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Injuries to the anterior cruciate ligament (ACL) are more likely to occur in females and sex hormones have been implicated in this sex-based injury disparity. Research suggests naturally produced sex hormones influence anterior knee laxity (AKL), a risk factor for ACL tears. A significant proportion of athletic females take hormonal oral contraceptive pills (OCPs), which reduce circulating levels of endogenously produced sex hormones. These pills contain a class of progesterone-like hormones called progestins, which exert both progestational and androgenic effects on soft tissue. The pharmacodynamic effect of these compounds varies across OCP brands as different types and doses of progestins are used in modern OCPs, and therefore the effect of OCP use on AKL is unclear. The purpose of this study three-fold: 1) determine an appropriate biochemical assay to measure progestins in the serum of active females on oral contraceptives. 2) compare the pharmacodynamic activity that was "prescribed" on OCP pill packages to the measured activity measured by the hormone assay, and 3) to examine the effects of progestins with different levels of androgenicity and progestational activity or potency on AKL Forty-five recreationally active females using OCPs were projected to be recruited and stratified and placed into three groups based on the pharmacodynamic activity of the OCPs used by the subjects. However, due to restraints imposed by COVID, a total of sixteen females were recruited, and group stratification was not possible. Blood samples were collected from participants during their third and fourth pill weeks. Concentrations of two common progestins, norethindrone and levonorgestrel were measured using a Waters Acquity Ultra Performance Liquid Chromatography system coupled to an LTQ Orbitrap XL Mass Spectrometer, and AKL was measured using the KT2000 knee arthrometer. Validation analyses commonly used in analytical chemistry were used to assess the performance of a UPLC-MS method created for this thesis. Simple bivariate correlational analyses between pill/serum potency and androgenicity and AKL were used to examine the associations between potency and androgenicity at the third pill week and the change in AKL from pill week three to pill week four (ΔAKL). Associations between pill/serum potency and androgenicity with AKL during the third pill week were largely non-significant. However, there was a significant association between peak serum androgenicity

and ΔAKL from week 3 to week 4. Additionally, in a sub-set of participants whose metabolized progestin was levonorgestrel (LNG), there was a significant positive association between peak serum potency/androgenicity and ΔAKL on the left leg. While there were few significant findings due to low statistical power, the results of this thesis lay a valuable and informative foundation from which to further examine the effects of OCPs on AKL in active women.

THE BIOLOGICAL EFFECT OF PROGESTINS ON ANTERIOR KNEE LAXITY IN FEMALES ON ORAL CONTRACEPTIVE PILLS

by

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CHAPTER I: INTRODUCTION

Injuries to the anterior cruciate ligament (ACL) commonly occur in the physically active, and females are 2-4 times more likely than males to experience non-contact ACL tears. Previous research suggests this gender-based disparity in ACL injury rates may be explained by anatomical, neuromuscular, environmental, and hormonal differences. Hormones have often been a target of research because the hormonal exposure in females is quite different than that of males. Specifically, females are exposed to greater levels of estradiol, relaxin, and progesterone along with lower levels of testosterone than males (Hackney 2016). The levels of these hormones change throughout the menstrual cycle. Consistent with these changes, the risk of ACL injury is also reported to change across the menstrual cycle, further implicating the possibility that hormones may play a role in ACL injury risk (Balachandar 2017; Hewett 2007). The mechanisms by which sex hormones are thought to influence ACL injury risk is through their influence on collagen structure and metabolism, which, in turn, dictate the structure and mechanical properties of the ACL. Female ACLs have been shown to have different structural organization than males. For example, the female ACL is smaller and has less collagen density, which may result in lower mechanical properties than the male ACL (Chandrashekar 2006; Hashemi 2008; Hashemi 2011) Sex hormones may affect both size and density by affecting collagen gene expression, collagen synthesis, collagen degradation, and crosslinking. Previous research has shown increased concentrations of the hormones estradiol and relaxin may result in decreased collagen gene expression (Yu 2001), collagen synthesis (Lee 2004;. Liu 1997; Liu 2005) crosslinking (Lee 2015) and increased rates of collagen degradation (Konopka 2016) all of which may result in lower ACL mechanical properties. On the other hand, limited evidence suggests that progesterone and testosterone may attenuate the effects of estradiol and relaxin on collagen, which may result in increased ACL mechanical properties (Dehghan 2014; Keller 2000; Romani 2016; Shultz 2004; Yu 2001)

Researchers have considered the possibility that hormonal oral contraceptives (OCPs) might provide a protective effect on ACL mechanical properties by lowering and stabilizing concentrations of endogenous hormones (Samuelson 2017). Though a large proportion of female

athletes use OCPs to regulate their menstrual cycles, research examining the effect of OCP use on ACL injury risk by comparing OCP users vs. non-users is equivocal (Beals 2002; Herzberg 2017; Miller 1999; Torstveit 2005). One challenge to such research is the fact that OCP brands vary considerably in the type and concentration of exogenous hormones delivered to the users yet have been treated as similar in research studies. Specifically, progestins, a class of hormones contained in modern OCPs vary considerably in their levels of androgenic and progestational activity, which may influence the extent to which these compounds counteract estrogenic effects on soft tissue (Greer 2005; Sitruk-Ware 2013). Therefore, stratification of OCP users based on the pharmacodynamic activity of their OCPs is needed to determine the extent to exogenous hormones may modulate ACL structure and ACL injury risk.

One mechanistic pathway through which we can understand the effects of OCPs on ACL injury risk is through the measurement of anterior knee laxity (AKL). AKL is a clinical measure of the mechanical properties of the ACL, and thus, an indirect measure of the structural quality of the ligament. Greater AKL has been associated with a structurally weaker ligament (Comerford 2005, Comerford 2006, Wang 2016, IP) as well as greater risk of ACL injury (Vacek 2016, Uhorchak 2003, Myer 2008). Females generally have greater AKL than males and AKL has been shown to change across the menstrual cycle (Hewett 2007, Herzberg 2017). Therefore, an examination of AKL may be an appropriate model to begin to understand the effect of different progestins on ACL mechanical properties.

Most of the research examining the relationship between sex hormones and AKL has been performed on normally menstruating women. Some evidence suggests women on OCPs have less AKL than eumenorrheic women (Martineau 2004, Lee 2014, Lee 2015) while others have not demonstrated a difference (Pokorny 2000). In order to clarify the effects of OCPs on AKL, there is a need to stratify females on OCPs based on the pharmacodynamic activity of those OCPs. Moreover, because the ACL literature does not yet contain a method for measuring the exogenous hormones found in OCPs in active women that allows for the examination of the pharmacodynamic activity of these hormones, there is a need to create a hormone assay with such a purpose. Therefore, the purpose of this study is three-fold: 1) determine an appropriate biochemical assay to measure progestins in the serum of active females on oral contraceptives. 2) compare the pharmacodynamic activity that was “prescribed” on OCP pill packages to the measured activity measured by the hormone assay, and 3) to examine the effects of progestins

with different levels of *androgenicity* and progestational activity or *potency* on AKL. It was hypothesized that: 1) ultraperformance liquid chromatography-mass spectrometry would be an appropriate analytical technique for the measurement of two common progestins in active women on birth control; 2) there would be a strong association between prescribed and serum measured androgenicity and potency and, 3) women on OCPs containing progestins with higher levels of androgenicity and potency will have lower AKL.

CHAPTER II: REVIEW OF THE LITERATURE

Section I: Sex Hormones and ACL Injury Risk

Injuries to the anterior cruciate ligament (ACL) are common among the athletic population in the United States and a large portion of ACL tears occur through non-contact mechanisms (Hewett 1999). Young athletic women[1] have a greater risk of experiencing a non-contact ACL tear than their male counterparts in gender compatible sports (Agel 2016, Beck 2017). Furthermore, women have been shown to be at a greater risk of experiencing ACL injuries that require surgery (Powell 2000). Risk factors for ACL injury in female athletes are typically categorized into broad categories of neuromuscular, environmental, anatomical, and hormonal (Hewett 2006). Sex hormones are one area where females and males differ greatly, as concentrations of sex hormones in males are quite different from concentrations in females (Styne 2016). Also, a female's hormones fluctuate throughout the course of a menstrual cycle while a male's hormones remain stable from day to day (Hackney 2017). These differences are important as sex hormones and are thought to have a profound effect on soft tissue properties. (Slauterbeck 1999, Dragoo 2009, Lee 2015, Romani 2016) The first portion of this literature review will present what is known about the effects sex hormones on ACL injury risk

The Effects of Sex Hormones on ACL Injury Risk

Hormones have often been implicated in the sex –based disparity in ACL injury rates because the hormonal environment of women is quite different than that of (Styne 2016). Adult women possess greater levels of estradiol, relaxin, and progesterone along with lower levels of testosterone than adult men (Styne 2016). Additionally, levels of all four hormones change throughout the course of a woman's menstrual cycle (Styne 2016, Hackney 2017). The menstrual cycle is divided into two phases, namely, the follicular and luteal phases, with an ovulatory event separating these phases (Hackney 2017). A number of systematic reviews have examined injury risk and the consensus is that a female's risk of ACL injury is not uniform across the menstrual

[1] In this thesis, the terms "young athletic women" or "athletic woman" is used to refer to athletic females between the ages of 15 and 25 who participate in either high school, collegiate, or professional sports.

cycle with risk generally being greater during the follicular phase of the menstrual cycle as compared to the luteal phase (Beynnon 2006, Hewett 2007, Balachandar 2017, Herzberg 2017). Therefore, it is logical to reason hormones may exert an influence on ACL injury risk.

The length of the menstrual cycle may vary from woman to woman, and even from cycle to cycle within a woman, which has made examining the effect of menstrual cycle phase on ACL injury risk difficult (Vescovi 2011). The sex hormones that are thought to have an influence on ACL injury risk include estradiol, progesterone, testosterone, and relaxin, all of which are secreted by the ovarian follicle and vary in magnitude during the menstrual cycle (Hackney 2017). Estradiol concentrations increase during the mid-follicular phase and reach their peak just prior to ovulation. At ovulation, the follicle becomes the corpus luteum and begins to secrete both estradiol and progesterone. During the luteal phase, progesterone levels reach their peak levels, and there is a second rise in estradiol concentrations. Levels of both hormones fall off near day 28 of the menstrual cycle (Hackney 2017). Testosterone, an androgenic hormone, reaches its peak near ovulation (Bui 2013) and relaxin rises and peaks briefly during the mid-luteal phase, shortly after the rise in progesterone (Bryant-Greenwood 1993, Stewart 1990, Casey 2018).

Two studies have prospectively examined actual hormone profiles in ACL injured populations compared to non-injured controls. In one case-control study, serum levels of progesterone, estradiol, and testosterone were compared in normally menstruating (eumenorrheic) women who had experienced an ACL rupture to eumenorrheic women who had examined an injury unrelated to the ACL (Stijak 2015). In this study, hormones were sampled at least three weeks after an ACL injury had occurred in the injured group, then in both groups, hormone samples were taken at the beginning of the follicular phase, the end of the follicular phase, 2 days prior to ovulation, during ovulation, and during the mid-luteal phases of the participants' cycles. Cycle phases were determined by calculating days from ovulation. Women in the ACL rupture group had lower levels of testosterone and higher levels of estradiol and progesterone than females who had not ruptured their ACL It should be noted that samples taken during ovulation and the luteal phase were analyzed together, which may affect the study's design as hormone levels during these periods may differ greatly (Vescovi 2011, Styne 2016). Nevertheless, this study suggests women with a certain hormonal profile, (i.e. low testosterone and high estradiol and progesterone), may be at increased risk of injury.

In another study, serum relaxin concentrations were collected in a group of collegiate women at the beginning of their freshman year. Hormones were sampled during the mid-luteal phase (6-8 days after LH surge that corresponds to ovulation). ACL injury was tracked prospectively over a four-year time period and relaxin levels greater than 6.0 pg/mL were associated with an increased risk of ACL injury (Dragoo 2011). However, this study is limited by the fact that relaxin concentrations were only sampled once in all participants, which may lead to inaccurate findings as relaxin levels peak for only a short time and peak levels may vary from cycle to cycle, as is true in the case of other sex hormones (Lenton 1983). Additionally, other hormones, (i.e. estradiol, testosterone, and progesterone) and other risk factors (i.e. environmental, neuromuscular, and anatomical) for ACL injury were not examined or controlled for in this study. Nevertheless, this study once again suggests a woman's hormonal profile over an extended period of time may influence her risk of experiencing an ACL tear.

This portion of the literature has presented what is known about the influence of sex hormones over ACL injury risk in young women. Recent systematic reviews suggest a woman's risk of injury is greatest during the follicular phase of the menstrual cycle, when estradiol levels are their highest (Herzberg 2017, Balachandar 2017, Hewett 2007). One study (Stijak 2015) suggests women with a certain hormone profile (i.e. low testosterone levels along with higher estradiol and progesterone levels) may have a higher risk of ACL injury, and another study (Dragoo 2011) suggests exposure to high levels of relaxin may increase a young woman's risk of injury. Thus, an understanding of the potential mechanisms behind these associations is warranted to gain a better understanding of whether hormone profiles should be a target to mitigate injury in young athletic women.

Section II: Hormonal Influences on Ligament Structure and Material Properties

ACL Composition and Metabolism

The primary structural component of the ACL is type I collagen, a fibrillar protein that provides most of its resistance to tensile forces (Sheffler 2012). The basic unit of a collagen is the tropocollagen triple helix, which is organized into collagen fibrils. Collagen fibers in the ACL are produced by cells called fibroblasts (Fratzl 2008). At least two factors may affect collagen structure and thus mechanical properties of the ACL; the balance between collagen synthesis and degradation, which may influence collagen fiber density; and, the degree of

crosslinking that occurs between tropocollagen helices, which may influence stiffness of a collagen fiber (Lee 2015). These two factors may affect ligament stiffness and load-to failure.

Sex Differences in Mechanical Properties of the ACL

Cadaveric studies have shown women have lower structural and material properties than men (Chandrashekar 2006, Hashemi 2008, Hashemi 2011). For example, a woman's ACL is smaller and has lower mechanical properties than a man's ACL, even when adjustments are made for body anthropometrics (Chandrashekar 2006). A woman's ACL also have lower ultrastructural properties (i.e., decreased fibril organization) than men, and these, may explain their lower mechanical properties (Hashemi 2008, Hashemi 2011, and Chandrashekar 2006). It should be noted that findings from cadaveric studies of the ACL are limited by the ages of participants. Age is one factor that can affect the mechanical properties of the ACL, and in all previously mentioned studies, participants from which ACLs were obtained were substantially older than the athletic population (Hashemi 2011). However, ages of male and female cadavers examined in these studies was comparable and therefore, it is reasonable to conclude the mechanical properties of women and men may differ at younger ages. Because lower mechanical properties of the female ACL may increase injury risk, research in the basic sciences have sought to examine the effect of sex hormones on ACL metabolism, structure, and mechanical properties.

Hormonal Influences on Fibroblast Proliferation and Collagen Gene Expression

An examination of hormonal influences on ACL structure should begin from the level of collagen gene expression. Hormones' effect on collagen metabolism are mediated by their receptors, and hormone receptors for estradiol, progesterone, testosterone, and relaxin have been identified on the human ACL (Faryniarz 2006, Liu 1996, Lovering 2005, Dragoo 2003). A few studies have examined the influence of hormones on collagen production, which is associated with the proliferation of ACL fibroblasts and collagen gene expression (Yu 2001, Lee 2004, Liu 2005, Liu 1997, Konopka 2016). Because these two factors are responsible for collagen production, it is reasonable to assume hormonal effects on fibroblast proliferation and gene expression may affect collagen structure and density.

Two studies have examined the effects of sex hormones on fibroblast proliferation. (Liu 1997, Yu 2001) One study examined rabbit ACL fibroblasts in response to physiological doses of estradiol (Liu 1997) and another examined the effect of estradiol and progesterone on fibroblast proliferation from cadaver tissue taken from two women (Yu 2001). In both studies,

increased estradiol exposure decreased fibroblast proliferation in a dose dependent manner. Interestingly, in one study, progesterone attenuated the estradiol- based decreases in fibroblast proliferation after prolonged exposure to both hormones, which suggests progesterone may oppose estradiol's effects on collagen metabolism to some degree (Yu 2001).

Effect of Sex Hormones on Collagen Gene Expression and Synthesis

Animal Studies

Three experimental animal studies have examined the effects of estradiol on collagen gene expression or collagen synthesis (Liu 1997, Lee 2004, Liu 2005). Two studies found a decrease in collagen production when ACL fibroblasts were exposed to estradiol in addition to mechanical stress (Lee 2004 and Liu 2005). The first study measured collagen gene expression when exposed to estradiol concentrations that were twice that of a normally menstruating female at any state of the menstrual cycle (10^{-9} M or about 272.38 pg/mL) (Liu 2005) while the other examined collagen gene expression over a range of estradiol concentrations (between 2.7 pg/mL and 2720 pg/mL) (Lee 2004). In both studies, increased exposure to estradiol in combination with mechanical stress decreased expression of type I collagen. Finally, one study examined the effect of administration of estradiol on collagen synthesis, which was done by measuring hydroxyproline content (Liu 1997). Rabbit fibroblasts were treated with varying levels of estradiol (0.025 to 25 ng/mL). Cells treated with 0.025 ng/mL, a physiologic dose of estradiol, had decreased collagen synthesis when compared to untreated controls. Collagen synthesis also decreased in cells treated with supraphysiologic doses (0.25- 25 ng/mL) of estradiol. However, the authors did not mention whether this decrease in collagen synthesis was significant. These studies may be limited by the fact that cells were treated with estradiol in the absence of other sex hormones, which does not allow for conclusions to be drawn regarding ACL fibroblasts under true physiologic conditions. Additionally, the fact that animal models were used limits their applicability to human. Nevertheless, these studies are consistent in directionality, and show that increased exposure of estradiol alone is associated with decreased type -1 collagen gene expression.

Human Studies

Two experimental human studies have examined the effects of sex hormones on collagen gene expression (Konopka 2016 and Yu 2001). In one study that examined the effect of relaxin and estradiol on collagen gene expression (Konopka 2016), fibroblasts were obtained from

females ACLs and these cells were divided into groups 6 groups: a control group, a relaxin-only group treated with 100 ng/mL relaxin, a group treated with estradiol and three groups of cells treated with a constant concentration of estradiol (1 μM estradiol, equivalent to 272 ng/mL estradiol) and varying levels of relaxin (1 ng/mL to 100 ng/mL). Type I collagen expression was significantly reduced in cells treated with 100 ng/mL of relaxin alone, and this effect was amplified in cells treated with both estradiol and 100 ng/mL relaxin. Another study (Yu 2001) examined the combined effects of progesterone and estradiol on collagen expression in ACL fibroblasts obtained from two women, aged 19 and 32. Cells were divided into three groups and were exposed to physiologic and supraphysiologic levels of estradiol (2.5 pg/mL, 25 pg/mL and 2500 pg/mL) and progesterone (1 ng/mL, 10 ng/mL 100 ng/mL). All cells were then analyzed for pro-collagen type I content after 1, 3, 5 and 7 days. Cells exposed to increased levels of estradiol for 1-, 3-, and 5-days experienced decreases in pro-collagen type-1 synthesis, but such decreases were attenuated by increasing concentrations of progesterone. When estradiol levels were controlled for in the analysis, it appeared increasing progesterone levels increased procollagen type I synthesis.

