The interplay between hormones, lipids, and dietary intake:  
The BioCycle Study

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ABSTRACT

Sunni Lyn Mumford: The interplay between hormones, lipids, and dietary intake: The BioCycle Study
(Under the direction of Anna Maria Siega-Riz)

Background: Exogenous estrogens (i.e. hormone therapy and oral contraceptives) have been shown to affect the lipid profile, leading to the hypothesis that endogenous estrogens may have similar effects. However, the relationship between estrogens and lipoproteins is complex, especially given that cholesterol is the initial precursor for steroid biosynthesis and dyslipidemia has been associated with altered hormone levels and anovulation. The effects of baseline cholesterol levels on anovulation are also not well understood. As fiber intake lowers both estrogen and lipoproteins, it is unknown whether estrogen may mediate the effect of fiber among premenopausal women.

Methods: The BioCycle study was a prospective cohort of 250 self-reported regularly menstruating women aged 18 to 44, followed for two menstrual cycles. Lipoprotein cholesterol and hormones were measured in serum collected at up to 16 visits timed using fertility monitors. Marginal structural models with inverse probability of exposure weights were used to model the associations of interest.

Results: Estradiol was positively associated with HDL cholesterol in acute effects models, and inversely associated with total and LDL cholesterol and triglycerides in persistent effects models. Total cholesterol levels preceding the predicted time of ovulation were weakly associated with an increased risk of anovulation, and luteinizing hormone:follicle-stimulating
hormone ratio, sex hormone binding-globulin, insulin, and acne were identified as significant predictors of anovulation. Fiber consumption at or above 22 g/day was associated with lower total and LDL cholesterol, independent of estrogen, however the controlled direct effect was reduced at high levels of estrogen.

**Conclusions:** Our results support the hypothesis that estrogen exerts beneficial effects on lipoprotein cholesterol levels and sheds light into the timing of these effects. The more atherogenic lipid profile and endocrine characteristics indicative of hyperandrogenism among anovulatory women are indicative of a mild undiagnosed phenotype of polycystic ovary syndrome, however further research is needed to elucidate the biological mechanisms. The reduced controlled direct effects of fiber on cholesterol at higher estrogen levels suggest that estrogen decreases fiber’s effect among premenopausal women. Cycle phase should be considered in the design and interpretation of studies in women of reproductive age due to estrogen’s influence on many biological processes.
ACKNOWLEDGEMENTS

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To my parents:

Who have taught me that I can do anything a boy can do—even though I get my period.
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LIST OF ABBREVIATIONS

AI  Adequate Intake
AIC  Akaike Information Criterion
ANOVA Analysis of Variance
AOR  Adjusted Odds Ratio
BMI  Body mass index
C   Celsius
CES-D Center for Epidemiologic Studies Depression Scale
CHD  Coronary heart disease
CI  Confidence interval
CL-EIA Chemiluminescent enzymatic immunoassay
CMP  Complete metabolic profile
CV  Coefficient of Variation
DAG  Directed acyclic graph
DELTA Dietary Effects on Lipoproteins and Thrombogenic Activity Study
DRI  Daily Reference Intake
DXA  Dual energy X-ray absorptiometry
E2  Estradiol
E3G  Estrone-3-glucuronide
EDTA Ethylenediamine tetraacetic acid
FFQ  Food frequency questionnaire
FSH  Follicle-stimulating hormone
g  Grams
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>g/day</td>
<td>Grams per day</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoproteins</td>
</tr>
<tr>
<td>HERS</td>
<td>Heart and Estrogen/progestin Replacement Study</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>Homeostatic metabolic assessment to predict insulin resistance</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate density lipoproteins</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IOM</td>
<td>Institute of Medicine</td>
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<tr>
<td>IPAQ</td>
<td>International Physical Activity Questionnaire</td>
</tr>
<tr>
<td>IPTW</td>
<td>Inverse-Probability-of-Treatment Weights</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>IUD</td>
<td>Intrauterine device</td>
</tr>
<tr>
<td>kcal</td>
<td>Kilocalories</td>
</tr>
<tr>
<td>kg/m²</td>
<td>Kilograms per meters squared</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoproteins</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>mcg</td>
<td>Microgram</td>
</tr>
<tr>
<td>METs</td>
<td>Metabolic equivalents</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>mg/dL</td>
<td>Milligrams per deciliter</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mmol/l</td>
<td>Millimoles per liter</td>
</tr>
<tr>
<td>MSM</td>
<td>Marginal structural model</td>
</tr>
<tr>
<td>NCC</td>
<td>Nutrition Coordinating Center</td>
</tr>
<tr>
<td>Acronym</td>
<td>full-form</td>
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<td>---------</td>
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<tr>
<td>NCEP</td>
<td>National Cholesterol Education Program</td>
</tr>
<tr>
<td>NDSR</td>
<td>Nutrition Data System for Research</td>
</tr>
<tr>
<td>OC</td>
<td>Oral contraceptives</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>P</td>
<td>Progesterone</td>
</tr>
<tr>
<td>PCOS</td>
<td>Polycystic ovary syndrome</td>
</tr>
<tr>
<td>pg/dL</td>
<td>Picograms per deciliter</td>
</tr>
<tr>
<td>PMDD</td>
<td>Premenstrual dysphoric disorder</td>
</tr>
<tr>
<td>RDA</td>
<td>Recommended daily allowance</td>
</tr>
<tr>
<td>REML</td>
<td>Restricted maximum likelihood</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SHBG</td>
<td>Sex hormone binding-globulin</td>
</tr>
<tr>
<td>U</td>
<td>Unmeasured variable</td>
</tr>
<tr>
<td>USDA</td>
<td>United States Department of Agriculture</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoproteins</td>
</tr>
<tr>
<td>WHI</td>
<td>Women’s Health Initiative</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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I. INTRODUCTION

Coronary heart disease (CHD) is the leading cause of death among women, primarily affecting postmenopausal women.\(^1\) The prevalence of CHD among women 20 to 39 years old is half the rate among men in the same age group (women, 7.8%; men, 15.9%). The difference in prevalence of CHD between men and women holds despite adjustment for various risk factors. However, as the prevalence of CHD among women increases with age, the difference between men and women drastically narrows.\(^1\) This has led to the hypothesis that circulating endogenous estrogens protect premenopausal women from CHD. With this in mind, hormone therapy was recommended to postmenopausal women as a means of cardio-protection.\(^2-5\) However, results from the Women’s Health Initiative (WHI) trial\(^6\) and the Heart and Estrogen/progestin Replacement Study (HERS)\(^7\) found that hormone therapy improved lipoprotein profiles but was associated with increased rates of CHD, which suggested that the role of exogenous/endogenous hormones on CHD was more complicated than originally thought.\(^6-8\)

The associations between exogenous sex hormones and lipoproteins seem to be well established. Estrogen is thought to exert a favorable effect on lipoprotein metabolism by increasing very low density lipoprotein (VLDL) synthesis, inhibiting hepatic lipase and lipoprotein lipase activity, and upregulating the low-density lipoprotein (LDL) receptors.\(^9-11\) The effect of endogenous sex hormones on lipoprotein levels in healthy premenopausal women however, remains uncertain, as well as whether these effects are chronic versus acute. Studies of lipoprotein metabolism during the menstrual cycle have yielded conflicting results,
and have failed to take into account circulating levels of other reproductive hormones such as progesterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) throughout the menstrual cycle, which could impact the association between estrogen and lipoprotein cholesterol levels. Understanding variations in lipoprotein cholesterol levels is important because there may be clinical implications regarding the appropriate timing of measurement during the cycle, as well as implications on the design and interpretation of studies in women of reproductive age. Further information regarding the effect of endogenous estrogen on lipoprotein metabolism may also help elucidate estrogen’s role in protection against atherosclerosis.

Not only are lipoprotein levels influenced by reproductive hormones, but total cholesterol is the initial precursor for steroid biosynthesis of sex hormones such as progesterone, estrogen, and testosterone. Reproductive hormone levels are intricately tied to ovulation through regulation of the hypothalamic-pituitary-ovarian axis. Sporadic anovulatory cycles may be fairly common among regularly menstruating women, although they are difficult to detect and the prevalence of anovulation has not been well described.\textsuperscript{12} The factors associated with anovulatory cycles are also not well understood among regularly menstruating women, but are of interest as sporadic anovulation might be associated with delayed time to pregnancy. As cholesterol plays a central role in the synthesis of sex hormones, lipoprotein cholesterol are hypothesized to be associated with anovulation.\textsuperscript{12} To our knowledge the association between lipoprotein cholesterol levels and anovulation has not been directly evaluated. Moreover, these relationships have not been prospectively evaluated in regularly menstruating women, free of diagnosed ovulatory disorders such as polycystic ovary syndrome (PCOS).\textsuperscript{13}
When studying lipid levels it is also important to consider dietary intake. Specifically, dietary factors such as fiber and fat intake have been shown to have an impact on serum cholesterol and lipoprotein levels. In fact, decreases of up to 20% in serum cholesterol have been observed with high fiber diets. This decrease is usually due to a reduction in LDL cholesterol. High-density lipoprotein (HDL) and triglyceride levels do not typically show the same effects.

However, there is evidence that pre- and post-menopausal women respond differently to fiber intake, with premenopausal women showing smaller reductions in lipoprotein cholesterol levels in response to fiber intake. It has therefore been hypothesized that estrogen could potentially mediate the effect of fiber on lipoprotein cholesterol levels in premenopausal women. High fiber intake has been associated with lower levels of estradiol, a result also confirmed in the BioCycle study in premenopausal women. Since estrogen and lipoproteins are intrinsically linked, as estrogen stimulates lipoprotein metabolism and cholesterol is the precursor for steroidogenesis, the implications of fiber’s association with estrogen on lipoprotein metabolism is evident. To date, however, there has been little research on how much of the observed effect of fiber on lipoprotein cholesterol levels is working independently, and not mediated by estrogen. As high fiber diets continue to be endorsed by the leading dietary and public health associations, a better understanding of the direct and indirect effects of fiber intake on lipoprotein cholesterol levels is essential. This knowledge could provide further insight regarding possible mechanisms, as well as valuable knowledge for the interpretation of studies of fiber intake among women of reproductive age.
This dissertation uses extant data from 259 regularly menstruating women enrolled in the BioCycle study, a prospective study of menstrual cycle function to address the following specific aims:

1. Evaluate the association between serum estrogen and short-term changes in lipoprotein cholesterol levels during the normal menstrual cycle.

2. Evaluate the association between serum lipoprotein cholesterol levels and risk of incident anovulation during the normal menstrual cycle.

3. Determine the controlled direct effect of dietary fiber intake on lipoprotein cholesterol levels not mediated by estrogen.
II. BACKGROUND

A. Menstrual Cycle Function

1. Reproductive Physiology

The menstrual cycle is a complex process involving multiple hormones which are regulated by intricate feedback mechanisms. Hormones, such as LH, FSH, estrogen, and progesterone follow a cyclical pattern which is coordinated by the hypothalamic-pituitary-ovarian axis. Typically, the cycle is divided into two distinct phases: follicular and luteal. The cycle begins with menstrual bleeding, and is then followed by the follicular phase. At the beginning of the follicular phase estrogen and progesterone levels are low, and FSH levels start to increase. Towards the end of the follicular phase estrogen levels begin to rise. Ovulation is stimulated by a surge in LH and FSH, and marks the end of the follicular phase and the beginning of the luteal phase. Estrogen levels also peak around this time, and progesterone levels start to increase. During the luteal phase, LH and FSH levels drop, and progesterone levels increase and remain high. In addition, estrogen levels are high during the luteal phase. If the egg is not fertilized, then both progesterone and estrogen levels drop and the cycle begins again. Figure 2.1 displays the normal hormonal pattern during the menstrual cycle not resulting in conception. Despite the consistent patterns that exist, there is considerable variation in “normal” menstrual cycle characteristics both within and between women.
2. **Menstrual Diary Studies**

Much of the data available regarding menstrual cycle characteristics comes from four seminal menstrual diary studies. These include the Matsumoto study of 701 Japanese women aged 13 to 52 who were followed for 2 years, the Treloar study of 2700 white American women aged 10 to 56 who were followed for up to 29 years, the Chiazze study of 2316 American and Canadian women aged 15 to 44 followed for two years, and the Vollman study of 691 Swiss women aged 11 to 58 who were followed for one to 39 years. The World Health Organization (WHO) also conducted a series of short menstrual diary studies, and several smaller studies have been conducted in specific populations across the world.

3. **Menstrual Cycle Characteristics**

Because hormone levels are usually difficult and expensive to monitor, menstrual cycle function is typically characterized by menstrual cycle length, phase length, absence of ovulation, or age at menarche. Less information is available on bleed duration and amount of flow. Menstrual disorders can be broadly placed into four categories: disruptions in bleeding patterns, ovarian function, and pain. Disruptions in bleeding patterns include menorrhagia (heavy or prolonged bleeding), oligomenorrhea (infrequent menstruation), polymenorrhea (frequent menstruation), or amenorrhea (cessation of menses). Two measures of ovarian function are anovulation and luteal phase deficiency, which are commonly defined as progesterone levels that do not reach 5 ng/mL for at least 5 days. Ovarian dysfunction may or may not result in altered bleeding patterns. Pain (dysmenorrhea) or other symptomatology (premenstrual syndrome) are also common menstrual disorders.
4. Cycle Length Variability

Menstrual cycle length is highly variable both between women and within a woman. Variability in cycle length tends to be greatest after menarche and shortly before menopause. Women ages 20 to 40 tend to experience less variability in their cycle length, but cycle length over this time tends to shorten from 30.1 to 27.3 days (Figure 2.2). It has been argued that the classic studies provide a limited understanding of cycle length variability because they assume that cycle length follows a normal distribution.

In the Agricultural Health Study, the prevalence of short cycles was estimated to be about 9.7%, of long cycles 3.2%, of irregular cycles 5%, with 13.3% of women experiencing intermenstrual bleeding or spotting. The prevalence would be assumed to vary in other populations.

Age is a major factor in determining menstrual cycle variability. Absolute weight and change in weight has been shown to influence menstrual function. This could be due to nutritional factors, or due to the effect of body fat on endocrine function. Fat tissue is a reservoir for steroid hormones and a site of estrogen production and could therefore directly influence endocrine function.

5. Cycle Phase Length Variability

There is very little data available on lengths of the follicular and luteal phases. They are difficult to measure because they require valid and accurate detection of the timing of ovulation. Ovulation may be classified differently based on whether the study used ultrasound, basal body temperature timing methods, or daily urine samples. Detection could also depend on the number of blood or urine samples collected and when they were collected.
With these limitations in mind, one study that evaluated phase lengths found that when long cycles were excluded, the follicular phase varied from 10 to 23 days, with a mean length of 13 to 15 days. The luteal phase was observed to vary from 8 to 17 days, with a mean of 12 to 14 days. When long cycles were included, the follicular phase was estimated to be longer with a mean of 17 to 18 days.\textsuperscript{38} It was also observed that the length of the follicular phase tended to decrease with age from about 14 days at ages 18 to 24 to 10.5 days at ages 45 to 60 among women still menstruating. Data from Vollman and Lenton suggest that once reproductive maturity is attained, the luteal phase remains relatively constant until menopause.\textsuperscript{34}

6. Cycle Length and Health Outcomes

Menstrual cycle characteristics have been associated with various reproductive outcomes, such as fertility, and spontaneous abortion.\textsuperscript{39} In addition, certain cycle characteristics have been associated with breast\textsuperscript{40-45} and ovarian cancer\textsuperscript{46,47}, uterine fibroids,\textsuperscript{48} diabetes,\textsuperscript{49} and cardiovascular disease.\textsuperscript{50,51} Menstrual irregularities have also been suggested to be associated with metabolic and hormonal abnormalities.

Long and irregular cycles have been associated with menarche after age 14, with depression, and with increasing body mass index (BMI).\textsuperscript{37,50} Length of the cycle has also been found to increase with increased parity, and decreased age, non-White race, and current smoking status.\textsuperscript{52} The likelihood of irregular cycles also tends to increase with age, BMI, and number of cigarettes smoked per day.\textsuperscript{52}

Long menstrual cycles have been associated with anovulatory cycles.\textsuperscript{35} In fact, short and long cycles are 10 to 30\% more likely to be anovulatory than cycles of 25 to 35 days. It
is estimated that for women aged 10 to 14 that approximately 50 to 60% of cycles are anovulatory, and for women aged 25 to 39 that approximately 2 to 7% of cycles are anovulatory, whereas, women above 50 years of age have approximately 34% anovulatory cycles. It has been hypothesized that this age-related change in incidence of anovulatory cycles is directly associated with changes in variability of menstrual cycle length throughout a woman’s reproductive life.

Menstrual cycle characteristics have also been associated with fertility and spontaneous abortion. Shorter cycles were less likely to be followed by conception, and likely reflect a shortened follicular phase.\textsuperscript{39} Cycles with lengths of 30 to 31 days preceded cycles with the highest fecundity. Conceptions after shorter or longer cycles were more likely to be spontaneously aborted as compared to 30 to 31 day cycles.

It is important to note that oral contraceptives (OCs) are often prescribed for menstrual irregularities. Therefore, studies of normally menstruating women which exclude OC users probably underestimate the prevalence of long and irregular cycles among all reproductive-aged women.\textsuperscript{37}

7. \textit{Validity of Self-Reported Menstrual Cycle Length}

Validity of self-reported cycle length was examined in a recent study by Small et al.\textsuperscript{39}. This study observed that 43\% of women reported their cycle length to be more than 2 days different from their actual mean length. When categorized into short (<26), normal (26 to 35), or long (>35) cycles, 21\% were misclassified. Accuracy of reporting was associated with older age, marriage, and higher income. Women with short and long cycles were less likely to report accurately, and as cycle variability increased the ability of a woman to
accurately report her cycle length also decreased. Another recent study by Jukic et al. found that on average, women overestimated their cycle length by 0.7 days. Systematic differences in reporting were found to be associated with sexual activity, history of infertility, parity, BMI, exercise, and prior treatment for irregular cycles.

B. Lipid Metabolism

1. Lipoprotein Structure and Function

Cholesterol is found in all tissues, and is required for the building and maintenance of cell membranes. Cholesterol is either derived from the diet through animal fats or synthesized in the endoplasmic reticulum. Transport in the blood is achieved through lipoproteins, specifically chylomicrons, intermediate density lipoproteins (IDL), VLDL, LDL, and HDL. Lipoproteins contain a core of neutral lipids, triglycerides, and cholesteryl esters, and an outer shell of more polar phospholipids, free cholesterol, and proteins. Apoproteins coat the surface of lipoproteins and function as lipid solubilizers, metabolic recognition sites, and coenzymes for catabolic enzymes. Lipoproteins all have the same basic structure, but differ in their relative contents of cholesterol, triglycerides, phospholipids, and apoproteins. Chylomicrons, VLDL, and LDL all transport cholesterol to peripheral tissues in the body, whereas HDL works to remove cholesterol through transport of cholesterol back to the liver. HDL metabolism is also known as reverse cholesterol transport. HDL is the smallest and densest of the lipoproteins and high levels have been associated with a lower risk of cardiovascular disease. High levels of LDL and total cholesterol on the other hand have been associated with an increased risk of cardiovascular disease. In general, individuals with a less atherogenic plasma lipid profile
are at lower risk for coronary heart disease. An atherogenic profile is one that is associated with higher levels of total cholesterol and LDL cholesterol, and lower levels of HDL cholesterol.

HDL functions to return cholesterol to the liver and to steroidogenic organs such as the adrenals, ovaries, and testes. HDL is removed using both direct pathways involving HDL receptors such as scavenger receptor BI and an indirect pathway mediated by cholesteryl ester transfer protein. This protein exchanges triglycerides of VLDL and cholesteryl esters of HDL. This exchange results in converting VLDL to LDL, which is subsequently removed by LDL receptors, and the breaking down of unstable triglycerides in HDL by hepatic lipase. These HDL particles are then available for uptake from the cells.

2. Role of Steroid Sex Hormones in Lipoprotein Metabolism

There is considerable evidence that women have an increased level of lipoprotein transport as compared to men. Much of this difference is attributed to sex hormones. In general, estrogens have been found to have favorable effects on lipoprotein metabolism by lowering LDL and increasing HDL. Progestins, on the other hand, have been associated with unfavorable effects on lipoprotein metabolism by increasing LDL levels and decreasing HDL. These differences have been attributed to estrogen’s impact on increasing VLDL synthesis and inhibiting hepatic lipase and lipoprotein lipase activity. Estrogen works to stimulate hepatic lipid production, as well as the formation of other proteins in the liver. Androgens, however, oppose all of the stimulatory effects of estrogen on protein synthesis. Estrogens also play a major role in VLDL production in women. Uptake and esterification of triglycerides and VLDL secretion have been shown to be increased in women, without a
subsequent increase in plasma levels, suggesting that rates of VLDL transport and removal are greater in women. Most LDL are formed from VLDL and VLDL remnants. With the stimulatory effects of estrogen on VLDL production, a similar increase in LDL and HDL would be expected. However, estrogen also directly affects the upregulation of the LDL receptors, thereby increasing the rate of LDL removal. Again, androgens have the opposite effect by downregulating the activity of the LDL receptor. Reductions in hepatic lipase activity due to estrogen have the effect of decreasing triglyceride removal from HDL, and HDL levels are typically higher among women than men. Since women have a greater rate of lipoprotein transport, it is thought that the effects of diabetes or obesity would lead to a greater increase in plasma lipoproteins in women when compared to men. In fact, those effects are observed when comparing lipoprotein levels in diabetic men and women with levels in normal men and women. This also helps in understanding why diabetes is a more significant risk factor for coronary heart disease in women.

