0.21 - 1.06) | 0.07 |

Model 2 - Adjusted for age (continuous), age at menarche (categorical) and physical activity (continuous); Model 1 - adjusted for age and smoking status

Outcome: 0= regular cycles and 1= irregular cycles; TBF - total body fat; ‡- also adjusted for %TBF in models 1 and 2

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# CHAPTER 3

# ASSOCIATION OF DIETARY FAT AND MENSTRUAL CYCLE CHARACTERISTICS IN YOUNG WOMEN

## 3.1 ABSTRACT

**Background**: Menstrual cycle length, pattern and bleed duration are important determinants of a woman's reproductive success. Although previous studies suggest total dietary fat intake may influence estrogen levels and possibly menstrual cycle features, little is known about the association between dietary intake of total omega-3 or omega-6 PUFA and menstrual cycle characteristics, particularly in young women. The current study examines the association of total dietary fat, omega-3 PUFA, omega-6 PUFA and the omega-6:omega-3 ratio with menstrual cycle characteristics in healthy young women.

**Methods**: The study subjects were a subset of 164 18- to 30-year-old women participants in the UMass Vitamin D Status Study who reported no use of hormonal contraceptives. Dietary intake of foods including fats was assessed using a validated food frequency questionnaire, and information on menstrual cycle characteristics (cycle pattern, length and bleed days) was obtained through a self-reported questionnaire.

**Results**: In multivariable linear regression analyses, higher intake of omega-6 PUFA was associated with short cycle length ($\beta$= 0.23, $p<0.01$), long cycle length ($\beta$=0.16, $p=0.06$) and irregular cycle pattern ($\beta$= 0.16, $p=0.05$). These associations were statistically significant for short cycle length in all models. However, total dietary fat, omega-3 PUFA and the omega-6:omega-3 PUFA ratio were not associated with any menstrual cycle characteristics.

Conclusion: Our findings suggest that high intakes of omega-6 PUFA are associated with abnormal menstrual cycle length and pattern in healthy young women. Further studies are warranted to replicate these findings and to investigate the underlying biological mechanisms.

## 3.2 INTRODUCTION

Menstrual cycle characteristics of length, pattern and bleed duration are important determinants of a woman's reproductive success and pregnancy outcomes. Epidemiologic evidence suggests abnormal menstrual cycle characteristics are associated with reduced fecundity and increased risk of miscarriage and gestational diabetes (Dishi et al., 2011; T. K. Jensen et al., 1999; Kolstad et al., 1999; Rowland et al., 2002; Small et al., 2010; Small et al., 2006; Steiner et al., 1999; Wise et al., 2011). Identifying modifiable nutritional factors that affect menstrual cycle characteristics can be a cost effective way to improve a woman's reproductive success and pregnancy outcomes.

The menstrual cycle involves a complex interplay of steroid hormones regulated by the hypothalamus-pituitary-ovarian (HPO) axis (Hawkins & Matzuk, 2008; Mihm et al., 2011; Speroff, 1999). Variation in menstrual cycle characteristics reflects underlying endocrine fluctuations, particularly of estrogen. Animal studies suggest that dietary polyunsaturated fatty acids (PUFA), particularly omega-6 (PUFA) and omega-3 PUFA, affect estrogen synthesis through their influence on cell membrane phospholipid composition and prostaglandin synthesis (Das, 2008; Larsson et al., 2004; Spector, 2000; Stocco et al., 2005; Wathes et al., 2007). Prostaglandins synthesized from the omega-3 or omega-6 PUFA may influence estrogen metabolism differentially by regulating

numerous genes, including those involved in estrogen metabolism, aromatase activity and conversion of androgen to estrogen (Wathes et al., 2007). Evidence in humans also suggests that changes in total dietary fat intake (high-fat diet to a low-fat diet) cause a reduction in estradiol levels (Forman, 2007; Wu et al., 1999) as well as menstrual cycle characteristics (Baines et al., 2007; Barr, 1999; Kato et al., 1999; Pedersen et al., 1991; Wu et al., 1999). Total dietary fat intake was positively associated with risk of cycle irregularity (Kato et al., 1999), but a low fat diet was associated with both an increase (Jones et al., 1987) and a decrease (Bagga et al., 1995) in cycle length in previous studies. These associations have not been observed consistently, and concurrent modification of other dietary components, such as fiber, make it hard to attribute the observations to changes in dietary fat intake alone (Forman, 2007; Hilakivi-Clarke et al., 2002; Ronco et al., 2010; Turner, 2011; Wu et al., 1999).

Although some previous studies suggest that total dietary fat intake may influence estrogen levels and possibly menstrual cycle features (Manna, Dyson, & Stocco, 2009; Stocco et al., 2005; Wathes et al., 2007), little is known about the association between dietary intake of total omega-3 or omega-6 PUFA and menstrual cycle characteristics, particularly in young women. The current study examines the association of intakes of total dietary fat, omega-3 PUFA, omega-6 PUFA and omega-6:omega-3 ratio with menstrual cycle characteristics in healthy young women.

## 3.3 METHODS

### 3.3.1 Study Design, Study Setting and Population

The current analyses uses data collected as part of the UMass Vitamin D Status Study, a cross-sectional study based at the University of Massachusetts Amherst, which was designed to assess vitamin D status in young women and to identify its dietary, environmental and lifestyle determinants. Between 2006 and 2011, 288 women between 18 to 30 years of age were enrolled in the study. Information about the women was collected during one study visit during the late luteal phase of their menstrual cycle. A research assistant obtained anthropometric information; demographic, health, and menstrual symptom data were collected using a self-administered questionnaire. Information on dietary intake was collected using the Willett food frequency questionnaire (FFQ), which has previously been validated (Willett et al., 1985). Women were ineligible for the study if they were currently pregnant or not menstruating, currently suffering from depression, had high blood pressure or other chronic diseases, or were taking medications known to influence vitamin D status, such as corticosteroids. Women using oral contraceptives (OC; n = 124) have regular cycles due to the hormonal composition of the OCs, and were, therefore, excluded from our primary analysis. The resulting sample size available was n=164. The UMass, Amherst institutional review board approved this study. All subjects provided written informed consent.

### 3.3.2 Menstrual cycle characteristics

For statistical modelling, menstrual cycle length, pattern and bleed duration were considered the exposure variables. Information regarding menstrual cycle characteristics was collected through a self-administered questionnaire. Menstrual cycle length categories were: <21 days, 21 to 25 days, 26 to 31 days, 32 to 39 days, 40 to 50 days, >50

days, too irregular to count. We collapsed these categories to ≤ 25 days (short), 26 to 31 days (typical) and ≥ 32 days (long). Menstrual cycle variability information was collected through a cycle pattern question with the following categories: extremely regular (no more than 1 to 2 days before or after expected), very regular (within 3-4 days), regular (within 5-7 days), usually irregular and always irregular. Cycle pattern was collapsed to regular (for ≤ 7 day variation) and irregular (for > 7 day variation). Bleed duration information was collected under the following categories: ≤ 3 days, 4-5 days, 6-7 days, ≥ 8 days. For statistical analyses, we collapsed bleed duration ≤ 3 days (short), 4 to 5 days (regular), >5 days (long).

### 3.3.3 Dietary fat assessment

We examined the relationship between menstrual cycle characteristics and dietary intake of total fat and omega-3 PUFA and omega-6 PUFA and omega-6:omega-3 PUFA ratio. At the study visit, dietary intake of food and supplements was assessed through a modified version of the Willett food frequency questionnaire (FFQ), which was used to collect information on diet during the two months prior to the study visit. The FFQ has been previously validated for use in US women (Feskanich et al., 1993; Hunter et al., 1992; Willett et al., 1985). The FFQ has 131 items including supplement use. FFQ consisted of 9 response categories ranging from "never or less than once per month" to "six or more per day". The participants were asked about type of fats and oils they used and how often they ate fried foods. The participants were also directed to provide information on fortified orange juice and soy products in an open response section

(Zagarins et al., 2012). The FFQ measured intake of total fat and various omega-3 and omega-6 PUFA.

The FFQ was analyzed by Harvard University using their Food Composition Database. The nutrient content of the foods was obtained by multiplying the food consumption frequency with the specified portion of each food item by its nutrient content. The values are added up to derive the overall nutrient intakes (Zagarins et al., 2012). For our study, we defined total omega-3 PUFA intake as alpha-linolenic + eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) + docosapentaenoic acid, total omega-6 PUFA as linoleic acid + arachidonic acid (AA). Total dietary fat, omega-6 PUFA and omega-3 PUFA intake was calculated as percent total calories (%). The dietary fats included total fat, total omega-3 PUFA, total omega-6 PUFA, and omega-6:omega-3 PUFA ratio. These intakes were explored as continuous variables.

### 3.3.4 Covariate Assessment (Anthropometry and Lifestyle Factors)

Information on covariates was collected through the study questionnaire and through anthropometric measures assessed during the study visit. BMI was calculated as weight in kilograms divided by height in meters squared. Weight was measured using a calibrated scale and height was measured using a wall-mounted stadiometer. Waist circumference (WC) was measured using a standard tape. Percent total body fat (%TBF) was measured by DXA whole body scan mode on a narrow angle fan GE Lunar Prodigy scanner (GE Lunar Corp., Madison, WI). The scan was performed with the subject lying in a supine position as per the manufacturer's specifications (Zagarins et al., 2012). Information concerning smoking, parity, and age at menarche was collected through self-

reported entry in the study questionnaire (Liu et al., 2004). Information on total fiber intake was assessed by the Harvard FFQ. Total caloric intake reflected the average daily caloric intake and was calculated using data from the FFQ

Physical activity was assessed in the study questionnaire, in which participants reported their average weekly physical activity from a modified version of the Nurses' Health Study II questionnaire, which has been previously validated (Wolf et al., 1994; Zagarins et al., 2012). Activities included walking, jogging, running, bicycling, aerobics/dance/rowing machine, tennis/racket sports, swimming, yoga/pilates, and weight training/resistance exercises. The response ranged from 0 to 11or more hours per week. Metabolic equivalents (MET) scores were assigned to each activity using the method used by Ainsworth et al., 2000 (Ainsworth et al., 2000). The intensity of physical activity was measured by weekly MET scores, with 1 MET defined as the amount of energy expended when a person is at rest (Ainsworth, Jacobs, & Leon, 1993).