These studies are both limited by the fact that supraphysiologic levels of both hormones were used. It is also important to note that the biomarker of interest was procollagen type I, not a mature collagen fibril. Nevertheless, these studies suggest the presence of multiple hormones may affect collagen metabolism within the ACL. Specifically, estradiol and relaxin may decrease collagen synthesis, resulting in decreased collagen density, while progesterone may attenuate estradiol-based decreases in collagen content, and even increase collagen production, thus potentially collagen density and mechanical properties of the ACL.

Effect of Sex Hormones on Matrix Metalloproteinases

Collagen degradation is regulated by the expression of matrix metalloproteinases (MMPs), a class of proteolytic enzymes. Some MMPs degrade type I collagen and have been shown to yield markers of collagen degradation (Garnero 2003). Several MMPs have been identified within ACL fibroblasts, including MMPs-1, -2, -3, -9, -13, and -14. (Foos 2001 and Konopka 2016) One cross-sectional animal study examined the relationship between MMP gene expression and ACL injury risk (Comerford 2006). This study examined MMP-2 expression in three canine breeds, the Labrador retriever, the golden retriever, and the greyhound (Comerford 2006). The first two breeds were known to be at increased risk of ACL tear, while greyhound

was a low-risk breed. High-risk breeds expressed more pro-MMP-2 than the greyhound, which may suggest a positive relationship between MMP-2 expression and ACL injury risk.

Hormones have been shown to influence the system of MMPs that exists in collagen producing cells. For example, progesterone has been shown to inhibit the production of MMP-2 in female stromal cells, which produce collagens that contribute to the development of the endometrium (Keller 2000, Curry 2012). Limited evidence suggests sex hormones may also exert an effect on the MMP system within ACL fibroblasts. A recent study examined the effects of estradiol and relaxin on MMP production in humans (Konopka 2016). ACL samples obtained from seven men and seven women were treated with estradiol and relaxin. Supraphysiologic levels of relaxin (10 ng/mL to 100 ng/mL), in combination with supraphysiologic levels of estradiol (1 μM or 2.72 ng/mL) significantly increased expression of MMP-1 and MMP-3 in cells obtained from the women. While this study suggests both estradiol and relaxin may increase MMP expression and thus lead to degradation of type-I collagen, the hormone concentrations these cells were exposed to were orders of magnitude greater what fibroblasts would be exposed to *in vivo.* To date, no study has examined the effect of physiological levels of estradiol or progesterone on ACL fibroblasts, and thus their effect on MMP expression is unknown. There is also a lack of data on the specific MMP isoforms that may be affected by progesterone. Nevertheless, this limited evidence suggests hormones may exert an effect on ACL metabolism over time by means of their influence on MMPs. Specifically, estradiol and relaxin may increase expression of MMP-1 and MMP-3 while progesterone may indirectly decrease expression of MMP-2. Unfortunately, there is a lack of research available regarding the effects of progestational and androgenic compounds on MMP expression.

The previously mentioned animal and human studies suggest increased relaxin and estradiol may result in both decreased collagen synthesis and increased collagen degradation, resulting in a balance of collagen metabolism toward degradation. (Konopka 2016, Yu 2001, Liu 1997, Lee 2004, Liu 2005). It is reasonable to assume this may lead to decreased collagen content within the ACL. Limited evidence also suggests progesterone may attenuate the effects of estradiol and relaxin on collagen metabolism (Yu 2001, Kellar 2000). In short, changes in the amount of collagen may lead to changes in ACL mechanical properties. The next section of this review will consider the proposed effects sex hormones may have on ACL mechanical properties.

Effect of Hormones on ACL Mechanical Properties

The Effect of Hormones on ACL Load to Failure

Three experimental animal studies have examined the effect of hormones on the mechanical properties of the ACL. (Slauterbeck 1999, Dragoo 2009, Romani 2016) Slauterbeck examined the effect of estradiol on ACL load to failure in ovarectomized rabbits (Slauterbeck 1999). Rabbits exposed to pregnancy-levels of estradiol (52 pg/mL) for 30 days experienced lower load to failure than controls. Another study examined the effects of estradiol and relaxin on ACL load to failure in Guinea pigs (Dragoo 2009). The ACLs were divided into three groups, control, a relaxin-only treatment group (8 mg/mL relaxin), and a group exposed to pregnancy levels of relaxin (12 to 17 ng/ml) in combination with estradiol (50 mg/mL). The treatment groups were exposed to each hormone for twenty-one days to allow the ACLs enough time to respond to hormone treatment. ACL load to failure was significantly lower in both the relaxin only group and the relaxin + estradiol treatment group when compared to load to failure of the ACLs in the control group. Both studies suggest estradiol decreases ligament load to failure, and one suggests estradiol in combination with relaxin may result in further decreases in load to failure.

One experimental animal study using a rodent model examined the effect of testosterone on stiffness and load to failure (Romani 2016). Two groups of male rats were examined in this study, one group with normal circulating levels of testosterone and another group that had been castrated. Rats with normal levels of testosterone had greater stiffness and load to failure than castrated male rats, which suggests testosterone may increase the mechanical properties of the ACL. However, this study is limited both by the use of an animal model, as well as the use of males instead of females. Additionally, studies on collagen gene expression, synthesis, and degradation have not been carried out to understand the mechanism behind the effects of testosterone on ACL mechanical properties in females.

The animal studies mentioned above have a number of limitations. In the studies performed on female rabbit ACLs (Slauterbeck 1999) and on female guinea pig ACLs (Dragoo 2009), the estradiol administered were at pregnancy-level concentrations. In women, pregnancy levels of estradiol can be three to four orders of magnitude greater than normal circulating levels (Schock 2016). Additionally, the study examining the effect of testosterone on ACL stiffness examined only the male gender. There is evidence that suggests the ACLs of men and women

may respond differently to hormones (Konopka 2016). Nevertheless, these studies continue to support the notion that estradiol or estradiol in combination with relaxin may reduce ACL structure and mechanical properties, while testosterone may increase ACL mechanical properties. The effect of progesterone on ACL mechanical properties has yet to be investigated to determine if the previously noted attenuating effects of progesterone on collagen synthesis (Yu 2001) would result in preservation of mechanical properties.

The Effect of Hormones on Collagen Crosslinking

There is evidence suggesting sex hormones may affect ACL mechanical properties by exerting their influence over collagen crosslinking reactions. Cross-linking is a process that induces covalent bonds between tropocollagen fibrils, thus increasing the mechanical properties of an entire collagen fiber (Lee 2015). Increases in collagen turnover have been associated with decreased ligament integrity, which may be due to a reduction in the number of collagen cross-links within a fibril (Comerford 2006). One experimental study suggests estradiol affects cross-linking of tropocollagen fibrils (Lee 2015). This study involved the use of ligaments engineered from fibroblasts of a small sample of young men and women. Ligaments in the experimental group were treated with three physiologic concentrations of estradiol over a period of 28 days, while those in the control group were treated with a low dose of estradiol for 14 days. Though collagen cross sectional area and content did not change in the experimental ligaments, high physiologic levels of estrogen decreased ligament stiffness and ultimate tensile strength. Further analysis revealed gene expression and enzymatic activity of lysyl oxidase (LOX), an enzyme responsible for inducing collagen cross-links, had decreased in response to high levels of estradiol. The functional implication of this finding is that decreased collagen cross-links may allow greater sliding of one fiber relative to another, leading to decreased mechanical properties of the ligament. This is supported by other work showing relaxin increases creep of collagenous tissues, which was thought to be facilitated by fibrillary sliding due to decreased cross-link formation (Wood and Luthin 2011). Therefore, it is reasonable estradiol and relaxin may decrease ligament integrity by decreasing number of crosslinks in the ligament's constituent collagen fibers.

Effect of Progesterone, Testosterone, and Estradiol on Expression of the Relaxin Receptor

Two studies have provided evidence that sex hormones exert an influence on relaxin receptors within the ACL. As previously mentioned, relaxin may decrease collagen content and

mechanical properties (Konopka 2016, Dragoo 2009, Wood 2011), thus hormones that regulate relaxin receptors may influence collagen content and mechanical properties. Two studies using rodent models suggest estradiol, progesterone, and testosterone influence relaxin receptor expression (Dehghan 2015, Dehghan 2014). One study has examined the effect of testosterone on relaxin receptor expression in the LCL and patellar tendon (Dehghan 2014). Relaxin receptor expression in both tissues decreased in a dose dependent manner in response to treatment with testosterone. Conversely, a study performed in the same tissues found treatment with progesterone and estradiol increased relaxin receptor expression (Dehghan 2015). It was the belief of the author that the differential manipulation of relaxin receptor expression in women may reduce their risk of non-contact ACL tears. However, these findings at present are limited to animal studies. Moreover, these studies only examined receptor expression, not relaxin concentrations or the interactions between relaxin and its receptor. Nevertheless, these studies suggest interactions between hormones and hormone receptors, especially the relaxin receptor, may partially explain the influence of hormones on ACL mechanical properties. More specifically, an androgenic compound, such as testosterone, may decrease relaxin receptor expression, and perhaps attenuate the effects of relaxin on collagen content and ligament properties (Dehghan 2014). On the other hand, estradiol appears to increase expression of the relaxin receptor, providing an explanation for the "priming" effect it exerts on tissues exposed to relaxin. Progesterone also resulted in an increase in relaxin receptor expression, both with and without the presence of estradiol. This would seem to be in opposition to other studies examining the effect of progesterone on collagen metabolism, which suggest progesterone attenuates estradiol's effects on fibroblast proliferation and collagen gene expression (Yu 2001). However it is important to remember that the interaction between hormones and collagen expression is complex and in the case of regulation of relaxin receptor expression, progesterone may perform the same role as estradiol. It is not known if progesterone's effect on relaxin receptor expression would negate its effects on collagen metabolism.

This section of the literature review has presented what is known about the effects of endogenous and hormones on collagen content, crosslinking, ligament mechanical properties. The ACL in women has different ultrastructural and lower mechanical properties than the male ACL, and there is evidence to suggest these differences may be due to hormonal differences between the two sexes (Chandrashekar 2006; Hashemi 2008; Hashemi 2011). Estradiol may

decrease collagen content (Liu 1997, Yu 2000, Lee 2004, Liu 2005), crosslinking, stiffness (Lee 2015), and load to failure (Slauterbeck 1999, Dragoo 2009), and these effects may be amplified in the presence of relaxin (Konopka 2016). While less is known about the effects of progesterone and testosterone on collagen content and ACL mechanical properties, these limited studies suggest progesterone may attenuate the effects of estradiol on collagen content, though no study has examined the effect of progesterone on ACL mechanical properties (Yu 2001, Kellar 2000). Testosterone may also decrease relaxin receptor expression, and increase ACL mechanical properties (Romani 2016, Dehghan 2014). However, it is important to note the applicability of these experimental studies to humans is still quite limited given 1) the use of animal models, 2) experimental treatments involving supraphysiologic levels of hormones, and 3) examination of only one or two sex hormones at a time. In humans, the relationship between hormones and collagen metabolism is very complex, as all four relevant sex hormones are present in various concentrations through the menstrual cycle. Therefore, studies examining the effect of progestational and androgenic hormones must acknowledge the fact that hormones do not act on soft tissue in isolation. Therefore, there is a need to examine the combined effects of all four hormones at normal physiological levels in humans if we are to fully understand the effects of these hormones on collagen metabolism and ACL mechanical properties.

Section III: Oral Contraceptives and ACL Injury Risk

Introduction to Oral Contraceptives

Though much of the research examining the effects of hormones on and ACL injury risk in young athletic women has centered on the study of this risk in eumenorrheic females, a large number of active and athletic women use oral contraceptive pills (OCPs). Epidemiological studies suggest a large portion at least 15.9% of women of reproductive age regulate their menstrual cycles using OCPs, (“NSFG - Listing C - Key Statistics from the National Survey of Family Growth,” 2018). Studies estimate the percentage of young athletic women who use OCPs may range from 27% to 52% depending on age range or sport. Other studies suggest there is no difference in OCP use between athletic and non-athletic populations (Beals 2002, Brynhyldesn 1997, Miller 1999, Paulus 2000, Torstviet 2005). Considering the prevalence of the use of OCPs among young women, studies examining the effects of these pills on injury risk are warranted. While researchers have often theorized that OCPs might provide a protective effect on the ACL by lowering and stabilizing endogenous hormone levels, there remains much uncertainty of their

actual impact on ligament properties and risk. This section of the literature review will first present what is known about the pharmacological effects of OCPs, with a specific focus on the pharmacodynamic effects of the hormones contained in these pills. Next, the literature examining the effect of OCPs on ACL injury risk will be presented, along with the gaps in this body of literature.

Pharmacology and Physiologic Effects of Oral Contraceptives

Oral contraceptives modify a woman's menstrual cycle by modulating the activity of the hypothalamic-anterior pituitary-ovarian axis (HPO) (Hackney 2017). Exposure to exogenous hormones contained in OCPs reduces the body's natural production of estradiol and progesterone (Hackney 2017). However, a fact that is often overlooked is the effect that exogenous hormones in OCPs also exert a biologic effect on soft tissue.

The most common type of oral contraceptive is the combination OCP, which contains the synthetic hormone ethinyl estradiol and a progestin, a class of progesterone-like compounds (Bruckner 2017). During pill cycle, OCPs are taken daily for three weeks. During the fourth week, the "off-pill week" a woman will either stop taking her OCP, or take a placebo pill, thus cutting off her exposure to exogenous hormones. when a female is not exposed to the synthetic hormones contained in the OCP (Bruckner 2017). OCPs are typically either monophasic or triphasic (Cedars 2002, Bruckner 2017). Monophasic OCPs deliver the same level of both ethinyl estradiol (EE) and a progestin during each pill week while triphasic OCPs increase the dose of their progestin from week to week (Cedars 2002, Bruckner 2017). Both the dosage of EE and progestin compounds may vary across OCP brands, (0.02-0.035 mg EE and 0.1 -1.0 mg of a given progestin) (Table A.1) This is important to note, as the pharmacodynamic activity of each type of progestin can vary (Stanczyk 2003).

Two relevant measures of an OCP's pharmacodynamic[2] activity are referred to as androgenicity and potency, both characteristics are determined by the dose and type of progestin compound used in a given OCP (Greer 2005). The term "androgenicity" refers to a progestin's ability to mimic the biological effects of natural testosterone by binding to the androgen receptor (AR) and displacing testosterone from sex hormone binding globulin (SHBG), a protein that transports steroid sex hormones through the bloodstream (Greer 2005). Potency refers to a

[2] **Pharmacodynamics** is the study of a drug's biochemical and physiologic effects on the body and **pharmacokinetics** the study of an organism's effect on a drug (i.e. metabolism). (Brucker 2017)

progestin's ability to bind to the progesterone receptor (Sitruk-Ware 2013 and Stanczyk 2003). Both properties vary from progestin to progestin, and because of this, OCPs can exert a wide range of androgenic and progestational effects (Greer 2005). There are currently six progestins that can be found in common combination OCPs and the pharmacodynamic activity of oral contraceptives is influenced greatly by the biochemical characteristics of each progestin. (Greer 2005 and Hall 2012). Two classes of progestins include the estrane group (norethindrone (NET), norethindrone acetate [NET-A], and ethynediol diacetate) and the gonane group (norgestrel, levonorgestrel (LNG), desogestrel, and norgestimate) (Bruker 2017. The most common progestins found in combination OCPs used by women in the United States are NET and LNG (Hall 2012). Because androgenicity and potency vary across OCP formulations, research examining the effects of OCPs on ACL mechanical properties must account take these two pharmacodynamic variables.[3]

One cross-sectional study suggests the effect of OCPs on endogenous hormone production varies depending on the formulation of the OCP (Elliot-Sale 2013). In this study, serum levels of estradiol and progesterone were compared among 95 females on 6 different OCP formulations. Blood was drawn when exogenous hormones delivered by the OCPs were at their peak. Mean endogenous progesterone and estradiol levels differed when brands were compared to each other. Though progesterone levels were reduced to near negligible levels, estradiol levels varied between undetectable levels and 90 pg/mL (Styne 2016). This may be due to differential effects on the HPO axis, but no explanation as to the mechanism behind this was offered (Hackney 2017). Regardless of the mechanism, this evidence suggests differences in the chemical composition of OCP brands may alter the bioavailability of hormone concentrations from both exogenous and endogenous sources. Therefore, when examining the effects of OCPs on risk factors for ACL injury, the pharmacodynamic activity of both exogenous and endogenous compounds should be accounted for.

Effects of Oral Contraceptives on ACL Injury Risk

The effects of OCP use on ACL injury is currently unclear. One early prospective study examining collegiate female soccer players suggests OCP use may decrease rates of musculoskeletal injury (Moller-Nielsen 1989). Since then, two studies have suggested OCPs

[3] For the sake of simplicity, the term pharmacodynamic variables (PDVs) will be used in Chapter 3 to cover both potency and androgenicity.

exert a protective effect on ACL injury (Rahr Wagner 2014 and Gray 2016) while two other studies suggest the opposite (Lefevre 2013 and Agel 2008). One study prospectively examined the prevalence of OCP use among a group of injured female skiers during one sport season (Lefevre 2013). Of the 172 injured skiers examined, 30.8% used some type of OCP. The percentage of injured skiers on an OCP was not significantly different from the eumenorrheic women. Another study examined the relationship between OCP use/non-use and non-contact ACL tears and ankle sprains among collegiate female basketball and soccer athletes over a period of three seasons (Agel 2008). This study contained a large sample of 3150 female collegiate athletes, with 1024 of the participants using either monophasic or triphasic OCPs. The proportion of athletes who experienced ACL tears or ankle sprains over the entire study period was not significantly different when OCP users and non-users were compared to each other (0.018 users vs. 0.016 for non-users). However, each of the studies mentioned above has its own limitations. For example, one lacked a control group, and therefore, comparisons in injury rates between injured and non-injured athletes could not be made (Levfevre 2013). In the study of collegiate soccer and basketball athletes (Agel 2008), the incidence of ACL injuries was low enough to where the study would have required nearly 45,000 participants in order to have enough power to detect differences between OCP users and non-users.