3. Differences in Lipid Levels by Age, Sex

Lipid profiles have been shown to differ between men and women throughout life. Interestingly, cholesterol levels tend to be higher among girls than among boys at birth, but this switches during the reproductive years. HDL cholesterol levels are also higher in women than in men at all ages. This has been used to explain the fact that women of reproductive age are at decreased risk for heart disease as compared to men. LDL levels rise with age in both men and women, and after 55 may be higher in women than in men. HDL declines slightly in women after 55 (corresponding to postmenopausal lowering of estrogen levels).
4. **Hormone Replacement Therapy**

The role of exogenous hormones on lipid metabolism has been well studied. The largest trial was conducted by the WHI, and investigated the hormone replacement therapy of estrogen plus progestin. In the WHI trial, postmenopausal women who received the estrogen plus progestin therapy showed an improved lipid profile, but no beneficial effect on heart disease. Specifically, women randomly assigned to estrogen plus progestin had greater reductions in total cholesterol, LDL, glucose, and insulin levels, and greater increases in HDL and triglycerides, than those on placebo. Women with higher baseline LDL levels appeared to have a greater excess risk of coronary heart disease with hormone therapy. WHI concluded that estrogen plus progestin hormone replacement therapy does not confer cardiac protection and may in fact increase the risk of coronary heart disease. In general, studies evaluating the impact of hormone replacement therapy on lipid metabolism have found that estrogen therapy reduces plasma LDL levels, and increases HDL and triglyceride levels. Addition of a progestin tends to attenuate some of the lipid benefits of estrogen, in particular, the increase in HDL levels.

5. **Oral Contraceptives**

Studies of the effect of OC’s on lipoprotein metabolism yield similar results to that of hormone replacement therapy. Estrogens are again in general associated with lower LDL levels and higher HDL levels, and progestins are associated with higher LDL and lower HDL levels. Estrogen-rich OC’s have also been found be associated with increased triglyceride levels, but those containing both estrogens and progestins typically did not have the same
A recent study found that two low dose monophasic OC’s containing ethinyl estradiol 30 mcg and either chlormadinone acetate 2 mg or 0.15 mg desogestrel had similar effects on lipoprotein levels (increased triglycerides, HDL, and decreased LDL and LDL/HDL ratio), although total cholesterol levels remained unchanged. On the other hand, a different study found no beneficial effects of low-dose OC’s containing 30 mcg ethinyl estradiol and 150 mcg levonorgestrel on HDL levels. Interestingly, in a study comparing OC users, nonusers, and levonorgestrel-releasing intrauterine device users, intrauterine device users displayed lower HDL and total cholesterol levels compared with the nonusers, whereas OC users had raised lipid levels (total cholesterol and triglycerides). The variable effects of OC’s on lipid metabolism are influenced by several factors. The dose and type of OC’s appears to make a difference in the changes in levels of plasma lipoproteins observed. In addition, studies differ by the age of the women studied, smoking, exercise, diet, alcohol, treatment for other conditions, and specific sampling procedures. Interestingly, the effects on lipid metabolism do not seem to be progressive with continued treatment, but rather go back to pretreatment concentrations after discontinuation.

6. Lipids and Menstrual Cycle Function

The effects of exogenous hormones on lipid metabolism, and the differences observed in lipoprotein transport between men and women all lend evidence to support that endogenous female sex hormones play a significant role in lipoprotein metabolism. The effect of endogenous hormones on lipoprotein metabolism is not well understood however, and not all studies have observed cyclic changes in lipoprotein levels across the menstrual cycle as would be expected. Some studies have shown that plasma lipid levels fluctuate
during the menstrual cycle, while others have not observed these changes. Within studies, it is also common to find that only certain measures of cholesterol (total, HDL, or LDL) differ between cycle phases. The contradictory results could be due to a number of possible factors. The changes in lipids between different days of the menstrual cycle are fairly small in magnitude. Studies with small sample sizes are then underpowered to detect these small effects. Inappropriate timing of sample collection to menstrual cycle phase could further mask the effects and complicate the results of these studies. Studies looking at differences in LDL often fail to account for the fact that LDL is not directly measured, but is in fact indirectly calculated using the Friedewald formula. In addition, analytical methods that appropriately account for intra-individual variability are rarely applied.

Total cholesterol and LDL are often found to be lower during the luteal phase of the menstrual cycle, as compared to the follicular phase. The luteal phase corresponds to the time of the cycle when estradiol and progesterone levels are high. Studies of this relationship have typically consisted of comparisons across only one cycle, with few measurements throughout the cycle. Timing of cholesterol measurements to menstrual cycle phase was made by determining hormone levels either by basal body temperature methods, ovulation charts, or blood samples. None of the past studies have utilized a fertility monitor to time ovulation during the cycle. Of the studies where blood samples were used, the sampling periods varied from 1 to 2 times per week, to every 5th day, to once per cycle phase as determined by menstrual cycle length, or up to 3 consecutive samples per phase. Analysis of these studies also was usually limited to paired t-tests, repeated measures analysis of variance (ANOVA), or correlation analyses, which were only able to compare mean cholesterol levels between the follicular and luteal phases of
the menstrual cycle. Sample sizes were typically very small. In fact, only three studies have been completed that included more than 50 participants. Of the three studies with more than 50 participants, one compared cholesterol levels between the follicular and luteal phases (n=54), one was cross-sectional and only studied the mid-follicular phase (n=177), and one consisted of daily salivary ovarian steroid concentrations (n=206). The largest study by Furberg et al. consisted of multiple hormone measurements, but only one lipid measurement taken at the beginning of the menstrual cycle. Proper timing of blood sampling for multiple cycles, along with multiple hormone and lipid measurements across the cycle would help to further elucidate the temporal variability in lipid levels.

Three studies used mixed modeling techniques to better understand variability in lipid levels across the cycle and to appropriately account for within-woman variability. These studies used modeling techniques similar to what will be utilized for the proposed analysis and therefore will be discussed in more detail. These studies were limited by very small sample sizes and uncertain timing of menstrual cycle phase. Wall et al. studied 12 women for one cycle, and found significant changes across the cycle. The strength of this study was the fact that they collected 20 blood samples throughout a single menstrual cycle. These samples were then divided into 6 phases and averaged within each phase. Even though daily blood samples were collected, there were no repeat measurements available from a second cycle, and daily measurements were averaged into 6 groups for the multi-level analysis. A study by Reed et al. compared intra-individual differences in lipid levels across the cycle among 39 premenopausal women enrolled in the Dietary Effects on Lipoproteins and Thrombogenic Activity (DELTA) Study. Blood samples were taken once per week for the last 4 weeks of each 8 week diet period for 3 non-consecutive cycles. Menstrual cycle
phase was determined based on the weekly hormone measurement and a personal menstrual calendar. The intra-individual variability among the premenopausal women was compared to the variability in 18 postmenopausal women, and 36 men under strict dietary conditions. They found that lipid levels followed a cyclical pattern for premenopausal women, but that the same patterns did not exist for men or postmenopausal women. However, the authors emphasized that the cycling only represents a small fraction of the total biologic variability, and that the intra-individual variability is so similar to that of postmenopausal women and men that timing of cholesterol measurements for premenopausal women is unnecessary.

Again this study was very small, and appropriate classification of measurements to menstrual cycle phase was not precise. No adjustment was made for analytical variability, and because they were testing the effects of 3 different diets, between-diet variability in lipids is inherently included in their analysis. In their analysis of lipid levels, they did not control for diet.

It is evident that there are several common limitations in the current literature. Sample collection needs to occur at appropriate times during the menstrual cycle, and measurement of both hormone levels and lipid levels at various points would be ideal. Measurement during the follicular and luteal phases, as well as during menstruation and ovulation are necessary to characterize the entire cycle. These design issues need to be employed in a large sample, with follow-up for more than one cycle. Appropriate statistical analysis will also help to elucidate this relationship.
C. Synthesis of Steroid Hormones

Cholesterol is the initial precursor for steroid biosynthesis and plays a central role in the synthesis of sex hormones such as progesterone, estrogen, and testosterone. Cholesterol is synthesized into steroid hormones from cholesterol that has been synthesized de novo, that has been acquired from the plasma, or that has been stored as esters.

1. Basic Hormone Structure

Steroid hormones are derived from cholesterol and are lipids that share a common nucleus comprised of 17 carbon atoms. Naturally occurring steroid hormones are named according to the structures of the parent compound which are differentiated by subtle modifications of the four fused rings of the sterol skeleton and side chain. In particular, cholestanes are made up of 17 carbons (e.g. cholesterol), pregnanes of 21 carbons (e.g. progesterone), androstanes of 19 carbons (e.g. testosterone), and estranes of 18 carbons (e.g. estradiol). Systemic names not only designate the parent structure, but also the number, location, and orientation of substituents attached to the central ring. Figure 2.3 shows the chemical structure of various steroid hormones. It is readily apparent that they share a common parent structure.

2. Steroid Hormone Production

Estrogen is primarily produced by developing follicles in the ovary and the corpus luteum, and is stimulated by FSH and LH. Estrogens are also produced in other tissues, but in smaller amounts. The synthesis of estrogens begins in the theca interna cells of the ovary and involves a series of sequential modifications of cholesterol. The first step in the
synthesis of ovarian hormones is the conversion of cholesterol to pregnenolone (Figure 2.4). Pregnenolone can then be converted to progesterone. Further metabolism results in synthesis of testosterone, aldosterone, and cortisol, and subsequently into estrogens. Both androstenedione and testosterone are substrates for estrogen production. Figure 2.4 shows the sequence of steroidogenesis, the process of generating various forms of steroid hormones by the transformation of other steroids, starting with the conversion of cholesterol.

Depending on the conversion process, there are three different naturally occurring types of estrogens that could be produced, including estradiol, estrone, and estriol. Estradiol is the primary estrogen present among premenopausal women, and estrone the primary estrogen present among postmenopausal women. Estradiol is secreted directly from the ovary and estrone is primarily derived from conversion of estradiol or androstenedione. Estrone can be further metabolized to estriol. The synthesis of androstenedione from cholesterol takes places in the theca interna cells in the ovary, and results in the formation of estrone or estradiol, either immediately or through testosterone. The structure of each type of estrogen is shown in Figure 2.5.

3. Role of Lipoproteins in Hormone Synthesis

Lipoproteins function to carry cholesterol to tissues for hormone synthesis. LDL is the primary way that cholesterol reaches the endocrine glands to be used for steroid hormone formation. Ovarian cells contain LDL receptors, which function to release cholesterol to be utilized for steroid biosynthesis. Once transported to steroidogenic organs by lipoproteins, cholesterol can then be synthesized into the steroid hormones.
D. Dietary Fiber

1. Health Benefits of Dietary Fiber Intake

Increased fiber intakes are recommended due to the many health promoting benefits associated with fiber consumption. In fact, diets high in fiber have been associated with reduced risk of various chronic diseases such as cardiovascular disease,89,90 diabetes,91 and breast cancer.92-95 In addition, fiber has been shown to lower cholesterol and lipoprotein levels,15 control weight96, reduce constipation,97 reduce blood pressure,98 and help control blood sugar levels.99,100

High fiber intakes have also been shown to be associated with lower levels of estrogen.20-27 It has been hypothesized that the effects of fiber on the development of breast cancer are potentially mediated by changes in estrogen levels.101 It is not well understood how much of the observed effect of fiber on cholesterol and lipoprotein levels is mediated by changes in estrogen.

2. Sources and Types of Fiber

Dietary fiber is the collective term for the carbohydrate substances found in plants that are indigestible by human enzymes. Dietary fiber is found in foods such as whole grain products, fruits, vegetables, and legumes. Sources of dietary fiber are often divided into soluble and insoluble components, based on whether or not they are water soluble. Soluble fiber is found in all plant foods, but in varying quantities. Sources of soluble fiber include legumes, oats, rye, barley, fruits such as plums and berries, vegetables such as broccoli, carrots, potatoes, sweet potatoes, onions, and psyllium seed husk. Sources of insoluble fiber include whole grain foods, bran, nuts, seeds, vegetables such as green beans, cauliflower,
zucchini, celery, and tomato skins. It was assumed that classifying fibers by their solubility
would help to distinguish between their biological responses. However, the physiological
response to sources of fiber is affected by more than solubility, but also by factors such as
fermentability, viscosity, and bile acid binding ability. Because of these additional factors,
the Institute of Medicine (IOM) has recommended new definitions for dietary fiber. Specifically, the new terms recommended for describing fiber intake are dietary fiber and
functional fiber. The term dietary fiber is used to describe the nondigestible carbohydrates
and lignin that are intrinsic and intact in plants, which is to be distinguished from functional
fiber which consists of the isolated nondigestible carbohydrates that have beneficial
physiological effects.

3. Recommended Intake

Current recommendations for fiber intake are 25 g per day for adult women ages 19
to 50 and 38 g for adult men ages 19 to 50. These recommendations from the IOM are
based on epidemiologic evidence showing a protective effect of lipids on cardiovascular
disease. It should also be noted that for fiber intake, only an “Adequate Intake (AI)” was
established. Since fiber is not absorbed, blood fiber levels cannot be measured, and a
Recommended Dietary Allowance (RDA) could not be calculated. The AI was based on the
median fiber intake that was observed to have the lowest risk of coronary heart disease. The
usual intake of dietary fiber in the United States is far below the recommended amount with
an average of only 15 g/day.
4. Effect of Fiber on Estrogen Levels

Increased consumption of dietary fiber has been associated with decreased levels of estrogen in several observational studies and randomized controlled trials.20-27 These studies were completed in various study populations, with varying levels of fiber consumption (mostly very high amounts of fiber intake), and usually with only a few measurements during the menstrual cycle. The most comprehensive study to date was done by Bagga et al. in which 12 women on a high fiber diet were followed for 2 menstrual cycles with serum was collected every other day.20 This study found 18% and 26% reductions in estradiol in the follicular and luteal phases, respectively, but no significant changes for other reproductive hormones. The most recent study followed 44 women for one menstrual cycle and evaluated daily estrogen levels in urine, as well as serum levels during the follicular phase.25 This study found a reduction in serum estrogen levels of 15% associated with high fiber/low fat diets, as well as reductions in the daily urine measurements of estrogen. The largest randomized controlled trial followed 213 women for one year and found a reduction of 7.5% in levels of estrogen among women on a high fiber diet, based on only one collected serum measurement per cycle.21 While these studies each have their own limitations, the evidence points towards an association between increased fiber consumption and reduced estrogen levels.

Dietary fiber intake is hypothesized to reduce levels of circulating estrogen through inhibiting deconjugation and reabsorption of estrogen from the colon.25 Additionally, all biliary estrogens are conjugated and have to be hydrolyzed before absorption.106 An increase in α-glucuronidase activity in feces associated with a high fat and low fiber diet leads to increased reabsorption of estrogen.106,107 The other proposed mechanism by which dietary
fiber intake reduces estrogen levels is through the elimination of estrogens by the fecal route because fiber binds estrogens, and works to speed up intestinal transit.

5. **Effect of Fiber on Estrogen Levels in the BioCycle Study**

The effect of fiber on estrogen levels was also evaluated in the BioCycle study. It was observed that increased consumption of dietary fiber was associated with decreased levels of estrogen as well as an increased risk of incident anovulation. These results remained consistent whether fiber intake was considered as a continuous variable, or categorized according to the AI. Dietary fiber consumption was inversely associated with all peak hormone concentrations (estrogen, progesterone, LH, FSH) \( (P<0.05) \), and with an increased risk of anovulation using random effects models adjusted for total calories, age, race, and vitamin E intake. Body mass index (BMI) was not found to be confounder in this study. Each 5 g/day increment in total fiber intake was associated with a 2.04 fold increased risk (95% confidence interval (CI) 1.26-3.28). Corresponding adjusted odds ratios (aORs) and (95%CI) for fruit and grain fiber were 7.98 (1.94-32.9) and 3.58 (1.50-8.52), respectively. Although the confidence intervals were wide, we found a greater risk of anovulation among women with fiber intakes at or above the AI (25 g/day) compared to those consuming less than 10 g/day (aOR=26.4, 95%CI 2.9-240.5).

6. **Lifestyle Measures to Reduce Cholesterol**

Several factors are associated with high cholesterol levels, such as obesity, physical inactivity, smoking, alcohol consumption, high-carbohydrate diets, diseases such as type 2 diabetes mellitus, chronic renal failure, and genetic predisposition. Several lifestyle
changes can be made in order to manage lipid levels. In particular, increased weight is a determinant of low HDL levels, and weight loss has positive effects on HDL cholesterol. Diet and exercise are two primary ways of lowering cholesterol, which can be used in addition to lipid-lowering medications for individuals who require prescription treatment. It is recommended that individuals avoid saturated fats, and include sources of soluble fiber in their diet. It has also been recommended that eating one-half cup of bran cereal that contains 5 g of soluble fiber could lower LDL levels by 5%, or one handful of almonds (70 g) could decrease LDL by 8%. Response to changes in diet vary considerably, but it can be expected that the adoption of a diet low in saturated fat will result in about a 8 to 10% change in cholesterol levels, and an additional lowering of 3 to 5% if dietary cholesterol is also reduced. Weight management and physical activity are also recommended to control cholesterol levels. Studies of the effects of physical activity have shown lower triglyceride levels (mean decreases of 24% for regular exercise) associated with exercise training. Total cholesterol and LDL cholesterol do not show changes associated with exercise, but are lowered due to changes in body weight, body fat, and dietary fat that usually accompany physical activity.

7. Effect of Fiber on Lipid Metabolism

Diets high in fiber have been shown to have an impact on serum cholesterol and lipoprotein levels. In fact, decreases of up to 20% in serum cholesterol have been observed with high fiber diets. The decrease is usually due to the reduction in LDL cholesterol. HDL and triglyceride levels do not typically show the same effect.
The mechanisms involved in the reduction of serum cholesterol levels due to increased fiber intake remain somewhat inconclusive. Potential mechanisms are displayed in Figure 2.6. Diets high in fiber could potentially be displacing foods that are high in saturated fat, and indirectly work to lower lipid levels. Another potential mechanism is through bile acid metabolism. Specifically, serum cholesterol levels are thought to decrease due to viscous polysaccharides acting in the gastrointestinal tract by decreasing cholesterol and fatty acid absorption, and by decreasing absorption of biliary cholesterol or bile acids. In this way, bile acid synthesis increases, and fecal excretion of bile acids is increased. One study in particular measured bile acid kinetics to test this hypothesis and observed that oat bran consumption lowered serum cholesterol in part by altering bile acid metabolism, and in part by decreasing the synthesis of cholesterol. Animal models have also shown that the water-soluble fiber most prevalent in oats and barley is the active agent causing the altered cholesterol metabolism. Although the mechanism is not fully understood, the evidence points to small increases in the fecal excretion of bile acids and neutral steroids that is not fully accounted for by de novo cholesterol synthesis. It has also been hypothesized that fiber may act through altered serum hormone concentrations or short-chain fatty acids that affect lipid metabolism.

E. Summary of Research Gaps

The purpose of this dissertation is to understand how the menstrual cycle interacts with other metabolic processes, specifically lipid metabolism. In particular we are interested in the effects of estrogen on lipid metabolism, the effects of baseline lipoprotein cholesterol
levels on the risk of incident anovulation, and the direct effects of dietary fiber intake on lipoprotein cholesterol not mediated by estrogen.

It is not well established whether lipoprotein levels fluctuate during the menstrual cycle, and whether estrogen is associated with lipoprotein cholesterol levels. Previous studies have been limited by several factors (small sample sizes, follow-up for a single cycle, few measurements during the cycle, single comparison between follicular and luteal phases, and differences in markers of ovulation to time lipid and hormone measurements). Further, the effect of lipoprotein cholesterol levels on risk of incident anovulation has not been evaluated prospectively in a group of regularly menstruating women. Although the effect of fiber on lipoproteins is fairly well established, menstrual cycle phase is generally not taken into account. It is unclear how much of the effect of fiber on lipoprotein cholesterol levels is mediated by the effect of fiber on estrogen.

This study seeks to improve upon limitations of previous studies in order to quantify and understand the variability and cyclical nature of both hormones and lipoprotein cholesterol levels during the normal menstrual cycle. The BioCycle study is a unique data source and is especially appropriate for addressing the questions of interest because of the quantity and quality of the data collected. Not only are biomarkers available, but various questionnaires regarding lifestyle and demographic factors were also administered. Biomarkers were measured in serum from 259 women at 16 time points across two menstrual cycles, and lifestyle questionnaires were also administered at multiple points during the study. This study also has the advantage of well-timed study visits because of the use of fertility monitors to track hormone levels throughout the cycle. Using this new technology, menstrual cycle phases were defined more accurately, representing a significant
improvement over previous studies. No previous study has had such a large sample and both hormone and lipoprotein cholesterol measurements at multiple time points. Basic descriptive analysis will be completed, along with the use of random effects models to account for both intra- and inter-individual variability. The longitudinal nature of the data allows us to also use mixed modeling techniques to better understand variability across the cycle and to appropriately account for within-woman variability. Marginal structural models also will be used to adequately account for time-dependent confounding due to changing hormone levels throughout the cycle.