### 3.3.5 Statistical Analysis

The characteristics of the study population are reported as means, standard deviations and ranges for continuous variables and number and percentages for categorical variables. All continuous outcome variables were examined for normal distribution; all were reasonably approximately normally distributed. Bivariate analysis was conducted to assess potential confounders by cross tabulating covariates against outcome and exposure variable categories. Significant differences in proportions were assessed using a chi square test, with Fisher's exact test used for cells with small

frequencies. For continuous variables, statistically significant differences in means were assessed using Student's t-test and ANOVA.

Univariable linear regression was conducted to estimate crude associations between various menstrual cycle characteristics and dietary fat. Multivariable linear regression, adjusted for energy intake, was used to model the association of the dietary fat intake with menstrual cycle pattern, bleed duration and cycle length (Willett, Howe, & Kushi, 1997). Variables significantly associated with the outcomes were included in the multivariable models if their inclusion yielded a 10% change or greater in the coefficient for dietary fat intake. Age and smoking were included in the model 1 a priori. We calculated beta coefficients and 95% confidence intervals. The p-values were considered statistically significant if $p < 0.05$. All statistical analyses were conducted using IBM SPSS version 22.

## 3.4 RESULTS

### Subject Characteristics

The descriptive statistics of the 164 women not using OCs who were included in the study are shown in **Table 3.1**. The study subjects were generally young and most (94.5%) were non-smokers. Although the mean BMI (23.0 ± 3.3 kg/m$^2$) and waist circumference were within the recommended ranges (18.5 to 24.9 kg/m$^2$ and 30.7 ± 3.6 inches, respectively), DXA measures of total body fat indicated that the mean percent body fat (% BF) was 32% ± 8.1%, which is higher than recommended. The mean level of physical activity of the group (51.5 ± 46.1 METs/week) is equivalent to 25 to 30 minutes of moderate intensity physical activity per day and meets the recommended physical activity guidelines of approximately 150 minutes per week ("Physical activity guidelines

for Americans," 2008). Most of the women (82.3%) in the cohort were greater than 6 years since menarche (YSM) and more than half of them (52.3%) achieved menarche at age 12 or less. The mean energy intake (2191 ± 864 kcal) and the fiber intake (32 ± 19 g) were within the current recommendations (**Table 3.1**).

The mean intake of total dietary fat was 74.6 g per day, or 30% ± 6.7% of total energy, which is within the acceptable macronutrient distribution range (Medicine, 2012). On average, omega-6 and omega-3 fatty acids accounted for 5.3% ± 1.7% and 0.8% ± 0.3% of energy intake, respectively, which meets the current recommendations for essential fatty acid intake (Medicine, 2012).

In bivariate analysis, omega-6 PUFA intake was higher in women with both short and long cycle lengths compared to women with average cycle lengths (**Table 3.2a**). No other dietary fat intake measures (total dietary fat, omega-3 PUFA and omega-6:omega-3 ratio) were associated with menstrual cycle length. Women with an irregular cycles were significantly older at menarche (13.5y vs. 12.4y; $p<0.003$) and reported fewer years since menarche (YSM: 7.1y vs. 9.2y; $p< 0.01$). Dietary intake of omega-6 PUFA was 19% higher in women with an irregular cycle pattern (6.16 vs. 5.17; $p < 0.01$). Menstrual cycle pattern was not associated with age, energy intake or physical activity (**Table 3.2b**).

Women in the highest tertile of intake of dietary fat had the lowest mean intake of dietary fiber as compared to the first and second tertiles (25.9g vs. 38g and 30.9g; $p<0.003$). In addition, mean total physical activity was highest in the lowest tertile of dietary fat intake (**Table 3.2b**). Overall, 11.6%, 40.2% and 38.6% of the women, respectively, reported irregular cycle patterns, abnormal cycle lengths, and short or long number of bleed days.

**Menstrual Cycle Characteristics**

***Menstrual Cycle Pattern:*** Whether the pattern of the menstrual cycle is regular, occurring at a predictable interval, or irregular is thought to be a measure of ovulatory function (van Hooff et al., 2004). Omega-6 PUFA intake was significantly associated with irregular cycle pattern in unadjusted, model 1 (adjusted for energy, smoking and age) and fully adjusted model 2 (adjusted for energy, fiber, % total body fat and age at menarche). Total dietary fat intake, omega-3 PUFA and the omega-6:omega-3 PUFA ratio was not associated with menstrual cycle pattern (**Table 3.3a**).

***Menstrual Cycle Length:*** Menstrual cycle length is the interval from the first day of menses to the start of the next menses. A short or a long cycle length may indicate an abnormal hormonal feedback mechanism that could affect menstrual phase lengths and, in turn, ovulation and oocyte quality (Fehring et al., 2006; Ouyang et al., 2007; Windham et al., 2002). Total omega-6 PUFA intake was significantly associated with short cycle length in unadjusted, model 1 (adjusted for age, energy and smoking) and fully adjusted model 2. Total omega-6 PUFA was significantly associated with long cycle length in unadjusted and model 1 (adjusted for age, energy and smoking). However, this association was attenuated and was no longer statistically significant in fully adjusted model 2 (**Table 3.3a**).

***Menstrual Cycle Bleed Days:*** Menstrual cycle bleed days is the duration of menstrual bleeding and is indicative of the endometrium development mediated by the ovarian hormones (Vitzthum, 2009). The number of menstrual cycle bleed days was not

associated with total dietary fat intake, omega-6 PUFA, omega-3 PUFA, or omega-6:omega-3 PUFA ratio (**Table 3.3b**).

## 3.5 DISCUSSION

In this cross-sectional study of young women, we found that higher intakes of omega-6 fatty acids were associated with both short and long cycle lengths. Additionally, higher intake of omega-6 fatty acid was marginally associated with having an irregular cycle pattern. However, the magnitude of our findings was small and it may not be clinically significant. Our findings are fairly consistent with those of five previous studies that explored the association of dietary fat with menstrual cycle length, although none of these examined the specific associations with omega-3 versus omega-6 fats. Among the three intervention studies that explored the association between a low fat (10 to 20% energy from fat) versus a high fat (30 to 40% of energy from fat) diet and menstrual cycle length (Goldin & Gorbach, 1988; Jones et al., 1987), only one very small (n=12) study, found a significant increase (1.3 days) in cycle length at the end of the low- fat intake period (Jones et al., 1987). In addition to its small size, this study differed from ours with respect to subject age and BMI as well as the type of dietary fats. One prospective study, by Nagata et al. (2006), examined the role of dietary fat (total fat intake and PUFA) and menstrual cycle length among 341 Japanese women aged 18- to 29 years and found that women in the highest quintile of PUFA intake had shorter cycle length compared to women in the lowest quintile of PUFA intake (31d vs. 33d; p for trend= 0.03). However, as in our study, total fat intake was not associated with menstrual cycle length (Nagata et al., 2006), and Nagata et al did not examine the association with omega-3 versus omega-6 PUFAs.

We also found higher intakes of omega-6 fatty acids tended to be associated with an irregular cycle pattern. Although data on the association between diet and menstrual pattern are scarce, one cross-sectional study by Kato et al., (1999) examined cycle pattern and its association with dietary fat in 4900 premenopausal women under age 45 years from the New York University Women's Health Study. They found that after adjusting for age, BMI, number of cigarettes smoked and energy intake, the risk of irregular cycles was positively associated with total dietary fat intake (OR: 1.14; $p < 0.05$). In our study, we did not find an association between irregular cycle pattern and total dietary fat intake. The contrasting findings may be related to the higher total fat intake of these women compared to those in our study. The mean intake of dietary fat (30.4%$\pm$ 6.7%) in our cohort was lower than the average intake of fat (approximately 43%) in the Kato et al., cohort of women (Wright et al., 2003). In addition, the women in the Kato et al, study were older (mean age 40 y) and may already have developed age-associated hormonal changes that may have affected their menstrual cycle characteristics (Vitzthum, 2009). In addition our study had a small sample size and may not have the power to detect a small association.

To our knowledge, no previous studies have examined the association between menstrual cycle characteristics and PUFAs intake in young healthy women, although some cautious parallels can be drawn from studies conducted in women with polycystic ovary syndrome (PCOS). For instance, a recent cross-sectional study in women (n=104) with PCOS found that a higher plasma omega-6 fatty acid concentration was associated with hyperandrogenemia, which is associated with the menstrual cycle irregularities seen in PCOS (Phelan et al., 2011). Our observed associations between omega-6 PUFA intake

and irregular cycles and short and long cycle length may be related to similar hormonal changes induced by omega-6 fatty acids.