Evidence from large epidemiological studies suggest OCPs may reduce a woman's risk of experiencing an ACL tear (Rahr-Wagner 2014 , Gray 2016, DeFroda 2019). These epidemiological case-control studies examined the relationship between OCP use in those who underwent ACL reconstruction using data compiled from insurance databases. In the first study, of the 13,335 women who underwent ACL reconstruction surgeries, those who had used OCPs at one point during their lifetime had a lower likelihood of undergoing ACL reconstructive surgery, with long-term and recent OCP users having the lowest risk of the procedure (Rahr-Wagner 2014). In other work comparing rates of ACL reconstruction among OCP users to non-users, 12,819 who received ACL reconstructive surgery were compared to 38,457 age-matched controls. Women on OCPs had 18% fewer ACL reconstructions than controls (Gray 2016). Finally, a study examining the risk of ACL reconstructive surgery among women between the ages of 15 and 49 in the Humana insurance database compared the number of women on OCPs who had received ACL reconstructive surgery to OCP-non-users. Women who used OCPs once again had a lower risk of experiencing ACL reconstructive surgery. This difference in injury

rates was driven by the differences in injury rates that existed in girls who were in late adolescence (15-19 years). Though these studies suggest a protective effect of OCP use on ACL injury it is important to remember not all ACL tears result in surgery, and therefore, injured females who do not receive ACL reconstructive surgery would not be included in these studies. These studies also did not account for population statistics to know if OCP use was similar within the two populations. Furthermore, these studies could not determine the level of physical activity of subjects included in the samples. Finally, neither study accounted for the type of OCP taken by subjects, and thus, the effect of OCP's biological activity on ACL reconstruction rates remains unknown. Nevertheless, the results of the three studies should not be taken lightly, considering their large sample sizes.

This section has presented current knowledge on OCP use and ACL injury. A large portion of young athletic women take OCPs, and some researchers believe the reduction in endogenous levels of possibility deleterious sex hormones may offer a protective effect on the ACL (Beals 2002, Miller 1999, Torstviet 2005, Samuelson 2017). While large epidemiological studies have reported women on OCPs may have fewer knee reconstructions than eumenorrheic women, thus supporting this theory, there remain significant limitations to confidently conclude OCP use reduces ACL injury risk. Specifically, the type and dose of progestins contained in modern OCPs widely vary, and thus their effect on soft tissue properties and ACL risk may differ.

Section IV: The Effects of OCPs on ACL Mechanical Properties

As previously mentioned, collagen metabolism and collagen density dictate the mechanical properties of collagenous tissue, and endogenous hormones have been shown to influence both properties. This section of the literature review will present what is known about the effects of OCPs on collagen metabolism, the relationship between OCPs and relaxin, and finally, the effects of OCPs on the mechanical properties of soft tissue. A specific focus will be placed on the effects of androgenic progestins on collagen metabolism and soft tissue properties.

Effects of OCPs on Collagen Metabolism

An understanding of the effect of OCPs on ligament mechanical properties should begin with an examination of what is known about the effect of OCPs on collagen metabolism. Three studies have examined the effects of OCP use on collagen metabolism in soft tissue (Hansen 2009, Wreje 2000, Shultz 2012). The biomarkers of interest in these studies included markers of

collagen synthesis (C-terminal propeptide of Type I collagen (CICP) and N-terminal pro-peptide of Type I collagen (PINP)) and markers of collagen degradation, C-terminal telopeptide of type I collagen (CTX) and Carboxyterminal telopeptide of type I collagen (ICTP). Two studies suggest OCPs reduce rates of collagen metabolism (Wreje 2000 and Hansen 2009). One study examined serum levels of PINP and ICTP in young women before they began taking OCPs, then at two time points (3 and 12 months) after the initiation OCP treatment (Wreje 2000). Concentrations of both biomarkers were reduced after three months of treatment, suggesting OCP use reduces collagen metabolism. Another study compared serum levels of PINP and CTX within the patellar tendons of moderately active women who were on OCPs and those who were not (Hansen 2009). In order to exaggerate the effect of the estrogens used in both groups, women the eumenorrheic control group were tested during the early follicular phase of the cycle and females in the OCP group were tested at the end of their pill cycle. Tendon collagen synthesis was estimated by tracing a radioactively labeled amino acid. Both tendon collagen synthesis and serum PINP levels were significantly lower in the OCP group than in the control group. However, there was no effect of OCP treatment on CTX. This result may be explained by a study that has shown MMPs do not yield CTX as a product after cleaving type-1 collagen, suggesting MMPs selectively cleave pro-collagen fibers at their N-terminal end (Garnero 2003).

The studies examining the effect of OCP use on collagen metabolism have a few limitations. The serum biomarkers examined reflect turnover in all tissues containing type-1 collagen, which include bone, tendon and ligament. Furthermore, studies performed in tendon may not be directly applicable to the ACL since the tendon possesses different proteomic and structural properties than the ACL (Little 2014). However, these studies suggest the hormones OCP users are exposed to are also biologically active and may influence rates of collagen metabolism when compared to eumenorrheic women.

One study examined the effects of androgenic progestins on collagen metabolism. (Shultz 2012). Serum levels of ICTP and CICP were measured daily in 10 women on OCPs and 10 eumenorrheic women across an entire menstrual cycle. A significant negative relationship between ICTP and the androgenicity of the progestins contained in the OCPs used by the subjects was found in this study. Though this study's OCP group was relatively small, its results suggest progestins with increased androgenicity may decrease collagen degradation, which over time, may lead to increased collagen density and improved ligament mechanical properties.

Effects of OCPS on Serum Relaxin Concentrations

Limited evidence suggests OCPs may attenuate the effects of relaxin on soft tissue. In one study, serum relaxin levels were examined in 23 eumenorrheic elite level athletes, 11 of whom had detectable levels of relaxin (Nose-Ogura 2017). Of these females, 5 had relaxin levels greater than 6.0 pg/mL and these subjects were recruited to take a combination OCP for two pill cycles. This was done as previous work suggests relaxin levels greater than 6.0 pg/mL may place a female at risk of ACL tear (Dragoo 2011). The type of pill used was a combination OCP that used norethindrone, a progestin of low androgenicity (Greer 2005). When serum hormone concentrations were re-assessed during the subjects' second pill cycle, relaxin levels in all athletes were reduced to undetectable levels, suggesting OCP use may reduce relaxin levels. As mentioned previously, relaxin works in concert with estradiol to reduce collagen synthesis, which may reduce ligament integrity. Thus, it may be possible for OCPs to influence ligament mechanical properties indirectly by reducing serum relaxin levels. While this study was severely limited to the inclusion of only 5 participants using "OCP therapy", comparable results were reported in another study examining the effect of natural testosterone on relaxin receptor expression (Dehghan 2015). In this study, testosterone decreased relaxin receptor expression, and its effects were mediated by the interaction of testosterone with the androgen receptor. These studies suggest androgenic compounds may reduce serum relaxin levels, either by their effects on the HPO axis, or in the case of highly androgenic OCPs, through an interaction with the androgen receptor, thus attenuating the effects of relaxin on soft tissue. This, in turn, may lead to greater ligament mechanical properties in OCP users.

Effects of OCPs on the Mechanical Properties of Soft Tissue

Evidence from human and animal suggests oral contraceptives may affect the structural properties of collagenous tissues. In one study, the ACLs of a group of female rats who were given combination OCPs that contained levonorgestrel were tested to failure and their properties, namely stiffness, energy absorbed to failure, and elongation to failure, were compared to eumenorrheic controls (Woodhouse 2007). ACL energy absorbed to failure and elongation to failure were significantly greater in the OCP group, though stiffness was significantly lower than controls. This suggests OCPs may have a long-term effect on ligament properties. Interestingly, this is the only study in which members of the OCP group all used OCPs that contained the same progestin dose and type.

One human study has examined the effects of OCP use on the mechanical properties of soft tissue in humans (Hansen 2013). This study examined the mechanical and biochemical properties of the patellar tendon (PT) in 30 sub elite handball athletes. The mechanical properties of the PT in fifteen OCP users, all of whom were on combined oral contraceptives for a period of 3-10 years, were compared to the mechanical properties of the PT in eumenorrheic. Biopsies of the tendons were obtained, and serum analysis of hormones and the cross-linking enzyme lysyl oxide was performed as well. The investigators found no effect of OCP use on tendon stiffness or young's modulus in comparison to eumenorrheic controls. However, the lack of any significant result may be in part due the OCP group using OCPs that contained 4 progestins, (levonorgestrel, gestodine, desogestrel, and dropirenonene), all of which have differing levels of androgenic and progestational activity (Greer 2005). Additionally, tendon samples were only taken at one time point, from one limb on each participant, which may have led to low statistical power. Therefore, the effect of OCPs on mechanical properties remains unclear and requires further study.

This section has presented what is known about the effects of OCPs on collagen metabolism and soft tissue mechanical properties. Studies have demonstrated collagen reduces rates of collagen degradation, which allows the maintenance of mature collagen crosslinks which, over time, allows for more stability in ligament properties (Comerford 2006, Wreje 2000, Hansen 2009). Therefore, these studies appear to suggest OCP use may improve ligament mechanical properties by reducing rates of collagen degradation. Studies examining the effects of OCP use on ligament mechanical properties are equivocal (Woodhouse 2007, Hansen 2009). Additionally, all studies are limited by the fact that measurements of collagen metabolism and mechanical properties often examined collagenous tissues, such as the patellar tendon, but not the ACL. Furthermore, as mentioned in the previous section, no study has stratified OCP users by the type of OCP used. Finally, studies of soft tissue properties have not been performed extensively in humans, and therefore, the effect of OCPs on ACL mechanical properties remains unclear.

Section V: Anterior Knee Laxity as a Model to Understand the Effects of OCPs the ACL

One mechanistic pathway through which we can understand effects of OCPs is through the measurement of anterior knee laxity (AKL), a clinical measurement of the ACL's mechanical properties. This metric describes the amount of anterior translation of the tibia relative to the

femur as an external load is placed on the posterior tibia. AKL is a relevant measure as females have greater AKL than males (Boguszewski 2015, Beynnon 2005, Shultz 2005, Vacek 2016). Females with greater AKL are more likely to rupture their ACL (Uhorchak 2003, Myer 2008). This section of the literature review will present what is known about AKL as a risk factor for ACL injury and the potential effects of sex hormones on AKL. Next, literature examining the relationship between collagen metabolism and AKL will be presented. Special focus will be placed on the proposed effects of androgenic and progestational hormones on AKL.

Anterior Knee Laxity as a Risk Factor for ACL Injury

Three prospective case-control studies suggest AKL is a risk factor for ACL injury (Uhorchak 2003, Vacek 2016, Myer 2008). A four-year prospective cohort study of 895 military cadets found increased knee laxity to be a risk factor for ACL injury in females (Uhorchack 2003). Anterior knee laxity, along with other proposed risk factors of non-contact ACL tear, were assessed at the beginning of the cadet's freshman year. Though AKL was not at significant predictor of ACL injury due to a lack of statistical power, women who had AKL values greater than one standard deviation above the mean were nearly three times more likely than women with average AKL to sustain an ACL tear. Additionally, the regression model that explained the greatest amount of variability in female injury risk (62.5% of the variability in injury risk) included BMI, femoral notch width, and AKL as predictors. In two additional studies, subjects were recruited from a cohort of subjects whose AKL was prospectively measured, and those who went on to tear their ACL were matched to uninjured teammates, either by bodyweight and height, or by age and sex (Myer 2008 and Vacek 2016). In both studies, multivariate models that best predicted ACL injury risk included AKL as a significant predictor of injury in combination with other risk factors such as knee hyperextension, prior injury, and BMI. These studies taken together suggest AKL, in combination with other risk factors, may predict ACL injury across a variety of sports, ages, and levels of competition.

Effect of Sex Hormones on Anterior Knee Laxity

Consensus is that AKL increases during the luteal phase of the menstrual cycle. (Zazulak 2006, Herzberg 2017, Balanchandar 2017). Though some studies have found no influence of menstrual cycle phase on AKL (Beynnon 2005 and Eiling 2007) others have indeed found a change in AKL over the course of a menstrual cycle (Shultz 2004, Shultz 2012, Romani 2003, Lovering 2005). One limitation to studies examining AKL in relation to sex hormones is

determination of cycle phase. In studies in which no change in AKL was found over the course of the menstrual cycle, estimates of cycle phase were based on counting days from ovulation (Beynnon 2005 and Eiling 2007). This may introduce error in menstrual cycle phase determination as females vary widely in the timing and magnitude of hormone concentrations, which requires one to sample actual hormone concentrations to accurately determine phase. Unfortunately, this is a methodological consideration that few studies have taken into account (Vescovi 2011).The specific time periods within the follicular phase during which AKL values are the greatest has varied from study to study, which may be due to the methods used to sample hormones and knee laxity in these studies (Herzberg 2017). Nevertheless, evidence from the strongest studies in this area of research suggest AKL fluctuates through the course of the menstrual cycle and is greatest during at the luteal phase of the menstrual cycle (Shultz 2004, Shultz 2012 and Herzberg 2017).

Two cross sectional studies have examined relationships between hormone levels and ACL stiffness at specific points during the menstrual cycle (Romani 2003 and Lovering 2015). ACL stiffness is the inverse of AKL and was defined by the authors as the slope of the line produced on a force-displacement curve when two different force values are applied to displace the tibia anteriorly. Both studies examined hormone levels in active eumenorrheic females during the early follicular phase, near ovulation, and during the luteal phase of their menstrual cycles. Estimates of subject's cycle phase were based on counting the number of days past ovulation. One study found a significant negative relationship between estradiol and ACL stiffness near ovulation and a positive relationship between progesterone and ACL stiffness near ovulation, once all other hormones, and sex hormone binding globulin (SHBG) levels were controlled for (Romani 2003). However, it is not clear from this study whether such a relationship would hold true at other points of the menstrual cycle. The second study examined testosterone concentrations and found no evidence of an effect of testosterone on ACL stiffness during any of the time points once all other hormones analyzed were accounted for (Lovering 2005). When analyzed at one time point, namely ovulation, one study suggests increased estradiol concentrations may result in decreases in ACL stiffness, while progesterone may exert the opposite effect (Romani 2003). However, the relationship between testosterone and ACL stiffness in isolation from other hormones remains unclear (Lovering 2005).

One series of studies performed on the same population has highlighted key relationships between absolute hormone levels and AKL (Shultz 2004 and Shultz 2006). In these studies, hormone levels of active normally menstruating females were sampled daily on each participant throughout one menstrual cycle, allowing for confirmation of menstrual cycle phase, as well as alignment of data by actual hormone fluctuations. The aim of one study was to examine the change in AKL in response to absolute hormone concentrations (Shultz 2006). In this study, lower minimum estradiol levels and higher minimum progesterone levels during the early follicular phase determined greater changes in AKL once estradiol and testosterone reached peak levels (Shultz 2006). These findings suggest AKL in females with a highly progestational hormone profile early in their cycle may be more responsive to changes in hormone concentrations during the luteal phase of the cycle. It may also suggest females who have lower progesterone levels and higher estradiol levels during the early follicular phase, may experience more stability in AKL post-ovulation. Additionally, estradiol, progesterone, and testosterone levels analyzed together explained more of the variability in AKL than either hormone analyzed alone (Shultz 2006). Interestingly, testosterone appeared to be a positive predictor of changes in AKL in one of these studies (Shultz 2004). However, it is important to remember testosterone and estradiol concentrations may be related, as testosterone can be converted to estradiol via aromatization, and thus, testosterone's relationship to AKL may manifest in its relationship to estradiol. These results highlight the complex relationship between hormones and AKL, and therefore any study examining the effect of hormones on AKL should take all relevant hormones into account.

Effect of Collagen Metabolism on AKL

As mentioned in previous sections, sex hormones have been shown to influence collagen metabolism (Liu 1997, Lee 2005, Yu 2001), and one of the mechanisms by which hormones affect collagen metabolism is through an effect on MMP expression (Konopka 2016, Curry 2003, Lee 2005). It stands to reason that changes in collagen metabolism may result in changes to mechanical properties and AKL. One cross sectional animal study has examined the relationship between pro-MMP expression and AKL in the Labrador retriever and the greyhound (Comerford 2005). A significant positive relationship between pro-MMP-2 levels and AKL was found. As previously mentioned, increased MMP levels, which may result from increased relaxin and concentrations, may decrease AKL over time (Konopka 2016). The fact that an animal

model was used in this study may limit its applicability to humans. Nevertheless, this study highlights the possibility that increased MMP gene expression may increase AKL. On the other hand, hormonal factors that decrease MMP gene expression may lead to decreases in AKL. As mentioned previously, progesterone has been shown to decrease expression of MMP-2 (Kellar 2000). Research examining the effects of progesterone on other MMPs was not found during this review, but this finding suggests other hormones with progestational properties may decrease AKL values by decreasing MMP gene expression.

One clinical cross-sectional study has examined the relationship between sex hormones, collagen metabolism, and AKL in active females (Shultz 2012). Ten eumenorrheic females were assessed over the course of one menstrual cycle, during which serum hormone concentrations of estradiol, progesterone, and testosterone, along with levels markers of collagen metabolism, and anterior knee laxity were measured daily. Then, five days representing each phase (Menstrual, Ovulatory, Early Luteal, and Late Luteal) were aligned by hormone events and used for analysis. The collagen markers included the C-terminal propeptide of type I collagen (CICP), a marker of collagen synthesis, and carboxyterminal telopeptide of type-1 collagen (ICTP), a marker of collagen degradation. Serum concentrations of CICP were a significant negative predictor of AKL across the entire cycle. This finding suggests decreased collagen synthesis may lead to increases in AKL. This this consistent with animal studies that have suggested estradiol decreases gene expression of type-1 collagen (Liu 1997, Lee 2004, Liu 2005), but the effect of estradiol on collagen synthesis was not analyzed in this study. Future studies may be needed to determine mechanistically whether estradiol increases AKL by decreasing markers of collagen synthesis.

This section of the literature review has presented what is known about the relationship between sex hormones, AKL, and ACL injury. Greater AKL values have been shown to be a consistent predictor of ACL injury risk. The strongest clinical studies in this area, which have sampled hormones daily over the course of a cycle, suggest hormones may influence AKL (Shultz 2004, Shultz 2012). However, the relationship between AKL and endogenous hormones with androgenic and progestational hormones is unclear (Shultz 2004, Romani 2003 Lovering 2005), and thus the implied effects of androgenic and progestational hormones on AKL requires further investigation.