This study adds a significant contribution to the literature because of the design, data quality, and improvements over previous studies. Understanding variations in lipoprotein cholesterol levels is important because there may be clinical implications regarding the appropriate timing of measurement during the cycle, as well as implications on the design and interpretation of studies in women of reproductive age. Further information regarding the effect of endogenous estrogen on lipoprotein metabolism may also help elucidate estrogen’s role in protection against atherosclerosis. This is also the first study to our knowledge to prospectively identify endocrine markers of sporadic anovulation in a group of regularly menstruating women. Understanding the factors associated with anovulation is important as anovulation might be associated with delayed time to pregnancy. Understanding the direct effects of dietary fiber intake on lipoprotein cholesterol levels, not mediated by estrogen, could provide further insight regarding possible mechanisms, as well as valuable knowledge for the interpretation of studies of fiber intake among women of reproductive age.
F. Tables and Figures

Figure 2.1. Pattern and timing of hormonal changes during the normal menstrual cycle.\textsuperscript{30}
Figure 2.2. Median menstrual cycle lengths from menarche to menopause.¹²
Figure 2.3. Chemical structure of cholesterol and various steroid hormones.
Figure 2.4. Structure of cholesterol.
Figure 2.5. Structure of three naturally occurring types of estrogen.

estradiol

estrone

estriol
Figure 2.6. Potential mechanisms for the effect of fiber on lipid metabolism.¹¹¹
III. SPECIFIC AIMS

This dissertation uses extant data from 259 regularly menstruating women enrolled in the BioCycle study, a prospective study of menstrual cycle function to address the following specific aims:

1. Evaluate the association between serum estrogen and short-term changes in lipoprotein cholesterol levels during the normal menstrual cycle.

2. Evaluate the association between serum lipoprotein cholesterol levels and risk of incident anovulation during the normal menstrual cycle.

3. Determine the controlled direct effect of dietary fiber intake on lipoprotein cholesterol levels not mediated by estrogen.
IV. METHODS

A. Study Design

The BioCycle study was designed to enroll 250 participants for 2 menstrual cycles, and to collect biologic samples, conduct physical measurements and administer questionnaires across two complete menstrual cycles among these women. In order to test the feasibility of the study and its measures, a pilot study of 9 subjects was conducted for 1 cycle prior to finalizing the study protocol. The full study involved biospecimen collection (blood and urine) at 8 key times per cycle for 2 cycles per participant. The collection times were selected to include points in the menstrual cycle with the most hormonal variation and include menstruation, the middle of the follicular phase, the estrogen peak, the LH and FSH surge, ovulation, progesterone elevation and peak, and immediately before menstruation. Based on an approximate 28-day cycle length, these would represent approximately days 2, 7, 12, 13, 14, 18, 22, and 27.

Figure 4.1 describes the expected hormonal variation occurring at each specific time during the cycle. Fertility monitors (Clear Blue™) were used to assist in the timing of specimen collection, and have been shown to improve timing of clinic visits. The strengths of these methods will be described in Section C. Cycle visits were routinely scheduled between 7:00 am and 8:30 am to allow for collection of fasting samples and to reduce diurnal variation. A complete hormonal profile and lipid profile were measured in serum collected at each clinic visit. Participants were also asked to complete questionnaires on the following topics: life style; health history; skin and body hair patterns; physical activity (IPAQ – The
International Physical Activity Questionnaire); family medical history questionnaire; Perceived Stress Scale (14 items); Depression Scale (Center for Epidemiologic Studies Depression Scale, CES-D); and occupational history. Nutrient data were collected using a 24 hour recall from the University of Minnesota database. Details regarding assessment of hormones, lipoproteins, and other covariates are described in each of the Dissertation Manuscript chapters.

B. Study Population

Healthy, normally menstruating, premenopausal women ages 18 to 44 were recruited from clinical practices in western New York. Specifically, recruitment efforts were focused at the University at Buffalo Department of Gynecology-Obstetrics practice plan and the University at Buffalo student health service. A total of 957 prospective participants called the clinical center to inquire about the study. Of these, 449 scheduled and completed a screening visit. Of these, 318 met the eligibility criteria and 276 enrolled. A diagram of the BioCycle recruitment is shown in Figure 4.2. In order to be eligible to participate, women could not have used oral contraceptives during the past 3 months, be currently pregnant, or be actively trying to conceive during the next 3 months. Only women reporting regular cycles and no history of gynecological or other chronic diseases were included. A complete list of exclusion criteria is shown in Table 4.4. A total of 259 women completed at least one cycle. Those eligible were fairly similar compared to those ineligible, although those eligible were slightly younger and more likely to report a white race.

Of the 276 who enrolled, 250 completed two cycles, 9 completed one cycle, and seventeen women dropped out of the study prior to completing at least 1 cycle. Among the
259 women who completed at least 1 cycle, the number of study cycle visits ranged from 5 to 8 per cycle (Table 4.2), with shorter menstrual cycle length as the primary reason for fewer than 8 visits completed per cycle. This would occur if a woman began menstruating before the last scheduled visit on day 27. Overall, 94% of all women completed 7 or 8 visits per cycle.

Women that participated in the BioCycle study were mostly white (60%), single (68%), college-educated (90%), below the age of 30 (68%), with a normal BMI (60%). Baseline demographics are summarized in Table 4.3. Overall, the mean age of participants was 27.3, and the mean BMI was 24.1. Only 12% of participants were current smokers, and 66% reported drinking at least 1 alcoholic drink per month in the past 6 months, with 37% reporting drinking at least 1 alcoholic drink per week. Self-reported menstrual cycle length ranged from 21 to 35 days with a mean (standard deviation (SD)) length of 28.4 (2.2) days.

This population has the advantage of having no reported gynecological problems, such as an abnormal Pap in the last 6 months with no subsequent normal result, laparoscopy confirmed endometriosis, treatment for infertility, uterine fibroids, or polycystic ovary disease. It is important to exclude women with these conditions, because these gynecological problems have an impact on circulating hormone levels. In addition, the age range of 18-44 allows us to study women who have regular periods, without the issues of low gynecological age, or women entering the perimenopausal phase.
C. Fertility Monitor

Each woman in the BioCycle study was given a fertility monitor (Clear Blue™) which was used to determine the timing of ovulation and to schedule clinic visits at specific phases of the menstrual cycle (Figure 4.3).

The monitors assessed fertility based on levels of estrone-3-glucuronide (E3G) (an estrone metabolite) and LH in urine. Testing began on approximately the 6th day following the start of the woman’s menstrual cycle and continued for 10 or 20 days depending on the timing of the LH surge and cycle length. Monitor indications of low, high and peak fertility were used to time mid-cycle visits, with peak day and the following 2 days being those that would approximately represent late follicular, LH surge, and ovulation dates. Based on an approximate 28 day cycle length, the cycle visits represent approximately days 2, 7, 12, 13, 14, 18, 22, and 27. Overall, the women that participated in the BioCycle study were very compliant with the study protocol. Table 4.5 shows that 48% of women did not miss a single test, and 80% missed only 0-2 days out of the entire cycle. In addition, about 76% of cycles showed peak LH levels (Table 4.6), indicating that blood samples were collected at appropriate times with respect to ovulation.

There may be some misclassification among the 20% without an observed LH peak. It is unclear to which direction this bias may impact the results. However, this rate of misclassification is much lower than other methods which are commonly used to time sample collection to menstrual cycle phase. In general, ovulation is said to occur on day 14 of a 28 day cycle. If we were simply to collect samples on day 14 of a woman’s menstrual cycle, then we would have missed a significant portion of the LH peaks, because the mean peak day observed in this sample was on day 16 for cycle 1 and day 15.
for cycle 2 (Table 4.6). The fertility monitor is able to capture a far greater proportion of the LH peaks than other methods including sampling on days 12 to 14 of the cycle, or taking the midpoint of the self-reported cycle and taking samples the day before and the day after the midpoint, or counting back 13 to 15 days from the end of the cycle (Figure 4.4).

Timing of biospecimen collection at 8 key times per cycle is one of the strengths of the study. However, the complexity involved in aligning the cycles so that measurements can be compared is quite complex. While use of the fertility monitor greatly improves timing of the cycle, there will of course be some variation in sample date collection.

D. Statistical Analysis

1. Overview

Basic descriptive analyses were completed, along with appropriate bivariate analyses. Mean and median levels of hormone, lipoprotein, and dietary fiber intake levels were calculated for each cycle day and then compared across the cycle. Distributional assumptions were evaluated graphically and using appropriate statistical tests, and transformations performed where necessary. Data were stratified by age and BMI to determine the impact of these factors on exposures and outcomes of interest during the cycle. ANOVA, repeated measures ANOVA, t-tests, and chi-square tests were used as appropriate to determine whether differences in hormones, lipoproteins, and dietary fiber intake varied significantly across the cycle.
Marginal structural models (MSMs) were used to assess the impact of time-dependent confounding through the use of inverse-probability of treatment weights (IPTW).\textsuperscript{115} Levels of diet, physical activity, smoking, and alcohol consumption were allowed to vary at multiple time points during the menstrual cycle. MSMs were used to evaluate the association between hormone and lipoprotein levels while accounting for the fact that these covariates vary over the cycle. Due to the intricate nature of the hormonal fluctuations during the cycle, these models adequately control for the effects of the hormonal feedback mechanisms during the menstrual cycle.

Weighted linear mixed effects models with random intercepts were used to estimate the coefficients of the marginal structural models. These models were used to account for both intra- and inter-individual variability. Linear mixed models have the advantage of being able to accommodate observations that are not independent, and are flexible in handling imbalanced longitudinal data. The inclusion of random intercepts allowed for each subject to have a different baseline level of response.

The main goals of the study analyses were: (Aim 1) to evaluate the association between serum estrogen and short-term changes in lipoprotein levels, (Aim 2) to evaluate the relationship between lipoprotein cholesterol levels and incident anovulation, and (Aim 3) to determine the direct and indirect effects of dietary fiber intake on lipoprotein cholesterol levels. Details regarding the specific analysis for each aim are included in the Methods section of each Dissertation Manuscript chapter.
### Table 4.1. Cycle visit schedule by cycle day (based on a 28-day cycle).

<table>
<thead>
<tr>
<th>Approximate Day of the Cycle</th>
<th>Phase of the Cycle</th>
<th>Expected Hormonal Variation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Menstrual</td>
<td>Low E2, Low P, Low LH/FSH</td>
</tr>
<tr>
<td>7</td>
<td>Mid follicular</td>
<td>Low E2, Low P, Low LH/FSH</td>
</tr>
<tr>
<td>12</td>
<td>Late Follicular</td>
<td>Peak E2, Low P, Low/Rising LH/FSH</td>
</tr>
<tr>
<td>13</td>
<td>LH/FSH surge</td>
<td>Peak LH/FSH, Low E2, Low P</td>
</tr>
<tr>
<td>14</td>
<td>Ovulation</td>
<td>Decline LH/FSH, Low E2, Rising P</td>
</tr>
<tr>
<td>18</td>
<td>Early Luteal</td>
<td>Moderate E2, High P, Low FSH/LH</td>
</tr>
<tr>
<td>22</td>
<td>Mid Luteal</td>
<td>Moderate E2, Peak P, Low FSH/LH</td>
</tr>
<tr>
<td>27</td>
<td>Late Luteal</td>
<td>Decline E2, Decline P, Low FSH/LH</td>
</tr>
</tbody>
</table>

*E2=Estradiol, P=Progesterone, LH=Luteinizing Hormone, FSH=Folicle Stimulating Hormone
Table 4.2. Number of visits per cycle for BioCycle participants.

<table>
<thead>
<tr>
<th>Number of visits</th>
<th>Cycle 1 (N=259)</th>
<th>Cycle 2 (N=250)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>3 (1.2)</td>
<td>2 (0.8)</td>
</tr>
<tr>
<td>6</td>
<td>14 (5.4)</td>
<td>7 (2.8)</td>
</tr>
<tr>
<td>7</td>
<td>57 (22.0)</td>
<td>52 (20.8)</td>
</tr>
<tr>
<td>8</td>
<td>185 (71.4)</td>
<td>189 (75.6)</td>
</tr>
</tbody>
</table>
Table 4.3. Baseline demographics of 259 women enrolled in the BioCycle study.

<table>
<thead>
<tr>
<th>Category</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
</tr>
<tr>
<td>&lt; 20</td>
<td>39 (15.1)</td>
</tr>
<tr>
<td>20-29</td>
<td>136 (52.5)</td>
</tr>
<tr>
<td>30-39</td>
<td>51 (19.7)</td>
</tr>
<tr>
<td>≥ 40</td>
<td>33 (12.7)</td>
</tr>
<tr>
<td><strong>Race</strong></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>154 (59.5)</td>
</tr>
<tr>
<td>African American</td>
<td>51 (19.7)</td>
</tr>
<tr>
<td>Other</td>
<td>54 (20.8)</td>
</tr>
<tr>
<td><strong>Education (highest grade completed)</strong></td>
<td></td>
</tr>
<tr>
<td>&lt; High school</td>
<td>33 (12.7)</td>
</tr>
<tr>
<td>Post-secondary</td>
<td>226 (87.3)</td>
</tr>
<tr>
<td><strong>Estimated median family income</strong></td>
<td></td>
</tr>
<tr>
<td>&lt;$19,999</td>
<td>55 (21.2)</td>
</tr>
<tr>
<td>$20,000-39,999</td>
<td>61 (23.6)</td>
</tr>
<tr>
<td>$40,000-74,999</td>
<td>72 (27.8)</td>
</tr>
<tr>
<td>$75,000-99,000</td>
<td>45 (17.4)</td>
</tr>
<tr>
<td>&gt;$100,000</td>
<td>24 (9.3)</td>
</tr>
<tr>
<td>Missing</td>
<td>2 (0.8)</td>
</tr>
<tr>
<td><strong>Marital Status</strong></td>
<td></td>
</tr>
<tr>
<td>Married/Living as married</td>
<td>66 (25.5)</td>
</tr>
<tr>
<td>Separated/Divorced</td>
<td>16 (6.2)</td>
</tr>
<tr>
<td>Single</td>
<td>177 (68.3)</td>
</tr>
<tr>
<td><strong>Body Mass Index (kg/m²)</strong></td>
<td></td>
</tr>
<tr>
<td>Underweight &lt;18.5</td>
<td>10 (3.9)</td>
</tr>
<tr>
<td>Normal 18.5 – 24.9</td>
<td>156 (60.2)</td>
</tr>
<tr>
<td>Overweight 25.0 – 29.9</td>
<td>65 (25.1)</td>
</tr>
<tr>
<td>Obese &gt; 30.0</td>
<td>28 (10.8)</td>
</tr>
</tbody>
</table>
Table 4.4. Inclusion/exclusion criteria for the BioCycle study.

**Inclusion Criteria**
- Age 18 to 44 at initial Screening Visit
- Premenopausal
- Self-reported cycle length between 21 and 35 days for each menstrual cycle for the past 6 months
- Willingness to provide regular fasting blood and urine specimens at our clinical center according to the proposed schedule of 8 visits per cycle times 2 cycles
- Willingness and ability to complete regular study questionnaires and diaries

**Exclusion Criteria**
- Depo-provera, norplant or Intrauterine Device (IUD) use in the past 12 months
- Oral Contraceptive use or other hormone supplement use in the past 3 months
- Planning to attempt to conceive in the next 3 months
- Reporting actively trying to conceive in the last 6 months
- Pregnant currently or in the last 6 months
- Breast feeding at any time in the last 6 months
- Abnormal Pap in last 6 months with no subsequent normal result
- Laparoscopy confirmed endometriosis
- Current uterine fibroids or removal of a fibroid in the last 12 months
- History of polycystic ovary disease
- History of Chlamydia infection or positive IgG screen at screening
- Untreated gynecologic infection or any genitourinary infection in the past 6 months
- Gynecologic surgery in the past year
- Sought treatment for infertility, ever (did not include male factor issues)
- History or clinical signs of gynecologic problems (i.e.
- Infection disease treated by a physician in the past 6 months
- Treatment for allergies with chronic medication (1 or more times per week in the last 3 months)
- Liver or kidney disease requiring treatment in the past year
- Psychiatric condition requiring medical therapy in the past year (including Premenstrual dysphoric disorder (PMDD))
- BMI (kg/m²) less than 18.0 or greater than 35.0 as measured in the clinic
- Plan to consume a restricted diet for intended weight loss or medical reason in the next 3 months
- Gastrointestinal conditions associated with malabsorption (i.e. Crohn’s disease)
- Unwilling to stop regular intake of vitamin and/or mineral supplements or herbal antioxidant supplements during cycle visit months
- Chronic use of certain medications, including lipid lowering drugs, anti-hypertensive medications and/or aspirin, among others
- Antibiotic use in the past 3 months
- History of chronic diseases such as heart disease, diabetes mellitus, cancer, inflammatory diseases, autoimmune, liver or kidney disease, thyroid disease or any other endocrine dysfunction
- History of alcohol abuse/dependency disorder or other drug/substance abuse
- Self-reported current regular illicit drug use in the past 30 days before baseline
- Diets high in phyto-estrogen content (i.e. soy based diet)
Table 4.5. Fertility monitor compliance among BioCycle study participants.

<table>
<thead>
<tr>
<th></th>
<th>Cycle 1</th>
<th>Cycle 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N     (%)</td>
<td>N     (%)</td>
</tr>
<tr>
<td>Incomplete monitor data</td>
<td>14    (5.6)</td>
<td>37    (16.0)</td>
</tr>
<tr>
<td>Number of missed tests out of first 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>120   (48.2)</td>
<td>98    (42.2)</td>
</tr>
<tr>
<td>1-2</td>
<td>83    (33.3)</td>
<td>77    (33.2)</td>
</tr>
<tr>
<td>3-4</td>
<td>23    (9.2)</td>
<td>27    (11.6)</td>
</tr>
<tr>
<td>5-10</td>
<td>22    (8.8)</td>
<td>18    (7.8)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1     (0.4)</td>
<td>12    (5.2)</td>
</tr>
<tr>
<td>Luteinizing hormone level</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak</td>
<td>189   (75.9)</td>
<td>180   (77.6)</td>
</tr>
<tr>
<td>No peak</td>
<td>49    (19.7)</td>
<td>29    (12.5)</td>
</tr>
<tr>
<td>Unknown</td>
<td>11    (4.4)</td>
<td>23    (9.9)</td>
</tr>
</tbody>
</table>
Table 4.6. LH peak day based on fertility monitor data.

<table>
<thead>
<tr>
<th></th>
<th>Cycle 1</th>
<th></th>
<th>Cycle 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=186)</td>
<td></td>
<td>(n=179)</td>
</tr>
<tr>
<td>Peak day</td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>3.4</td>
<td>15</td>
</tr>
<tr>
<td>Visit on peak day</td>
<td>N (73.7%)</td>
<td></td>
<td>N (81.0%)</td>
</tr>
</tbody>
</table>
Figure 4.1. Timing of blood sample collection in relation to pattern and timing of hormonal changes during the normal menstrual cycle.\textsuperscript{30}
Figure 4.2. BioCycle recruitment flow diagram.

957 Calls Received

449 Screening Visits Done

484 Not Interested or Self-screened out

24 Recruitment Ended Before Able to Join

318 Eligible

131 Ineligible

276 Enrolled

42 Not Enrolled

9 completed 1 cycle

250 completed 2 cycles

17 withdrew
Figure 4.3. ClearBlue® Fertility Monitor.
Figure 4.4. Observation of LH peak based on different menstrual cycle timing methods.
V. Dissertation Manuscript #1: A Longitudinal Study of Serum Lipoproteins in Relation to Endogenous Reproductive Hormones during the Menstrual Cycle: Findings from the BioCycle Study

A. Abstract

It has been hypothesized that circulating endogenous estrogens protect premenopausal women from coronary heart disease. The purpose of this study was to evaluate the association between lipoprotein and hormone levels across the normal menstrual cycle. Lipoprotein cholesterol and hormone levels were measured in serum from 259 healthy, regularly menstruating women in the BioCycle study. Fasting blood samples were collected at up to 16 points across 2 menstrual cycles, with collection scheduled using fertility monitors. We evaluated the relationship between estradiol and lipoproteins using marginal structural models with inverse probability of exposure weights. Total and low-density lipoprotein (LDL) cholesterol were lower during the luteal phase as compared to the follicular phase (P<0.001), and high-density lipoprotein (HDL) levels were highest around ovulation (P<0.001). More women were classified above the desirable range (LDL≥130 mg/dL or total cholesterol≥200 mg/dL) when measured during the follicular phase. Estradiol was positively associated with HDL cholesterol in acute effects models (beta=0.016, 95% confidence interval (CI): 0.012, 0.019), and inversely associated with total cholesterol (beta=-0.015, 95% CI: -0.018, -0.012), LDL cholesterol (beta=-0.024, 95% CI: -0.028, -0.019), and triglycerides (beta=-0.015, 95% CI: -0.027, -0.004) in persistent effects models. In conclusion, we observed that lipoprotein cholesterol levels varied across the cycle and were significantly associated with endogenous estrogen levels. This study supports the
beneficial effects of estrogen on lipoprotein cholesterol levels, and sheds light into the timing and mechanisms of estrogen’s effects, suggesting that they are not acute. Cyclic variations have clinical implications regarding the appropriate timing of measurement during the cycle and may need to be considered in the design and interpretation of studies in women of reproductive age.