Dietary fatty acid intake affects the lipid composition of the lipid bilayer in cell membranes (Spector, 2000; Wathes et al., 2007), and the make-up of these membrane lipids influences which prostaglandins are generated when eicosanoid synthesis is triggered (Larsson et al., 2004; Spector, 2000; Wathes et al., 2007). Arachidonic acid—an omega-6 fatty acid— is the principal precursor of prostaglandins, which play a role in ovarian hormone steroidogenesis ("Hormone Biosynthesis, Metabolism, and Mechanism of Action," 1999; Stocco et al., 2005; Wathes et al., 2007). In addition, longer chain PUFAs derived from omega-6 and omega-3 fats, such as arachidonic acid (AA) and EPA/DHA, respectively, compete for inclusion in membrane phospholipids (Larsson et al., 2004; Spector, 2000; Stocco et al., 2005), where they are crucial to the synthesis of prostaglandins and other lipid biomediators. Recent studies have indicated that the AA-derived eicosanoids are involved in the expression and release of steroidogenic acute regulator (STAR) protein, which is a rate regulator in the ovarian steroidogenesis pathway (Manna et al., 2009; Stocco et al., 2005; Wathes et al., 2007). Release of the STAR protein initiates steroidogenesis (Phelan et al., 2011; Stocco et al., 2005; Wathes et al., 2007).

We did not find an association between omega-3 PUFA intake or the omega-6:omega-3 fatty acid intake ratio with any menstrual cycle characteristic. A recent study by Phelan et al. using bovine theca cells found that AA derived from omega-6 fatty acids upregulated luteinizing hormone (LH)-induced androgen production, whereas treatment of bovine cells with EPA and DHA derived from omega-3 fatty acids did not cause any

change in androgen production (Phelan et al., 2011). These observations suggest biologically plausible mechanisms through which omega-6-derived AA may alter the hormonal milieu, thereby influencing menstrual cycle length or pattern. This biological mechanism may provide an explanation for the association seen in our study between omega-6 fatty acid intake and menstrual cycle characteristics.

To our knowledge, our study is one of the first to explore the association between omega-6 and omega-3 PUFA intake and menstrual cycle characteristics healthy young women. Some of the strengths of our study include a thorough assessment of nutrient intakes using a validated food frequency questionnaire. We also collected information on demographic and lifestyle factors that may influence dietary fat intake or menstrual cycle characteristics. Although the data on menstrual cycle characteristics were self-reported and therefore subject to recall bias, all study visits occurred during the late luteal phase of the women's menstrual cycles, which may make women more likely to recall their cycle characteristics accurately. In addition, women in our study were at an ideal age since they are less likely to be affected by age-related hormonal changes than younger or older women might be. An important limitation of our study is the relatively small sample size and the homogeneous nature of our population, which may limit the generalizability of our findings. In addition, although we attempted to adjust in our analyses for most known confounders, residual confounding may still be present. However, the magnitude of our associations is small and may not be clinically relevant. Additional studies in larger cohorts are needed to confirm our findings and to provide information on the association between dietary fat intake and serum hormonal levels. Identifying modifiable

dietary factors may be a safe and cost-effective way to improve a woman's menstrual cycle characteristics, which may improve her chances of reproductive success.

Table 3.1 Descriptive statistics of study participants n=164)

| **Characteristics** | | **Mean ± SD[a]** |
|---|---|---|
| Age (years) | | 21.5 ± 3.1 |
| Total fat intake (% calories) | | 30 ± 6.7 |
| n-6 PUFA (% calories) | | 5.3 ± 1.7 |
| n-3 PUFA (% calories) | | 0.8 ± 0.3 |
| n-6:n-3 PUFA ratio | | 7.3 ± 2.3 |
| Fiber intake (g) | | 32.0 ± 19.0 |
| Saturated fat (g) | | 24.0 ± 11.4 |
| Monounsaturated fat (g) | | 27.0 ± 14.4 |
| Polyunsaturated fat (g) | | 15.5 ± 9.0 |
| Energy (kcal) | | 2191.0 ± 864.0 |
| Physical Activity (METs) | | 51.5 ± 46.1 |
| | | **n (%)** |
| **Menstrual Cycle Pattern** | | |
| | Regular (≤7 days) | 145 (88.4) |
| | Irregular (>7 days) | 19 (11.6) |
| **Menstrual Cycle Length** | | |
| | Short (≤25 days) | 32 (19.5) |
| | Typical (26 to 31days) | 98 (59.8) |
| | Long (≥32 days) | 34 (20.7) |
| **Menstrual Bleed days** | | |
| | Short (≤3 days) | 25 (15.2) |
| | Regular (4 to 5 days) | 100 (61.3) |
| | Long (>5 days) | 38 (23.3) |
| **Age at Menarche:** | ≤12years | 86 (52.4) |
| | >12 years | 78 (47.6) |
| **%Total Body fat (TBF):** | ≤32 | 86 (54.8) |
| | >32 | 71 (45.2) |
| | Missing | 7 |
| **Waist circumference:** | ≥35 inches | 23 (14) |
| | < 35 inches | 141 (86) |
| **YSM** | ≤6years | 29 (17.7) |
| | >6years | 135 (82.3) |
| **Current smokers:** | Yes | 9 (5.5) |
| | No | 154 (94.5) |
| | Missing | 1 |

[a] SD - standard deviation; YSM - years since menarche

Table 3.2a Distribution of dietary fat intake across menstrual cycle characteristics (n=164)

| Menstrual Cycle Characteristics | Total dietary fat (%calories) | Omega-3 PUFA (%calories) | Omega-6 PUFA (% calories) | Omega-6 :Omega-3 PUFA ratio |
|---|---|---|---|---|
| | Mean±SD[a] | | | |
| **Cycle Pattern** | | | | |
| Regular | 29.9 ± 6.4 | 0.8 ± 0.3 | 5.2 ± 1.43 | 7.3 ± 2.3 |
| Irregular | 30.0 ± 8.5 | 0.9 ± 0.4 | 6.2 ± 2.8 | 7.4 ± 2.2 |
| p-value[c] | 0.79 | 0.06 | **0.01*** | 0.84 |
| **Cycle length** | | | | |
| Short | 30.8 ± 7.2 | 0.8 ± 0.3 | 5.8 ± 1.9 | 7.7 ± 3.1 |
| Typical | 29.6 ± 6.6 | 0.7 ± 0.3 | 4.9 ± 1.3 | 7.1 ± 2.0 |
| Long | 30.4 ± 6.7 | 0.8 ± 0.3 | 5.7 ± 2.3 | 7.5 ± 2.0 |
| p-value[b] | 0.62 | 0.50 | **0.02*** | 0.69 |
| **Bleed Days** | | | | |
| Short | 29.6 ± 6.7 | 0.8 ± 0.3 | 5.5 ± 1.9 | 7.0 ± 1.9 |
| Regular | 30.0 ± 7.3 | 0.8 ± 0.3 | 5.3 ± 1.7 | 7.2 ± 2.5 |
| Long | 30.1 ± 5.2 | 0.7 ± 0.3 | 5.3 ± 1.5 | 7.7 ± 1.8 |
| p-value[b] | 0.95 | 0.56 | 0.85 | 0.12 |

[a] SD - standard deviation; * p-value <0.05

[b] p-value - one way analysis of variance F test

[c] p-value - two sample t-test

Table 3.2b Distribution of menstrual cycle characteristics by covariates (n=164)

| | Menstrual Cycle Length | | | | Cycle Pattern | | | Bleed Days | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Short | Typical | Long | | Regular | Irregular | | Short | Regular | Long | |
| | n=32 | n=98 | n=34 | | n=145 | n= 19 | | n=25 | n=100 | n=38 | |
| | Mean ± SD[a] | | | [b]P | Mean±SD[a] | | [c]P | Mean±SD[a] | | | [b]P |
| **Age (y)** | 21.2 ± 2.8 | 21.8 ± 3.4 | 21.0 ± 2.0 | 0.42 | 21.6 ± 3.1 | 20.6 ± 2.1 | 0.14 | 21.7 ± 3.1 | 21.3 ± 2.9 | 21.8 ± 3.3 | 0.71 |
| **Energy (kcal)** | 2110 ± 749 | 2172 ± 852 | 2318 ± 1000 | 0.59 | 2179 ± 830 | 2284 ± 1114 | 0.62 | 2194 ± 809 | 2181 ± 894 | 2211 ± 850 | 0.98 |
| **Fiber intake (g)** | 30.1 ± 14.8 | 32.2 ± 20.2 | 33.1 ± 19.6 | 0.81 | 31.0 ± 16.9 | 39.3 ± 30.3 | 0.07 | 34.7 ± 22.1 | 32.0 ± 19.9 | 30.1 ± 14.3 | 0.67 |
| **Physical Activity (METs)** | 49.8 ± 44.5 | 50.2 ± 43.3 | 56.9 ± 41.7 | 0.74 | 50.5 ± 46.3 | 59.3 ± 45.2 | 0.43 | 65.9 ± 54.9 | 49.8 ± 43.7 | 47.4 ± 45.9 | 0.24 |
| **%TBF** | 31.2 ± 7.2 | 32.8 ± 8.2 | 29.5 ± 9.0 | 0.15 | 32.3 ± 8.0 | 27.8 ± 8.6 | **0.04** | 28.6 ± 6.3 | 32.8 ± 5.0 | 31.0 ± 7.6 | 0.09 |
| **Age at Menarche** | 12.5 ± 1.1 | 12.5 ± 1.5 | 12.9 ± 1.7 | 0.33 | 12.4 ± 1.4 | 13.5 ± 1.7 | **0.01*** | 12.7 ± 1.4 | 12.4 ± 1.5 | 12.6 ± 1.4 | 0.81 |
| **YSM (y)** | 8.8 ± 2.8 | 9.3 ± 3.9 | 8.2 ± 2.6 | 0.25 | 9.2 ± 3.6 | 7.1 ± 2.6 | **0.01*** | 9.0 ± 3.5 | 8.9 ± 3.5 | 9.2 ± 3.7 | 0.87 |