Section VI: Oral Contraceptive Use and Anterior Knee Laxity

Limited evidence suggests females on OCPs have lower AKL values than eumenorrheic females. In three of these studies, women on OCPs had lower AKL values than eumenorrheic women (Martineau 2004, Lee 2014, Lee 2015). However, two studies found no difference in AKL between OCP users and non-users (Pokorny 2000 and Hicks-Little 2007). This section of the literature will highlight the differences in study design that have produced these contradictory results. One study that examined the effects of the pharmacodynamic activity of progestins on AKL will be presented as well.

An assessment of AKL across the menstrual cycle must involve confirmation of menstrual cycle phase via hormone concentrations as accurate determination of menstrual cycle phase requires multiple measurements over the course of a cycle, due to the inter- and intra-subject variability female's cycles (Vesocovi 2011). This is a methodological consideration that few studies have considered. In two studies, AKL was measured on a single occasion (Pokorny 2000 and Martineau 2004). In these studies, subjects were compared based on counting cycle days, while no confirmation of cycle phase by hormonal measurement was made. In the third study, AKL measurements were taken on 5 consecutive days, but the menstrual cycle phase of eumenhorreic controls was not considered (Lee 2015). Two of these studies found OCP users had lower AKL values than non-users (Martineu 2004 and Lee 2015) while one study found no difference in AKL between the two groups (Pokorny 2004). The aforementioned characteristics of these studies makes it difficult to determine whether differences, or the lack of differences in laxity values, were due to true hormone differences or sampling inconsistencies. Additionally, none of these studies controlled for the type of OCP used by subjects, which may impact results given the different progestational and androgenic effects of different OCPs as previously mentioned.

Two studies have examined the differences in AKL between OCP users and non-users at specific phases of the menstrual cycle (Lee 2014, Hicks-Little 2007). The first study, performed on young, moderately active women had AKL measurements taken at four time points corresponding to four points of the menstrual cycle (Lee 2014). AKL in OCP users was measured during the same time points to align their test days with subjects in the non-OCP group. Estradiol and progesterone levels were also measured in all subjects. AKL values were lower in the OCP group than in the eumenorrheic at all time points. In the second study, AKL

measurements were taken on days 1, 13, and 23 of the cycles of both subject groups (Hicks-Little 2007). However, no differences in laxity were found. Part of the reason for this is likely due to the large amount of inter-subject variability in menstrual cycle length (Vescovi 2011). Both studies assumed all eumenorrheic women experienced transitions between cycle phases on the exact same day, when in reality, this is not true (Vescovi 2011, Shultz 2004). Moreover, confirmation of cycle phase could not be made in this study as hormones were not sampled.

Only one study has compared differences in AKL between women who use OCPs and eumenorrheic women over the course of an entire menstrual cycle (Shultz 2012). In this study, AKL in ten women on OCPs was compared to AKL values in ten eumenorrheic females. AKL measurements and hormone measurements were taken daily over a complete menstrual cycle. Then, five days representing each phase (Menstrual, Ovulatory, Early Luteal, and Late Luteal) were aligned by hormone events and used for analysis. AKL remained stable across the cycle for the OCP group, while it fluctuated in the eumenorrheic group. This suggests eumenorrheic women may be more sensitive to factors that affect AKL in the short term, such as hormonal effects on crosslinking, by either estradiol or relaxin, while OCP users may not be as sensitive to such changes (Lee 2015, Wood 2003). Additionally, there was less variability in ICTP and CICP in the OCP group compared to the non-OCP group. The reduced variability in markers of collagen metabolism found in OCP users in may explain their reduced variability in AKL. Though, this study provides the strongest evidence that AKL values in OCP users are lower than those of eumenorrheic women, it is important to note this study was limited a relatively small OCP group, which contained subjects who were all on different OCPs, containing progestins with different levels of pharmacodynamic activity.

This section of the literature review has presented what is known about the effects of OCPs on AKL. Though the aforementioned studies suggest active women on OCPs may have less AKL than eumenorrheic women, subjects in all studies used a variety of OCP brands, each with a different type and quantity of progestin (Pokorny 2000, Martineau 2004, Shultz 2012, Lee 2014, Lee 2015). Thus, the androgenicity and potency of the OCPs used in these studies varied greatly. Most importantly, only one study stratified OCP users based on the pharmacodynamic activity of the progestins in the subjects' OCPs (Shultz 2012). Such stratification is important to consider since all progestins differ by level of progestational and androgenic activity, which may

cause progestins to act on soft tissue and AKL differently (Sitruk-Ware 2013 and Stanczyk 2003). Thus, further research is required to elucidate the effects of OCP use on AKL.

Literature Review Conclusion

This literature has presented what is known about the effects of OCPs on ligament properties and ACL injury risk. Naturally produced sex hormones may influence a young woman's risk of ACL injury by influencing collagen metabolism and ACL mechanical properties (Hewett 2007, Balachander 2017). Hormones such as relaxin and estradiol are thought to decrease ligament mechanical properties, while progesterone and testosterone may attenuate the effects of these hormones.

A large proportion of the female athlete population use hormonal oral contraceptives to regulate hormone levels (Beals 2002, Miller 1999, Torstviet 2005). OCPs contain progestins, synthetic forms of the hormone progesterone, which vary according to their progestational and androgenic properties (Stanczyk 2003). At present, the effects of androgenic and progestational hormones is very limited (Keller 2000, Yu 2001, Lovering 2015, Shultz 2004). Additionally, most studies presented in this literature review have examined OCP users as a homogenous group, regardless of the pharmacodynamic activity of the progestins contain in subjects' OCPs, and because of this, the effects of OCPs on ACL injury risk remains unclear. An examination of anterior knee laxity, a clinical measure of the mechanical properties of ligament, may serve as a viable model for the analysis of the effects of OCPs on ACL mechanical properties. Research is needed to compare progestins with high vs. low progestational and androgenic properties relative to AKL. Such studies would further elucidate the effect of OCP use on AKL and ACL injury risk. These studies may also elucidate the effects of both progestational and androgenic hormones on ACL mechanical properties.

CHAPTER III METHODS

The method section of this thesis is organized as follows: Presented first is a description of participant inclusion and exclusion criteria. Next is the description of participant laboratory visits and data collection procedures. Third is a detailed description of the method development and implementation of hormone assays used to measure progestin levels in participants after data collection had been completed. The method section will conclude with a description of data reduction and analysis for each aim of the thesis.

Participants

Forty-four young (18-30 years) recreationally active women were projected to be recruited for this study. "Recreationally active" was defined as participation in aerobic or resistance training exercise between 1.5 and 10 hours per week (Shultz 2012). All study participants were to have at least one healthy knee, no history of knee ligament injury, non-smoking, nulliparous, with a BMI less than 30. Participants did not use other hormone modifying medication besides an oral contraceptive pill (OCP).

Participants were included if they used a monophasic or triphasic OCP for a period of at least six months before beginning the study. Participants for this study were purposefully sampled to obtain a sample who used a broad range OCPs containing progestins relative to androgenicity and potency: levonorgestrel (LNG), norgestimate, norgestrel, norethindrone-acetate (NET-A). A list of acceptable OCP brands participants could have taken can be found in Appendix A.

The rationale behind the selection of these progestins was two-fold. LNG and NET are the two most often used progestins in modern OCPs (Hall 2012) and are the biologically active forms of a few other progestins (Shindler 2003). Specifically, NET-A is a pro-drug that is converted into NET (Shindler 2003) and norgestimate is a pro-drug that is converted into LNG (Shindler 2003, Kuhl 2005). LNG is a highly potent and androgenic progestin.

Table 1: Pill Pharmacodynamic Variables (Greer 2005)

Progestin (per 1 mg Norethindrone)	Progestational Activity	Androgenic Activity
Norethindrone	1	1
Norethindrone-acetate	1.2	0.6
Levonorgestrel	5.3	8.3

Progestin (per 1 mg Norethindrone)	Progestational Activity	Androgenic Activity
Norgestimate	1.3	1.9

Modifications to Data Reduction and Statistical Approach Due to COVID-19

Unfortunately, the COVID-19 Pandemic of 2020 and 2021 introduced various challenges to subject recruitment. Specially, recruitment of participants was halted in March 2020 and as a result, the thesis was limited to the sample collected to date (N=16). This required statistical approach to Aims II and III to be modified to best meet the study objectives based on this limited sample. These data collection, data reduction and analyses procedures as described reflect these changes.

Data Collection Procedures

Before a participant's first day of testing, she attended a participant orientation session in the Applied Neuromechanics Laboratory where she had the study's purpose and procedures explained to her. She read and signed an informed consent form and completed a Physical Activity and Health Questionnaire, a Marx Physical Activity Rating Scale and a Female Hormonal History questionnaire (Appendix A). These forms included questions regarding her height, weight, age, current level of physical activity, type of OCP taken, duration of OCP use, and time of OCP ingestion. She was also asked to bring a copy of her OCP pill package so that its progestin type and dose could be confirmed. At the end of the orientation session, she was familiarized with the laxity measurements that were to be taken during laboratory visits. Finally, her two laboratory visits were scheduled based on her OCP cycle. Participants who were enrolled were encouraged to maintain their normal pattern of physical activity and OCP use. Participants testing days were counterbalanced to control for order effects.

Participant Laboratory Visits

Study participants were tested on two occasions: once between days 19 and 21 of their pill cycle (end of third week), and once between days 26 and 28 (end of the fourth week of their pill cycle). Days 19-21 were chosen since participants would have had the greatest and most prolonged exposure to exogenous progestins during this period. Days 26-28 were chosen since participants would have been off progestins for several days and progestin levels would be lowest. Testing consistently took take place between the hours of 7:00 AM and 9:00 AM, when

endogenous hormones were expected to be at their lowest. A female research assistant was always present to assist with data collection.

Blood Draws and Serum Processing

Blood draws were performed by a trained technician in the UNCG Exercise Endocrinology (E^2) laboratory. As participants typically ingested their pill at bedtime, where an average of 10.6 ± 3.3 hours elapsed between the time of a pill ingestion and their visit. Twenty milliliters of blood were collected from the antecubital vein from each participant on each laboratory visit to assay hormone levels. Blood was allowed to clot for at least 15 minutes before centrifugation at 3500 revolutions per minute (RPM) for 15 minutes.[4] Afterwards, the supernatant fluid (serum) was collected, separated into six aliquots and stored in a -80°C freezer in preparation for sample analysis. Aliquots of serum were labeled with the date, day of cycle, and subject code.

Measurement of Joint Laxity

The next portion of the laboratory visit involved an assessment of joint laxity. The first battery of tests involved examination of a participant's general joint laxity, the "looseness" of various joints, using the Beighton and Horan Joint Mobility Index (Beighton 1973). The participant was asked to extend her first and fifth metacarpophalangeal (MCP) joints. A score of 1 was given to the participant if her thumb was able to contact her wrist (1^{st} MCP) and 0 if not. For the 5^{th} MCP, joint angle of 90° or less was assigned a score of 1, and joint angle of 91° or greater was assigned a score of 0. The participant was then asked to fully extend both her elbows, stand with both knees fully extended/locked. If the joint angle of either of these was greater than 10°, she was assigned a score of 1. If these angles were less than 10°, she would be assigned a score of 0. Finally, she was asked to touch the floor with her palms while her knees locked. A score of 1 was assigned if she was able to touch the floor with her palms, and a score of 0 was assigned if she could not.

Anterior knee laxity was measured by the KT2000 Knee Arthrometer (MedMetric Corp, San Diego, CA). Participants were asked to lie on athletic table in supine position with knees on top of a bolster to place the knee is approximately 25-30^o flexion, and the device strapped to one leg. A posterior load of -90 N to anterior load of 133 N was exerted against the tibia for three

[4] Centrifugation of blood collected from participants and storage of serum aliquots took place after the last participant had left for the day.

continuous cycles to record posterior and anterior tibial displacement respectively. The test was consistently conducted on the right leg first, then repeated on the left leg. AKL values of the second and third cycles were averaged for each leg and each test days. Data reduction of AKL data was performed using MatLab (MathWorks, Natick, Mass).

Prior to the start of this project, one tester (Foli) was trained to use the arthrometer for this project. AKL values were repeated on 10 participants on 2 consecutive days to determine intratester reliability and precision (ICC [2,k= 0.94 SEM=0.66 mm).

Aim I: Development of a Modified UPLC-MS Method for the Quantitation of Levonorgestrel, Norethindrone, Testosterone, and Progesterone

Before proceeding to the primary research questions, it was first necessary to demonstrate that UPLC-MS ultraperformance liquid chromatography-mass spectrometry (UPLC-MS), the selected method for hormone analysis, could measure LNG, NET, T, and P_4 in the serum of participants in this study. UPLC-MS was chosen for the following reasons: First, because all steroid hormones are structurally similar and derived from cholesterol, there would be a risk of cross-reactivity with antibodies used in direct ligand-binding assays, such as the ELISA, which could result in an overestimation of the concentration of certain analytes. Mass spectrometry-based assays have greater specificity than ligand binding assays, which have been used to measure steroid hormones in the ACL literature (Stanczyk 2010). Secondly, mass spectrometry allows for the analysis of multiple analytes within one sample, while direct ligand binding assays do not (Stanczyk 2010).

To address this aim, the data reduction and analyses are described in three Sub-Aims, with details to follow:

Purpose of Aim IA: Develop and determine the efficacy of a modified UPLC-MS method, based on previously published UPLC-MS methods for the measurement of endogenous steroid hormones to measure NET, LNG, P_4, and T in the study's participants.

- Hypothesis Aim IA: The modified UPLC-MS method would be sufficient to measure LNG and NET in the study's participants.

Purpose of Aim IB: Measure NET, LNG, P_4 and T in the serum of active women on birth control pills that contained progestins whose active metabolites are LNG and NET

- Hypothesis Aim IB: The modified UPLC-MS method developed in Aim IA would measure exogenous and endogenous hormone levels participants with sufficient precision and accuracy, in accord with the standards used in analytical chemistry

Difficulties that arose when measuring endogenous estradiol (17 -β estradiol) in the study's participants in Aim IB resulted in the addition of a third aspect of Aim I

Purpose of Aim IC: To confirm the presence of and measure relative amounts of 17-β estradiol in the study's participants using tandem mass spectrometry (MS/MS).

- Hypothesis Aim IC: The mass spectrum and extracted ion chromatogram produced by the final UPLC-MS/MS experiment that could confirm the presence of 17β- estradiol in the study's participants.

It is important to note that, at times, tasks under Aim I were conducted concurrently with other aspects of this projects or even before other aims had been completed. A detailed timeline of the events that took place under this aim is featured in Figure 1.

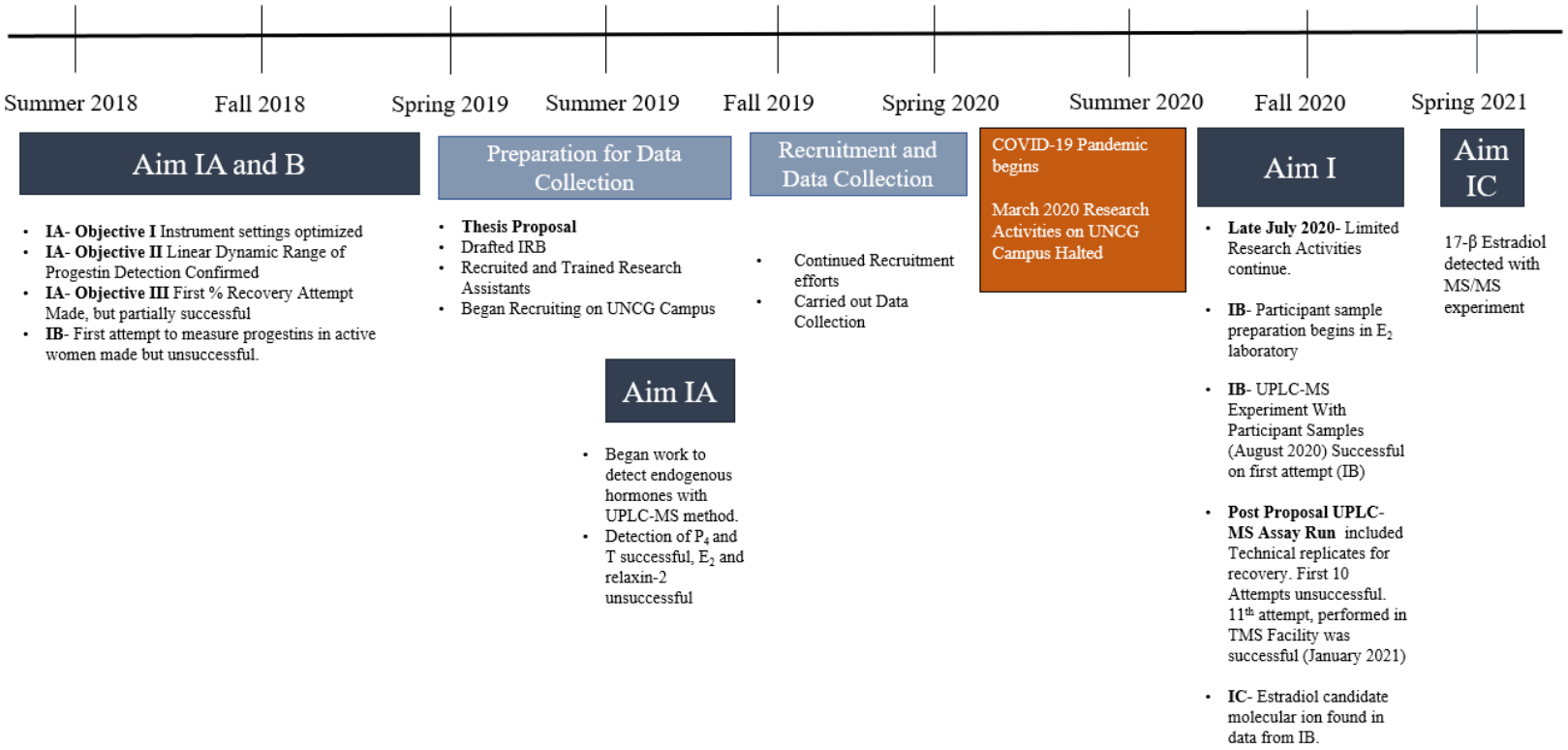


Figure 1 Timeline of Events for EXO-AKL Study

An Introduction to UPLC-MS Methods

Ultraperformance liquid chromatography-mass spectrometry (UPLC-MS) involves separation of analytes within a sample using a liquid chromatography system followed by the identification of the analytes based on their mass-to-charge (*m/z*) ratio using a mass spectrometer. UPLC-MS has been used in clinical chemistry and in pharmacokinetic studies to quantify concentrations of ethinyl estradiol (EE) and progestins in human serum (Wong 1999; DiLiberti 2011; Gong 2012; Edeleman 2016; Huang 2016; Praditpan 2017; Blue 2019;).[5]

The first step in an UPLC-MS assay is *chromatographic separation* by a liquid chromatography system, which separates analytes within a sample based on their polarity. In brief, a sample is injected onto an LC column, a device containing tightly packed particles known as the *stationary phase* and is eluted using a liquid solvent known as the *mobile phase*. Compounds within the sample interact with the stationary phase to varying degrees, thus causing them to separate from one another. This study will used *reverse phase liquid chromatography,* where the stationary phase is non-polar, and the mobile phase is polar. The time it takes for the analyte to elute from the column is referred to as the analyte's *retention time.*

The second phase of the UPLC-MS assay involves ionization and detection of the analytes contained within the sample. The analyte is transferred from the UPLC system in the form of a fluid, to the ion source on the mass spectrometer (Makin 2010). An ion source produces gas phase ions of the molecules in the UPLC eluent. Depending on the instrument settings, these ions can be either positive or negative ions. The ions are then guided through the instrument using a series of electromagnetic fields until they are sorted by their *m/z* ratio in the mass analyzer. In this project, *electrospray ionization* (ESI) was used as the ion source and an Orbitrap was used as the mass analyzer[6].