B. Introduction

Heart disease is the leading cause of death among women, primarily affecting postmenopausal women. The prevalence of coronary heart disease (CHD) among women 20 to 39 years old is half the rate among men in the same age group (women, 7.8%; men, 15.9%). The difference in prevalence of CHD between men and women holds despite adjustment for various risk factors. However, as the prevalence of CHD among women increases with age, the difference between men and women drastically narrows. This has led to the hypothesis that circulating endogenous estrogens protect premenopausal women from CHD. With this in mind, hormone therapy was recommended to postmenopausal women as a means of cardio-protection. However, results from the Women’s Health Initiative (WHI) trial and the Heart and Estrogen/progestin Replacement Study (HERS) found that hormone therapy improved lipoprotein profiles but was associated with increased rates of CHD, which suggested that the role of exogenous/endogenous hormones on CHD was more complicated than originally thought.

The associations between exogenous sex hormones and lipoproteins seem to be well established. Estrogen is thought to exert a favorable effect on lipoprotein metabolism by increasing very low density lipoprotein (VLDL) synthesis, inhibiting hepatic lipase and
lipoprotein lipase activity, and upregulating the LDL receptors. The effect of endogenous sex hormones on lipoprotein levels in healthy premenopausal women however, remains uncertain, as well as whether these effects are chronic versus acute. Therefore, the objective of this study was to evaluate the association between serum estrogen and lipoprotein cholesterol levels during the normal menstrual cycle in a large prospective study of menstrual cycle function, with multiple measures of lipoprotein cholesterol and hormones throughout the cycle.

C. Methods

Study Sample

The BioCycle study was a prospective cohort of 259 women who were followed for one (n=9) or two (n=259) cycles. Participants were recruited from healthy, premenopausal volunteers, aged 18 to 44, from western New York. Exclusion criteria included current use of oral contraceptives or other medications including lipid lowering drugs, anti-hypertensive medications, and/or aspirin, pregnancy in the last 6 months, or a self-reported body mass index (BMI) at screening less than 18 or greater than 35. Further details on inclusion and exclusion criteria have been reported elsewhere. The University at Buffalo Health Sciences Institutional Review Board approved the study. All participants provided written informed consent.
Data Collection

The study involved up to 8 clinic visits per cycle for 2 cycles with visits timed using fertility monitors (Clearblue® Easy Fertility Monitor, Inverness Medical, Waltham, MA, USA) so that biospecimen collection occurred during specific phases of the cycle. Monitor indications of low, high, and peak fertility were used to time mid-cycle visits. Visits corresponded to biologically relevant windows including menstruation, mid- and late-follicular phase, LH/FSH surge, ovulation, and early-, mid-, and late-luteal phase. Participants were highly compliant with the study protocol—the number of visits ranged from 5 to 8 per cycle, with 94% of all women completing at least 7 visits per cycle.

Hormone Assessment

Estradiol, progesterone, LH, and FSH, were measured in fasting serum samples collected at each visit. Estradiol, the principal estrogen secreted by the ovary, was measured using a radioimmunoassay. Progesterone, LH, and FSH were measured using Solid phase competitive Chemiluminescent Enzymatic Immunoassay by Specialty Laboratories, Inc. (Valencia, CA) on the DPC Immulite®2000 analyzer (Siemens Medical Solutions Diagnostics, Deerfield, IL). The analytical imprecision of these assays was monitored using 3-level quality control materials as part of the laboratory’s quality assurance program. Across the study period the imprecision of all three quality control material levels was <5% coefficient of variation (%CV) for estradiol, LH, and FSH, and <10% CV for progesterone.
Lipoprotein Assessment

A complete lipid profile was performed for each cycle visit. The lipid profile included analysis of total cholesterol, HDL cholesterol, and triglycerides, using a Beckman LX20 automated chemistry analyzer at the Kaleida Center for Laboratory Medicine, Buffalo, NY. LDL cholesterol was determined indirectly using the Friedewald formula. The analytical imprecision across the study period was <5% for all lipid and lipoprotein assays.

Covariate Assessment

Participants were asked to complete questionnaires on lifestyle (smoking status), physical activity (International Physical Activity Questionnaire long form 2002), and reproductive history. Dietary intake was assessed using the 24-hour dietary recall methodology. Recalls were conducted 4 times per cycle using the Nutrition Data System for Research (NDSR) software version 2005 developed by the Nutrition Coordinating Center (NCC), University of Minnesota, Minneapolis, MN. Physical and anthropometric measures were done according to standardized protocols and included height, weight, and waist circumference.

Statistical Analysis

Descriptive statistics were computed for all study variables according to tertile of baseline HDL and LDL cholesterol levels, as well as according to whether baseline LDL cholesterol levels were above the desirable range (≥ 130 mg/dL) according to the National Cholesterol Education Program (NCEP) guidelines. Baseline HDL and LDL levels were measured on the second day of menses during the first cycle under study. Exact chi-square
tests and analysis of variance were used to test for associations between demographic variables and lipoprotein levels. Median and interquartile range (IQR) levels of hormones and lipoprotein cholesterol were calculated for each clinic visit. Both lipoprotein cholesterol and hormone levels were log transformed for normality. Linear mixed models were used to compute the p-values for comparisons between the mean log values of hormones and lipoproteins across the cycle.

Marginal structural models were used to model the association between lipoprotein cholesterol and estradiol levels measured on the same day (acute effects) or with estrogen levels at one visit predicting lipoprotein cholesterol levels at the next visit (persistent effects). Persistent effects models are intended to represent prolonged exposure to estrogen (approximately two days), and were considered to demonstrate temporality of effects. Lipoprotein cholesterol and hormone levels were allowed to vary over time and all models included hormone and lipoprotein cholesterol concentrations throughout the cycle, including up to 8 measurements per cycle. Models were fit for each lipoprotein parameter (total, HDL, and LDL cholesterol, triglycerides, total cholesterol/HDL ratio). In addition, since cholesterol is the initial precursor for steroid biosynthesis, we also evaluated the effect of baseline lipoprotein levels on hormone levels throughout the cycle.

Weighted linear mixed effects models with random intercepts were used to estimate the variables of the marginal structural model using inverse probability of exposure weights to appropriately adjust for time-dependent confounding. In order to estimate the stabilized weights for each cycle visit under study, the conditional density of estradiol levels at each cycle visit while adjusting for other factors was obtained by ordinary least-squares regression and estimated by the normal distribution. The choice of covariates in the weight
models was determined by a review of the literature and included age, BMI, and progesterone, LH, and FSH. Additional measures of dietary intake, physical activity, smoking, alcohol use, and race were considered as potential covariates, but did not appreciably alter the estimates.

Lastly, the percentage of women with cholesterol levels above the desirable ranges, as identified by the NCEP were calculated overall, and for each cycle day. Minimum and maximum lipoprotein cholesterol measurements across the cycle were calculated for each woman. The percentage of women with minimum and maximum lipoprotein cholesterol measurements was calculated for each cycle day. All statistical analyses were carried out using SAS version 9.1 (SAS Institute, Cary, NC, USA).

D. Results

Baseline characteristics varied somewhat by tertile of baseline HDL and LDL cholesterol levels and by whether a woman was considered to have “high” levels of LDL based on the NCEP guidelines (Table 5.1). Specifically, older ages and a higher BMI and waist circumference were associated with higher levels of LDL cholesterol. Higher levels of energy intake (kcais) and physical activity were observed among women with the most optimal lipid profiles.

Both hormone and lipoprotein cholesterol levels were observed to vary across the cycle (Table 5.2). Hormone levels showed expected patterns of variation. Total, LDL cholesterol, and triglycerides were highest during the follicular phase and declined during the luteal phase. Specifically, median total cholesterol declined by 3.0% (p<.0001) and LDL cholesterol by 4.9% (p<.0001) from the mid-follicular phase to the mid-luteal phase. HDL
cholesterol levels were highest during ovulation, but did not meaningfully change across other phases (p=1.0).

In acute effects models, estradiol was positively associated with levels of total (p=0.0003) and HDL cholesterol (p<.0001), and inversely associated with the total cholesterol/HDL ratio (p<.0001) (Table 5.3). In persistent effects models, estradiol was significantly inversely associated with total (p<.0001) and LDL cholesterol (p<.0001), total cholesterol/HDL ratio (p<.0001), and triglycerides (p=0.01). Estradiol was also positively associated with HDL cholesterol (p=0.57), though this association was not significant. In general, baseline lipoprotein levels were not significantly associated with hormone levels (results not shown).

The absolute changes in median lipoprotein cholesterol levels between women across the cycle were small (total cholesterol, -3.0%; HDL, 2.0%; LDL, -4.9%), however, the mean change within a woman over the cycle was much greater (i.e. total cholesterol, 19%). In fact, the range of total cholesterol values within a woman over the 8 cycle visits was between 8 and 85 mg/dL, with a mean of 27.7 (SD 11.1).

In this study, only 5% (n=13) of women had total cholesterol levels above 200 mg/dL at all eight visits. A total of 51 women (19.7%) had levels above 200 mg/dL on at least one cycle visit. When measured during the late luteal phase the smallest percentage of women would be classified as having high cholesterol (total cholesterol, 7.9%; LDL cholesterol, 10.5%), whereas the largest percentage are above the desirable levels during the follicular phase (total cholesterol, 14.3%; LDL cholesterol, 17.8%) (Table 5.4). 45.0% of women had their maximum total cholesterol level throughout the cycle during the follicular phase; and 45.4% of women had their minimum level during the luteal phase (Figure 5.1). The
minimum between woman variability for these lipid measures was observed to occur during
menses and during or immediately following ovulation (Figure 5.2). Triglycerides and HDL
cholesterol were observed to be highly variable throughout the cycle.

E. Discussion

In this longitudinal study of serum lipid profiles, we found that lipoprotein cholesterol
levels varied across the cycle. Specifically, total and LDL cholesterol were highest during
the follicular phase and declined during the luteal phase, whereas HDL cholesterol was
highest at ovulation. In addition, we observed that increased levels of endogenous estrogen
were associated with an improved lipid profile. It appears that estrogen has a rapid effect on
increasing HDL cholesterol levels. However, the effects of estrogen on total and LDL
cholesterol do not appear to be acute, as only prolonged exposure (approximately two days)
was associated with decreased levels of total and LDL cholesterol.

We observed significant changes in lipoprotein cholesterol measurements and
variability throughout the cycle. In fact, more women would be classified above the
desirable range when tested during the follicular phase of the cycle. Between-woman
variability was reduced during menses and immediately following ovulation. These
differences in both mean and variance across the cycle suggest that cycle phase should be
taken into account when evaluating lipoprotein cholesterol levels among reproductive aged
women. While treatment decisions may still require repeated samples above the
recommended level, standardizing the timing of measurements may improve the
interpretability of results and consequently reduce the overall number of tests. It is
interesting to note that these changes in classification and variability across the cycle were
observed among healthy women with no other risk factors for CHD. It is possible that the variability in lipoprotein cholesterol could be much greater among women with multiple risk factors for CHD. In fact, among obese women over 40 years of age in our study we observed increased variability across the cycle.

These results are in line with several studies that have shown total and LDL cholesterol levels to be lower during the luteal phase of the cycle (when estrogen and progesterone levels are high), as compared to the follicular phase. In studies of exogenous hormones, however, progestins are usually considered to oppose the stimulatory effects of estrogen on lipoprotein metabolism. The luteal phase could be considered as a natural state of “opposed estrogen”, thus suggesting that endogenous progesterone may not exhibit the same opposing effects as exogenous progesterone. We also observed HDL cholesterol levels to be highest around the time of ovulation, a finding that only a few previous studies have observed. This could be due to the fact that measurements compared between the follicular and luteal phases would tend to miss the peak levels of HDL cholesterol we observed to occur around ovulation. Most previous studies observed no association between hormones and triglycerides, but not all. We observed an effect of hormones on triglycerides only in persistent effects models, suggesting that there may be a delay in the impact of estrogen on triglyceride levels. Our results comparing acute and persistent effects of estrogen have not been previously addressed in the literature due to limited measures of hormones and lipoprotein cholesterol across the cycle in past studies.

As far as potential mechanisms, it is becoming clearer that endogenous estrogen affects lipoprotein metabolism at many points and in many beneficial ways, most
consistently with a decrease in plasma LDL cholesterol and an increase in HDL cholesterol.\textsuperscript{67, 80, 122-125} We know that, to some degree, the rates of formation of all lipoprotein fractions are increased under the influence of estrogen, but their removal rates are variably increased or decreased.\textsuperscript{9} Lipoprotein cholesterol levels are, however, imperfect markers of lipoprotein metabolism. There is growing evidence that improvements to the atherogenic nature of the plasma lipid profile are in response to endogenous or exogenous estrogen. These changes are a function of effects including upregulation of the LDL receptors which act to increase the clearance of LDL cholesterol,\textsuperscript{126} the upregulation of ABCA1 (the ATP binding cassette transporter) and Apo-A1 which increases HDL synthesis,\textsuperscript{127} and the suppression of hepatic SR-BI (class B scavenger receptors) expression leading to decreased hepatic selective cholesterol uptake from HDL\textsuperscript{128} in addition to further effects on LDL. The results of the present study support these findings in that we also observed estradiol to be associated with higher levels of HDL cholesterol in acute effects models and lower levels of LDL cholesterol in persistent effects models.

These results suggest that estrogen has an acute effect on increasing HDL cholesterol. The accompanying rise observed in total cholesterol levels is most likely due to this increase in HDL cholesterol as we observed no accompanying associations with LDL. As exposure to estrogen increased (persistent effects models), we observed a decline in total and LDL cholesterol, which could be indicative of a delayed impact of estrogen on upregulation of the LDL receptors. Estrogen also stimulates removal of triglycerides from HDL which could explain the inverse association between estrogen and triglycerides, and the null association between estrogen and HDL cholesterol, we observed in persistent effects models.
Intensive monitoring on a large number of participants throughout 2 cycles, with multiple clinic visits timed using fertility monitors, are unique strengths of our study. Our multiple measurements of both hormones and lipoproteins enabled us to more precisely model the association between estradiol and lipoprotein cholesterol levels, and to evaluate both acute and persistent effects. Through the use of weighted linear mixed effects models we were also able to account for the correlation between and within women throughout the cycle, while taking levels of other reproductive hormones into account (adjusting for time-varying confounding). The prospective design and exclusion criteria at baseline strengthen the ability to draw inference, having reduced the potential for bias from factors known to be associated with lipoprotein levels. Standardized assessment of a wide variety of participant and dietary characteristics increased the ability to adjust for potential confounding factors in the weight models.

There are several limitations worth noting. Residual confounding is a possibility, since it can be very difficult to capture effects of dietary intake and exercise. The fact that adjustment for these factors did not appreciably alter the results for any of the models could partially be due to misclassification of these covariates. While our study sample population was restricted to normally menstruating women to exclude potential confounders by design, such restrictions could also limit the generalizability of our findings. There may be some selection bias due to loss to follow-up over the cycle as not all women completed 8 visits per cycle, however, marginal structural models account for this loss to follow-up through inverse probability of exposure weighting. Also, there is the potential for some misclassification of cycle phase as not all LH peaks were captured on the monitor. The increase in total cholesterol observed during the follicular phase could be due, at least in part, to the reduction
in plasma volume observed during this phase of the cycle which we were unable to
evaluate.\textsuperscript{63, 73, 129} The observed changes in HDL cholesterol around ovulation, however,
appear to be independent from the expected increase in plasma volume. Although we cannot
completely account for temporality of the effects of hormones and lipoproteins, we observed
no significant effects of baseline lipoprotein levels on hormones throughout the cycle.
Finally, while the use of marginal structural models was a significant improvement over
traditional covariate assessment, they are based on certain strong assumptions such as no
unmeasured confounding, positivity, correct model specification, and consistency. These
assumptions are generally hard to meet and should be taken into consideration when
interpreting results.\textsuperscript{115, 120}

In conclusion, cholesterol levels varied and were associated with endogenous
estrogen levels across the cycle. This study confirms the hypothesized beneficial effects of
endogenous estrogen on lipoprotein cholesterol levels, and suggests that the effects of
estrogen on lowering total and LDL cholesterol are not acute. This study is the first to
evaluate the association between endogenous estrogen and lipoproteins using multiple and
longitudinal serum measures of estrogen and lipoproteins, while comprehensively
considering potential impacts from other reproductive hormones. More importantly, this
research helps to fill an etiological gap in understanding the relationship between lipoprotein
cholesterol changes induced by hormonal variations during the menstrual cycle. Cyclic
variations in lipoprotein cholesterol levels observed in the present study have clinical
implications regarding the appropriate timing of lipoprotein cholesterol measurement during
the menstrual cycle and should be accounted for in the design and interpretation of studies in
women of reproductive age. Our research on the effects of estrogen on lipid profiles, a
significant risk factor for cardiovascular disease, helps clarify the role of endogenous
estrogen in cardio-protection and should be used to inform the direction of future research in
pre- and post-menopausal women.
### Tables and Figures

Table 5.1. Characteristics of women in the BioCycle study by tertile of baseline HDL and LDL cholesterol (n=259).

<table>
<thead>
<tr>
<th></th>
<th>HDL Cholesterol tertile</th>
<th>LDL Cholesterol tertile</th>
<th>NCEP Guidelines - LDL Cholesterol*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Overall</td>
<td>&lt;44 mg/dL</td>
<td>44-54 mg/dL</td>
</tr>
<tr>
<td>N=259 n=86 n=85 n=86 n=87 n=84 n=86</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age – yr†</td>
<td>27.3 ± 8.2</td>
<td>26.4 ± 7.8</td>
<td>27.7 ± 8.2</td>
</tr>
<tr>
<td>BMI – kg/m²</td>
<td>24.1 ± 3.9</td>
<td>25.3 ± 3.9</td>
<td>23.7 ± 3.4</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>74.7 ± 8.7</td>
<td>76.9 ± 9.0</td>
<td>74.9 ± 7.3</td>
</tr>
<tr>
<td>Race – n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>154 (59)</td>
<td>45 (52)</td>
<td>52 (61)</td>
</tr>
<tr>
<td>African American</td>
<td>51 (20)</td>
<td>19 (22)</td>
<td>14 (16)</td>
</tr>
<tr>
<td>Other</td>
<td>54 (21)</td>
<td>22 (26)</td>
<td>19 (22)</td>
</tr>
<tr>
<td>Less than high school education – n (%)</td>
<td>33 (13)</td>
<td>16 (19)</td>
<td>7 (8)</td>
</tr>
<tr>
<td>Married – n (%)</td>
<td>66 (26)</td>
<td>14 (16)</td>
<td>24 (28)</td>
</tr>
<tr>
<td>Physical activity category – n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>25 (10)</td>
<td>9 (10)</td>
<td>8 (9)</td>
</tr>
<tr>
<td>Moderate</td>
<td>92 (36)</td>
<td>34 (40)</td>
<td>28 (33)</td>
</tr>
<tr>
<td>High</td>
<td>142 (55)</td>
<td>43 (50)</td>
<td>49 (58)</td>
</tr>
<tr>
<td>Energy (kcal)</td>
<td>1603.5 ± 397.7</td>
<td>1582.8 ± 383.1</td>
<td>1604.9 ± 421.1</td>
</tr>
<tr>
<td>Nulliparous – n (%)</td>
<td>187 (74)</td>
<td>64 (75)</td>
<td>61 (73)</td>
</tr>
<tr>
<td>Current smoker – n (%)</td>
<td>10 (3.9)</td>
<td>3 (3)</td>
<td>3 (3)</td>
</tr>
</tbody>
</table>

BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein; NCEP, National Cholesterol Education Program; SD, standard deviation.

*LDL Cholesterol <130 mg/dL is considered “optimal” or “near optimal/above optimal” and ≥130 mg/dL is considered “borderline high,” “high,” or “very high” according to the NCEP; †Values are mean ± SD; ‡HDL p-value <0.05; §LDL p-value <0.05; ‖NCEP p-value <0.05
Table 5.2. Serum hormone and lipoprotein cholesterol concentrations among women in the BioCycle study (n=259) by menstrual cycle phase.