[a] SD - standard deviation; YSM - years since menarche; * p-value < 0.01; TBF- total body fat

[b] P - p-value for one way analysis of variance F

[c] P- p-value two sample t-test

Table 3.2c Distribution of covariates by dietary fat intake (% calories) intake (n=164)

| Covariates | Tertiles of omega-6 PUFA %calories | | | | Tertiles of omega-3 PUFA %calories | | | | Tertiles of total fat intake %calories | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1st | 2nd | 3rd | | 1st | 2nd | 3rd | | 1st | 2nd | 3rd | |
| | Mean±SD[a] | | | [b]P | Mean±SD[a] | | | [b]P | Mean±SD[a] | | | [b]P |
| **Age (y)** | 21.3 ± 3.2 | 21.6 ± 2.8 | 21.6 ± 3.1 | 0.86 | 21.5 ± 2.8 | 21.5 ± 3.3 | 21.6 ± 3.1 | 0.99 | 21.6 ± 3.1 | 21.4 ± 2.8 | 21.7 ± 3.2 | 0.88 |
| **Calories (kcal)** | 2102 ± 824 | 2195 ± 958 | 2278 ± 805 | 0.58 | 2321 ± 928 | 2109 ± 700 | 2144 ± 941 | 0.39 | 2187 ± 764 | 2285 ± 929 | 2217 ± 908 | 0.46 |
| **Fiber intake (g)** | 31.2 ± 16.5 | 30.0 ± 19.5 | 34.8 ± 21.0 | 0.39 | 29.2 ± 17.2 | 30.9 ± 16.1 | 35.6 ± 22.7 | 0.18 | 38.0 ± 22.1 | 30.9 ± 16.2 | 25.9 ± 15.9 | **0.01*** |
| **Physical Activity (METs)** | 51.1 ± 50.7 | 55.7 ± 50.4 | 47.3 ± 35.7 | 0.63 | 52.1 ± 53.7 | 51.4 ± 44.8 | 50.9 ± 39.8 | 0.99 | 63.5 ± 53.3 | 49.4 ± 46.8 | 39.6 ± 31.5 | **0.02** |
| **% TBF** | 34.6 ± 8.9 | 29.8 ± 7.2 | 31.8 ± 7.4 | **0.01** | 33.9 ± 7.7 | 30.7 ± 7.4 | 31.5 ± 8.8 | 0.10 | 31.8 ± 8.3 | 33.6 ± 7.1 | 30.8 ± 8.7 | 0.23 |
| **Age at Menarche (y)** | 12.5 ± 1.4 | 12.5 ± 3.6 | 12.7 ± 1.4 | 0.67 | 12.4 ±1.5 | 12.5 ± 1.4 | 12.7 ± 1.5 | 0.47 | 12.4 ± 1.3 | 12.6 ± 1.5 | 12.7 ± 1.5 | 0.42 |
| **YSM (y)** | 8.9 ± 3.5 | 9.2 ± 3.5 | 8.9 ± 3.6 | 0.91 | 9.1 ± 3.4 | 9.0 ± 3.7 | 8.9 ± 3.5 | 0.93 | 9.2 ± 3.5 | 8.8 ± 3.4 | 8.9 ± 3.8 | 0.81 |

[a] SD - standard deviation; * p-value < 0.01

[b] P- p-value for one way analysis of variance F test; TBF- total body fat; YSM -years since menarche

Table 3.3a Association of dietary fat and menstrual cycle characteristics using unadjusted and adjusted multivariable linear regression (n=164)

| Dietary fat Intake | Menstrual Cycle Pattern | | | Menstrual Cycle Length | | | | |
|---|---|---|---|---|---|---|---|---|
| | Regular | Irregular | | Short | | Typical | Long | |
| | | Beta (95% CI) | P | Beta (95%CI) | P | | Beta (95%CI) | P |
| **Total dietary Fat** | | | | | | | | |
| Crude | Ref | 0.001 (-3.20, 3.20) | 0.99 | 0.07 (-1.48, 3.90) | 0.37 | Ref | 0.05 (-0.6, 1.12) | 0.53 |
| Model 1[a] | Ref | 0.02 (-2.9, 3.5) | 0.85 | 0.03 (-2.1, 3.20) | 0.69 | Ref | 0.06 (-0.55, 1.16) | 0.48 |
| Model 2[b] | Ref | 0.04 (-2.3, 4.0) | 0.61 | 0.10 (-0.95, 4.20) | 0.21 | Ref | 0.02 (-0.74, 0.92) | 0.83 |
| **Omega-6 PUFA** | | | | | | | | |
| Crude | Ref | 0.20 (0.2, 1.8) | **0.01** | 0.191 (0.14, 1.40) | **0.02** | Ref | 0.165 (0.01, 0.40) | **0.04** |
| Model 1[a] | Ref | 0.20 (0.23, 1.8) | **0.01** | 0.164 (0.02, 1.30) | **0.04** | Ref | 0.164 (0.01, 0.40) | **0.04** |
| Model 2[b] | Ref | 0.16 (0, 1.69) | **0.05** | 0.23 (0.29, 1.60) | **0.01** | Ref | 0.152 (-0.01, 0.42) | 0.06 |
| **Omega-3 PUFA** | | | | | | | | |
| Crude | Ref | 0.15 (-0.01, 0.3) | 0.06 | 0.09 (-0.05, 0.18) | 0.27 | Ref | 0.055 (-0.02, 0.05) | 0.50 |
| Model 1[a] | Ref | 0.18 (0.02, 0.27) | **0.03** | 0.06 (-0.09, 0.13) | 0.67 | Ref | 0.057 (-0.02, 0.05) | 0.49 |
| Model 2[b] | Ref | 0.08 (-0.07, 0.22) | 0.30 | 0.11 (-0.03, 0.19) | 0.16 | Ref | 0.046 (-0.03, 0.05) | 0.57 |
| **Omega-6:Omega-3 PUFA ratio** | | | | | | | | |
| Crude | Ref | 0.02 (-0.99, 1.2) | 0.85 | 0.10 (-0.35,1.50) | 0.22 | Ref | 0.07 (-0.17, 0.40) | 0.39 |
| Model 1[a] | Ref | 0.01 (-1.00, 1.10) | 0.95 | 0.12 (-0.23,1.60) | 0.14 | Ref | 0.07 (-0.20, 0.42) | 0.41 |
| Model 2[b] | Ref | 0.06 (-0.71, 1.60) | 0.45 | 0.10 (-0.35,1.52) | 0.22 | Ref | 0.07 (-0.18, 0.43) | 0.41 |

Outcomes - total dietary fat (% calories); omega-6 PUFA ( % calories); omega-3 PUFA (%calories); omega-6:omega-3 PUFA ratio

[a]Model 1- adjusted for energy, age, smoking status; [b] Model 2 - adjusted for energy, fiber, %body fat, age at menarche

Table 3.3b Association of dietary fat and menstrual cycle characteristics using unadjusted and adjusted multivariable linear regression (n=164)

| Dietary fat Intake | Menstrual Bleed Days | | | | |
|---|---|---|---|---|---|
| | Short | | Regular | Long | |
| | Beta (CI) | P | | Beta (CI) | P |
| **Total dietary Fat** (%) | | | | | |
| Crude | -0.02 (-3.40, 2.60) | 0.78 | Ref | 0.01 (-2.5, 2.6) | 0.95 |
| Model 1[a] | -0.01 (-3.10, 2.67) | 0.88 | Ref | 0.02 (-2.2, 2.74) | 0.83 |
| Model 2[b] | -0.01 (-3.00, 2.60) | 0.88 | Ref | -0.08 (-3.5, 1.2) | 0.33 |
| **Omega-6 PUFA** (%) | | | | | |
| Crude | 0.05 (-0.50, 0.95) | 0.57 | Ref | 0.003 (-0.6, 0.64) | 0.97 |
| Model 1[a] | 0.06 (-0.45, 0.99) | 0.46 | Ref | 0.01 (-0.57, 0.66) | 0.88 |
| Model 2[b] | 0.04 (-0.59, 0.92) | 0.68 | Ref | -0.04 (-0.78, 0.50) | 0.67 |
| **Omega-3 PUFA** (%) | | | | | |
| Crude | 0.04 (-0.09, 0.16) | 0.60 | Ref | -0.07 (-0.15, 0.6) | 0.43 |
| Model 1[a] | 0.06 (-0.75, 0.17) | 0.46 | Ref | -0.04 (-0.13, 0.08) | 0.60 |
| Model 2[b] | 0.004 (-0.12, 0.13) | 0.96 | Ref | 0.08 (-0.16, 0.06) | 0.34 |
| **Omega-6:Omega-3 PUFA ratio** | | | | | |
| Crude | -0.03 (-1.18, 0.83) | 0.73 | Ref | 0.09 (-0.38,1.33) | 0.27 |
| Model 1[a] | -0.03 (-1.2, 0.81) | 0.71 | Ref | 0.08 (-0.44, 1.27) | 0.34 |
| Model 2[b] | 0.01 (-0.95, 1.10) | 0.89 | Ref | 0.08 (-0.45, 1.30) | 0.34 |

Outcomes - total dietary fat, omega-6 PUFA ( % calories); omega-3 PUFA (%calories); omega-6:omega-3

Model 1[a]- adjusted for energy, age, smoking status; Model 2[b] - adjusted for energy, fiber, %body fat, age at menarche;

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# CHAPTER 4

# ASSOCIATION OF SERUM 25- HYDROXYVITAMIN D AND MENSTRUAL CYCLE CHARACTERISTICS IN COLLEGE-AGED WOMEN

## 4.1 ABSTRACT

**Background**: Vitamin D may be involved in synthesis in sex steroid hormones, which could affect menstrual cycle function, yet few studies have explored its association with menstrual cycle characteristics in young women. The current study examines the association between serum 25-hydroxyvitamin D (25-OH vitamin D), a biomarker for vitamin D status, and menstrual cycle characteristics.