The outputs from the UPLC-MS instrument are *mass spectra* and the *chromatograms* (Figure 2)**.** A mass spectrum is a graphical of the *relative abundance* or *signal intensity* of all molecular ions detected within a sample during a given UPLC-MS run. The chromatogram displays *chromatographic peaks* for analytes of interest at retention time of the analyte. An

[5] An expanded glossary of terms related to UPLC-MS can be found in Appendix B

[6] A detailed explanation of the capabilities of the Orbitrap mass analyzer and a comparison to other commonly used mass analyzers, can be found at https://www.thermofisher.com/us/en/home/industrial/mass-spectrometry/liquid-chromatography-mass-spectrometry-lc-ms/lc-ms-systems/orbitrap-lc-ms.html

extracted ion chromatogram (XIC) displays chromatographic peaks associated with a specific *m/z* value within a specified mass tolerance (five parts per million in the case of the assays used for this thesis), which is automatically set by the software used to process MS data. *Peak area*, the integral of the retention time vs. relative abundance curve, is a measure of the response of the mass spectrometer to a given amount of that ion, and with an external calibration curve, can be used to determine the concentration of an analyte in a sample.[7]

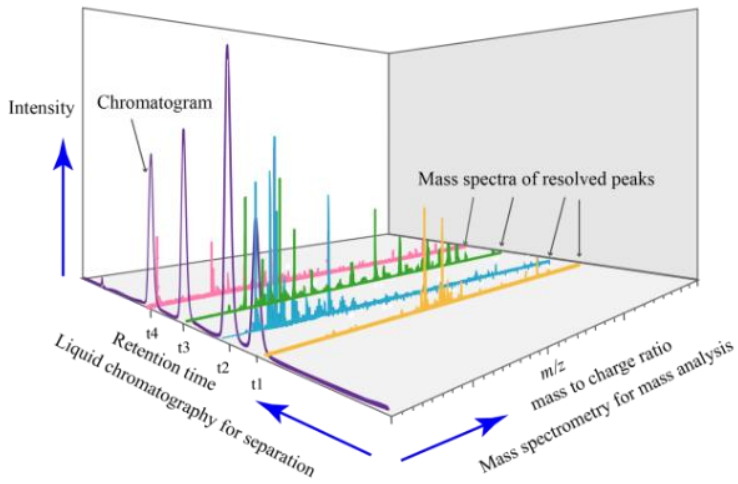


Figure 2 Example Mass Spectrum and Chromatogram (Norena-Caro 2017)

Aim IA: UPLC-MS Method Development Study

Purpose Statement: Prior to the initiation of this study, pilot work was performed to determine whether previously validated LC-MS methods used to measure ethinyl estradiol, 17β-estradiol, testosterone, and progesterone could be modified and used to measure LNG and NET in an organic matrix (Huang 2016; Schofield 2017; Yuan 2019) This work, which was performed in a stepwise fashion, consisted of three objectives: 1) Determination of instrument settings that would best allow for chromatographic separation and mass spectrometric detection of LNG and

[7] Though an external calibration curve is needed to determine the concentration of an analyte, peak areas of the same analyte can be compared to one another, within each assay, thus providing information about the relative abundance of the analyte within the samples of a given assay.

NET 2) Determination of the linear dynamic range of NET and LNG and 3) Determining the % recovery that could be expected from the sample preparation method that would be used to prepare participant serum samples.

The hypothesis for Aim IA Objectives I-III was that the modified UPLC-MS method would be sufficient to measure LNG and NET in the serum of participants on OCPs that would be metabolized into these active progestins.

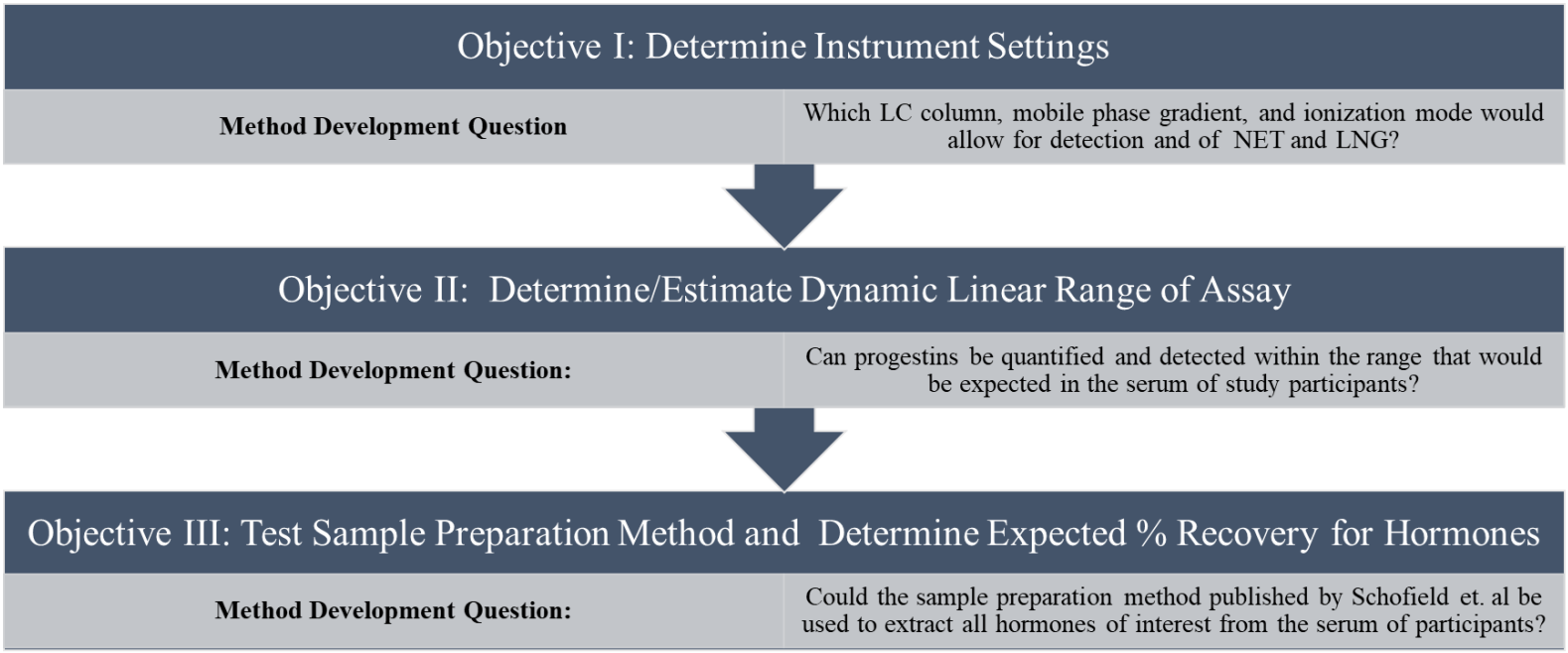


Figure 3 Method Development Plan for Aim IA

Objective I: Determination of Instrument Settings for Modified UPLC-MS Assay

Purpose Statement for Objective I: Determine the LC column, mobile phase gradient, and ionization mode that would allow for detection of LNG and NET.

Hypothesis for Aim-IA Objective I: LNG and NET would be detectable at a concentration of 50 µg/mL with the instrument settings used in the modified UPLC-MS method created under this objective.

Materials and Reagents

The reference standard LNG were purchased from Millipore Sigma (Burlington, MA) and the reference Standard for NET was purchased from APExBIO (Houston, TX). Blank human serum from a female of reproductive age, but not on any hormonal oral contraceptives was obtained from BioChemMed (Winchester, VA)

Preparation of Standards

Stock solutions of both NET and LNG were created by dissolving 0.003 grams (g) of each standard powder into 3 mL of 100% MeOH to yield a 3 mL stock solution of NET and LNG at a concentration of 1 mg/mL. Two solutions of high concentrations of NET and LNG were prepared by diluting each stock solution down to 50 µg/mL. These solutions were then transferred into an UPLC sample vial. A 10 µL aliquot of each solution was injected into the UPLC-MS system.

Instrumentation and Instrument Settings

Chromatographic settings: Chromatographic separation for this project was carried out on an Acquity Ultra Performance Liquid Chromatographic (UPLC) system (Waters, Milford, MA, USA). A Raptor Biphenyl column (Phenomenex, Torrance, CA, USA) was selected for the separation of the sex steroids of interest. Solvent flow rate was set to 0.3 mL/min for all UPLC runs. Samples were eluted from the column using a binary solvent system consisting of water (H_2O) containing 0.1% formic acid and acetonitrile (CH_3CN) containing 0.1% formic acid using the following gradient (ratios represent H_2O in 0.1% Formic Acid/CH_3CN): 0 to 0.5 min, 80:20, 0.5 to 7.5 min 40:60, 7.5 min to 90.10 min 0:100, 90.10 min to 10 min, 80:20. This gradient was based on a LC method used a biphenyl column for steroid hormone profiling (Phenomenex).

Mass spectrometric settings. The mass spectrometer used in this project was the LTQ Orbitrap XL mass spectrometer. During the first assay, the instrument was set to negative ionization mode. The second assay was that was set to positive ionization mode. (ThermoFisher Scientific, Waltham, MA). The optimized settings for the MS were as follows: spray voltage, 3.80 kV; source temperature 350 ℃, sheath gas flow, 30; auxiliary gas flow, 20; tube lens voltage, 100 V; capillary voltage, 20.0 V. The scan range was set to 125 – 750 *m/z* and the resolving power was set to 30,000.

Objective II: Determination of the Dynamic Linear Range of Detection of Assay

Purpose Statement- Determine whether LNG and NET can be detected and quantified within the concentration range that would be expected in the study's participants

Hypothesis for Aim IA- Objective II: The instrument settings used in Objective I would be able to detect and measure LNG and NET within a concentration range of, that could be expected in the study's participants

Preparation of External Calibration Curve

Calibrators, standards for the external calibration curve, were prepared by dissolving pure reference standards of LNG, NET and powder in 100% methanol to obtain a stock solution with a concentration of 1 mg/mL. This solution was diluted to 500 ng/mL, and a series of two-fold dilutions use used to create ten-point standard curves for NET and LNG between 0.25 and 250 ng/mL. These concentrations are based on reference ranges from validated LC-MS methods for the measurement of ethinyl estradiol, norgestimate, norelgestromine[8], and levonorgestrel (Huang 2016; Theron 2004).

Instrumentation and Instrument Settings

The instrumentation settings used in this objective were based on the results of the previous completed objective. A blank, or zero calibrator, comprised only of optima grade methanol was added to the UPLC-MS sequence at the start of each run and sampled five times

[8] Norelgestromine, a metabolite of norgestimate, also goes by the names, levonorgestrel-3-oxime (LNG-3-oxime) and 17-deacetyl norgestimate (Huang 2016). It was not realized, until all UPLC-MS assays had been run, that this progestin may have more important to measure along with LNG (See Aim II: Examination of the Relationship Between Pill and Serum Pharmacological Parameters)

before the calibrator of the lowest concentration.[9] Each calibrator was sampled by single injection. Thermo XCalibur (ThermoFisher Scientific, Waltham MA) and Microsoft Excel (Microsoft Corporation, Redmond WA) were used for data acquisition and processing.

Objective III: Determination of Sample Preparation Technique

Purpose Statement for Aim IA-Objective III- Determine whether a previously published sample preparation method used to extract testosterone and estradiol could be used to extract and measure NET and LNG from the serum of participants (Schofield 2017).

Hypothesis for Aim IA- Objective III: LNG and NET would be extracted from blank human serum spiked with a known concentration of each progestin, thus allowing for quantitation of the progestins in human samples.

Preparation of solutions for sample preparation

Extraction solution- A 10:90-ethyl acetate: hexane extraction solution was prepared by mixing 10 mL of ethyl acetate with 90 mL of hexane in a glass graduated cylinder.

Sodium Chloride Solution A 100 mM sodium chloride solution (NaCl) was prepared by dissolving 0.584 g of sodium chloride into 100 mL of purified H_2O and mixing well.

Diluent- Optima grade methanol was used as the diluent whenever dilutions were required.

Preparation of an External Calibration Curve

Calibrators between 0.25 ng/mL and 250 ng/mL were prepared according to the procedure established in Objective II.

Preparation of Technical Replicate for Recovery Experiment

To determine % recovery for each progestin, a technical replicate was produced by spiking previously dissolved LNG and NET into 1 mL sample blank human serum from a female who did not use any hormonal oral contraceptives. The expected concentration of each progestin was 31.25 ng/mL. Two-point five milliliters mL of the extraction solution and 50 µL of 100 mM NaCl were added to the technical replicate, then shaken for 15 minutes. Next, the technical replicate was transferred into glass centrifuge tubes, covered with parafilm, and placed in a centrifuge set to 3500 revolutions per minute (rpm) for 5 minutes to separate the organic and

[9] All UPLC-MS runs in this thesis were conducted such that a blank was injected first, and calibrators were sampled from lowest concentration to highest concentration to prevent analyte carryover.

aqueous layers. Finally, the technical replicate was flash frozen in a dry ice -acetone bath, and the organic layer was decanted into a 25 mL glass recovery vial then for dried under nitrogen stream. After the organic layer had dried, each the technical replicate was reconstituted in 80 µL of a 1:1 H_2O: MeOH solution, before being transferred into UPLC vial insert, then to a 2.0 mL UPLC vial.

Instrumentation and Instrument Settings

The settings used in Objective I were used for this objective. The zero calibrator (a methanol blank), progestin calibrators, and technical replicates were sampled by single injection.

Post Proposal UPLC-MS Assay

Purpose Statement: Refinements to Objectives II and III were made based on the data collected from these objectives and further pilot work (unreported) that was completed during the Summer 2019. All these refinements combined into one UPLC-MS run. Therefore, the purpose of this assay was used to determine whether modifications made to Objectives II and III would be suitable to measure P_4, T, NET, and LNG in samples of serum spiked with known amounts of all four hormones.

Hypothesis: This assay was that it would be sufficiently rigorous, according to standards commonly used in analytical chemistry, to measure hormones in human serum. The rigor this assay was assessed by an examination of the following parameters: Coefficient of determination of the calibration curves, precision and accuracy, mass accuracy, lower limits of detection and quantitation, % recovery and reproducibility of technical replicates.

Refinements made to the External Calibration Curve (Objective II)

Two endogenous hormones, T and P_4, were confirmed to be detectable and measurable under the instrument settings established in Aim I based on work completed during the Summer of 2019 (See Figure 1). Calibrators for the external calibration curves were prepared by creating four 1.0 mg/mL stock solutions of LNG, NET, P_4 and E_2 in optima grade methanol. Testosterone was already shipped in a 1.0 mg/mL stock solution in acetonitrile. Ten microliters of each stock solution were added to 950 µL of MeOH to make a 10 µg/mL solution containing all five hormones. This solution was then diluted once more by adding 50 µL of the second stock solution to 1,950 µL to make a solution with a concentration of 250 ng/mL. One milliliter of this solution was set aside for the refined recovery experiment discussed in the next section, while the other was pipetted into a glass UPLC autosampler vial. A 14-point external calibration curve was

prepared by performing two-fold dilutions from 250 ng/mL to 0.03 ng/mL. A detailed figure of the dilution scheme for this experiment can be found in Appendix B (Figure B.3)

Refinements Made to Preparation of Technical Replicates for Recovery Experiment (Objective III)-

Four 2- mL technical replicates and one serum blank were prepared according to the procedure established in the pre-proposal pilot work. The previously prepared 250 ng/mL stock solution was used to spike 2.5 mL of blank human serum from a female who did not use hormonal birth control. Two replicates were spiked with 32 µL of the stock solution and two were spiked with 80 µL. The 2.5 mL of the extraction solution and 50 µL of 100 mM NaCl were added to all technical replicates and the blank, then placed on a mechanical shaker for 15 minutes. Next, all technical replicates were transferred into glass centrifuge tubes, covered with parafilm, and placed in a centrifuge set to 3500 revolutions per minute (rpm) for 5 minutes to separate the organic and aqueous layers. Finally, the samples were flash frozen in a dry ice - acetone bath, and the organic layer was decanted into a 25 mL glass recovery vial then for dried under nitrogen stream. After the organic layer had dried, each sample was reconstituted in 80 µL of a 1:1 H_2O: MeOH solution, before being transferred into UPLC vial insert, then to a 2.0 mL UPLC vial. The insert was used to the small volumes of sample. The calculations for the expected recoveries for all replicates can be found in Appendix B (Equation 8)

Instrumentation and Instrument Settings

Instrument settings again matched the setting established in Objective I. The zero calibrator was run at the beginning of the sequence and between injections of calibrators or technical replicates. Calibrators were sampled in triplicate and technical replicates were sampled in duplicate. A table of the detailed UPLC-MS sequence can be found in Appendix B: Table B.1

Aim IB: Measurement of Participant Serum Hormone Levels

Purpose statement: The purpose of this aim was to quantify P_4, T, LNG, and NET in the study's participants.

The *hypothesis* for this aim was that the UPLC-MS method developed in Aim IA would measure exogenous and endogenous hormone levels participants in accord with the standards used in analytical chemistry.