<table>
<thead>
<tr>
<th></th>
<th>Menses</th>
<th>Mid-Follicular</th>
<th>Late Follicular</th>
<th>LH/FSH Surge</th>
<th>Ovulation</th>
<th>Early Luteal</th>
<th>Mid-Luteal</th>
<th>Late Luteal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen (pg/mL)†</td>
<td>33.0 (20.0)</td>
<td>48.0 (37.0)</td>
<td>121.0</td>
<td>116.0</td>
<td>117.0</td>
<td>117.0</td>
<td>122.0</td>
<td>75.0</td>
</tr>
<tr>
<td>Progesterone (ng/mL)†</td>
<td>0.5 (0.3)</td>
<td>0.4 (0.3)</td>
<td>0.6 (0.6)</td>
<td>0.9 (1.2)</td>
<td>1.7 (3.0)</td>
<td>7.4 (10.0)</td>
<td>8.9 (7.4)</td>
<td>4.0 (6.3)</td>
</tr>
<tr>
<td>LH (ng/mL)†</td>
<td>3.9 (2.4)</td>
<td>4.7 (3.0)</td>
<td>7.6 (10.6)</td>
<td>10.3 (14.6)</td>
<td>8.5 (10.9)</td>
<td>6.4 (6.2)</td>
<td>4.3 (4.6)</td>
<td>4.0 (3.4)</td>
</tr>
<tr>
<td>FSH (mIU/mL)†</td>
<td>6.3 (2.5)</td>
<td>6.4 (2.5)</td>
<td>6.2 (4.0)</td>
<td>6.8 (5.3)</td>
<td>6.3 (5.3)</td>
<td>4.4 (3.8)</td>
<td>3.1 (2.5)</td>
<td>3.4 (2.3)</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dL)†</td>
<td>160.0 (36.0)</td>
<td>166.0 (39.0)</td>
<td>164.0 (38.0)</td>
<td>164.0 (34.0)</td>
<td>162.0 (34.0)</td>
<td>161.0 (35.0)</td>
<td>161.0 (36.0)</td>
<td>157.0 (40.5)</td>
</tr>
<tr>
<td>HDL Cholesterol (mg/dL)†</td>
<td>48.0 (17.0)</td>
<td>50.0 (17.0)</td>
<td>52.0 (17.0)</td>
<td>52.0 (16.0)</td>
<td>52.0 (16.0)</td>
<td>52.0 (16.0)</td>
<td>51.0 (16.0)</td>
<td>50.0 (17.0)</td>
</tr>
<tr>
<td>LDL Cholesterol (mg/dL)†</td>
<td>98.0 (32.0)</td>
<td>102.0 (33.0)</td>
<td>100.0 (32.0)</td>
<td>98.5 (32.0)</td>
<td>98.0 (30.0)</td>
<td>96.0 (31.0)</td>
<td>97.0 (34.0)</td>
<td>96.0 (34.5)</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)§</td>
<td>51.5 (32.0)</td>
<td>56.0 (33.0)</td>
<td>55.0 (30.0)</td>
<td>51.0 (28.0)</td>
<td>53.0 (27.0)</td>
<td>53.0 (31.0)</td>
<td>51.0 (28.0)</td>
<td>49.0 (28.0)</td>
</tr>
<tr>
<td>Total Cholesterol/HDL Ratio‡</td>
<td>3.3 (1.0)</td>
<td>3.3 (1.2)</td>
<td>3.2 (1.1)</td>
<td>3.1 (1.0)</td>
<td>3.1 (1.0)</td>
<td>3.1 (0.9)</td>
<td>3.1 (1.0)</td>
<td>3.2 (1.1)</td>
</tr>
</tbody>
</table>

FSH, follicle stimulating hormone; HDL, high density lipoprotein; IQR, interquartile range; LDL, low density lipoprotein; LH, luteinizing hormone.

*Results presented are Median (IQR)
† P < 0.0001; § P < 0.01; ‡ P < 0.10
Table 5.3. Association between log(estrogen) and log(lipoprotein cholesterol) levels among women participating in the BioCycle study (n=259). Results are based on marginal structural models with inverse probability of exposure weights.

<table>
<thead>
<tr>
<th></th>
<th>Effects</th>
<th>Beta†</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol (mg/dL)</td>
<td>Acute</td>
<td>0.0049</td>
<td>(0.0022, 0.0076)</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td>Persistent</td>
<td>-0.0150</td>
<td>(-0.0178, -0.0122)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>HDL Cholesterol (mg/dL)</td>
<td>Acute</td>
<td>0.0156</td>
<td>(0.0121, 0.0192)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>Persistent</td>
<td>0.0011</td>
<td>(-0.0026, 0.0047)</td>
<td>0.57</td>
</tr>
<tr>
<td>LDL Cholesterol (mg/dL)</td>
<td>Acute</td>
<td>-0.0012</td>
<td>(-0.0053, 0.0029)</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>Persistent</td>
<td>-0.0236</td>
<td>(-0.0278, -0.0194)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Total Cholesterol/HDL</td>
<td>Acute</td>
<td>-0.0120</td>
<td>(-0.0151, -0.0088)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>Persistent</td>
<td>-0.0178</td>
<td>(-0.0210, -0.0145)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>Acute</td>
<td>-0.0021</td>
<td>(-0.0132, 0.0090)</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>Persistent</td>
<td>-0.0169</td>
<td>(-0.0282, -0.0056)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

BMI, body mass index; CI, confidence interval; HDL, high density lipoprotein; LDL, low density lipoprotein.

*Acute effects models model the association between estrogen and lipoproteins measured on the same day. Persistent effects models model the association between estrogen measured at one visit and lipoprotein cholesterol levels at the next visit.

†Inverse probability of exposure weights were used to adjust for confounding. Weight models adjusted for hormone levels on previous days of the cycle. All weight models adjusted for age and BMI, weights for triglyceride models also adjusted for physical activity, race, alcohol use, smoking, and trans fatty acid intake.
Table 5.4. Women participating in the BioCycle study (n=259) above desirable ranges of total, HDL, and LDL cholesterol at each cycle visit during cycle one as defined by the National Cholesterol Education Program (NCEP), n (%).\textsuperscript{119}

<table>
<thead>
<tr>
<th>n (%)</th>
<th>Menses n=257</th>
<th>Mid-Follicular n=258</th>
<th>Late Follicular n=259</th>
<th>LH/FSH Surge n=258</th>
<th>Ovulation n=257</th>
<th>Early Luteal n=252</th>
<th>Mid-Luteal n=244</th>
<th>Late Luteal n=191</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol ≥200 mg/dL</td>
<td>26 (10.1)</td>
<td>37 (14.3)</td>
<td>29 (11.2)</td>
<td>28 (10.9)</td>
<td>27 (10.5)</td>
<td>23 (9.1)</td>
<td>23 (9.4)</td>
<td>15 (7.9)</td>
</tr>
<tr>
<td>Total Cholesterol ≥240 mg/dL</td>
<td>5 (1.9)</td>
<td>4 (1.6)</td>
<td>6 (2.3)</td>
<td>6 (2.3)</td>
<td>5 (1.9)</td>
<td>5 (2.0)</td>
<td>4 (1.6)</td>
<td>4 (2.1)</td>
</tr>
<tr>
<td>LDL Cholesterol ≥130 mg/dL</td>
<td>34 (13.2)</td>
<td>46 (17.8)</td>
<td>36 (13.9)</td>
<td>27 (10.5)</td>
<td>29 (11.3)</td>
<td>28 (11.1)</td>
<td>27 (11.1)</td>
<td>20 (10.5)</td>
</tr>
<tr>
<td>LDL Cholesterol ≥160 mg/dL</td>
<td>5 (1.9)</td>
<td>5 (1.9)</td>
<td>5 (1.9)</td>
<td>5 (1.9)</td>
<td>6 (2.3)</td>
<td>4 (1.6)</td>
<td>4 (1.6)</td>
<td>5 (2.6)</td>
</tr>
<tr>
<td>HDL Cholesterol &lt;40 mg/dL</td>
<td>43 (16.7)</td>
<td>42 (16.3)</td>
<td>32 (12.4)</td>
<td>34 (13.2)</td>
<td>32 (12.5)</td>
<td>36 (14.3)</td>
<td>38 (15.6)</td>
<td>39 (20.4)</td>
</tr>
</tbody>
</table>

FSH, follicle stimulating hormone; HDL, high density lipoprotein; LDL, low density lipoprotein; LH, luteinizing hormone; NCEP, National Cholesterol Education Program.
Figure 5.1. Day of maximum or minimum total cholesterol concentration across the cycle among women participating in the BioCycle study (n=259).
Figure 5.2. Between woman variance of total, HDL, and LDL cholesterol and triglycerides by cycle visit among women participating in the BioCycle study (n=259).
A. Abstract

Sporadic anovulatory cycles may be fairly common among regularly menstruating women, and reproductive hormone levels are intricately tied to ovulation. As cholesterol is the initial precursor for steroid biosynthesis, lipoprotein cholesterol levels are hypothesized to be associated with anovulation, however this remains to be tested. The objective of this study was to evaluate the association between lipoprotein cholesterol levels and incident anovulation among women reporting regular menstruation. The BioCycle Study was a prospective cohort study conducted at the University at Buffalo from September 2004 to 2006, which followed 250 self-reported regularly menstruating women aged 18 to 44, for two menstrual cycles. Women with at least one anovulatory cycle displayed higher mean levels of total and LDL cholesterol and triglycerides (adjusted for age and BMI), as well as several markers of endocrine function. Total cholesterol levels preceding the predicted time of ovulation were weakly associated with an increased risk of incident anovulation (OR per 5 mg/dL: 1.09, 95% CI: 1.01-1.18), however after adjustment for age at menarche and the LH:FSH ratio there was further attenuation (OR per 5 mg/dL: 1.06, 95% CI: 0.98-1.15). Markers of endocrine function (increased LH:FSH ratio, acne, and insulin levels and decreased SHBG) were significant predictors of anovulation. In conclusion, anovulatory women showed a more atherogenic lipid profile and endocrine characteristics indicative of
hyperandrogenism—findings that are consistent with retrospective data reported for women with polycystic ovary syndrome (PCOS). Lipoprotein cholesterol levels were weakly associated with risk of anovulation, however we identified that LH:FSH ratio, SHBG, insulin, and acne were predictors of incident anovulation. Collectively, these markers are consistent with mild hyperandrogenism coincident to anovulation, and may be indicative of a mild undiagnosed PCOS phenotype. This is the first study to our knowledge to prospectively identify endocrine markers of sporadic anovulation in a group of regularly menstruating women. Further studies are needed to elucidate the biological mechanisms that lead to sporadic anovulation.

B. Introduction

Sporadic anovulatory cycles may be fairly common among regularly menstruating women, although they are difficult to detect and the prevalence of anovulation has not been well described. The factors associated with anovulatory cycles are also not well understood among regularly menstruating women, but are of interest as anovulation might be associated with infertility. Ovulatory disorders are one of the leading causes of female infertility, affecting between 18 and 30% of infertile couples. Reproductive hormone levels are intricately tied to ovulation through regulation of the hypothalamic-pituitary-ovarian axis. As cholesterol is the initial precursor for steroid biosynthesis and plays a central role in the synthesis of sex hormones, lipoprotein cholesterol are hypothesized to be associated with anovulation.

There is some evidence of a more atherogenic lipid profile (increased levels of total cholesterol, low-density lipoprotein (LDL) cholesterol, and triglycerides, and decreased
levels of high-density lipoprotein (HDL) cholesterol) among women with certain ovulatory disorders, specifically among women with polycystic ovary syndrome (PCOS). However, the association between lipoprotein cholesterol and anovulation, in the absence of diagnosed PCOS, has not been well characterized. A broad spectrum of severity and variability in PCOS has been reported, and according to recently updated diagnostic criteria it appears that there exist subgroups of women with milder forms of the syndrome that may differ from classic PCOS in gonadotropin and hormone secretion patterns, as well as degree of dyslipidemia. Past studies have generally relied on the case-control design to evaluate these relationships, however they have described lipid profiles only between PCOS and non-PCOS women. To our knowledge the association between lipoprotein cholesterol levels and anovulation has not been directly evaluated. Moreover, these relationships have not been prospectively evaluated in regularly menstruating women, free of diagnosed PCOS.

Therefore, the objective of this study was to prospectively evaluate the association between serum lipid and lipoprotein cholesterol levels at baseline, and at multiple time points preceding the predicted time of ovulation, and incident anovulation among a group of regularly menstruating women.

C. Methods

Study Sample

The BioCycle study was a prospective cohort of 259 women who were followed for one (n=9) or two (n=250) cycles. Participants were recruited from healthy premenopausal volunteers aged 18 to 44 from the western New York region. Exclusion criteria included current use of oral contraceptives, or other medications including lipid lowering drugs, anti-
hypertensive medications, and aspirin, pregnancy in the last 6 months, chronic disease, or a self-reported body mass index (BMI) at screening less than 18 or greater than 35. Full details on inclusion and exclusion criteria have been reported elsewhere. The University at Buffalo Health Sciences Institutional Review Board approved the study and all participants provided written informed consent.

Data Collection

The study involved 5 to 8 clinic visits per cycle (94% of all women completed at least 7 visits per cycle) for up to 2 cycles, with visits timed using fertility monitors (Clearblue® Easy Fertility Monitor, Inverness Medical, Waltham, MA, USA) so that biospecimen collection occurred during specific phases of the menstrual cycle. Monitors measured estrone-3-glucuronide and luteinizing hormone (LH) in urine daily starting on the 6th day following the start of the woman’s menstrual cycle. Monitor indications of low, high, and peak fertility were used to time mid-cycle visits. If by day 14 there was no positive indication on the monitor, a visit was scheduled the following day while the participant continued daily monitor testing for 10 additional days. This new technology was used to more accurately define menstrual cycle phase, representing a significant improvement over previous studies. Visits corresponded to biologically relevant windows including menstruation, mid- and late-follicular phase, LH and follicle stimulating hormone (FSH) surge, ovulation, and early-, mid-, and late-luteal phase.
Laboratory Assays

Estradiol, progesterone, LH, FSH, sex hormone-binding globulin (SHBG), glucose, and insulin were measured in fasting serum samples collected at each visit. Fasting morning blood draws were collected between 7:00 a.m. and 8:30 a.m. at each visit and processed according to standardized protocols. Samples were then frozen at -80°C and sent as complete participant cycle batches to Kaleida Health Center for Laboratory Medicine (Buffalo, NY) for analysis of hormone concentrations. Estradiol was measured using a radioimmunoassay. Progesterone, LH, FSH, SHBG, and insulin were measured using a solid phase competitive chemiluminescent enzymatic immunoassay by Specialty Laboratories, Inc. (Valencia, CA) on the DPC Immulite®2000 analyzer (Siemens Medical Solutions Diagnostics, Deerfield, IL). The analytical imprecision of these assays was monitored using 3-level quality control materials as part of the laboratory’s quality assurance program. Across the study period the imprecision was <5% for the inter-assay variability in estradiol, LH, and FSH levels, and <10% for levels of progesterone, SHBG, and insulin. Fasting plasma glucose was assayed using a hexokinase based methodology on a Beckman LX20 autoanalyzer and inter-assay variability was <3%. Insulin resistance (HOMA-IR) was calculated based on the homeostasis model using the equation: fasting insulin (µU/ml) x fasting glucose (mmol/l) / 22.5.137

A complete lipid profile was also performed for each participant at each cycle visit. The lipid profile included analysis of total cholesterol, HDL cholesterol, and triglycerides, and was measured using a Beckman LX20 automated chemistry analyzer at the Kaleida Center for Laboratory Medicine (Buffalo, NY). LDL cholesterol was determined indirectly using the Friedewald formula.84 The analytical imprecision across the study period was <
5% for all lipid and lipoprotein assays. Baseline total cholesterol, HDL, LDL, and triglyceride levels were assessed on the second day of menses during the first cycle under study.

Classification of Anovulation

Menstrual cycles were initially classified as anovulatory if the peak progesterone concentration across the cycle was $\leq 5$ ng/mL ($n=65$).\textsuperscript{138,139} To minimize misclassification, we employed a conservative definition for ovulation in which cycles with progesterone concentrations $\leq 5$ ng/mL and an observed serum LH peak on the mid- or late luteal phase visit were considered ovulatory cycles. Based on this algorithm, 42 of the 509 cycles (8.3%) in this study were classified as anovulatory. Overall, 35 (13.5%) women had at least one anovulatory cycle; 28 (10.8%) women had one anovulatory cycle and 7 (2.7%) women had two anovulatory cycles.

Covariate Assessment

Participants were asked to complete questionnaires on demographic characteristics, lifestyle (smoking status), physical activity (International Physical Activity Questionnaire long form 2002),\textsuperscript{117} body hair patterns (modified Ferriman-Gallwey hirsutism score),\textsuperscript{140} acne, and reproductive history. Physical and anthropometric measures were according to standardized protocols and included height, weight, and waist and hip circumference.\textsuperscript{118} Dietary intake was assessed on several of the same days as biologic sample collection using the 24-hour recall methodology. Recalls were conducted 4 times per cycle, for a total of up to 8 recalls, using the Nutrition Data System for Research (NDSR) software version 2005
developed by the Nutrition Coordinating Center (NCC), University of Minnesota, Minneapolis, MN. Average dietary consumption across the cycle was calculated for each nutrient of interest. Cycle length was defined as the number of days from the first day of bleeding (menstruating by 4:00 p.m.) until the day before the next onset of bleeding.

Statistical Analysis

Descriptive statistics were computed for all study variables and compared between women with 0, 1, or 2 anovulatory cycles. Analysis of variance (ANOVA) was used to test for differences in means, and chi-square tests were used to test for differences in categorical variables. Mean levels of baseline lipoprotein cholesterol, and levels of lipoprotein cholesterol at multiple points across the cycle, were compared between ovulatory and anovulatory women, both unadjusted and adjusted for age and BMI. Linear mixed models were used to calculate predicted mean values and 95% confidence intervals (CIs) of age- and BMI-adjusted lipoprotein cholesterol levels at baseline according to ovulatory status, as well as age- and BMI-adjusted markers of endocrine function according to ovulatory status. Geometric means are presented for non-normally distributed variables.

Generalized linear mixed models with random intercepts were used to model the association between lipoprotein cholesterol levels (total cholesterol, HDL, and LDL cholesterol, and triglycerides) and the probability of incident anovulation across two menstrual cycles. Adjusted odds ratios (aORs) and CIs were calculated to represent a 5 mg/dL change in lipoprotein levels. We considered the effects of baseline lipoprotein cholesterol levels on anovulation, as well as the effects of lipoprotein cholesterol levels up to the time of expected ovulation and anovulation in a given cycle. At baseline we assumed
that lipoprotein cholesterol levels would not be affected by reproductive hormones, as hormone levels are typically low during menses and any influence should be minimal at this point. All models were adjusted for age, BMI, fiber intake, and total energy. We also compared results after adjusting for age at menarche, and each LH:FSH ratio, HOMA-IR, insulin, SHBG, hirsutism score, and current acne, in order to determine whether the observed associations could be explained by confounding due to underlying endocrine disturbances.

For models of the association between lipoprotein cholesterol levels up to the time of expected ovulation and anovulation, we included up to 4 lipoprotein cholesterol measurements per cycle for each woman, corresponding to menses, mid- and late-follicular phase, and the LH/FSH surge. Stabilized inverse probability of exposure weights were used to adjust for time-dependent confounding due to changing hormone levels across the cycle. Stabilized weights were estimated by the conditional probability density of lipoprotein cholesterol levels at each visit given a woman’s age, BMI, fiber intake, total energy, and levels of estrogen, LH, and FSH over the cycle. Estimates were obtained by ordinary least squares regression and estimated by the normal distribution.\(^{115,120}\) Weight model misspecification was explored by checking the distribution of the weights for different choices of covariates in the weight model. The choice of covariates was determined by literature review.

We further evaluated the women in our study with both an ovulatory and anovulatory cycle (n=23). We compared day 2 lipoprotein cholesterol levels between cycles using paired t-tests and generated ORs and 95% CIs for the association between lipoprotein cholesterol levels and anovulation adjusted for the LH:FSH ratio. SAS version 9.1 (SAS Institute, Cary, NC, USA) was used for all statistical analyses.
D. Results

The women in the BioCycle study were on average 27.3 years of age (range 18 to 44) and consisted mainly of single, nulliparous, normal weight, White women with some post-secondary education. Number of anovulatory cycles (0, 1, 2) differed significantly according to age, marital status, parity, and years since menarche (Table 6.1). Women with anovulatory cycles were on average younger, single, nulliparous, and with fewer years since menarche. Women with two anovulatory cycles also had shorter cycles on average. Age at menarche was similar between the three groups, although we noted a slight increase among the women with two anovulatory cycles (0.5 years older). After adjustment for age and BMI, we detected an upward trend in baseline triglyceride levels with increasing number of anovulatory cycles. Similarly, women with anovulatory cycles tended to have higher total and LDL cholesterol levels at baseline, however these differences were not statistically significant. When multiple measures of lipoprotein cholesterol levels were evaluated across the cycle, we observed significantly higher levels of total (p=0.01) and LDL (p=0.06) cholesterol and triglycerides (p=0.0002) in anovulatory as compared to ovulatory women (results not shown). No differences were observed with HDL cholesterol (p=0.8).

Several markers of endocrine function were evaluated and compared between anovulatory and ovulatory women, after adjusting for age and BMI (Table 6.2). Hirsutism scores and waist-to-hip ratios did not differ significantly between women with 0, 1, or 2 anovulatory cycles. However, prevalence of current acne was significantly higher among women with one or two anovulatory cycles, with the prevalence increasing with the number of anovulatory cycles observed. LH:FSH ratios were significantly higher (for day 2, day 7,
and the average of days 2 and 7), and SHBG levels (day 2) were significantly lower among anovulatory women, with dose-response relationships observed corresponding to the number of anovulatory cycles. Glucose, insulin, and HOMA-IR levels were not significantly different between the three groups, although women with at least one anovulatory cycle had higher insulin and HOMA-IR values on average.