**Methods**: The study subjects were a subset of 164 18- to 30-year-old women participants in the UMass Vitamin D Status Study who reported no use of hormonal contraceptives. Serum 25-OH vitamin D was measured in fasting venous samples, and information on menstrual cycle characteristics (cycle pattern, length and bleed days) was obtained through a self-reported questionnaire.

**Results**: After controlling for age, season and age at menarche, the odds of having sufficient vitamin D status (serum 25-OH vitamin D concentration >50 nmol/L), was 80% lower among women with irregular cycle pattern compared to those with a regular cycle [OR-0.19; 95% CI: 0.04, 0.92]. Menstrual cycle length and bleed days were not associated with vitamin D status.

**Conclusion**: Insufficient vitamin D status was significantly associated with irregular cycles in young women. Although our findings indicate that optimal vitamin D status

may play a role in mediating ovarian function and menstrual cycle regularity, additional prospective studies are needed to determine whether improving vitamin D status also improves menstrual function.

## 4.2 INTRODUCTION

The menstrual cycle is an important aspect of women's reproductive health and is governed by the hypothalamus-pituitary-ovarian axis (Mihm et al., 2011). Underlying ovarian hormonal fluctuations are reflected in variations in menstrual cycle characteristics (Dishi et al., 2011; T. K. Jensen et al., 1999; Kolstad et al., 1999; Rowland et al., 2002; Small et al., 2010; Small et al., 2006; Steiner et al., 1999; Wise et al., 2011). Whether the pattern of the menstrual cycle is regular, occurring at a predictable interval, or irregular is thought to be a measure of ovulatory function (van Hooff et al., 2004). Menstrual cycle length is the interval from the first day of one menses to the start of the next menses. A short or long cycle may indicate abnormal hormonal feedback, which may affect menstrual phase lengths and, in turn, ovulation and the quality of the oocyte (Fehring, Schneider, and Raviele, 2006; Ouyang et al., 2007; Windham et al., 2002). Menstrual bleed days indicate the duration of menstrual bleeding and reflect endometrial development, which is mediated by the ovarian hormones (Vitzthum, 2009). Abnormal menstrual cycle characteristics are associated with reduced fecundity and increased risk of miscarriage (Kolstad et al., 1999; Small et al., 2010; Small et al., 2006).

Suboptimal vitamin D status, characterized by decreased serum 25-hydroxycholecalciferol (25-hydroxyvitamin D) concentrations, has been reported in 35% of reproductive age women (Yetley, 2008). Vitamin D is classically known for its role in

calcium homeostasis and bone health (Holick, 2011). However, recent studies suggest a role for vitamin D in women's reproductive health and fertility (Grundmann & von Versen-Hoynck, 2011; Lerchbaum & Obermayer-Pietsch, 2012), and evidence from animal studies support vitamin D's involvement in fertility and estrogen synthesis (Halloran & DeLuca, 1980; Kwiecinksi et al., 1989). Other reports indicate that seasonal changes in sunlight exposure influence menstrual cycle length and pattern (Danilenko et al., 2011; Stumpf & Denny, 1989; Sundararajan et al., 1978). Vitamin D is made endogenously in the skin in response to sunlight (UVB radiation) through cutaneous synthesis and varies with season due to UVB availability (Holick, 2011). Vitamin D is activated by hydroxylation reactions that take place in the liver to form 25-hydroxyvitamin D and in the kidney to produce the hormonally active 1,25-dihydroxyvitamin D. Thus, one mechanism by which seasonal changes may influence menstrual cycle regularity could be through differences in vitamin D status.

The biological actions of vitamin D are mediated through 1,25-dihydroxyvitamin D-dependent activation of the vitamin D receptor (VDR) protein, a nuclear transcription factor. Recent studies demonstrate the presence of the VDR in various reproductive tissues, including the ovarian granulosa cells, which are a site for estrogen synthesis (Parikh et al., 2010). Additional support for a role of vitamin D in sex hormone synthesis is provided by findings in VDR knockout mice. VDR null mice had decreased P450 aromatase enzyme activity and decreased expression of the *cyp19* gene, which encodes the P450 aromatase enzyme, a key enzyme required in estrogen synthesis from androgens (Kinuta et al., 2000). Vitamin D deficiency in animals causes a substantial decrease in fertility and fecundity and disturbed their estrus cycling (Halloran & DeLuca, 1980).

Estrus cycle abnormalities, including arrested follicular development, prolonged cycles and disturbance in estrus staging patterns, were observed in vitamin $D_3$-deficient *cyp*27b1 knock-out mice (Dicken et al., 2012). The *cyp27b1* gene encodes the 1α-hydroxylase enzyme needed to activate 25-hydroxyvitamin D to the hormonal 1,25-dihydroxyvitamin D metabolite.

Despite evidence that vitamin D status may influence female reproductive function, little is known about the association between vitamin D status and menstrual cycle characteristics in women, particularly young women. Because abnormal menstrual cycle characteristics are associated with poor reproductive outcomes, (Kolstad et al., 1999; Small et al., 2010; Small et al., 2006), understanding the role of nutritional status in these events could inform interventions aimed at enhancing reproductive success. The current study evaluates the association of three menstrual cycle characteristics (cycle pattern, cycle length and bleed days) with vitamin D status in young women participating in the UMass Vitamin D Status Study (Bertone-Johnson et al., 2010).

## 4.3 METHODS

### 4.3.1 Study Design, Study Setting and Population

The study uses data collected as part of the UMass Vitamin D Status Study, a cross-sectional study designed to assess vitamin D status in young women and to identify its dietary, environmental and lifestyle determinants. The current analysis examines the association between serum 25-hydroxyvitamin D concentration, a biomarker for vitamin D status and menstrual cycle length, pattern and bleed days.

The UMass Vitamin D Status Study is based at the University of Massachusetts, Amherst. The study subjects consisted of 288 female students of all ethnicities between 18 to 30 years of age. Women on campus were recruited through brochures distributed at various classes and displayed on bulletin boards in various departments in the university and through references from women who participated in the study. Information about women was collected during one study visit in the late luteal phase of their menstrual cycle. During the study visit, the participant completed a study questionnaire that collected information on demographics, health history, and menstrual symptoms. The participant also completed a Harvard food frequency questionnaire (FFQ) to record dietary intake (Willett et al., 1985).

Women were ineligible for the study if they were currently pregnant or not menstruating, currently suffering from depression, had high blood pressure, kidney or liver disease, bone diseases (osteopenia or osteomalacia), rheumatologic diseases, multiple sclerosis, cancer, diabetes type I/II, hyperparathyroidism, hyperlipidemia, polycystic ovaries, digestive disorders such as celiac disease, Crohn's disease or ulcerative colitis and thyroid diseases. Women taking medications such as corticosteroids (e.g. prednisone; anabolic steroids), anticonvulsants (e.g. Depakote), Tagamet or propranolol were also excluded. Women with the above conditions were excluded as each of the conditions could potentially influence vitamin D status. Women using oral contraceptives (OC) have regular cycles due to the hormonal content in the pill, which is not indicative of the true nature of their menstrual cycle before OC use. For these analyses, therefore, we excluded women using OCs or other hormonal contraceptives

(n=124). In our cohort of women, OC users differed significantly from non- users only with respect to their menstrual cycle characteristics.

### 4.3.2 Serum 25-hydroxyvitamin D

Overnight fasting venous blood samples (~45 mL) were collected from the participants in the late luteal phase of their menstrual cycle. The blood samples were collected by a trained phlebotomist from the University Health Services. The collected blood samples were kept on ice and processed as serum, plasma and whole blood no later than 1 hour after the blood draw. These samples were stored at -80º C until the assay for 25-hydroxyvitamin D was conducted. Serum 25-hydroxyvitamin D concentration were assessed by a FDA-approved and previously validated (Hollis, 2000) radioimmunoassay kit manufactured by Diasorin, Inc. (Stillwater, MN). A gamma counter (Beckman Gamma 4000 counter, Beckman Coulter, California, USA) was used to quantify gamma irradiation $I^{125}$ (in counts per minute; cpm). The CPM was converted to concentration units using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA) (Bertone-Johnson et al., 2010). Serum 25-hydroxyvitamin D was characterized as both a continuous and a categorical variable. The categories included "Insufficient" ($\leq$ 50 nmol/L) and "Sufficient" (>50 nmol/L) serum 25-hydroxyvitamin D. Serum 25-hydroxyvitamin D concentration below 50 nmol/L are considered to be inadequate for bone health and general health in healthy individuals 25-hydroxyvitamin D ("Vitamin D: Fact Sheet for Health Professionals," 2011). We assessed the season from the month of blood draw, as season affects vitamin D status due to maximum vitamin D availability from sunlight during certain seasons at higher latitudes (Holick, 2011). The following

categories were developed for season: Winter months (November to March 14) and Non-winter months (March 15 to October). This information was collected from the clinic study questionnaire, which was administered at the time of the study visit.