Preparation of External Calibration Curve Standards

An 11-point external calibration curve for each analyte was prepared in a manner that differed slightly from the calibration curve prepared under IA-Objective III, *Refinements made in Post-Proposal Work*. As was the case in all experiments, 1.0 mg/mL stock solutions for P, NET, and LNG were prepared. The progestins were diluted and combined at 10 µg/mL before being diluted to 500 ng/mL. Progesterone and testosterone were also diluted and combined at 10 µg/mL before being diluted to 100 ng/mL. The two hormone mixtures were combined in a 1:1 dilution such that the highest concentration calibrator for LNG and NET was 250 ng/mL, and the highest concentration calibrator for P_4 and T was 50 ng/mL. A series of two-fold dilutions were carried out to the lowest concentration calibrator for each set of hormone standards. A detailed figure of the preparation of this calibration curve can be found in Appendix B: Figure B.7 Dilution Scheme for Preparation of External Calibration Curves (Aim IB)

Preparation of Participant Samples

Participant serum samples were prepared according to the same procedure used to prepared technical replicates in Aim IA- Objective III and the Post-Proposal Assay (Schofield 2017), with the following differences: All sample preparation was performed in the UNCG Exercise Endocrinology laboratory. Secondly, participant samples were frozen overnight in a -80 ℃ freezer instead of flash frozen. Finally, drying was performed in larger glass vials instead of the glass recovery vials used in the Post-Proposal Assay.

Instrument Settings

Instrument settings match those that were established in Aim IA-Objective I. A zero calibrator was sampled at the beginning and end of the UPLC-MS run as well as between the calibrators and participants samples. Participant samples were sampled by single injection and calibrators were sampled by triplicate injection.

Aim IC: Qualitative Analysis of Participant Samples Containing 17β-Estradiol

Endogenous estradiol (17 β- estradiol or E_2) may influence anterior knee laxity (Shultz 2006) and was one of the hormones that was to be included in the UPLC-MS method for this thesis. However, as mentioned previously, 17β-estradiol was thought to be undetectable using the modified UPLC-MS method developed for this thesis because it had not been detected in any of the UPLC-MS runs performed prior to the assay run under Aim IB. Chemical derivatization, the chemical addition of another compound to enhance the signal of an analyte, has been used in

previously published UPLC-MS methods for E_2, but it was decided not to perform this due to the low recoveries that are associated with derivatization (Nelson 2004).

Fortunately, a re-examination of the data obtained in Aim IB revealed XICs that contained a compound that matched the theoretical m/z (273.185) of E_2 in Participants 10-16 at a retention time of 4.03-4.04 minutes. If this was indeed estradiol, it could aid in the explanation of the influence of an additional endogenous sex hormone on AKL. Therefore, it was decided to confirm the presence of E_2 in these subject samples by tandem mass spectrometry (MS/MS). The purpose of this experiment was to 1) determine the retention time and fragmentation pattern[10] of an E_2 standard and 2) assess the retention time and fragmentation pattern of the XIC of a participant whose XIC obtained in Aim IB contained a peak for the *m/z* 273.185 molecular ion at 4.03-4.04 minutes

Preparation of an Estradiol Standard

Estradiol was difficult to detect with certainty at concentrations less than 250 ng/mL, so it was decided to make a high concentration standard (250 µg/mL) that would produce a higher signal on the mass spectrometer. This solution was prepared, first by making a 1.0 mg/mL solution, then diluting it down to 250 µg/mL using MeOH.

Sample Preparation

Serum from Participant 16, (Pill Week Three sample only) was used in this analysis, since this subject had the greatest quantity of serum of all available among all participants. The extraction solution and NaCl solution were prepared in the same way the solutions in Aim IA were prepared, and this participant's serum was prepared in the same way all samples were prepared in Aim IB.

Instrument Settings

The mass spectrometer was set with the same settings used in Aims IA and IB, with the addition of a fragmentation step in the sequence mass spectrometry settings. Specifically, the analyte of interest was fragmented with a normalized collision energy of 35. The 250 µg/mL E_2 standard and prepared serum from Participant 16 were sampled by duplicate injection.

[10] **Fragmentation by Collision Induced Dissociation (CID**) is a technique forces a collision of an analyte of interest (precursor ion) with a chemically inert gas, thus producing fragments that are unique to the compound. Fragmentation pattern, or unique "fingerprint" of an analyte, based on the chemical groups that form the fragments of the molecule is used to in qualitative analysis and can be used to determine the identity of the precursor ion.

Aim II: Examination of the Relationship Between Pill and Serum Pharmacological Parameters

Purpose Statement: To understand the effects of progestins on AKL, it is important to know how much of a progestin contained in an OCP is reaching systemic circulation. The purpose of this aim was to examine the strength of the association between pill potency and pill androgenicity to serum potency and serum androgenicity as measured by UPLC-MS in AIM I.

The determination of the two pharmacodynamic variables (PDV) of interest requires the calculation of the androgenicity and potency in the pill and in serum. Pill PDVs were calculated by multiplying the progestin dose of each subjects OCP by its potency or androgenicity relative to 1 mg of NET as described in Greer 2005 (Table 1). The variable of choice for serum is *peak serum concentration (*C_{peak}*).* These concentrations were not measured directly due to the timing of participants' laboratory visits. However, C_{peak} can be estimated by back calculation using Equation 1.

Equation 1: Integrated Rate Law Describing for the First Order Elimination of Progestins

$$C_{measured} = C_{peak} e^{-k_e(T_{measured} - T_{max})}$$

where $C_{measured}$ is the concentration of progestin measured by UPLC-MS, k_e is the elimination rate constant[11] (k_e) was calculated by dividing the average elimination half-life ($t_{1/2}$)[12] of the progestin by the natural log of 2, $T_{measured}$ is the number of hours between pill ingestion and the participants' blood draw, and T_{max} is the literature value from the time from pill ingestion to C_{peak} (Loftsson 2015).

Peak serum potency and androgenicity were calculated first by reducing each progestin's potency and androgenicity score from the milligram scale to the picogram scale (Greer 2005), then by multiplying peak serum progestin concentration by the metabolized progestin's transformed androgenicity or potency value relative to 1 pg/mL of NET.

[11] **Elimination rate constant** is the rate at which a drug is removed from systemic circulation by the body

[12] **Elimination half-life**- the time required for half of a drug to be removed from systemic circulation by the body.

Table 2 Pill Pharmacodynamic Variable Multipliers (Greer 2005)[13]

Progestin (per 1 mg Norethindrone)	Progestational Activity	Androgenic Activity
Norethindrone	1	1
Norethindrone-acetate	1.2	0.6
Levonorgestrel	5.3	8.3
Norgestimate	1.3	1.9

Table 3 Serum Pharmacodynamic Variable Multipliers (Greer 2005)

Progestin (per 1 pg Norethindrone)	Progestational Activity	Androgenic Activity
Norethindrone	1	1
Levonorgestrel	5.3	8.3

Equation 2: Example Pharmacokinetic Calculations for Participant 1, week 3

$$\boldsymbol{k_e = \frac{\ln 2}{25.4\ hours} = \frac{0.0252}{hr}}$$

$$\boldsymbol{C_{peak}} = \frac{181.79\frac{pg}{mL}}{\left[\!\left[(e)\right]\!\right]^{\left(-\frac{0.0252}{hr}\right)(9.75\ hours - 1.3\ hours)}} = \left(224.9\frac{pg}{mL}\right)$$

Equation 3: Example Pharmacodynamic Variable Calculations for Participant 1, week 3

$$\boldsymbol{Pill\ Potency = (0.1\ mg\ LNG) * \frac{5.3\ mg\ NET}{1\ mg\ LNG} = 0.53\ mg\ NET}$$

$$Pill\ Androgenicity = (0.1\ mg\ LNG) * \frac{8.3\ mg\ NET}{1\ mg\ LNG} = 0.83\ mg\ NET$$

$$Serum\ Potency = \left(224.9\frac{pg}{mL}\ \ LNG\right) * \left(\frac{5.3\ pg\ NET}{1\ pg\ LNG}\right) = 1191.7\ \ pg\ NET\frac{pgNET}{mL}$$

$$Serum\ Androgenicity = \left(\ 224.9\frac{pg}{mL}\ \ LNG\right) * \left(\frac{5.3\ pg\ NET}{1\ pg\ LNG}\right) = 1866.3\ \ pg\frac{NET}{mL}$$

Hypothesis for Aim II: It was hypothesized that, among all participants, there would be a significant positive correlation between pill pharmacodynamic parameters and serum pharmacodynamic parameters.

[13] The data contained in this table can also be found in Table 1

Aim III: Examination of the Relationship Between Pill and Serum Pharmacodynamic Variables and Anterior Knee Laxity during On Pill and Off Pill Weeks

Purpose Statement for Aim III: The purpose of this aim was to examine the relationship, if any exists, between pill and serum pharmacodynamic parameters of progestins and anterior knee laxity among active women who take OCPs.

Hypothesis Aim III A negative relationship exists between potency/androgenicity and AKL at week 3. It was also hypothesized that due to these hormone effects, females on OCPs with high androgenicity and potency would see a greater increase in AKL from week 3 (3rd pill week) to week 4 (off pill week)

Data Reduction and Statistical Analysis by Aim

Aim I Development of a Modified UPLC-MS Method for the Quantitation of Levonorgestrel, Norethindrone, Testosterone, and Progesterone

Aim IA UPLC-MS Method Development Study

Analytical Approach for Pre-Proposal Method Development

Objective I: This objective was assessed by producing XICs and mass spectra for LNG and NET. Prior to examining the XICs for each analyte, the *mass tolerance*, or maximum distance between a measured analyte's *m/z* and its theoretical *m/z* value, was limited to five parts per million, thus ensuring a measurement of accurate mass for each standard. The estimated LOD was determined by an assessment of the mass spectrometer's response to the 0.25 ng/mL calibrator. The theoretical *m/z* values for the progestins to four decimal places of precision are 313.2162 and 299.2006 for LNG and NET respectively.

Objective II: The hypothesis for this aim was tested by: 1) determining the lowest point at which either progestin could reasonably be detected above the blank (zero calibrator), and 2) determining coefficient of determination (r^2) for calibration curves of NET and LNG, which ranged between 0.25 ng/mL and 250 ng/mL. External calibration curves were generated by plotting the concentration of each calibrator against the peak area from the accompanying XIC.

Objective III: This objective was assessed by determining the % recovery or extraction efficiency of the sample preparation process, which was determined by the series of equations below:

Equation 4 Calculations of % Recovery

Step 1. Determine Peak Area Difference from Methanol Blank

$$Difference\ from\ blank = Technical\ Replicate\ PA - Blank\ Peak\ Area$$

Step 2. Measured concentration of technical replicate

$$Measured\ concentration = \frac{Difference\ from\ blank - Y\ Intercept\ of\ Cal.\ Curve}{slope\ of\ calibration\ curve}$$

Step 3. Recovery percentage

$$\%Recovery = \frac{Measured\ concentration}{(Expected\ concentration)} * 100$$

The reproducibility of this experiment was determined by calculating %CV for each injection.

Analytical Approach for Post Proposal Assay

The analytical approach for the assay was more rigorous than the work performed before this thesis' proposal. The following measurements would assess instrument accuracy and precision (mass accuracy and chromatography), intra-assay accuracy and precision, calibration curve goodness-of-fit, assay limits of quantitation and detection, and the accuracy and precision of the sample preparation process.

A mass error (or tolerance) of 5 ppm was the established limit for accurate mass for all analytes. This assessment was automatically performed by Thermo XCalibur. The theoretical *m/z* values for the hormones of interest are listed below:

- P_4 - 315.2319
- T - 289.2162
- NET - 299.2006
- LNG - 313.2162

The temporal deviation of chromatographic peaks for all analytes across calibrators was acceptable if deviations were less than 0.1 minutes. Within the XICs of technical replicates, chromatographic peaks outside the temporal boundaries set by the calibrators were not measured.

Calibration curve goodness-of-fit (r^2)- based on the work completed in Objectives I and II, it was deemed reasonable to expect an r^2 value greater than 0.95 for all calibration curves.

Intra-assay accuracy was assessed by calculating using % relative error (%RE) of each calibrator which was calculated by the equation below:

Equation 5: % Relative error calculation

$$RE\ \% = \frac{measured\ conentration - expected\ concentration}{expected\ concentration} * 100$$

The relative errors would be averaged to determine the relative error of each analyte's calibration curve. The calibration curve was considered acceptable if relative errors of all calibrators on the points of the calibration curve for each hormone was less than 20%

Intra-assay precision, which was assessed by calculating the percent coefficient of variation (% CV) for each series of triplicates at each concentration for each analyte.

Equation 6: %CV Calculation

$$\%\ CV = \frac{SD\ of\ Peak\ Area}{Average\ Peak\ Area} * 100$$

Precision for each analyte was determined by averaging the CV% for each point on its calibration curve. It was hypothesized that the average of the % CVs for all points on the calibration curve for each hormone would be less than 20%

The lower limit of quantitation (LLOQ), or the lowest concentration at which the assay could quantify a given analyte within acceptable limits (≤ 20% intra-assay precision and accuracy). Since serum samples would be concentrated by a factor of 40, LLOQs were

determined to be sufficient if greater than 0.5 ng/mL for endogenous hormones (12.5 pg/mL unconcentrated)[14], greater than 1 ng/mL (25 pg/mL unconcentrated serum) for the progestins.

The lower limit of detection (LLOD), or lowest concentration at which there was a response from the instrument for at least one of the injections of each analyte (Wells 2011). No expected LLODs were established prior to the UPLC-MS run.

Percentage of recovery, or extraction efficiency for each analyte was determined by subtracting the peak area of the analyte in the specified technical replicate from the serum blank. This value was then inserted into the regression equation produced from each calibration curve, and the equation was solved for concentration. Examples of the calculations for specific analytes can be found in Appendix B (Equation 9).

Equation 7 Calculations of % Recovery

1. Calculated Peak Area Difference from Blank

$$Difference\ from\ serum\ blank = Technical\ Replicate\ PA - Blank\ Peak\ Area$$

2. Measured concentration of technical replicate

$$measured\ concentration = \frac{Difference\ from\ blank - Y\ Intercept\ of\ Cal.\ Curve}{slope\ of\ calibration\ curve}$$

3. Recovery percentage

$$\%Recovery = \frac{Measured\ concentration}{(Expected\ concentration)} * 100$$

The reproducibility of the % recovery for the technical replicates was calculated by examining the % CV for each hormone.

[14] In this study, the lowest progesterone concentration found in females on a OCP that could be taken by a participant in this study was 0.5 ng/mL as measured by ELISA (Elliott-Sale 2013). The reference intervals for free testosterone among females on OCPs is between 0.8655 pg/mL and 7 pg/mL (Pesant 2012). Thus, it was be possible for testosterone to fall below the LLOQ of this assay.

Aim IB Measurement of Participant Serum Hormone Levels

Analytical Approach

The same analyses performed on the data (calibration curve goodness of fit, intra-assay precision and accuracy, mass accuracy) from Aim IA- *Post-Proposal UPLC-MS Assay* were used to confirm of the robustness of the assay used to measure hormone levels in this study's participants. Within each sample, chromatographic peaks in the XICs that were outside the temporal boundaries set by the assay's calibrators were ignored. Recovery percentages for each analyte were assumed to be the same between the two assays. Participant hormone concentrations would be calculated inserting the peak area of each participant sample in the regression equation for each hormone's calibration curve. Sample calculations can be found in Appendix B (Equation 10)

Aim IC Qualitative Analysis of Participant Samples Containing 17β-Estradiol

Analytical Approach

Confirmation of the presence of 17β-estradiol in Participant samples 10-16 required the following:

1. Data from the analytical standard of 17β-estradiol
   - A mass spectrum of the standard containing the molecular precursor ion, and any adducts, or fragments produced by the MS/MS experiment[15]
   - A mass spectrum of the MS/MS experiment displaying the fragmentation pattern of the analytical standard
   - An XIC displaying the retention time of the analytical standard and any fragments or adducts it produces MS/MS experiment.
2. The following data from Participant 16
   - A mass spectrum containing molecular ions that match that of the standard

[15] An **adduct** is an ion formed when another ion is added to the precursor ion in the mass spectrometer. Common adducts involve the addition of sodium, ammonium, and potassium ions, which may come from additives in the mobile phase, glassware, or other parts of the instrument (Kruve 2017). A **fragment** is simply a smaller molecular ion that breaks off the precursor ion because of the energy of the ionization source or due to high energy collisions that are part of MS/MS experiments.

- A mass spectrum of the MS/MS experiment that displays the same fragmentation pattern of the analytical standard.
- An XIC that displays the same retention time of the analytical standard and any fragments or adducts produced by the MS/MS experiment

Aim II Examination of the Relationship Between Pill and Serum Pharmacological Parameters

Statistical Analysis for Aim II: Bivariate correlations between pill and serum potency as well as pill and serum androgenicity were performed. Statistical analyses were performed using SPSS (version 21, IBM, Chicago).

Aim III: Examination of the Relationship Between Pill and Serum Pharmacodynamic Variables and Anterior Knee Laxity during On Pill and Off Pill Weeks

Statistical Approach for Aim III: Bivariate correlations were used to compare left (L) and right (R) AKL during Pill week three and the change in L and R AKL (Δ) from week 3 to week 4 to the pill and serum pharmacodynamic variables. Delta scores were calculated by subtracting each knee's week 3 AKL value from its week 3 value (AKL Week 4 – AKL Week 3). Statistical analyses were performed using SPSS (version 21, IBM, Chicago)

CHAPTER IV: RESULTS

Subject Demographics

Sixteen recreationally active women completed the study (Age: 21.8 ± 2.7 years, Weight: 61.8 ± 14 kg, Height: 163.8 ± 7.9). Of the participants who were recruited, three had injuries to the ligaments on at least one limb and therefore their data for that limb was not included. Demographic data can be found in Table 4.