Baseline lipoprotein cholesterol levels were not found to be associated with risk of anovulation for any of the lipoprotein cholesterol parameters, regardless of model choice (Table 6.3). When levels of lipoprotein cholesterol were allowed to vary up to the time of expected ovulation, we saw weak evidence of an association between total cholesterol and risk of anovulation (OR per 5 mg/dL: 1.09, 95% CI: 1.01, 1.17). However, these results were attenuated after adjustment for age at menarche (OR per 5 mg/dL: 1.07, 95% CI: 0.99, 1.15). Further adjustment for the LH:FSH ratio did not appreciably alter the results (OR per 5 mg/dL: 1.07, 95% CI: 0.99, 1.16). We also adjusted for HOMA-IR, insulin, SHBG, hirsutism score and current acne, but the results remained unchanged (data not shown). We did not observe any associations between HDL, LDL, triglycerides and anovulation regardless of covariate choice. In all models we observed that in addition to age, an increased LH:FSH ratio on day 2 and decreased SHBG on day 2 were predictive of sporadic anovulation in a given cycle, when considering changing levels of lipoprotein cholesterol. Elevated insulin levels were predictive of anovulation in models of total and LDL cholesterol, but not triglycerides, and presence of current acne was predictive of anovulation in models of total cholesterol and triglycerides. Hirsutism score was not found to be predictive of anovulation. Odds ratios for these covariates are displayed in Figure 6.1.
To account for time-invariant measured and unmeasured confounders, we evaluated the 23 women with both an ovulatory and anovulatory cycle during the study period. We observed that mean day 2 total cholesterol, LDL cholesterol, and triglyceride levels were higher preceding the anovulatory cycle. The mean paired difference was 7.6 mg/dL for total cholesterol (p=0.03), 5.1 mg/dL for LDL cholesterol (p=0.07), and 10.3 mg/dL for triglycerides (p=0.05). There was no difference in HDL cholesterol levels (p=0.9). Overall, higher total and LDL cholesterol, and triglyceride level were associated with an increased risk of anovulation among these women, even after adjustment for the LH:FSH ratio (Figure 6.2).

E. Discussion

In this prospective cohort of normally menstruating women, the women who experienced an anovulatory cycle showed a more atherogenic lipid profile along with several endocrine characteristics consistent with hyperandrogenism. We observed a weak association between total cholesterol levels preceding the predicted time of ovulation and anovulation, that was attenuated after adjustment for age at menarche and the LH:FSH ratio. Further, among women with both an ovulatory and anovulatory cycle during the study period, lipoprotein cholesterol levels on day 2 of menses were higher preceding anovulatory cycles and were associated with an increased risk of anovulation. Increased day 2 LH:FSH ratio, levels of insulin, the presence of acne, and decreased levels of SHBG were found to be predictive of an anovulatory cycle. Collectively, these markers are consistent with mild hyperandrogenism and may be indicative of an undiagnosed mild PCOS phenotype. To our
knowledge, this is the first study to prospectively identify endocrine markers of sporadic anovulation in a group of regularly menstruating women.

Associations between lipid profiles and anovulation are typically described among women with PCOS, a disorder associated with menstrual disruption (such as irregular ovulatory cycles) and hormonal abnormalities characterized by hyperandrogenism, in the absence of related disorders.\textsuperscript{132, 141, 142} According to recently updated diagnostic criteria, the diagnosis of PCOS has broadened and only requires presence of two of the following three criteria: hyperandrogenism, ovulatory dysfunction, and the presence of polycystic ovaries.\textsuperscript{141} This expanded definition has led to the identification of four potential PCOS phenotypes (anovulatory PCOS-with or without polycystic ovaries (‘classic’ PCOS), ovulatory PCOS, and nonhyperandrogenic), representing a spectrum of severity and variability in metabolic parameters, body composition, and cardiovascular disease risk.\textsuperscript{143} The more atherogenic lipid profile we observed among the anovulatory women in our study is consistent with previous research among women diagnosed with PCOS,\textsuperscript{144-148} including those exhibiting a mild phenotype.\textsuperscript{133} The degree of dyslipidemia also appears to depend on phenotype, with ovulatory PCOS women displaying a milder form of atherogenic dyslipidemia than anovulatory PCOS women.\textsuperscript{133} In a recent retrospective study which evaluated the extent of atherogenic dyslipidemia it was observed that total cholesterol levels were highest among anovulatory PCOS women (mean 189 mg/dL), followed by ovulatory PCOS women (mean 178 mg/dL), and controls (166 mg/dL).\textsuperscript{133} After adjustment for age and BMI, the 35 women in our study with anovulatory cycles demonstrated a lipid profile more atherogenic than those 224 women with two consecutive ovulatory cycles, though not to the extent often reported for women with ‘classic’ PCOS. The latter is likely a consequence of our strict study
inclusion/exclusion criteria which was intended to exclude women at high risk or diagnosed with PCOS. In fact, lipoprotein cholesterol levels (as well as glucose, insulin, HOMA-IR, and LH:FSH ratio) among the anovulatory women in our study were of the same range as healthy controls in other studies of PCOS when the age and BMI ranges were similar, although much lower than control groups age and/or BMI-matched to older, obese PCOS women.

Not all previous studies observed a more atherogenic lipid profile among PCOS women, and differences could be due to small sample sizes, or differences in diagnostic criteria for classifying PCOS as there has been much debate regarding the diagnostic criteria for PCOS. It is interesting to note that while lipoprotein cholesterol levels among women with PCOS typically remain in the normal range according to the National Cholesterol Education Program criteria, women with PCOS have an increased risk of cardiovascular events. We also did not observe a significant decrease in HDL cholesterol that many previous studies have noted. This could be due in part to the fact that the full metabolic disturbances may not be present in the absence of obesity, and the women in our study were mostly of normal weight.

Based on the PCOS literature, a more atherogenic lipid profile would be expected among women with ovulatory disorders. These previous studies however were case-control studies by design, and unable to prospectively evaluate the effects of lipoprotein cholesterol on anovulation and rule out the possibility of reverse causality. In our prospective study we did not observe an association between baseline lipoprotein cholesterol levels and anovulation. However, when we considered levels prior to the time of expected ovulation, total cholesterol was weakly associated with anovulation in that cycle. After
adjusting for age at menarche and the LH:FSH ratio this result was attenuated towards the null. The fact that we did not observe associations between baseline lipoprotein cholesterol and anovulation could be due to the small number of anovulatory cycles in this study which limited statistical power, or to the limited range of lipoprotein cholesterol levels by inclusion of only healthy, regularly menstruating women.

Through multiple sensitivity analyses, we sought to determine whether the weak association we observed between total cholesterol levels and anovulation could be due to underlying endocrine disturbances. First, we adjusted our results for several different markers of endocrine function (LH:FSH ratio, current acne, hirsutism score, SHBG, insulin, HOMA-IR), in order to control for possible confounding by underlying endocrine disturbances. The results of the associations between lipoprotein cholesterol levels and anovulation adjusting for the LH:FSH ratio and other markers were essentially the same (Table 6.3). The fact that the results did not change after adjustment might suggest that these findings are not fully explained by underlying endocrine disturbances. However we cannot rule out this possibility as the LH:FSH ratio, and the other markers we measured, are not perfect proxies for these conditions. In addition, we did not have ultrasound test results to detect presence of polycystic ovaries or other more diagnostic criteria.

Second, in order to further account for the effect of both measured and unmeasured chronic factors (age, BMI, underlying PCOS, and other unmeasured chronic factors), we did a paired analysis of the 23 women in our study for which we had day 2 lipoprotein cholesterol levels during both an ovulatory and anovulatory cycle. In these comparisons, each woman was essentially matched to herself (i.e., case-crossover study), thus controlling by design for age, age at menarche, BMI, underlying PCOS, and any other factor that does
not vary over time, even those that went unmeasured. On average, lipoprotein cholesterol levels were higher preceding the anovulatory cycle, and 16 of the 23 women (70%) had higher day 2 lipoprotein cholesterol levels preceding their anovulatory cycle. This did not seem to be affected by time ordering of the cycles in that we observed higher levels whether the anovulatory cycle preceded the ovulatory cycle or vice versa. It remains an alternative possible explanation to the findings that there is a time-varying effect that is driving these associations, however we observed similar results after adjusting for several factors that may change over time, such as glucose, insulin, SHBG, and the LH:FSH ratio (Figure 6.2). Androgen levels were not measured in this study though and could potentially account for some of this association. These results are again suggestive of an association between lipoprotein cholesterol levels and anovulation that is independent of underlying endocrine disturbances, but are not conclusive.

In addition to raised lipoprotein cholesterol levels, we also observed that the anovulatory women in our study displayed several characteristics of mild hyperandrogenism and other endocrine disturbances, although assessment of hyperandrogenemia was limited in our study as we did not measure androgen levels. Phenotypically, self-reported hirsutism score assessments based on a modified Ferriman-Gallwey scale with 5 sites were not different between anovulatory and ovulatory women, but the prevalence of current acne increased with number of anovulatory cycles observed. Moreover, anovulatory women in this study demonstrated higher levels of several non-specific markers for androgen bioactivity including higher LH:FSH ratios, higher follicular phase LH, and lower SHBG levels, all consistent with, but not diagnostic of, hyperandrogenism. Increased LH:FSH ratios are indicative of hypothalamic-pituitary-ovarian dysfunction, which is
characteristic among women with PCOS. In addition, insulin levels were increased to a
degree among anovulatory women, an observation consistent with the insulin
resistance that frequently accompanies PCOS.\textsuperscript{158}

To investigate these associations further, we evaluated whether these endocrine
parameters characteristic of PCOS were significant predictors of sporadic anovulation
adjusting for lipoprotein cholesterol levels. We found that increased levels of day 2 LH:FSH
ratio and acne, and reduced levels of day 2 SHBG were predictive of anovulation in a given
cycle. We also observed day 2 insulin levels to be a significant predictor only of LDL levels,
although in all cases elevated day 2 insulin levels were associated with an increased risk of
anovulation. These findings raise the possibility of a very mild phenotype of PCOS among
our anovulatory women. More research is needed to identify the pathophysiology of incident
anovulation, particularly in the context of the spectrum of PCOS. The variability that we
observed in gonadotropin and hormone secretion patterns even among a group of women
reporting ‘regular’ menstrual cycles further emphasizes the need for additional research into
risk factors for anovulation in larger populations of premenopausal women.

To our knowledge this is the first study to prospectively evaluate the association
between baseline lipoprotein cholesterol levels, and lipoprotein cholesterol levels prior to the
time of expected ovulation, and anovulation. By preserving temporality we were able to
further evaluate whether the associations we observed could be due to biological effects of
lipoprotein cholesterol on anovulation, rather than just being symptomatic of an underlying
condition which leads to both ovulatory dysfunction and a more atherogenic lipid profile. In
addition, we were also able to evaluate the possibility of confounding by underlying
endocrine disturbances by adjustment for several markers of endocrine function. Through
the use of weighted generalized linear mixed models, we were also able to take into account the effects of varying reproductive hormone levels over the cycle. This study is further distinguished from previous research by its strict inclusion/exclusion criteria to include a group of normally menstruating women with no diagnosed cases of PCOS.

While this study allowed us to expand upon previous studies in this area, we were limited by a small number of anovulatory cycles, and an imperfect measurement of ovulation. The small number of anovulatory cycles was partly a consequence of our employment of a conservative definition for anovulation, and limited the power we had to detect effects. This could partially explain why we did not observe significant associations between lipoprotein cholesterol levels and anovulation. While we did have multiple serum hormone measurements to aid in classifying ovulation, along with the use of fertility monitors measuring LH daily in urine, daily measures of progesterone and transvaginal ultrasounds (the gold standard) were not available, and thus misclassification of ovulation is possible. However, a sensitivity analysis to evaluate misclassification of ovulation using a less conservative definition of anovulation was performed and yielded similar results. We were also unable to directly measure androgen levels, and had to rely on several non-specific markers. In particular, the hirsutism questionnaire used in this study was based on only 5 sites and has not been previously validated, and cannot be directly compared with results from previous studies. As this was a study among women reporting regular menstrual cycles with no history of PCOS, we have no ultrasound examinations for presence of polycystic ovaries.

In conclusion, we observed that women with at least one anovulatory cycle tended to display a more atherogenic lipid profile, along with several characteristics consistent with
mild hyperandrogenism. This is the first study to prospectively evaluate the effects of lipoprotein cholesterol levels leading up to ovulation and anovulation, while taking into account possible effects due to underlying PCOS by design and by analysis. While we observed a weak association between total cholesterol and anovulation, this association became non significant after adjustment for age at menarche and several markers of endocrine function. However, we did find that the LH:FSH ratio, SHBG, current acne, and insulin levels were predictors of incident anovulation among regularly menstruating women. Collectively, these markers are consistent with a small degree of hyperandrogenism and may be indicative of a mild undiagnosed PCOS phenotype. Further prospective studies are needed to elucidate the biological mechanisms that lead to sporadic anovulation and mild PCOS.
**F. Tables and Figures**

Table 6.1. Baseline demographic characteristics and lipoprotein cholesterol levels of women enrolled in the BioCycle study by ovulatory status (n=259).

<table>
<thead>
<tr>
<th>Number of Anovulatory Cycles</th>
<th>Overall</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Women</strong> n=259</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>224</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>28</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Demographics</strong></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs: mean ± SD</td>
<td>27.3 ± 8.2</td>
<td>28.0 ± 8.3</td>
<td>23.2 ± 6.1</td>
<td>19.4 ± 0.9</td>
<td>0.0004</td>
</tr>
<tr>
<td>BMI, kg/m²: mean ± SD</td>
<td>24.1 ± 3.9</td>
<td>24.2 ± 3.9</td>
<td>23.8 ± 4.0</td>
<td>21.6 ± 2.1</td>
<td>0.21</td>
</tr>
<tr>
<td>Cycle 1 length, days: mean ± SD</td>
<td>28.9 ± 4.6</td>
<td>29.0 ± 4.5</td>
<td>30.0 ± 5.3</td>
<td>24.4 ± 2.6</td>
<td>0.02</td>
</tr>
<tr>
<td>Cycle 2 length, days: mean ± SD</td>
<td>28.7 ± 3.5</td>
<td>28.8 ± 3.2</td>
<td>29.1 ± 5.7</td>
<td>25.7 ± 3.1</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>Physical Activity: n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.75</td>
</tr>
<tr>
<td>Low</td>
<td>25 (9.7)</td>
<td>22 (9.8)</td>
<td>2 (7.1)</td>
<td>1 (14.3)</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>92 (35.5)</td>
<td>81 (36.2)</td>
<td>8 (28.6)</td>
<td>3 (42.9)</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>142 (54.8)</td>
<td>121 (54.0)</td>
<td>18 (64.3)</td>
<td>3 (42.9)</td>
<td></td>
</tr>
<tr>
<td><strong>Race: n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.92</td>
</tr>
<tr>
<td>White</td>
<td>154 (59.5)</td>
<td>134 (59.8)</td>
<td>15 (53.6)</td>
<td>5 (71.4)</td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>51 (19.7)</td>
<td>43 (19.2)</td>
<td>7 (25.0)</td>
<td>1 (14.3)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>54 (20.9)</td>
<td>47 (21.0)</td>
<td>6 (21.4)</td>
<td>1 (14.3)</td>
<td></td>
</tr>
<tr>
<td>≤ High school education: n (%)</td>
<td>33 (12.7)</td>
<td>29 (13.0)</td>
<td>2 (7.1)</td>
<td>2 (28.6)</td>
<td></td>
</tr>
<tr>
<td>Current smoker: n (%)</td>
<td>10 (3.9)</td>
<td>9 (4.0)</td>
<td>0 (0.0)</td>
<td>1 (14.3)</td>
<td></td>
</tr>
<tr>
<td>Married: n (%)</td>
<td>66 (25.5)</td>
<td>65 (29.0)</td>
<td>1 (3.6)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>Nulliparous: n (%)</td>
<td>187 (73.9)</td>
<td>154 (70.0)</td>
<td>26 (100.0)</td>
<td>7 (100.0)</td>
<td></td>
</tr>
<tr>
<td>Past OC Use: n (%)</td>
<td>140 (54.9)</td>
<td>127 (57.2)</td>
<td>11 (42.3)</td>
<td>2 (28.6)</td>
<td></td>
</tr>
<tr>
<td>Age at menarche, yrs: mean ± SD</td>
<td>12.5 ± 1.2</td>
<td>12.4 ± 1.2</td>
<td>12.5 ± 1.4</td>
<td>13.1 ± 1.5</td>
<td>0.32</td>
</tr>
<tr>
<td>Years since menarche: mean ± SD</td>
<td>14.9 ± 8.3</td>
<td>15.7 ± 8.3</td>
<td>10.5 ± 6.9</td>
<td>6.3 ± 1.5</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

**Baseline Lipoprotein Cholesterol Levels (mg/dL)†**

| Total Cholesterol: mean ± SD | 163.4 ± 29.0 | 163.6 ± 29.6 | 163.1 ± 26.2 | 158.1 ± 22.2 | 0.92 |
|                             | (162.8, 167.2)| (159.1, 166.5)| (156.5, 177.9)| (146.8, 188.9)| 0.69 |
| Adjusted for age, BMI‡      | (50.0, 50.0) | (50.0, 50.0) | (50.0, 50.0) | (50.0, 50.0) | 0.91 |
| HDL Cholesterol: mean ± SD  | 50.1 ± 11.5 | 50.0 ± 11.7 | 50.0 ± 10.7 | 51.4 ± 9.5 | 0.91 |
| Adjusted for age, BMI       | (48.5, 51.5)| (46.2, 54.8)| (42.4, 59.4)| (42.4, 59.4)| 0.96 |
| LDL Cholesterol: mean ± SD  | 101.5 ± 25.7| 101.7 ± 26.0| 101.1 ± 24.2| 96.3 ± 22.4| 0.75 |
| Adjusted for age, BMI       | (97.8, 104.4)| (94.8, 113.7)| (84.2, 121.6)|              |
| Triglycerides: mean ± SD    | 59.2 ± 27.9 | 59.0 ± 27.8 | 60.1 ± 30.8 | 62.3 ± 20.3 | 0.83 |
| Adjusted for age, BMI       | (58.5, 62.4)| (52.1, 72.7)| (50.2, 90.9)| (50.2, 90.9)| 0.43 |

BMI, body mass index; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; OC, oral contraceptives; SD, standard deviation

*p value for continuous variables calculated using ANOVA, and for categorical variables using Fisher’s exact test.

†Baseline levels were measured at the first visit of the first cycle (day 2 of menses).

‡Values are predicted means (95% CI) after adjusting for age and BMI.
Table 6.2. Markers of endocrine function among women in the BioCycle study (n=259).

<table>
<thead>
<tr>
<th></th>
<th>Overall*</th>
<th>0</th>
<th>Number of Anovulatory Cycles†</th>
<th>1</th>
<th>2</th>
<th>p value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=259</td>
<td>n=224</td>
<td>n=28</td>
<td>n=7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hirsutism score</td>
<td>3.03 ± 2.58</td>
<td>3.02 (2.68, 3.36)**</td>
<td>3.24 (2.26, 4.21)</td>
<td>2.43 (0.49, 4.38)</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>Currently have acne: n(%)§</td>
<td>72 (30.5)</td>
<td>58 (28.4)</td>
<td>9 (36.0)</td>
<td>5 (71.4)</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Waist-to-Hip Ratio</td>
<td>0.75 ± 0.05</td>
<td>0.75 (0.75, 0.76)</td>
<td>0.76 (0.74, 0.79)</td>
<td>0.74 (0.68, 0.80)</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>LH:FSH Ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 2</td>
<td>0.61 ± 0.44</td>
<td>0.61 (0.57, 0.65)</td>
<td>0.86 (0.71, 1.03)</td>
<td>0.92 (0.64, 1.33)</td>
<td>0.0008</td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>0.71 ± 0.47</td>
<td>0.71 (0.67, 0.75)</td>
<td>0.88 (0.75, 1.04)</td>
<td>0.92 (0.66, 1.28)</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Average of day 2 and day 7</td>
<td>0.70 ± 0.45</td>
<td>0.67 (0.64, 0.71)</td>
<td>0.93 (0.79, 1.09)</td>
<td>0.93 (0.67, 1.27)</td>
<td>0.0003</td>
<td></td>
</tr>
<tr>
<td>SHBG nmol/L: Day 2</td>
<td>44.50 ± 29.05</td>
<td>45.07 (42.56, 47.73)</td>
<td>37.07 (31.47, 43.68)</td>
<td>28.27 (20.35, 39.27)</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>Glucose mg/dL: Day 2</td>
<td>87.00 ± 7.00</td>
<td>87.31 (86.57, 88.05)</td>
<td>86.46 (84.35, 85.57)</td>
<td>86.12 (81.90, 90.35)</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>Insulin uIU/mL: Day 2</td>
<td>6.00 ± 4.50</td>
<td>5.79 (5.42, 6.18)</td>
<td>6.99 (5.80, 8.42)</td>
<td>6.49 (4.47, 9.43)</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>HOMA-IR: Day 2</td>
<td>1.32 ± 0.56</td>
<td>1.29 (1.21, 1.38)</td>
<td>1.49 (1.22, 1.81)</td>
<td>1.43 (0.97, 2.13)</td>
<td>0.39</td>
<td></td>
</tr>
</tbody>
</table>

FSH, follicle-stimulating hormone; HOMA-IR, homeostatic metabolic assessment to predict insulin resistance; LH, luteinizing hormone; SHBG, sex-hormone binding globulin
*Values are mean ± SD for overall population.
†Values are predicted means (95% CI) after adjusting for age and BMI. LH:FSH ratio, SHBG, Insulin, and HOMA-IR are geometric means.
‡p value for continuous variables calculated using ANOVA, for acne calculated using a chi-square test.
§Acne: 5 or more pimples, pustules, or nodules on the face (except nose) during the last 3 months.
Table 6.3. Results of weighted non-linear mixed effects models of the association between lipoprotein cholesterol levels preceding predicted ovulation and incident anovulation among women in the BioCycle study (n=259).