### 4.3.3 Menstrual Cycle Characteristics

Information on menstrual cycle characteristics was collected through a self-reported study questionnaire. Menstrual cycle length categories were: <21 days, 21 to 25 days, 26 to 31 days, 32 to 39 days, 40 to 50 days, >50 days, or "too irregular to count". We collapsed these categories to ≤ 25 days (short), 26 to 31 days (regular) and ≥ 32 days (long). Menstrual cycle variability information was collected through a cycle pattern question with the following categories: extremely regular (no more than 1 to 2 days before or after expected), very regular (within 3-4 days), regular (within 5-7 days), usually irregular and always irregular. Cycle pattern was collapsed to regular (for ≤7 day variation) and irregular (for > 7 day variation). Bleed duration information was collected under the following four categories: ≤3 days, 4-5 days, 6-7 days, ≥8 days. For statistical analyses, we collapsed bleed duration into three categories: ≤3 days (short), 4 to 5 days (regular), >5 days (long).

### 4.3.4 Covariate Assessment (Anthropometric and Lifestyle)

Information on covariates was collected through a health history questionnaire completed during the study visit. Anthropometric measures were collected during a single study visit by a trained research assistant. Weight was measured using a calibrated scale, and height was measured using a stadiometer. BMI was calculated as weight, in

kilograms, divided by the square of height, in meters. Waist circumference was measured at the umbilicus using a standard tape. We measured total body fat percent using DXA whole body scan mode on a narrow angle fan GE Lunar Prodigy scanner (GE Lunar Corp., Madison, WI). The scan was performed with the subject lying in a supine position as per the manufacturer's specifications (Zagarins et al., 2012). Information concerning smoking, parity, age at menarche was collected through self-reported entry in the questionnaire (Liu et al., 2004). Physical activity can affect menstrual cycle function, and information on participant's physical activity was assessed via a questionnaire (Orio et al., 2013). Participants reported their average weekly physical activity from a modified version of the Nurses' Health Study II questionnaire (Willett et al., 1985; Zagarins et al., 2012). Activities included walking, jogging, running, bicycling, aerobics/dance/rowing machine, tennis/racket sports, swimming, yoga/pilates, and weight training/resistance exercises. The response ranged from 0 to 11 or more hours per week. Metabolic equivalents (MET) scores were assigned to each activity using the method used by Ainsworth et al., 2000 (Ainsworth et al., 2000).

### 4.3.5 Statistical Analysis

All statistical analyses were conducted using IBM SPSS, version 22. The characteristics of the study population were reported using means, standard deviations, and ranges for continuous variables (age, physical activity score, %TBF, age at menarche, years since menarche) and counts and percentages for categorical variables (menstrual cycle length, cycle pattern, bleed duration, age at menarche, oral contraceptive

use, smoking status, season). We examined the distribution of continuous outcome variables to assess normality and all variables were sufficiently normally distributed.

Bivariate analyses were conducted to evaluate the potential confounders by cross tabulating covariates against menstrual cycle characteristics and serum 25-hydroxyvitamin D concentration. Differences in means were assessed using t-tests and ANOVA, and differences in proportions were assessed using Chi square tests and Fisher's exact test for cells with small frequencies.

Univariable linear regressions were conducted to provide unadjusted associations between the various menstrual cycle characteristics and serum 25-hydroxyvitamin D concentrations. Variables that was significantly associated with serum 25-hydroxyvitamin D were evaluated for inclusion in the multivariable models. Multivariable linear regression analysis was used to study the association of the menstrual cycle pattern, bleed duration and cycle length and serum 25-hydroxyvitamin D, controlling for those covariates that caused a 10% change in the coefficient for serum 25-hydroxyvitamin D. We calculated beta coefficients and 95% confidence intervals. We also conducted logistic regression and estimated odds ratio and 95% confidence intervals for the association of menstrual cycle characteristics and vitamin D status. We tested several models with various covariates including physical activity, %TBF, BMI, age at menarche, season and we only included covariates that caused a 10% change in the estimates of the serum 25-hydroxyvitamin D in our final model. We considered a $p < 0.05$ to be statistically significant.

## 4.4 RESULTS

**Subject Characteristics**

The current analysis included 110 women with available serum 25-hydroxyvitamin D. The descriptive statistics of the 110 eligible women from the UMass Vitamin D Study are shown in **Table 4.1**. The mean (± SD) age of this young adult group was 21.6 (± 3.3) years. Anthropometric measurements indicated that the average BMI was 23.1 (± 3.1) kg/m$^2$, which is within the recommended normal BMI range (18.5-24.9 kg/m$^2$), as was the mean waist circumference (30.9 ± 3.3 inches). Total body fat (TBF) —as measured by DXA—indicated that the mean %TBF in this group was 32% (±7.9%), which is higher than the recommended level of < 32% (Pi-Sunyer, 2000). Overall, the mean level of physical activity of the group (54.5 ± 48.3 METs/week), calculated from subject estimates of type and duration of daily activities, was considered moderate by government guidelines ("Physical activity guidelines for Americans," 2008). The mean (±SD) serum 25-hydroxyvitamin D level was 69.9 (±27.4) nmol/L, which is above the recommendations of > 50 nmol/L required for optimal vitamin D status. More than two-thirds of the women had sufficient 25-hydroxyvitamin D (79.1%) and 20.9% of the women had 25-hydroxyvitamin D levels ≤ 50 nmol/L, indicating insufficiency ("Vitamin D: Fact Sheet for Health Professionals," 2011). Irregular menstrual cycle pattern, cycle length and number of bleed days were reported by 7.3%, 39.1% and 36.4% of the women, respectively (**Table 4.1**).

In bivariate analysis, 25-hydroxyvitamin D sufficiency was associated with a more regular menstrual cycle (**Table 4.2a**). Women with insufficient serum 25-hydroxyvitamin D (≤ 50 nmol/L) were nearly four times more likely to report irregular cycles (17.4 vs. 4.6%, p =0.06). Serum 25-hydroxyvitamin D concentration was not

associated with menstrual cycle length (**Table 4.2a**). In addition to serum 25-hydroxyvitamin D, other subject characteristics were associated with menstrual cycle pattern. Women with irregular cycles had a later mean age at menarche (13.7 vs. 12.6, $p<0.02$) and reported fewer years since menarche (YSM: 6.1 vs. 9.2, $p<0.02$). Menstrual cycle pattern was not associated with age, physical activity, %TBF or season. No covariates were associated with menstrual cycle length (**Table 4.2b**). No covariates were associated with serum 25-hydroxyvitamin D concentrations (**Table 4.2c**)

**Menstrual Cycle Characteristics**

***Menstrual Cycle Pattern:*** To further evaluate the impact of potential covariates on the association of serum 25-hydroxyvitamin D with menstrual cycle pattern, we developed adjusted linear regression models (**Table 4.3a**). In all linear regression models, serum 25-hydroxyvitamin D concentration was not associated with irregular cycle pattern.

In logistic regression analyses, unadjusted, model 1(adjusted for BMI and physical activity) and model 2 (adjusted for age, season and age at menarche) women with irregular cycles had an 80% lower odds of having sufficient serum 25-hydroxyvitamin D concentration compared to women with regular cycles [model 2: OR= 0.19; 95% CI (0.04,0.92)]. These associations were statistically significant in all three models (**Table 4.3b**).

***Menstrual Cycle Length:*** No significant associations were found in unadjusted and adjusted models (model 1 and 2) between serum 25-hydroxyvitamin D and menstrual cycle length in linear and logistic regression models **(Table 4.3a, 4.3b).**

***Menstrual Cycle Bleed Days:*** In all unadjusted and adjusted linear and logistic regression models, no association was found between the number of menstrual cycle bleed days and serum 25-hydroxyvitamin D concentration (**Table 4.3a and table 4.3b**).

## 4.5 DISCUSSION

In this cross-sectional study of young, healthy women, we found that odds of having an irregular menstrual cycle was 80% lower in women with adequate vitamin D status (serum 25-hydroxyvitamin D concentration >50 nmol/L), compared to vitamin D-insufficient women. However, vitamin D status was not associated with menstrual cycle length or bleed days.

To our knowledge, our study is one of the first to explore the association between vitamin D status and menstrual cycle characteristics in women age 30 and younger. A cross-sectional study by Jukic et al., conducted in mostly late reproductive-aged (30 to 49 years) mostly African American women, examined the association between plasma 25-hydroxyvitamin D concentration and menstrual cycle regularity and length (Jukic, Steiner, & Baird, 2015). After controlling for age, race, BMI, age at menarche and physical activity, they found that a decrease of 25 nmol/L plasma 25-hydroxyvitamin D was associated with a nearly twofold increase in the odds of irregular cycles (OR=1.9; 95% CI: 1.0, 3.4). Although the sample of women from the Jukic et al. study (Jukic et al., 2015) differed from ours in age and race, their observation of an association between lower vitamin D status and menstrual cycle irregularity is similar to our findings in this study, which was conducted among younger, mostly Caucasian women.

Our study did not provide sufficient biomarker information to gain insight into the possible biological mechanism underlying the association between vitamin D status and menstrual cycle characteristic. However, our observations corroborate with findings made in animals fed vitamin D-deficient diets, which were associated with abnormal estrous cycles (Dicken et al., 2012; Halloran & DeLuca, 1980). Moreover, the presence of the vitamin D receptor in several reproductive tissues, including the granulosa cells of the ovary, a site for aromatase activity, suggests a possible role for vitamin D in steroid synthesis (Parikh et al., 2010). As ours was a cross sectional study, we cannot establish a temporal relationship; however, the underlying biology suggests that menstrual cycle characteristics are more likely affected by 25-hydroxyvitamin D concentrations than vice versa.