Table 4 Participant Demographics

Participant	Age	Mass	Height	Primary Form of Physical Activity
1	21	60	155	Recreational Strength training
2*	18	60	169	CrossFit, Varsity High School Softball
3	19.83	64	161	Recreational Running
4	26.67	40	158	Recreational Cycling and Strength Training
5	21.67	72	158	Recreational Tennis
6	25.92	97	188	Recreational Strength training
7*	24.25	69	164	Recreational running and strength training
8	20.41	48	156	Recreational strength training and dance
9*	18.33	57	158	Recreational Basketball and Volleyball
10	21.67	53	165	Varsity Collegiate Volleyball
11	19.41	61	166	Recreational Running and Strength Training,
12	21.91	52.2	162	Recreational Flag Football
13	21.92	50	164	Recreational Basketball, Volleyball
14	21.83	85	168	Recreational Strength Training
15	19.75	60	169	Recreational Strength Training
16	26.58	60	159	General Strength Training

***participants with ACL injury (Participants 2 and 9 – left; Participant 7- right)**

Table 5 Participant OCP Data

Participant	OCP Brand	Years of OCP Use	Collection Order by Pill Week	Day of Week Three Visit (Pill Day)	Day of Week Four Visit (Pill Day)	Time Between Pill Ingestion and Blood Draw (hours) Week Three Only
1	Lessina 1/20	2	4	20	26	9.75
2*	Tri-Lo-Sprintec	4	3	19	26	9
3	Tri-Lo-Marzia	2.83	4	18	27	12
4	Cryselle	6.67	3	20	26	2.5
5	Sprintec	5.67	3	19	26	9.5
6	Tri-Previfem	2.5	4	19	27	13
7*	Trisprintec	5.83	3	19	26	9
8	Sprintec	4.41	4	19	26	12
9	Tri-Femynor	1.08	3	20	27	9
10	Sprintec	3.42	3	21	28	11
11	Ortho-Tri-Cyclen	3.41	4	19	28	11
12	Cryselle	5.91	3	21	28	9.5
13	Junel 1/20 Fe	3.92	4	19	26	19
14	Junel 1/20 Fe	4.63	3	19	26	11
15	Junel 1/20 Fe	3.75	4	20	25	12
16	LoLoestrin Fe	11.58	3	19	26	10.5

Examination of both the pill and serum PDV data made it clear that two groups existed within the sample that was obtained. One group, Participants 1-12 (N= 12) used LNG, norgestrel, or norgestimate, progestins that would take the biologically active form of LNG once metabolized in the liver (Kuhl 2005). This group was characterized by 4.2 ± 0.18 mg NET potency and 0.62 ± 0.3 mg NET androgenicity. A second group, participants 13-16 (N= 4) that used norethindrone-acetate (NET-A), which was metabolized to NET. This group was characterized by 1.2 mg NET potency and 0.6 mg NET androgenicity. Additionally, two participants (4 and 13) had substantially higher hormone concentrations than other participants. For one of these participants, Participant 4, this was likely due to ingesting her pill in the morning hours (just a few hours before testing) compared to the majority of participants who ingested their pills at bedtime. It is unknown why the other participant would have high values.

Therefore, the results for Aim 2 and 3 were presented in the following manner:

- Primary Analysis- All Participants (N = 16)
- Secondary Analyses
    - Levonorgestrel (LNG) Group (N = 12)
    - NET Group (N=4)

Aim I Development of a Modified UPLC-MS Method for the Quantitation of Levonorgestrel, Norethindrone, Testosterone, and Progesterone

Aim IA UPLC-MS Method Development Study

Objective I of UPLC-MS Method Development

Both analytes failed to ionize in negative mode but ionized in positive mode. Both standards were found within a 5-ppm mass tolerance of their theoretical *m/z* values (Figure 4 and Figure 5). The retention time for NET was 4.75 minutes and 5.49 minutes for LNG thus confirming sufficient chromatographic peak separation between NET and LNG (Figure 6)

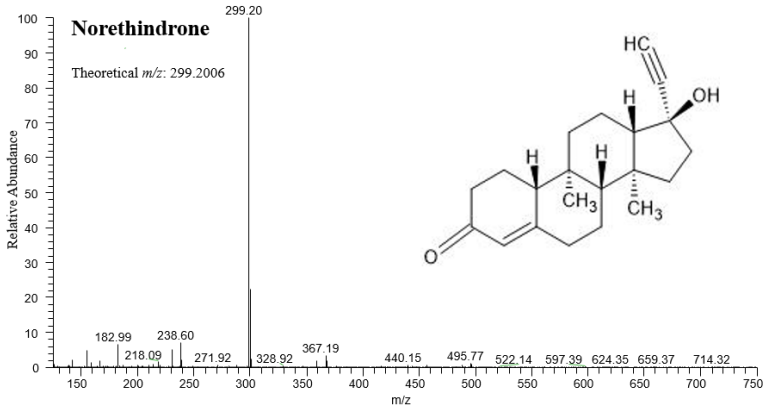


Figure 4 Mass Spectrum for NET

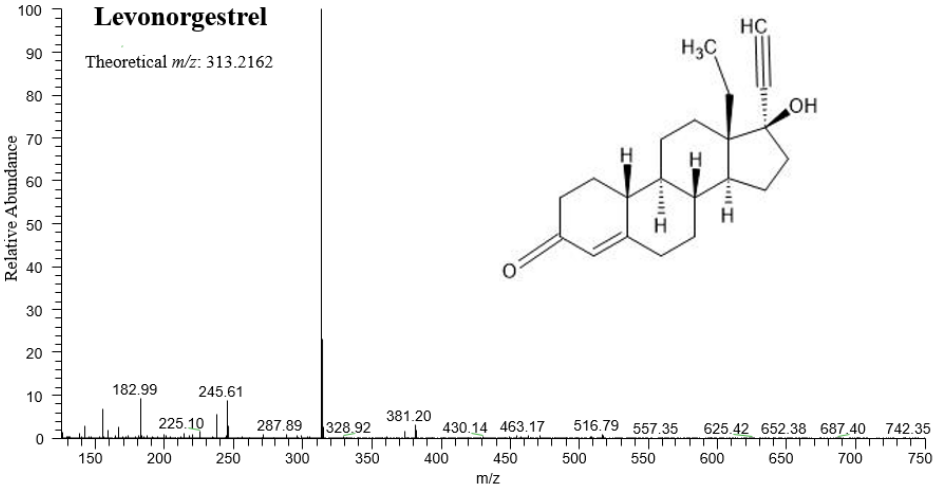


Figure 5 Mass Spectrum for LNG

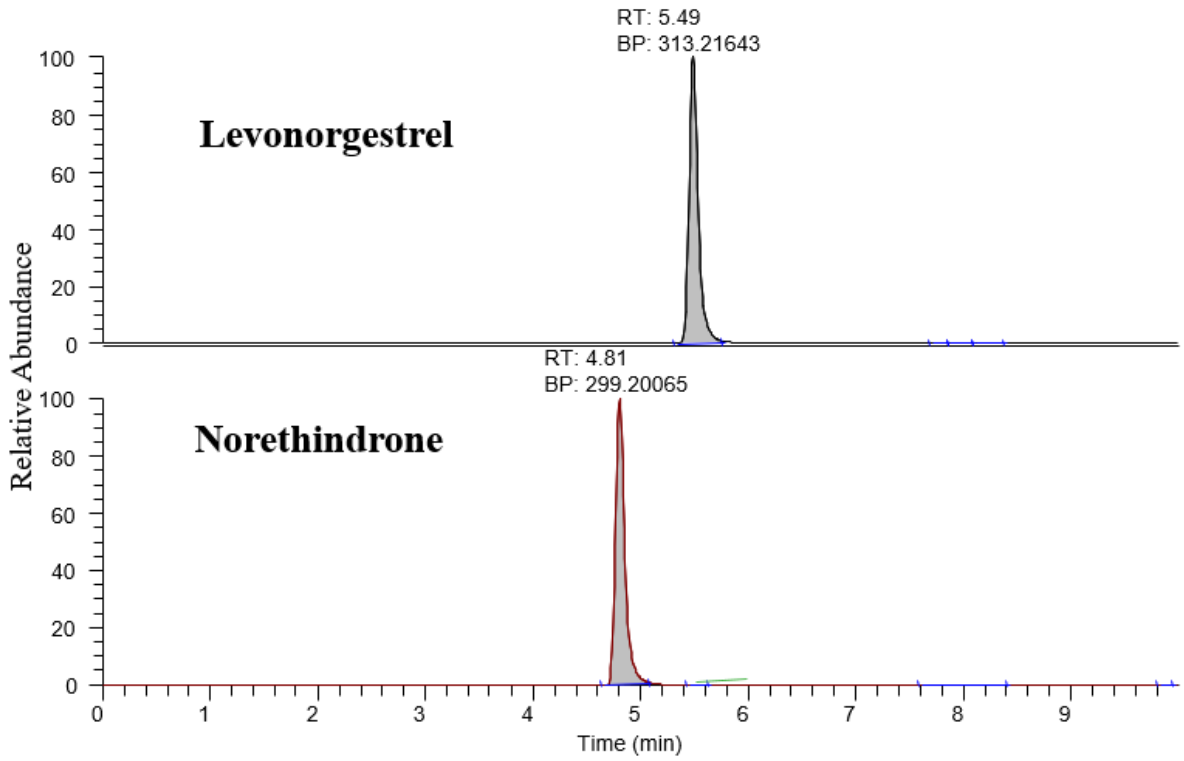


Figure 6 Extracted Ion Chromatograms (XICs) for LNG and NET

Objective II of UPLC-MS Method Development

The calibration curves produced by the runs of both LNG and NET were linear between 0.25 and 250 ng/mL (r^2 = 0.9917-0.9993) (Figure 7-Figure 8). None of the zero calibrators produced a signal for an XIC of *m/z* 299.2006 or 313.2162. Therefore, it was reasonable to believe the 0.25 ng/mL calibrator, which had the lowest concentration was detectable.

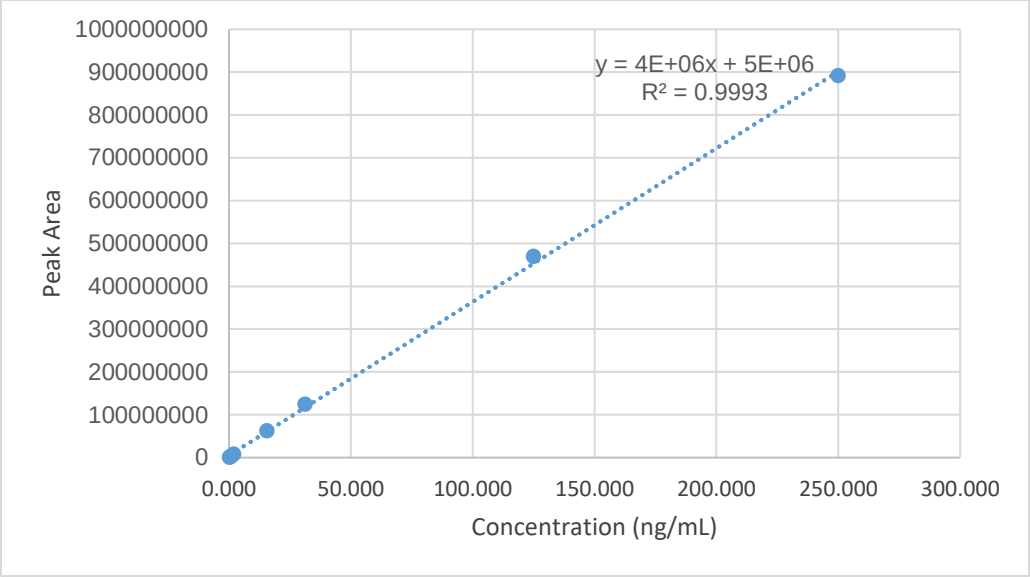


Figure 7 Calibration Curve for NET (Aim IA Objective II)

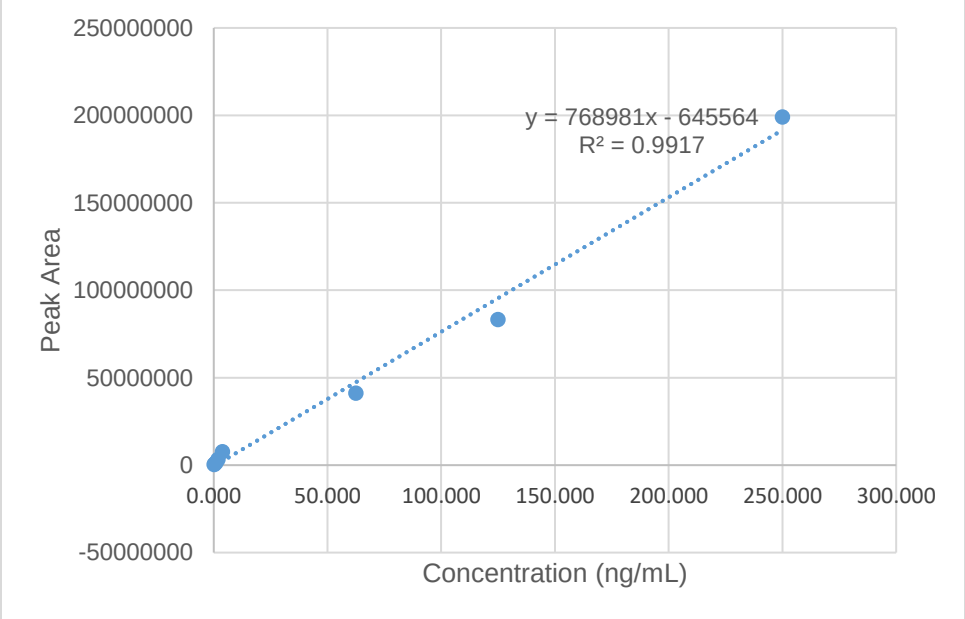


Figure 8 Calibration Curve for LNG (Aim IA Objective II)

Objective III of UPLC-MS Method Development

The % recoveries for LNG and NET were 69.3% and 182.0% respectively (Table 6). Though the recovery for LNG was acceptable, the recovery for NET was much greater that what should have been expected. The %CVs for the replicates were 11% and 5% for LNG and NET respectively (Table 6).

Table 6 Recovery Experiment Results Aim IA-Pre-Proposal

Analyte	Expected Concentration (ng/mL)	Injection 1 Peak Area	Injection 2 Peak Area	Average Peak Areas	Peak Area Subtracted from Blank	St. Dev	%CV	Measured Concentration	% Recovery
Blank	0	ND	946	946	N/A	N/A	N/A	N/A	N/A
LNG	31.5	1317530	1133885	1225708	1224762	19856.9	11%	21.65	69.30%
Blank	0	124032	76232	100132	N/A	N/A	N/A	N/A	N/A
NET	31.5	56507510	52554926	54531218	54431086	2794899.3	5%	59.20	189.44%

Post Proposal UPLC-MS Assay

Mass accuracy was within 5 ppm mass error all analyte's theoretical molecular ion *m/z* values. Retention times within the calibrators for all analytes were within ±0.01 seconds for all injections.

The r^2 values for the calibration curves for all quantifiable analytes ranged from 0.9996 to 1. Intra-assay precision ranged from 2% to 14% for all analytes intra-assay accuracy ranged from -2% and 4% (Table 7). The LLOQs ranged between 0.49 ng/mL and 3.19 ng/mL An XIC for a 250 ng/mL calibrator can be found in Appendix B: Figure . Full calibration curves and raw data tables can be found in Table B.2- Table B.6.

Finally, the percentage of each hormone recovered from serum for all analytes, except for E_2 ranged from 18.60%- 39.5%. The precision of the measurements for all technical replicates 1.4% to 6.4). An XIC for the serum blank for the extraction (Figure B.5), and a technical replicate for the extraction (Figure B.6) can be found in Appendix B: Aim I-Aim IB Additional Figures. On average, the sample preparation method used in Aim IA-Post-Proposal Assay concentrated participant serum by a factor of 40.

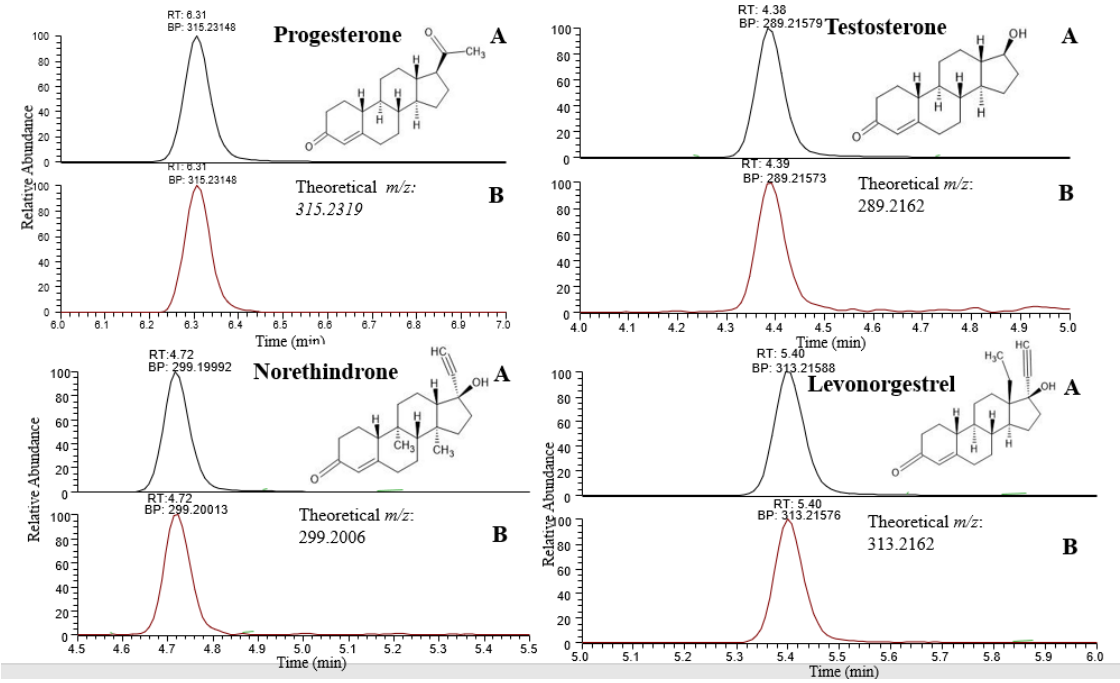


Figure 9 Extracted Ion Chromatograms (XIC) For Progesterone, Testosterone, Norethindrone, and Levonorgestrel

Image A represents the XIC for the 250 ng/mL calibrator. Image B represents the XIC for Technical Replicate 1. RT- Retention Time, BP- Base Peak

Table 7 Limits of Detection and Quantitation, Retention Time, % CVs, Accuracy, and % Recovery for All Analytes Post Proposal UPLC-MS Run

Analyte	LLOD (ng/mL)	LLOQ (ng/mL)	Retention Time (min)	%CV	% Relative Error	Average Recovery ± %CV
E2	65	65	3.79	14%	-2%	N/A
P4	0.03	0.49	6.30-6.31	2%	-1%	37.28 ± 1.8%
T	0.24	0.24	4.38-4.39	5%	-4%	24.9 ± 6.1%
NET	0.24	3.91	4.71-4.72	11%	4%	26.4 ± 1.3%
LNG	0.49	0.98	5.40-5.41	3%	-3%	35.61 ±1.4%

Aim IB Measurement of Participant Serum Hormone Levels

Mass accuracy was within 5 ppm mass error all analyte's theoretical molecular ion *m/z* values. Retention times for all analytes ranged between 0.02-0.05 seconds for all replicates (

Table 8).

The r^2 values for the calibration curves for all quantifiable analytes ranged from 0.9997 to 1. Intra-assay accuracy ranged between -8% and 20% for all analytes. Lower limits of quantitation ranged between 0.39 ng/mL and 1.49 ng/mL (Table 8). The calibration curves prepared for this sub-aim (Table B.10 – Table B.13), and the calculations used to determine serum hormones concentrations, which account for the concentration of each sample and each hormone's % recovery can be found in Appendix B Equation 10.