<table>
<thead>
<tr>
<th></th>
<th>Baseline lipoprotein cholesterol levels and anovulation*</th>
<th>Levels of lipoprotein cholesterol up to predicted time of ovulation and anovulation†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR 95% CI p-value</td>
<td>OR 95% CI p-value</td>
</tr>
<tr>
<td><strong>Total Cholesterol per 5 mg/dL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 1‡</td>
<td>1.04 (0.97, 1.12) 0.27</td>
<td>1.09 (1.01, 1.17) 0.03</td>
</tr>
<tr>
<td>Model 2§</td>
<td>1.03 (0.95, 1.11) 0.51</td>
<td>1.07 (0.99, 1.15) 0.09</td>
</tr>
<tr>
<td>Model 3║</td>
<td>1.02 (0.95, 1.11) 0.56</td>
<td>1.07 (0.99, 1.16) 0.07</td>
</tr>
<tr>
<td><strong>HDL Cholesterol per 5 mg/dL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 1</td>
<td>1.02 (0.86, 1.20) 0.86</td>
<td>1.03 (0.87, 1.22) 0.71</td>
</tr>
<tr>
<td>Model 2</td>
<td>1.01 (0.85, 1.19) 0.92</td>
<td>1.02 (0.86, 1.20) 0.86</td>
</tr>
<tr>
<td>Model 3</td>
<td>1.01 (0.85, 1.20) 0.90</td>
<td>1.02 (0.86, 1.20) 0.86</td>
</tr>
<tr>
<td><strong>LDL Cholesterol per 5 mg/dL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 1</td>
<td>1.03 (0.95, 1.12) 0.42</td>
<td>1.07 (0.98, 1.16) 0.13</td>
</tr>
<tr>
<td>Model 2</td>
<td>1.02 (0.94, 1.11) 0.66</td>
<td>1.05 (0.96, 1.14) 0.28</td>
</tr>
<tr>
<td>Model 3</td>
<td>1.02 (0.94, 1.11) 0.60</td>
<td>1.04 (0.95, 1.13) 0.39</td>
</tr>
<tr>
<td><strong>Triglycerides per 5 mg/dL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 1</td>
<td>1.06 (0.99, 1.14) 0.10</td>
<td>1.04 (0.99, 1.10) 0.09</td>
</tr>
<tr>
<td>Model 2</td>
<td>1.05 (0.97, 1.13) 0.23</td>
<td>1.04 (0.99, 1.09) 0.16</td>
</tr>
<tr>
<td>Model 3</td>
<td>1.01 (0.93, 1.10) 0.76</td>
<td>1.04 (0.99, 1.10) 0.16</td>
</tr>
</tbody>
</table>

BMI, body mass index; FSH, follicle-stimulating hormone; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; LH, luteinizing hormone; OR, odds ratio; SD, standard deviation

*Baseline levels of lipoprotein cholesterol levels were measured on day 2 of menses during the first cycle under study. These models evaluate the association between baseline lipoprotein cholesterol and anovulation using generalized linear mixed models.

‡Levels of lipoprotein cholesterol were allowed to vary up to the time of predicted ovulation, and included up to 4 measurements per woman per cycle. These models evaluate the association between lipoprotein levels preceding ovulation and anovulation in that cycle using weighted generalized linear mixed models.

¶Model 1 is adjusted for age, BMI, fiber intake, and total energy. In weighted models for the association of lipoprotein cholesterol levels up the predicted time of ovulation, models also adjusted for levels of other reproductive hormones. Measurements taken on day 2 of menses are considered to be unaffected by other hormone levels.

§Model 2 adjusts for age at menarche in addition to the covariates included in Model 1.

║Model 3 adjusts for LH:FSH ratio on day 2 in addition to the covariates included in Model 2.
Figure 6.1. Odds ratios and 95% confidence intervals for associations between endocrine factors and risk of anovulation adjusted for total cholesterol, age, age at menarche, BMI, fiber intake, and total energy.

FSH, follicle stimulating hormone; HOMA-IR, homeostatic metabolic assessment to predict insulin resistance; LH, luteinizing hormone; SHBG, sex-hormone binding globulin
Figure 6.2. Odds ratios and 95% confidence intervals for day 2 lipoprotein cholesterol levels and anovulation in 23 women experiencing an ovulatory and anovulatory cycle during the study period; models unadjusted and adjusted for LH:FSH ratio.

HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol
VII. Dissertation Manuscript #3: Direct Effects of Dietary Fiber Intake on Lipoprotein Cholesterol Levels in Premenopausal Women

A. Abstract

High fiber diets are associated with the lowering of lipoprotein cholesterol levels. It has been hypothesized that estrogen could potentially mediate this effect in premenopausal women. Healthy, regularly menstruating women enrolled in the BioCycle study were followed for up to 2 menstrual cycles (n=259), with lipoprotein cholesterol and hormone levels measured in serum at up to 16 points, with clinic visits timed using fertility monitors. Marginal structural models with inverse probability of exposure weights were used to estimate the controlled direct effect of fiber on lipoprotein cholesterol in premenopausal women when controlling for time dependent estrogen set at specific levels. We observed reductions in total and LDL cholesterol and triglycerides, not mediated through estrogen, among women consuming only at or above 22 g/day, when controlling for estrogen at specified levels in the population (corresponding to oral contraceptive use and postmenopausal levels). The controlled direct effects of fiber intake were reduced at higher estrogen levels, suggesting that estrogen mediates the association between fiber and lipoproteins by slightly diminishing the effect. While the controlled direct effects we observed were not of clinical significance, these results provide further insights regarding the possible biological mechanisms of the effects of fiber on lipoprotein cholesterol, and support the hypothesis of a direct effect of fiber on cholesterol that might work through bile acid
metabolism. More research is needed to elucidate these mechanisms among premenopausal women.

B. Introduction

Diets with increased fiber intake are recommended due to the many health promoting benefits associated with high fiber consumption.\textsuperscript{15,159} In particular, evidence from randomized controlled trials, observational studies, and animal models, have all shown that dietary fiber lowers cholesterol and lipoprotein levels,\textsuperscript{15-17,159,160} which are common risk factors for cardiovascular disease (CVD).\textsuperscript{15,89,90,159,160} These overall decreases in total cholesterol are usually attributed to a reduction in low density lipoprotein (LDL) cholesterol, as high density lipoprotein (HDL) and triglyceride levels have not shown similar effects.

However, there is also evidence that pre-and post-menopausal women respond differently to fiber intake,\textsuperscript{18,19} with premenopausal women showing smaller reductions in lipoprotein cholesterol levels in response to fiber intake. It has therefore been hypothesized that estrogen could potentially mediate the effect of fiber on lipoprotein cholesterol levels in premenopausal women. High fiber intake has been associated with lower levels of estradiol,\textsuperscript{20-27} a result also confirmed in the BioCycle study in premenopausal women.\textsuperscript{28} Since estrogen and lipoproteins are intrinsically linked, as estrogen stimulates lipoprotein metabolism and cholesterol is the precursor for steroidogenesis, the implications of fiber’s association with estrogen on lipoprotein metabolism is evident. To date, however, there has been little research on how much of the observed effect of fiber on lipoprotein cholesterol levels is working directly, and not mediated by estrogen.
Therefore, the objective of this study was to determine the controlled direct effect of dietary fiber intake on lipoprotein cholesterol levels, among healthy, regularly menstruating women in the BioCycle study. As high fiber diets continue to be endorsed by the leading dietary and public health associations, a better understanding of the direct and indirect effects of fiber intake on lipoprotein cholesterol levels is essential. This knowledge could provide further insight regarding possible mechanisms, as well as valuable knowledge for the interpretation of studies of fiber intake among women of reproductive age.

C. Methods

Study Sample

The BioCycle study was a prospective cohort of 259 women who were followed for one (n=9) or two (n=250) cycles. Participants were recruited from healthy premenopausal volunteers aged 18 to 44 from the western New York region. Exclusion criteria included current use of oral contraceptives, vitamin and mineral supplements, or other medications including lipid lowering drugs, anti-hypertensive medications, and/or aspirin, pregnancy in the last 6 months, a recent history of infections or diagnosis of chronic conditions, including history of menstrual and ovulation disorders and gastrointestinal conditions (e.g. Crohn’s Disease). Women with a self-reported body mass index (BMI) at screening less than 18 or greater than 35 were excluded, as were women planning to restrict their diet for weight loss or medical reasons. Full details on inclusion and exclusion criteria have been reported elsewhere. The University at Buffalo Health Sciences Institutional Review Board approved the study and all participants provided written informed consent.
Data Collection

The study involved 5 to 8 clinic visits per cycle (94% of all women completed at least 7 visits per cycle) for up to 2 cycles, with visits timed using fertility monitors (Clearblue® Easy Fertility Monitor, Inverness Medical, Waltham, MA, USA) so that biospecimen collection occurred during specific phases of the menstrual cycle. Monitors measured estrone-3-glucuronide and LH in urine daily starting on the 6th day following the start of the woman’s menstrual cycle. Monitor indications of low, high, and peak fertility were used to time mid-cycle visits. This new technology was used to accurately define menstrual cycle phase, representing a significant improvement over previous studies. Visits corresponded to biologically relevant windows including menstruation, mid- and late-follicular phase, luteinizing hormone (LH) and follicle stimulating hormone (FSH) surge, ovulation, and early-, mid-, and late-luteal phase.

Dietary Assessment

Dietary intake was assessed on the same days as sample collection using the 24-hour dietary recall methodology. Recalls were conducted 4 times per cycle, for a total of up to 8 recalls, using the Nutrition Data System for Research (NDSR) software version 2005 developed by the Nutrition Coordinating Center (NCC), University of Minnesota, Minneapolis, MN. This program computed the nutrients (i.e. total energy, vitamin E) and non-nutrients (i.e. dietary fiber) from each day of intake. The majority of women completed 4 dietary recalls per cycle (87%).
Hormone Assessment

Estradiol, progesterone, LH, and FSH were measured in fasting serum samples collected at each visit. Fasting morning blood draws were collected between 7:00 a.m. and 8:30 a.m. at each visit and processed according to standardized protocols. Samples were frozen at -80°C and sent as complete participant cycle batches to the Kaleida Health Center for Laboratory Medicine (Buffalo, NY) for analysis of hormone concentrations. Estradiol was measured using a radioimmunoassay. Progesterone, LH, and FSH were measured using a solid phase competitive chemiluminescent enzymatic immunoassay by Specialty Laboratories, Inc. (Valencia, CA) on the DPC Immulite®2000 analyzer (Siemens Medical Solutions Diagnostics, Deerfield, IL). The analytical imprecision of these assays was monitored using 3-level quality control (QC) materials as part of the laboratory’s quality assurance program. Across the study period, the imprecision of all three QC material levels was <5% inter-assay coefficient of variation (%CV) for estradiol, LH, and FSH, and <10% CV for progesterone.

Lipoprotein Assessment

A complete lipid profile was performed for each cycle visit. The lipid profile included analysis of total cholesterol, HDL cholesterol, and triglycerides, and was measured using a Beckman LX20 automated chemistry analyzer at the Kaleida Center for Laboratory Medicine (Buffalo, NY). LDL cholesterol was determined indirectly using the Friedewald formula. The analytical imprecision across the study period was < 5% CV for all lipid and lipoprotein assays.
Covariate Assessment

Participants were asked to complete questionnaires on lifestyle (smoking status), physical activity (International Physical Activity Questionnaire long form 2002), and reproductive history. High, moderate, and low physical activity categories were formed based on standard IPAQ cut points. Cycle length was defined as the number of days from the first day of bleeding (menstruating by 4:00 p.m.) until the day before the next onset of bleeding. Physical and anthropometric measures were done according to standardized protocols and included height and weight, which were used to calculate BMI. All covariates assessed had at least a 95% response rate.

Controlled Direct Effects

A simple directed acyclic graph (DAG) in Figure 7.1 displays the direct effect of fiber on lipoprotein cholesterol, and the indirect effect of fiber on lipoprotein cholesterol working through estrogen. Additionally, the hypothesized confounders of the fiber-lipoprotein association, the fiber-estrogen association, and the estrogen-lipoprotein association are also displayed. In this analysis we are interested in estimating controlled direct effects, which are the direct effects when setting estrogen to a given level, and by so doing effectively blocking the effects of fiber on estrogen (and any other effects on estrogen), as is shown in Figure 7.1. The proposed methods assume that we can intervene on the mediator (estrogen) and change the value of estrogen, so that direct effects can be estimated at given levels of estrogen (i.e. controlled direct effects). In theory there are as many direct effects as there are levels of estrogen. Here we considered the controlled direct effects where estrogen is set to the mean level among postmenopausal women (11 pg/mL, equivalent to intervening and performing a
hysterectomy), and the mean level among premenopausal women on oral contraceptives (45 pg/mL, equivalent to intervening and giving women oral contraceptives). Controlled direct effects are to be distinguished from natural direct effects which block the effect of fiber on estrogen, but allow estrogen levels to vary among individuals because the effect of other factors on estrogen are not blocked.

Statistical Analysis

Descriptive statistics were computed for all study variables and exact chi-square tests and analysis of variance (ANOVA) were used to test for associations between demographic variables and levels of fiber intake, while taking into account multiple cycles per woman. Average levels of hormones across the cycle (estrogen, LH, FSH), average luteal phase progesterone, and baseline lipoprotein cholesterol levels (second day of menses during the first cycle under observation) were compared across levels of fiber intake using repeated measures ANOVA with Bonferroni adjusted comparisons. No significant differences were found in dietary fiber intakes across each cycle, therefore the average daily intake of fiber was calculated per cycle. Fiber intake was categorized into multiple groups of equal size and compared using linear mixed models adjusted for age, BMI, and energy intake to determine whether there was evidence of a threshold effect of fiber intake. Fiber intake was subsequently categorized according to whether a woman consumed at or above 22 g/day. Predicted mean levels of hormones and lipoproteins were calculated for each visit by fiber intake after adjusting for age, BMI, and total energy, using linear mixed models with random intercepts.
Marginal structural models (MSMs) with inverse probability of exposure weights were applied to estimate the controlled direct effect of fiber intake on lipoprotein levels.\textsuperscript{165} Weighted linear mixed effects models were used to model the association, allowing lipoprotein levels to vary over time, and treating fiber intake as a dichotomous variable indicating whether the woman consumed at or above 22 g/day during each cycle. Stabilized weights were obtained by estimating two sets of weights, one for fiber intake and one for estrogen levels. Weights for dichotomous fiber intake were obtained by logistic regression, and for continuous estrogen levels by linear regression replacing the probabilities with values from a probability density function.\textsuperscript{115} Weight models included age, BMI, energy intake, physical activity, vitamin E, LH, and FSH levels (see Figure 7.1). Confidence intervals were bootstrapped.\textsuperscript{165}

Further, we also evaluated the effects of meeting the daily reference intake (DRI) of fiber intake on lipoprotein cholesterol levels, with the DRI based on the Institute of Medicine’s recommendation of 14 grams of fiber per day per 1,000 kcal.\textsuperscript{105} We calculated a woman’s individual DRI for fiber intake based on her estimated total energy intake from the 24-hour recall, as well as based on her estimated energy requirements according to her age, weight, and height. Fiber intake was then categorized as at or above the individual DRI for each woman. SAS version 9.1 (SAS Institute, Cary, NC, USA) was used for all statistical analyses.

D. Results

The distribution of demographics, hormone levels, baseline lipoprotein levels, and dietary factors by fiber intake are displayed in Table 7.1. Fiber intake above and below 22
g/day varied significantly according to age and race/ethnicity, with younger and minority women tending to consume less fiber. BMI and physical activity were not significantly associated with fiber intake. Average estrogen levels across the cycle, and average luteal phase progesterone levels were lower among women consuming at or above 22 g/day. Baseline total and LDL cholesterol, and triglyceride levels were lowest among women consuming at or above 22 g/day. There were no significant differences in HDL cholesterol levels between fiber intake categories. Analysis of fiber intake according to quartiles of intake produced similar results for demographic and dietary characteristics (data not shown).

Fiber intake was inversely associated with estrogen levels, as shown in Figure 7.2 which displays the predicted means of estrogen concentrations according to fiber intake at each clinic visit across the menstrual cycle after adjusting for age, BMI, and total energy. Women consuming at or above 22 g/day had lower mean estrogen levels across the cycle, as well as lower luteal phase progesterone, and a lower LH peak (Figure 7.2). There were no differences in FSH levels across the cycle by fiber intake category. Women consuming at or above 22 g/day also displayed lower total and LDL cholesterol across the cycle. Triglyceride levels were lower during menses, the follicular phase, and around the time of expected ovulation (Figure 7.3). No consistent pattern was noticed for HDL cholesterol.

Women consuming at or above 22 g/day were observed to have significantly lower total and LDL cholesterol levels using linear mixed models, with evidence of a threshold effect of fiber intake (data not shown). When fiber intake was grouped into quantiles (tertiles, quartiles, quintiles, 8, 10, 12, 15), we only observed significant associations between high fiber intake and total and LDL cholesterol levels when the cut point for creation of the highest category was above 21.8 g/day pointing to a possible threshold effect. In particular,
the highest category was significantly different from each of the other categories of lower intake, with no differences in lipoprotein cholesterol level between the lower categories. Fiber intake was not significantly associated with HDL cholesterol or triglycerides.

Using MSMs, the controlled direct effects of fiber intake ($\geq 22$ versus $< 22$ g/day) on lipoprotein cholesterol levels were statistically significant for total and LDL cholesterol and triglycerides (Table 7.2). In other words, high fiber intake had a significant controlled direct effect on lowering lipoprotein cholesterol levels when setting estrogen at levels corresponding to levels of women on oral contraceptives, and to levels of postmenopausal women. High fiber intake did not have a controlled direct effect on HDL cholesterol. The controlled direct effects were small, and consuming fiber above 22 g/day was only associated with a decrease in lipoprotein cholesterol levels of about 1 mg/dL on average. The controlled direct effect of fiber intake on total cholesterol was significant at low levels of estrogen, but only marginally significant at high estrogen levels. Controlled direct effects on LDL were consistent at both levels of estrogen evaluated, whereas effects on triglycerides were only significant at low levels of estrogen.

We did not observe significant reductions in lipoprotein cholesterol levels in response to meeting the DRI of fiber intake based on either a woman’s estimated total energy intake from the 24-hour recall, or her estimated energy requirements according to her age, weight, and height (data not shown). We observed a wide range of energy intakes in this population (515 to 3717 kcal), corresponding to DRI’s of fiber intake between 7 and 52 g/day. Estimated energy requirements based on the Mifflin criteria averaged 1378 kcal, with a range of 1093 to 1819 kcal, corresponding to DRI’s of fiber intake between 15 and 26 g/day).166, 167
E. Discussion

We found that fiber consumption only at or above 22 g/day was associated with lower total and LDL cholesterol and triglyceride levels, independent of estrogen, when estrogen was set at levels corresponding to levels of women on oral contraceptives, and levels of postmenopausal women. While high fiber intakes were associated with reduced levels of total and LDL cholesterol and triglycerides, the controlled direct effect was reduced at higher levels of estrogen. These results suggest that estrogen mediates the association between fiber and lipoproteins by slightly decreasing the effect among premenopausal women. The effects we observed were small and not clinically important, but do provide further insights regarding the possible biological mechanisms of the effects of fiber on lipoprotein metabolism, and suggest that fiber has an independent or direct effect on lowering lipoprotein cholesterol levels.

The observed associations between high fiber intake and reduced total and LDL cholesterol levels are in line with several randomized controlled trials and observational studies that have found fiber intake to be associated with a less atherogenic lipid profile.\textsuperscript{15,96,159,168-170} The effects of fiber intake on lipoprotein cholesterol levels are generally observed at high levels of intake. In randomized controlled trials of fiber intake, supplementation was usually designed so that women would be consuming high amounts of fiber (generally greater than 20 g/day). In observational studies, typically the highest quintile or the 4\textsuperscript{th} and 5\textsuperscript{th} quintiles of fiber intake were associated with lower total cholesterol levels.\textsuperscript{168,170} Cut points for these high levels of intake were similar to the threshold level we observed around 22 g/day. The apparent threshold association could be explained by the fact that fiber intakes in this population were low, and so we could only observe significant effects at high levels.
However, the fact that we observed no reductions in lipoprotein cholesterol levels for women meeting the DRI requirements also supports the presence of a threshold effect of fiber intake. In particular, when we evaluated the effects of meeting the DRI requirements, using approaches based on calculating a woman’s individual DRI from her estimated total energy intake from the 24-hour recall, or from her estimated energy requirements according to her age, weight, and height, we observed no reductions in lipoprotein cholesterol levels. It seems that the effects of fiber intake on lowering lipoprotein cholesterol independent of estrogen levels are in response to high levels of intake, and not in response to meeting the recommendations or consuming a certain portion of fiber from the diet. Even at high levels of fiber intake though the controlled direct effects we observed were small, and not of clinical importance (consuming at or above 22 g/day was only associated with a decrease of about 1 mg/dL). It should be noted that the total effect of high fiber intake in this population was also low (only about 5 mg/dL).