Some of the limitations of our study include the homogenous nature of our population. All study participants were within a narrow age window (18 to 30 years old) and 83% of them were Caucasians, which may limit the generalizability of our results. However, the similarity of our findings and those of Jukic et al., conducted in older and mostly non-Caucasian women, would argue in favor of a greater degree of generalizability. Another potential limitation to the study is that we used self-report to assess menstrual cycle characteristics, which can cause measurement errors that would bias the results toward the null. However, menstrual cycle information was collected in the late luteal phase of their menstrual cycles, which could make them more likely to recall their cycle characteristics accurately and thereby reduce measurement errors. In addition, although we attempted to adjust for most known confounders, residual confounding may still be present.

In conclusion, we found that one out of five young, healthy, Caucasian women in our study were vitamin D deficient, based on current standards adopted by the Institute of Medicine ("Vitamin D: Fact Sheet for Health Professionals," 2011). Moreover, women with vitamin D deficiency were at substantially increased risk of irregular menstrual cycles. Overall, our findings indicate that optimal vitamin D status may be important in controlling menstrual cycle regularity. However, our study was cross-sectional and had a relatively small sample size, which limits our conclusions. A vitamin D supplementation trial in women with chronic menstrual cycle irregularities would be needed to provide further mechanistic and cause-and-effect evidence of this relationship.

Table 4.1 Descriptive statistics of study participants (n=110)

| Characteristics | | Mean ± SD[a] |
|---|---|---|
| **Age (years)** | | 21.6 ± 3.3 |
| **BMI (kg/m$^2$)** | | 23.1 ± 3.1 |
| **Waist circumference (inches)** | | 30.9 ± 3.3 |
| **% Total Body Fat** | | 32.0 ± 7.9 |
| Missing | | 6 |
| **Serum 25-OH D (nmol/L)** | | 69.9 ± 27.4 |
| **Physical Activity (METs/week)** | | 54.5 ± 48.3 |
| **Age at menarche (years)** | | 12.6 ± 1.3 |
| **Years since menarche (YSM)** | | 9.0 ± 3.7 |
| | | **n (%)** |
| **Menstrual Cycle Pattern** | | |
| Regular | | 102 (92.7) |
| Irregular | | 8 (7.3) |
| **Menstrual Cycle Length** | | |
| Short | | 19 (17.3) |
| Regular | | 67 (60.9) |
| Long | | 24 (21.8) |
| **Bleed days** | | |
| Short | | 17 (15.5) |
| Regular | | 70 (63.6) |
| Long | | 23 (20.9) |
| **Serum 25-OH D (nmol/L):** | ≤ 50 nmol/L | 23 (20.9) |
| **Age at Menarche:** | ≤12 years | 52(47.3) |
| **Waist circumference:** | ≥35 inches | 17(15.5) |
| **Current smokers:** | Yes | 7 (6.4) |

[b]p-values - chi square test of association; [a]SD - standard deviation
* p-value ≤ 0.05; %TBF-percent total body fat;25-OH D- 25-hydroxyvitamin D

Table 4.2a Distribution of menstrual cycle characteristics across serum 25-OH D concentrations (n=110)

| | Serum 25-OH D concentration | | | | |
|---|---|---|---|---|---|
| | Grouped | | | Continuous (nmol/L) | |
| | Insufficient n=20 | Sufficient n=76 | | n=110 | |
| | n (%) | | P[a] | Mean ± SD[c] | P[b] |
| **Menstrual Cycle Pattern** | | | | | |
| Regular (108) | 19 (82.6) | 83 (95.4) | 0.06[d] | 71.1 ± 27.8 | 0.12 |
| Irregular (8) | 4 (17.4) | 4 (4.6) | | 55.3 ± 15.7 | |
| **Cycle Length** | | | | | |
| Short (19) | 5 (21.7) | 14 (16.1) | 0.74 | 74.3 ± 37.8 | 0.70 |
| Regular (67) | 14 (60.9) | 53 (60.9) | | 68.4 ± 25.5 | |
| Long (24) | 4 (17.4) | 20 (23) | | 70.5 ± 23.4 | |
| **Bleed Days** | | | | | |
| Short (17) | 2 (8.7) | 15 (17.2) | 0.59 | 79.4 ± 28.2 | 0.28 |
| Regular (70) | 16 (69.6) | 54 (62.1) | | 67.7 ± 24.9 | |
| Long (23) | 5 (21.7) | 18 (20.7) | | 69.6 ± 32.9 | |

[a]p-values - chi square test of association; [b] p-value - one way analysis of variance F test

[c] SD - standard deviation; * p-value $\leq$ 0.05; [d]p-values- Fisher's exact test

25-OH D: 25-hydroxyvitamin D

Table 4.2b Distribution of menstrual cycle characteristics by covariates (n=110)

| | Menstrual Cycle Length | | | | Menstrual Cycle Pattern | | | Menstrual Bleed Days | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Short | Regular | Long | | Regular | Irregular | | Short | Regular | Long | |
| | n=19 | n=67 | n=24 | | n=102 | n=8 | | n=17 | n=70 | n=23 | |
| | Mean ± SD[a] | | | P[b] | Mean ± SD[a] | | P[b] | Mean ± SD[a] | | | P[b] |
| **Age (yr)** | 21.6±3.4 | 21.8±3.6 | 21.1±1.8 | 0.65 | 21.8±3.3 | 20.0±1.2 | 0.11 | 21.7±3.6 | 21.4±3.1 | 22.2±3.5 | 0.58 |
| **Physical Activity (METs/week)** | 49.1±46.4 | 53.9±50.2 | 60.3±45.6 | 0.74 | 53.2±47.3 | 71.6±61.7 | 0.30 | 64.5±67.2 | 55.1±48.8 | 45.4±38.0 | 0.47 |
| **Age at Menarche** | 12.8±0.9 | 12.6±1.4 | 12.8±1.6 | 0.74 | 12.6±1.3 | 13.7±1.8 | **0.02** | 12.5±1.4 | 12.6±1.4 | 12.8±1.2 | 0.84 |
| **YSM** | 8.8±3.5 | 9.2±4.1 | 8.3±2.5 | 0.57 | 9.2±3.7 | 6.1±1.3 | **0.02** | 9.2±4.1 | 8.7±3.6 | 9.4±4 | 0.71 |
| **BMI (kg/m$^2$)** | 23.4±2.9 | 23.0±2.9 | 23.2±3.7 | 0.93 | 23.1±3.1 | 23.5±3.1 | 0.75 | 22.9±2.4 | 23.4±3.4 | 22.5±2.8 | 0.42 |
| **TBF (%)** | 32.6±7.2 | 32.0±7.6 | 31.3±9.4 | 0.88 | 31.8±7.7 | 33.4±11 | 0.59 | 30.1±6.9 | 32.7±8.2 | 30.4±5.8 | 0.45 |
| | n (%) | | | P[c] | n (%) | | P[c] | n (%) | | | P[c] |
| **Season** | | | | | | | | | | | |
| Winter months | 7 (36.8) | 22 (32.8) | 8 (33.3) | 0.95 | 33 (33.4) | 4 (50) | 0.44 | 7 (41.2) | 19 (27.1) | 11 (47.8) | 0.15 |
| Non-winter months | 12 (67.2) | 45 (67.2) | 16 (66.7) | | 69 (67.6) | 4 (50) | | 10 (58.8) | 51 (72.9) | 12 (52.2) | |
| **Age at Menarche** | | | | | | | | | | | |
| ≤ 12 years | 7 (36.8) | 34 (50.7) | 11 (45.8) | 0.56 | 50 (49) | 2 (25) | 0.17 | 10 (58.8) | 31 (44.3) | 11 (47.8) | 0.56 |
| > 12 years | 12 (63.2) | 33 (49.3) | 13 (54.2) | | 52 (51) | 6 (75) | | 7 (41.2) | 39 (55.7) | 12 (52.2) | |

[a] SD - standard deviation; TBF - total body fat; winter months- November to March 14 and non-winter months-March 15 to October

[b] p-value - one way analysis of variance F test

[c] p-value - chi-square test of association

YSM- years since menarche

Table 4.2c Distribution of covariates by serum 25-OH D† concentration (n=110)

| | Serum 25-OH D† concentration | | |
|---|---|---|---|
| | ≤ 50nmol/l | >50nmol/l | |
| | Mean ± SD[a] | | p-value[b] |
| **Age (years)** | 22.0 ± 3.7 | 21.5 ± 3.2 | 0.52 |
| **Physical Activity (METs/week)** | 53.6 ± 50.5 | 54.7 ± 48.0 | 0.92 |
| **Age at menarche (y)** | 12.6 ± 1.7 | 12.6 ± 1.3 | 0.96 |
| **YSM** | 9.4 ± 4.5 | 8.9 ± 3.5 | 0.56 |
| **BMI (kg/m$^2$)** | 23.5 ± 3.2 | 23.0 ± 3.1 | 0.56 |
| **TBF (%)** | 34.0 ± 7.3 | 31.4 ± 8.1 | 0.19 |
| | **Serum 25-OH D† concentration** | | |
| | Mean ± SD[a] | p-value[c] | |
| **Season** | | | |
| Winter months | 64.3 ± 28.8 | 0.13 | |
| Non-winter months | 72.8 ± 26.4 | | |
| **Age at Menarche** | | | |
| <= 12y | 70.1 ± 27.9 | 0.94 | |
| > 12 y | 69.7 ± 27.1 | | |

[a] SD - standard deviation; [b] p-value - chi square test association

[c] p-value - independent sample t-test; MET-metabolic equivalents

winter months - November -March 14; non-winter months: March 15 –October

†25-OH D: 25-hydroxyvitamin D; YSM-years since menarche.