We also observed that the controlled direct effects of fiber on total cholesterol and triglycerides were reduced at high levels of estrogen, while the effects of fiber on LDL cholesterol were similar at both high and low levels of estrogen. The controlled direct effects we estimated, however, assume that we can intervene and set the estrogen levels of all women in the population to a given value. We chose to estimate controlled direct effects at the level of estrogen we would expect to see among postmenopausal women versus the level we would expect to see if we intervened and put all women on oral contraceptives. While we did not directly compare pre- and post-menopausal women in this study, our findings are similar to studies comparing pre- and post-menopausal women that have observed reduced responses to fiber supplementation among premenopausal women.\textsuperscript{18,19} In a study among 8
premenopausal and 11 postmenopausal women, psyllium fiber supplementation (15 g/day) significantly decreased total cholesterol levels from baseline among postmenopausal women, but not in premenopausal women. Similarly, a crossover trial which included 23 premenopausal and 21 postmenopausal women found that total cholesterol decreased modestly during the fiber period (3.8%) among postmenopausal women, but only by 0.6% among premenopausal women, although these differences were not significant. While there were several important differences between this crossover trial and our study (i.e. older and more obese study population, trial evaluating fiber supplementation, and higher fiber intakes on average before supplementation), together these findings suggest that there are differences in the impact of fiber intake on lipoprotein cholesterol, potentially mediated by estrogen levels.

The mechanisms involved in the reduction of serum cholesterol levels related to increased fiber intake support the presence of multiple pathways, with estrogen as a possible mediating factor. Although the mechanisms remain somewhat inconclusive, the major direct mechanism of fiber’s effect on lipoprotein cholesterol levels is hypothesized to work through bile acid metabolism. Fiber decreases absorption of biliary cholesterol and bile acids, increases bile acid synthesis, and enhances fecal excretion of bile acids, thereby leading to a decrease in cholesterol levels. However, as increased fiber intake does not always lead to an increased fecal output of bile acids, it has been suggested that the reduction in cholesterol levels may be working through another mechanism as well. As an alternative, dietary fiber may act on cholesterol through altering serum hormone concentrations or short-chain fatty acids which could in turn affect lipid metabolism. As discussed, high fiber intakes have been associated with lower levels of estradiol, presumably due to a reduction in β-
glucuronidase activity in feces in response to fiber intake, which subsequently leads to a decline in the reabsorption of estrogen in the colon. As increased estradiol tends to have beneficial effects on the lipid profile, the reduction in estradiol in response to fiber intake would presumably lead to an increase in lipoprotein cholesterol levels. This could potentially explain why we observed a decreased controlled direct effect of fiber intake on lipoprotein cholesterol at high levels of estrogen as compared with low levels.

To our knowledge this is the first study to evaluate the independent effects of fiber on lipoprotein cholesterol levels, and to estimate controlled direct effects (i.e. the effect of fiber when estrogen is set at specified levels—here corresponding to levels among women on oral contraceptives versus levels typically seen among postmenopausal women). MSMs were used to estimate the controlled direct effects of fiber intake on lipoprotein cholesterol levels because they offer several important advantages over standard approaches. Perhaps the most important advantages of the MSM approach are that MSMs can control for time-varying confounders affected by prior exposure, as well as accommodate interactions between fiber and estrogen. First, MSMs adequately adjust for time-modified confounding caused by changing reproductive hormone levels during the menstrual cycle. Second, standard approaches for effect decomposition are not valid in the presence of interactions, and would require that there be no women in the population where fiber had a causal effect on both estrogen and lipoprotein levels. Based on our knowledge of the effects of fiber on both estrogen and lipoproteins, absence of such an interaction seems biologically implausible. This is an especially important consideration, given that the use of standard methods would never be appropriate in this situation given the interaction between fiber and estrogen. When such an interaction is present, the total effect cannot be partitioned into direct and indirect
effects using standard approaches and requires that additional assumptions be met when using MSMs. Had we analyzed the association using the standard approach (comparing the effect estimate adjusted for potential confounders to an estimate adjusted for the same confounders plus the hypothesized mediating variable) we would not have observed any mediation by estrogen, and would have concluded only that fiber had a direct effect on lipoprotein cholesterol levels. The BioCycle study offers a unique opportunity to apply these methods and to adjust for the effects of potential confounding by reproductive hormones due to the prospective, longitudinal nature of the study, with multiple measures of dietary intake, hormones, and lipoprotein cholesterol levels.

Our analysis is restricted by the assumptions of MSMs which limit our interpretation of the effects. In estimating controlled direct effects we have assumed that we can intervene and set estrogen levels to be a certain value. Although this is a possible intervention through the use of oral contraceptives, it is not necessarily a practical one. In addition, MSMs assume positivity, in that the probabilities in the denominators of the weights cannot be zero, as well as no unmeasured confounding (i.e. exchangeability). Specifically we assume no unmeasured confounding of the treatment-outcome relationship, and the mediator-outcome relationship, assumptions that are hard to verify in practice, but assumed in standard analysis methods as well. Despite the fact that we had standardized assessments of a wide variety of participant and dietary characteristics which increased our ability to adjust for potential confounders, it is still possible that there is unmeasured confounding. Our study sample population was restricted to healthy normally menstruating women in order to exclude potential confounders by design, but such restrictions could also limit the generalizability of our findings. Although the average fiber
intake among women in this study was 13.6 g/day, which is comparable to the average fiber intake in the U.S. (13.8 g fiber/d for reproductive-aged women),\textsuperscript{105,159} these intakes were substantially lower than the recommendations, and we were limited by observing only a small number of women consuming at or above 22 g/day. In addition, follow-up was limited for two cycles, and fiber intake was based on estimates obtained from 24-hour recalls.

In conclusion, we observed reductions in total and LDL cholesterol and triglycerides, not mediated through estrogen, only among women consuming at or above 22 g/day, when controlling estrogen at specified levels in the population corresponding to levels of women on oral contraceptives and among postmenopausal women. The lipid-lowering effects of fiber intake were only observed at high levels of intake, indicating the importance of consuming a high amount of fiber. The controlled direct effects of fiber intake were reduced at higher estrogen levels, suggesting that estrogen mediates the association between fiber and lipoproteins by slightly diminishing the effect. Despite the reduced controlled direct effect of fiber on lipoproteins due to estrogen, high fiber consumption was independently associated with an improved lipid profile. Similar approaches could be applied to determine the direct effects of other dietary factors that are also associated with estrogen levels (i.e. soy intake, fat intake). While the controlled direct effects we observed were not of clinical significance, these findings add to the current understanding regarding the possible biological mechanisms of fiber’s effect on lipoproteins. In addition, these results suggest that fiber has an independent effect on lowering lipoprotein levels that is partially mediated by estrogen, and support the hypothesis of a direct effect of fiber on cholesterol that might work through bile acid metabolism. More research is needed to elucidate these mechanisms among premenopausal women.
F. Tables and Figures

Table 7.1. Characteristics of participants in the BioCycle study according to dietary fiber intake at or above 22 g/day (n=509 cycles).

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Fiber Intake</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Cohort</td>
<td>&lt; 22 g/day</td>
</tr>
<tr>
<td>n (number of cycles)</td>
<td>509</td>
<td>468</td>
</tr>
<tr>
<td>Age, yrs: mean (SD)</td>
<td>27.4 (8.2)</td>
<td>27.4 (8.3)</td>
</tr>
<tr>
<td>BMI, kg/m²: mean (SD)</td>
<td>24.1 (3.9)</td>
<td>24.2 (3.8)</td>
</tr>
<tr>
<td>Race: n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>302 (59.3)</td>
<td>269 (57.5)</td>
</tr>
<tr>
<td>Black</td>
<td>101 (19.8)</td>
<td>98 (20.9)</td>
</tr>
<tr>
<td>Other</td>
<td>106 (20.8)</td>
<td>101 (21.6)</td>
</tr>
<tr>
<td>≤ High school education: n (%)</td>
<td>65 (12.8)</td>
<td>63 (13.5)</td>
</tr>
<tr>
<td>Married: n (%)</td>
<td>131 (25.7)</td>
<td>118 (25.2)</td>
</tr>
<tr>
<td>Nulliparous: n (%)</td>
<td>367 (73.6)</td>
<td>336 (73.4)</td>
</tr>
<tr>
<td>Current smoker: n (%)</td>
<td>20 (3.9)</td>
<td>20 (4.3)</td>
</tr>
<tr>
<td>Physical Activity: n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>48 (9.5)</td>
<td>44 (9.4)</td>
</tr>
<tr>
<td>Moderate</td>
<td>182 (36.0)</td>
<td>163 (34.8)</td>
</tr>
<tr>
<td>High</td>
<td>275 (54.5)</td>
<td>261 (55.8)</td>
</tr>
<tr>
<td>Past OC Use: n (%)</td>
<td>275 (54.7)</td>
<td>248 (53.7)</td>
</tr>
</tbody>
</table>

Reproductive hormones, mean (SD)

<table>
<thead>
<tr>
<th>Reproductive hormones, mean (SD)</th>
<th>Fiber Intake</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average estradiol, pg/mL</td>
<td>112.3 (91.0)</td>
<td>114.4 (92.2)</td>
</tr>
<tr>
<td>Average luteal progesterone, ng/mL</td>
<td>7.3 (5.5)</td>
<td>7.4 (5.5)</td>
</tr>
<tr>
<td>Average LH, ng/mL</td>
<td>9.6 (11.6)</td>
<td>9.6 (11.7)</td>
</tr>
<tr>
<td>Average FSH, mIU/mL</td>
<td>6.4 (4.2)</td>
<td>6.4 (4.2)</td>
</tr>
</tbody>
</table>

Baseline lipoprotein cholesterol levels, mean (SD)

<table>
<thead>
<tr>
<th>Baseline lipoprotein cholesterol levels, mean (SD)</th>
<th>Fiber Intake</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>163.4 (29.0)</td>
<td>164.7 (29.1)</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>50.1 (11.5)</td>
<td>50.1 (11.6)</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>101.5 (25.7)</td>
<td>102.6 (25.8)</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>59.2 (27.9)</td>
<td>60.1 (28.4)</td>
</tr>
</tbody>
</table>

Dietary intake, mean (SD)

<table>
<thead>
<tr>
<th>Dietary intake, mean (SD)</th>
<th>Fiber Intake</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total energy, kcal</td>
<td>1608.1 (405.0)</td>
<td>1578.7 (390.0)</td>
</tr>
<tr>
<td>Total fiber, g/day</td>
<td>13.6 (6.0)</td>
<td>12.3 (4.0)</td>
</tr>
<tr>
<td>Insoluble fiber, g/day</td>
<td>9.6 (4.7)</td>
<td>8.6 (3.1)</td>
</tr>
<tr>
<td>Soluble fiber, g/day</td>
<td>3.8 (1.4)</td>
<td>3.5 (1.1)</td>
</tr>
</tbody>
</table>

BMI, body mass index; FSH, follicle stimulating hormone; HDL, high density lipoprotein; LDL, low density lipoprotein; LH, luteinizing hormone; OC, oral contraceptives; SD, standard deviation.
Table 7.2. Results of marginal structural models for estimating the controlled direct effect of fiber intake (≥ 22 g/day versus < 22) on log lipoprotein levels using weighted linear mixed effects models among women participating in the BioCycle study.

<table>
<thead>
<tr>
<th></th>
<th>Estrogen levels*</th>
<th>Estimate†</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Cholesterol (mg/dL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postmenopausal 11 pg/mL</td>
<td>-0.060</td>
<td>(-0.106, -0.018)</td>
<td></td>
</tr>
<tr>
<td>Premenopausal 45 pg/mL</td>
<td>-0.044</td>
<td>(-0.087, -0.012)</td>
<td></td>
</tr>
<tr>
<td><strong>HDL Cholesterol (mg/dL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postmenopausal 11 pg/mL</td>
<td>0.000</td>
<td>(-0.047, 0.049)</td>
<td></td>
</tr>
<tr>
<td>Premenopausal 45 pg/mL</td>
<td>0.015</td>
<td>(-0.024, 0.052)</td>
<td></td>
</tr>
<tr>
<td><strong>LDL Cholesterol (mg/dL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postmenopausal 11 pg/mL</td>
<td>-0.068</td>
<td>(-0.130, -0.011)</td>
<td></td>
</tr>
<tr>
<td>Premenopausal 45 pg/mL</td>
<td>-0.068</td>
<td>(-0.121, -0.028)</td>
<td></td>
</tr>
<tr>
<td><strong>Triglycerides (mg/dL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postmenopausal 11 pg/mL</td>
<td>-0.161</td>
<td>(-0.282, -0.031)</td>
<td></td>
</tr>
<tr>
<td>Premenopausal 45 pg/mL</td>
<td>-0.069</td>
<td>(-0.158, 0.022)</td>
<td></td>
</tr>
</tbody>
</table>

BMI, body mass index; CI, confidence interval; FSH, follicle stimulating hormone; LH, luteinizing hormone.
*Controlled direct effects are calculated at a given level of estrogen; here we present controlled direct effects at mean postmenopausal and mean premenopausal levels of estrogen.
†Estimates are adjusted for age, BMI, physical activity, total energy intake, vitamin E intake, LH, FSH through the use of inverse probability of exposure weights.
Figure 7.1. Directed acyclic graph of the controlled direct effect of fiber intake on lipoprotein cholesterol which is estimated by setting estrogen levels to a fixed value and thus blocking all of the effects on estrogen.$^{161}$
Figure 7.2. Predicted mean levels of estradiol, progesterone, LH, and FSH across the menstrual cycle according to fiber intake at or above 22 g/day among women in the BioCycle study. Predicted means are based on linear mixed effects models with random intercepts adjusted for age, BMI, and total energy.
Figure 7.3. Predicted means levels of total, LDL, and HDL cholesterol, and triglycerides across the menstrual cycle according to fiber intake at or above 22 g/day among women in the BioCycle study. Predicted means are based on linear mixed effects models with random intercepts adjusted for age, BMI, and total energy.
VIII. CONCLUSIONS

The purpose of this dissertation was to understand how the menstrual cycle interacts with other metabolic processes, specifically lipid metabolism. In particular, we were interested in the effects of estrogen on lipid metabolism, the effects of baseline lipoprotein cholesterol levels on the risk of incident anovulation, and the direct effects of dietary fiber intake on lipoprotein cholesterol not mediated by estrogen.

We observed that lipoprotein cholesterol levels varied across the menstrual cycle and were associated with endogenous estrogen levels, confirming the hypothesized beneficial effects of endogenous estrogen on lipoprotein cholesterol levels. Our results also suggest that the effects of estrogen on lowering total and LDL cholesterol are not acute.

We also found that the anovulatory women in our study displayed a more atherogenic lipid profile and endocrine characteristics indicative of hyperandrogenism. While lipoprotein cholesterol levels were only weakly associated with the risk of anovulation, we identified several predictors of incident anovulation (the LH:FSH ratio, SHBG, insulin, and acne). These markers, along with sporadic anovulation, may be indicative of a mild undiagnosed PCOS phenotype even among women reporting ‘regular’ menstrual cycles.

Dietary fiber intake was shown to have an independent effect on lipoprotein cholesterol levels, not mediated by estrogen. Specifically, we observed a direct effect of fiber intake on reducing total and LDL cholesterol and triglycerides, among women consuming at or above 22 g/day, when setting estrogen at levels corresponding to levels of
women on oral contraceptives and among postmenopausal women. The controlled direct effects of fiber intake were reduced at higher estrogen levels, suggesting that estrogen mediates the association between fiber and lipoproteins by slightly diminishing the effect. Despite the reduced controlled direct effect of fiber on lipoproteins due to estrogen, high fiber consumption was independently associated with an improved lipid profile at all levels of estrogen.

This study is the first to evaluate the association between endogenous estrogen and lipoproteins using multiple longitudinal serum measures of estrogen and lipoproteins, while comprehensively considering potential impacts from other reproductive hormones. More importantly, this research helps to fill an etiological gap in understanding the relationship between lipoprotein cholesterol changes induced by hormonal variations during the menstrual cycle. Cyclic variations in lipoprotein cholesterol levels observed in the present study have potential clinical implications regarding the appropriate timing of lipoprotein cholesterol measurement during the menstrual cycle and may need to be accounted for in the design and interpretation of studies in women of reproductive age. In addition, this is the first study to our knowledge to prospectively identify endocrine markers of sporadic anovulation in a group of regularly menstruating women. Further studies are needed to confirm these results and help elucidate the biological mechanisms that lead to sporadic anovulation in healthy women. Understanding the factors associated with anovulation is important as anovulation might be associated with infertility. Lastly, while the controlled direct effects of fiber intake on lipoprotein cholesterols were not of clinical significance, these findings add to the current understanding regarding the possible biological mechanisms of fiber’s effect on
lipoproteins, and suggest that fiber has an independent effect on lowering lipoprotein levels. More research is needed in this area to better understand the biological mechanisms.

In conclusion, these findings provide insight into the complex associations between hormones and lipoproteins during the menstrual cycle, and how dietary factors affect these relationships. Menstrual cycle phase should be considered in the design and interpretation of studies in women of reproductive age due to estrogen’s influence on many biological processes.

A. Strengths

The BioCycle study offered a rich and unique data source for better understanding the biological processes of the menstrual cycle, and for improved characterization of hormonal profiles, menstrual cycle characteristics, lipid profiles, and dietary intake across the cycle. In particular, the BioCycle study consisted of multiple serum measurements of both hormone and lipoprotein cholesterol levels across two menstrual cycles. Because we had multiple measures of both hormones and lipoproteins we were able to more precisely model the association between estradiol and lipoprotein cholesterol levels. Due to the prospective nature of this study, we were also able to preserve temporality which had a number of benefits. In particular, we were able to evaluate both acute and persistent effects of estradiol on lipoproteins, which yielded further insight into the effects of estrogen on lipoprotein metabolism.

Use of fertility monitors to time clinic visits was a distinguishing feature of this study, and a significant improvement over previous studies. This new technology was used to more accurately define menstrual cycle phase, leading to more accurate characterizations of
hormonal and lipid fluctuations across the cycle. Participants were intensively monitored throughout two cycles, and standardized assessment of a wide variety of participant and dietary characteristics increased our ability to adjust for potential confounding factors. In the analysis of the association between lipoprotein cholesterol and anovulation, we were able to evaluate the possibility of confounding by underlying endocrine disturbances because we had measures of several markers of endocrine function. The prospective design and exclusion criteria at baseline strengthened our ability to draw inference among a group of healthy, regularly menstruating women. Not only is this especially important in understanding the underlying biological mechanisms between estradiol and lipoprotein metabolism, but we were also able to evaluate the association between lipoprotein cholesterol levels and anovulation among a group of normally menstruating women with no diagnosed cases of PCOS.

The analysis is set apart by the use of weighted generalized linear mixed effects models, which offer several advantages over traditional methods. First, we were able to account for the correlation between and within women throughout the cycle. Second, we were able to adequately adjust for time-modified confounding due to changing hormone levels across the cycle. A special application of marginal structural models (MSMs) was used to estimate the controlled direct effects of fiber intake on lipoprotein cholesterol levels because they offer several important advantages over standard approaches. Perhaps the most important advantages of the MSM approach are that MSMs can control for time-varying confounders affected by prior exposure, as well as accommodate interactions between fiber and estrogen.
B. Limitations

There are several limitations worth noting. First, our study sample population was restricted to healthy normally menstruating women in order to exclude potential confounders by design, but such restrictions could also limit the generalizability of our findings. In addition, the women in this study were fairly young on average, had a low BMI, were very physically active, mostly non-smokers, and not on oral contraceptives, which could further limit generalizability. Despite the fact that we had standardized assessments of a wide variety of participant and dietary characteristics which increased our ability to adjust for potential confounders, it is still possible that there is unmeasured confounding. In particular, we were not able to adjust for measures of plasma volume which could account for some of the observed changes in lipoprotein cholesterol levels across the cycle. We were also unable to directly measure androgen levels, and had to rely on several non-specific markers in our assessment of hyperandrogenism. Ultrasound examinations for presence of polycystic ovaries were also unavailable. Residual confounding is also a possibility, since it can be very difficult to capture effects of dietary intake and exercise for example, and some misclassification of these covariates is likely.

Using the fertility monitors to time visits was a significant improvement over previous studies, however there is still potential for some misclassification of cycle phase as not all LH peaks were captured on the monitor. We were also limited by a small number of anovulatory cycles and an imperfect measurement of ovulation. The small number of anovulatory cycles was partly a consequence of our employment of a conservative definition for anovulation, and our strict study inclusion criteria, and limited the power we had to detect effects. While we did have multiple serum hormone measurements to aid in classifying
ovulation, along with the use of fertility monitors measuring LH daily in urine, daily measures of progesterone and transvaginal ultrasounds (the gold standard) were not available, and thus misclassification of ovulation is possible. While there may be some selection bias due to loss to follow-up over the cycle as not all women completed 8 visits per cycle, marginal structural models account for this loss to follow-up through inverse probability of exposure weighting.

Finally, while the use of marginal structural models was a significant improvement over traditional covariate assessment, they are based on certain strong assumptions such as no unmeasured confounding, positivity, correct model specification, and consistency. These assumptions are generally hard to meet and should be taken into consideration when interpreting results. In the case of effect decomposition, the assumptions of MSMs also limit our interpretation of the effects. In estimating controlled direct effects we have assumed that we can intervene and set estrogen levels to be a certain value. Although this is a possible intervention through the use of oral contraceptives, it is not necessarily a practical one. Despite these limitations, the BioCycle study offers several improvements over previous studies and significantly adds to the research in this area.
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