Table 4.3a Associations of serum 25-OH D concentration and menstrual cycle characteristics using unadjusted and adjusted multivariable linear regression (n=110)

| | Serum 25-OH D concentration (nmol/L)[1] | | | | | |
|---|---|---|---|---|---|---|
| | **Crude** | | **Model 1** | | **Model 2** | |
| **Menstrual Cycle Characteristics** | Beta (95% CI) | p-value | Beta (95% CI) | p-value | Beta (95% CI) | p-value |
| **Menstrual Cycle Length** | | | | | | |
| Short | 0.08 (-8.2, 20) | 0.41 | 0.09 (-7.7, 20.6) | 0.37 | 0.09 (-7.4, 20.9) | 0.35 |
| Regular | Referent | | Referent | | Referent | |
| Long | 0.03 (-3.6, 5) | 0.75 | 0.03 (-3.7, 4.9) | 0.78 | 0.03 (-3.7, 4.9) | 0.78 |
| **Menstrual Cycle Pattern** | | | | | | |
| Regular Pattern | Referent | | Referent | | Referent | |
| Irregular Pattern | -0.15 (-35, 4) | 0.12 | -0.16 (-36.4, 3.3) | 0.10 | -0.16 (-37.1, 3.7) | 0.11 |
| **Menstrual Bleed Days** | | | | | | |
| Short | 0.16 (-2.9, 26.4) | 0.12 | 0.14 (-3.9, 25.5) | 0.15 | 0.16 (-2.4, 27) | 0.10 |
| Regular | Referent | | Referent | | Referent | |
| Long | 0.03 (-11,14.9) | 0.77 | 0.02 (-11.5,14.7) | 0.81 | 0.05 (-9.8 to 16.6) | 0.61 |

Model 1- Covariates adjusted in the model include: physical activity and BMI;
25-OH D: 25-hydroxyvitamin D

Model 2- Covariates adjusted in the model include: BMI, physical activity, season and age at menarche

Table 4.3b Associations of serum 25-OH D concentration and menstrual cycle characteristics using unadjusted and adjusted multivariable logistic regression (n=110)

| | **Serum 25-OH D concentration[1] (nmol/L) OR (95% Confidence Interval)** | | | | | |
|---|---|---|---|---|---|---|
| **Menstrual Cycle Characteristics** | **Unadjusted** | **p-value** | **Model 1** | **p-value** | **Model 2** | **p-value** |
| **Menstrual Cycle Pattern** | | | | | | |
| Regular | Referent | | Referent | | Referent | |
| Irregular | **0.23 (0.05,0.99)** | **0.05** | **0.22 (0.05, 0.99)** | **0.05** | **0.19 (0.04, 0.92)** | **0.04** |
| **Menstrual Cycle length** | | | | | | |
| Short | 0.74 (0.23, 2.4) | 0.61 | 0.75 (0.23, 2.44) | 0.63 | 0.63 (0.23, 2.45) | 0.63 |
| Regular | Referent | | Referent | | Referent | |
| Long | 1.32 (0.38,4.49) | 0.65 | 1.33 (0.39, 4.54) | 0.65 | 1.28 (0.37, 4.44) | 0.69 |
| **Menstrual Bleed days** | | | | | | |
| Short | 2.2 (0.46, 10.7) | 0.32 | 2.16 (0.44, 10.54) | 0.34 | 2.5 (0.51,12.37) | 0.26 |
| Regular | Referent | | Referent | | Referent | |
| Long | 1.06 (0.34, 3.32) | 0.91 | 1.03 (0.33, 3.25) | 0.96 | 1.29 (0.39, 4.24) | 0.67 |

Model 1- adjusted for BMI and physical activity; [1]Outcome 25-OH D variable coding : Control =0 $\leq$ 50nmol/l and Case =1 >50nmol/l; Model 2-adjusted for age, season and age at menarche;

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# CHAPTER 5

# CONCLUSION AND FUTURE DIRECTION

## 5.1 Conclusions

Menstrual cycle characteristics serve as indicators of optimal ovarian function and have been linked to a woman's reproductive success. Several factors can affect ovarian function and menstrual cycle attributes. The findings of the three current studies highlight the possible influence of nutritional factors in regulating menstrual cycle characteristics, particularly menstrual cycle pattern.

Our first study, which examined the association of adiposity—as measured by DXA scans—with menstrual cycle characteristics, led to several important findings. First, we found that general body fatness as well as fat distribution was associated with both menstrual cycle pattern and the number of bleed days. Women with low %TBF were more likely to report irregular cycle patterns than were women with higher %TBF. In addition, women with less gynoid fat or a lower G/A ratio were more likely to have irregular cycles.

We also found that proxy measures of adiposity, such as BMI and waist circumference, were not associated with cycle characteristics. Furthermore, we found that BMI, although convenient and noninvasive, underestimates the prevalence of obesity when compared to obesity defined in terms of %TBF. This disparity underscores the inadequacy of BMI as a measure of body fat especially in our cohort of healthy, young, active women. Because the actual quantity of adipose tissue influences the underlying biological mechanisms associated with menstrual cycle regularity (Balen et al., 2007; Frisch, 1996), selecting a method that accurately measures

adiposity is critical to understanding the nature of the association of body fat with cycle regularity. Our study suggests that DXA measurements may more accurately distinguish among levels of adiposity than does BMI. In addition, DXA measurements provide valuable information on regional adiposity, which appears to be important in regulating menstrual cycle function. Further investigations are warranted to study the underlying biological mechanisms.

Among other nutritional factors, our findings also highlight the role of dietary fat intake, particularly omega-6 PUFA, and menstrual cycle characteristics. High intake of omega-6 PUFA was associated with both short and long cycle length. Additionally, high intakes of omega-6 PUFA were associated with irregular cycles. To our knowledge, our study is the first to explore the association between omega-6 and omega-3 PUFA intake and menstrual cycle characteristics. Making simple dietary modifications may help improve menstrual cycle function in young women.

Our third study examined the association of menstrual cycle characteristics with vitamin D status. We found that one out of five young healthy women in our study had suboptimal serum levels of 25-hydroxyvitamin D, the biomarker for vitamin D status, and women with a high %TBF tended to have lower serum 25-hydroxyvitamin D concentrations than leaner women. We also found that women with sufficient serum 25-hydroxyvitamin D concentration (> 50 nmol/L) were 80% less likely to have irregular cycles. These findings are especially interesting in light of those from our first study, in which we found that higher adiposity appears protective of cycle regularity, yet women with a higher %TBF were more likely to have insufficient vitamin D status, which was associated with irregular cycles. These findings suggest that sufficient vitamin D status along with a certain level of adiposity may be needed for optimal menstrual function. Among the cycle characteristics examined, all nutritional factors were

consistently associated with cycle pattern but not menstrual cycle bleed days or menstrual cycle length.

In conclusion, nutritional factors including omega-6 PUFA intake, serum 25-hydroxyvitamin D concentrations and adiposity may affect menstrual cycle regularity. Among the various menstrual cycle characteristics studied, we found cycle pattern was most associated with all the nutritional factors examined in our study. One-third of women of reproductive age in the U.S. have sub-optimal vitamin D status (Yetley, 2008) and the intake of omega-6 PUFA has increased in the western diet over the last few decades (Blasbalg, Hibbeln, Ramsden, Majchrzak, & Rawlings, 2011). Simple dietary and lifestyle modification may help improve a woman's menstrual cycle function. However, this is a pilot study with a small sample size and the magnitude of our finding for omega-6 PUFA are small and may not be clinically significant. Further studies are warranted to replicate our findings in larger cohorts.

### 5.2 Future Directions

Our results were promising, however further research needs to be done to better understand these associations. Future research should consider the following improvements:

- The cross-sectional nature of our study does not allow us to determine the temporal relationship. Future studies should choose a prospective study design to evaluate these associations.
- Menstrual cycle characteristics assessed via self-report can be subject to inaccuracies. Future studies will benefit from collecting these prospectively. Studies suggest a 90 or 120-day observation period during which time a woman can record information on start of menses and number bleed days (Vitzthum, 2009). This observation period will help

record at least two cycle lengths among women with 36 or up to 40 days of usual cycle length. Cycle length and variability (pattern) can be assessed from these records. Maintaining diaries to document cycle characteristics over a period of three menstrual cycles will help optimally assess cycle characteristics.

- In addition, future studies need to understand the underlying biological mechanisms. Adiposity, omega-6 PUFA and serum 25-hydroxyvitamin D may likely affect menstrual function by modulating ovarian hormones (Balen et al., 2007; Frisch, 1996; Parikh et al., 2010; Wathes et al., 2007). Collecting serum hormonal markers of estradiol and other ovarian hormones at key time points during the follicular and luteal phases will help elucidate underlying biological mechanisms.
- The cohort had a small number of women who were mostly Caucasians. It will be interesting to see how race affects menstrual cycle characteristics in women and its association with nutritional factors. Future studies should target a large diverse population sample to increase generalizability.
- Changing nutritional factors is an easy and cost effective way to modify menstrual function. Future dietary intervention trials with nutrients including vitamin D may help provide cause-and-effect evidence for this association.

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