Participant derived serum progestin concentrations can be found in Table 9

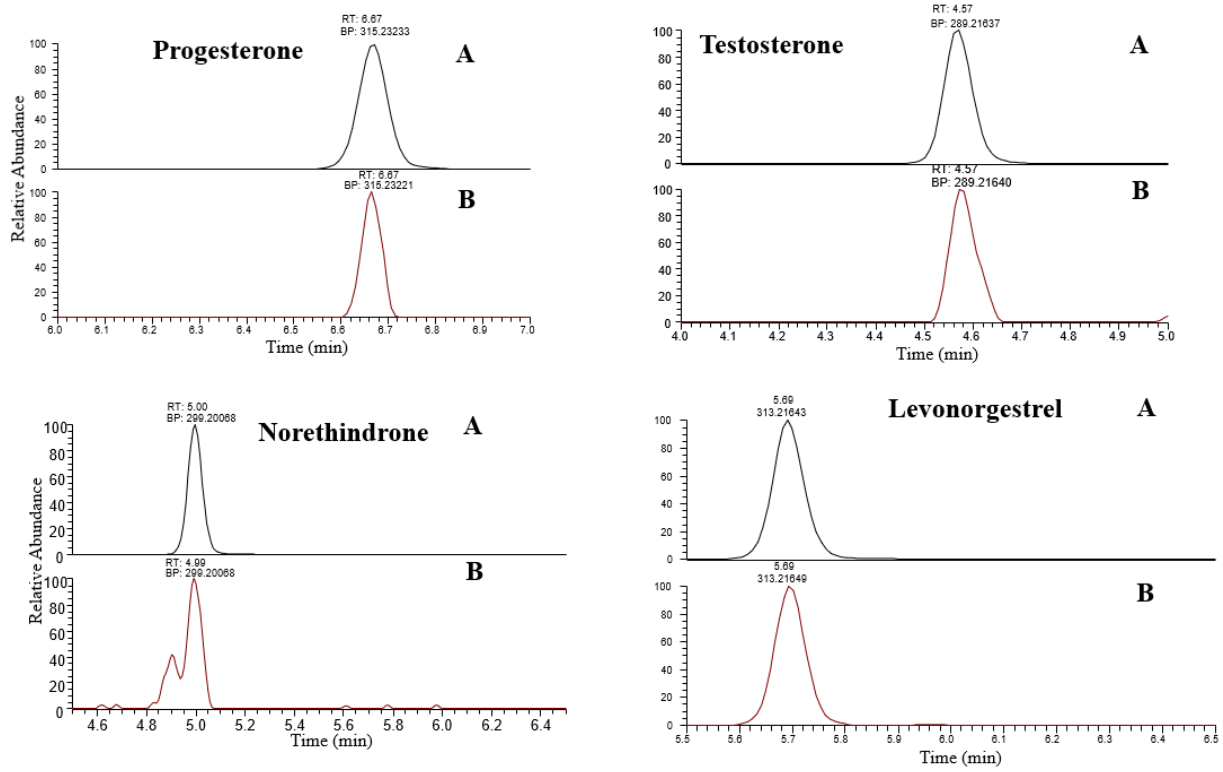


Figure 10 Extracted Ion Chromatograms for Progesterone, Testosterone, Norethindrone, and Levonorgestrel

Images from 250 ng/mL calibrator (A) above participant samples from Pill Week 3 (B). Example XICs for progesterone, testosterone and levonorgestrel came from Participant 1, and the example XIC for norethindrone came from Participant 13.

Table 8 Limits of Detection and Quantitation, Accuracy, and Precision for All Analytes (Aim IB)

Analyte	LLOD (ng/mL)	LLOQ (ng/mL)	Retention Time (min)	%CV	RE%
P4	0.10	0.78	6.64-6.69	20%	-1%
T	0.05	0.39	4.55-4.57	14%	1%
NET	0.24	0.98	4.96-5.00	6%	1%
LNG	0.24	1.94	5.64-5.69	8%	-3%

Images from 250 ng/mL calibrator (A) above participant samples from pill week three (B). Example XICs for progesterone, testosterone and levonorgestrel came from Participant 1, and the example XIC for norethindrone came from Participant 13.

Table 9 Participant Hormone Concentrations Measured by UPLC-MS [Condensed Table] (Aim IB)

	Progesterone				Testosterone			
	Pill Week Three		Pill Week Four		Pill Week Three		Pill Week Four	
Participant	Measured concentration (ng/mL)	Concentration in serum (pg/mL)	Measured concentration (ng/mL)	Concentration in serum (pg/mL)	Measured concentration (ng/mL)	Concentration in serum (pg/mL)	Measured concentration (ng/mL)	Concentration in serum (pg/mL)
1	1.14	2.37	ND	ND	2.37	16.29	2.58	16.76
2	<LOQ	<LOQ	ND	ND	ND	ND	11.00	71.61
3	ND	ND	ND	ND	3.37	21.38	17.33	160.48
4	1.29	1.72	ND	ND	1.72	12.93	10.57	121.18
5	<LOQ	<LOQ	<LOQ	<LOQ	3.50	22.78	6.54	58.33
6	ND	ND	ND	ND	ND	ND	2.23	19.88
7	ND	ND	ND	ND	ND	ND	7.94	51.69
8	<LOQ	<LOQ	ND	ND	ND	ND	9.57	62.27
9	ND	1.98	ND	ND	1.98	19.11	16.07	193.46
10	1.72	18.90	ND	ND	18.90	122.99	7.75	50.42
11	<LOQ	<LOQ	ND	ND	25.99	169.13	8.82	88.52
12	ND	12.59	ND	ND	12.59	81.95	ND	ND
13	ND	20.47	ND	ND	20.47	224.04	3.55	35.61
14	<LOQ	<LOQ	ND	ND	10.86	104.56	5.63	50.25
15	<LOQ	<LOQ	<LOQ	<LOQ	ND	ND	9.91	72.31
16	<LOQ	<LOQ	ND	ND	ND	ND	ND	ND

	Norethindrone				Levonorgestrel			
	Pill Week Three		Pill Week Four		Pill Week Three		Pill Week Four	
	Measured concentration (ng/mL)	Concentration in serum (pg/mL)	Measured concentration (ng/mL)	Concentration in serum (pg/mL)	Measured concentration (ng/mL)	Concentration in serum (pg/mL)	Measured concentration (ng/mL)	Concentration in serum (pg/mL)
1	ND	ND	ND	ND	22.34	181.79	ND	ND
2	ND	ND	ND	ND	10.83	128.49	2.37	18.21
3	ND	ND	ND	ND	16.99	127.31	9.09	99.52
4	ND	ND	ND	ND	93.16	829.16	ND	ND
5	ND	ND	ND	ND	27.14	208.94	7.56	79.71
6	ND	ND	ND	ND	11.44	85.72	<LOQ	<LOQ
7	ND	ND	ND	ND	9.99	72.97	<LOQ	<LOQ
8	ND	ND	ND	ND	21.30	163.96	2.04	15.68
9	ND	ND	ND	ND	19.52	222.36	ND	ND
10	ND	ND	ND	ND	25.20	194.00	<LOQ	<LOQ
11	ND	ND	ND	ND	44.14	339.74	ND	ND
12	ND	ND	ND	ND	27.04	208.13	ND	ND
13	150.38	1432.74	ND	ND	ND	ND	ND	ND
14	90.68	760.30	ND	ND	ND	ND	ND	ND
15	82.57	665.61	ND	ND	ND	ND	ND	ND
16	31.73	255.76	ND	ND	ND	ND	ND	ND

*(ND= Not Detected, <LOQ= Analyte detected lower than LLOQ)

Measured Concentration- Concentration, measured in which was sampled in an 80 µL UPLC vial. Concentrated about 40x by sample preparation process. Expanded tables that include the peak areas of the MS experiment can be found in Appendix B

Aim IC Qualitative Analysis of Participant Samples Containing 17β-Estradiol

The retention time of the 273.18 *m/z* E_2 standard was 3.80 minutes, with a source fragment that at *m/z* 255. 16, likely due to the removal of water during ionization (Figure 11: Images A and B). The mass spectrum also shows a sodium adduct at *m/z* 309.15 found in greater abundance than the 273.16 m/z E_2 molecular ion (Figure 11; Image C) The mass spectrum of the fragmented estradiol molecular ion produced fragments at *m/z* 135.04, 159.01, 173.07 and 255.15. These fragments, (134.99,159.09, 173.08, 255.19) were found at low abundance in the pill week three sample from Participant 16. When a time delay of 0.27 seconds was applied to the experiment 3 XIC for the *m/z* 273.16 mass for participant[16], it overlapped the chromatogram from the m/z 273.16 from the same participant's XIC produced under Aim IB (Figure 13). Finally, the 255.19 *m/z* source fragment eluted from the column at the same time as the 273.16 *m/z* precursor ion in the assay run under Aim IB (Figure 14). Interestingly, this same fragment appeared in the Aim IB XICs of Participants 10-15. Table 10 lists the obtained estradiol serum hormone concentrations for all participants.

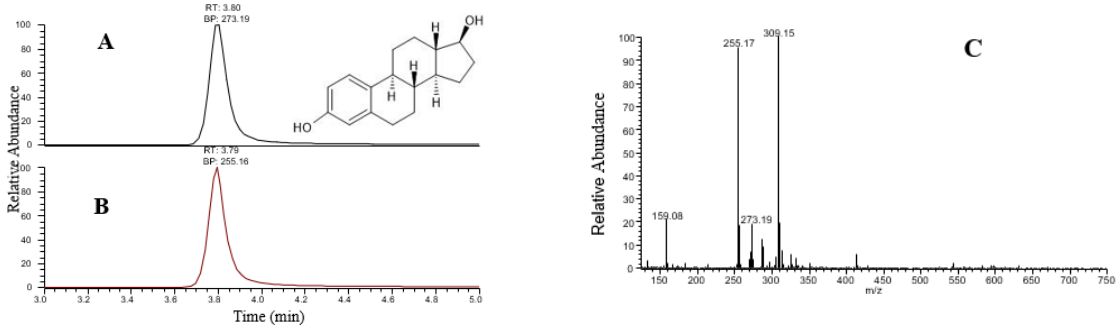


Figure 11 XIC and Mass Spectrum for 250 µg/mL E2 standard

A. XIC for the molecular ion; B. XIC for estradiol source fragment (a fragment produced by the electrospray source); C. The mass spectrum for the estradiol standard

[16] This time delay was applied due to a change in LC columns between the time Aim IB and Aim IC were carried out. The LC column used in Aim IB was rendered useless due to improper sample prep during piloting. A new biphenyl column, which was also used in the Aim IA-Post Proposal Assay, shifted analyte retention times down by ~0.27 seconds.

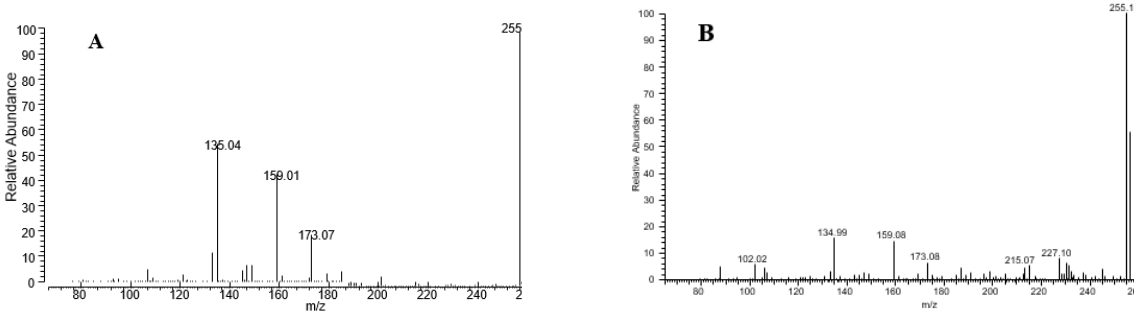


Figure 12 Mass Spectra for MS/MS Experiment

A. Fragmentation pattern of 250 µg/mL E_2 Standard. B fragmentation pattern of m/z 273.18 molecular ion Participant 16, Week 3

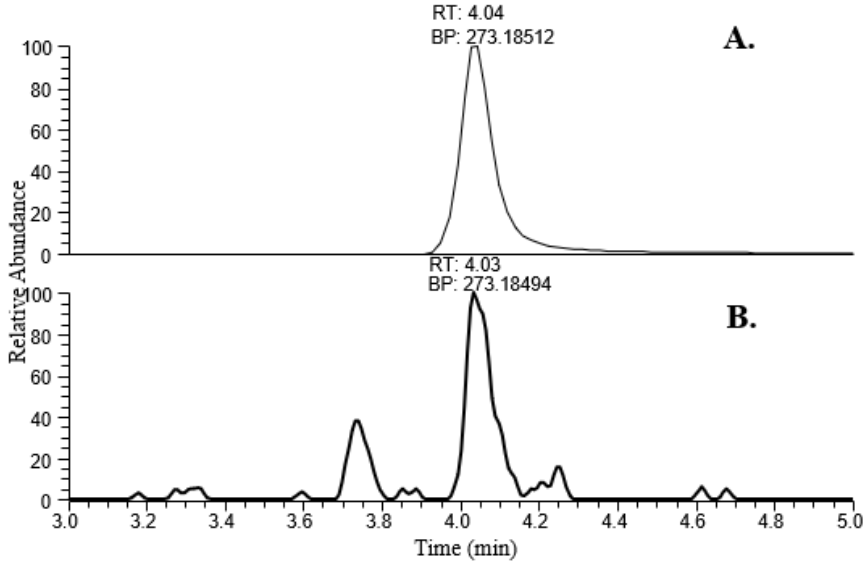


Figure 13 XICs for Participant 16, Week 3

Image A. is a depiction of the XIC for the participant from Aim IC.
Image B depicts the same participants' XIC from Aim IB

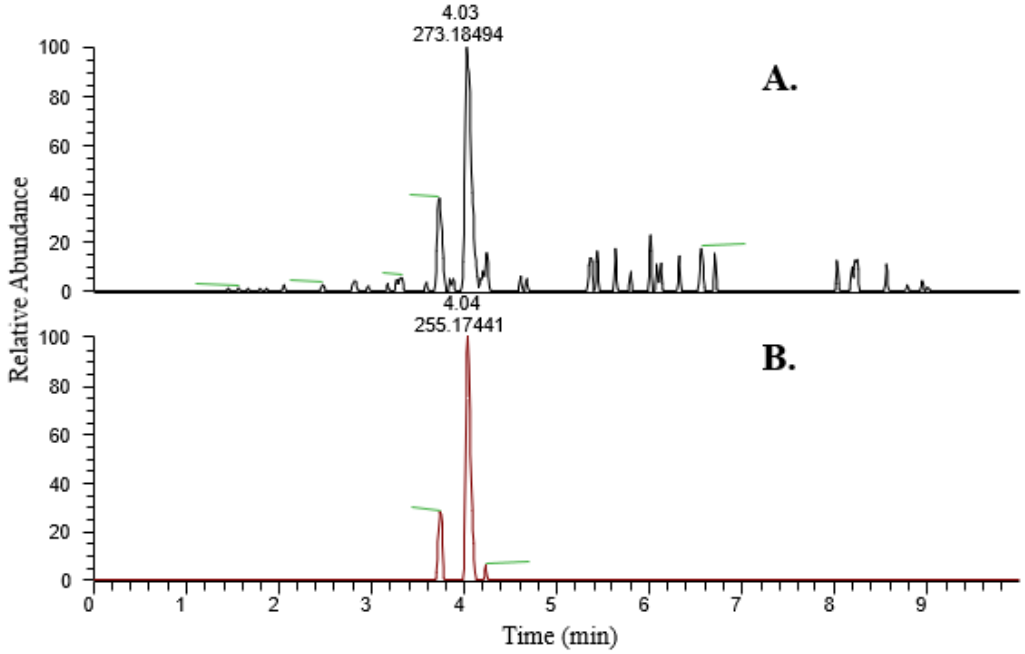


Figure 14 Estradiol XIC for Participant 16, Week 3 from Sub-Aim 2

Image A is the original peak for molecular ion with *m/z* 273.185. Image B is a depiction of the source fragment.

Table 10 Relative Estradiol Amounts in All Subjects (Aim IC)

	Pill Week Three				Pill Week Four			
Participant	Initial Serum Volumes (mL)	Peak Area	Peak Area (Normalized to 1 mL serum)	Normalized Peak Area x 10000	Initial Serum Volume	Peak Area	Peak Area (Normalized to 1 mL serum)	Normalized Peak Area x 10000
1	3.5	ND	ND	ND	3.7	490665	132612	13.26
2	2.4	ND	ND	ND	3.7	439588	118808	11.88
3	3.8	ND	ND	ND	2.6	361156	138906	13.89
4	3.2	ND	ND	ND	2.1	400305	190621	19.06
5	3.7	ND	ND	ND	2.7	ND	ND	ND
6	3.8	ND	ND	ND	2.7	117038	43347	4.33
7	3.9	ND	ND	ND	3.7	324546	87715	8.77
8	3.7	ND	ND	ND	3.7	147216	39788	3.98
9	2.5	ND	ND	ND	2	513656	256828	25.68
10	3.7	546920	147816	14.78	3.7	260587	70429	7.04
11	3.7	479015	129464	12.95	2.4	320215	133423	13.34
12	3.7	411625	111250	11.13	3.7	637925	172412	17.24
13	2.2	330636	150289	15.03	2.4	133141	55475	5.55
14	2.5	330636	132254	13.23	3.7	ND	ND	ND
15	2.6	226870	87258	8.73	2.5	319863	127945	12.79
16	2.6	311264	119717	11.97	2.4	792370	330154	33.02

ND= Analyte not Detected

Aim II Examination of the Relationship Between Pill and Serum Pharmacological Parameters

Tables 11-13 list the individual participant pill and serum pharmacokinetic and pharmacodynamic parameters. When all participants were examined together, there was a positive, but non-significant association between pill potency and peak serum potency (Table 14 and Figure 15 A) and a significant positive association between pill androgenicity and peak serum androgenicity (Figure 16 A). It is important to note the peak serum PDV values from Participants 4 and 13, influenced the strength of these associations (Figure 15). However, these values were not removed since they were not statistical outliers.

Similar findings occurred among participants within the LNG only group (N=12). Within this group there were significant moderate associations between pill potency and serum potency (Figure 15 C) and between pill androgenicity and serum androgenicity (Figure 16 C).

Correlations between pill and serum PDVs could not be made among the participants in whose metabolized progestin was NET due to a lack of variability in the pill PDV data among them.

Table 11 Participant Pill Dose and Metabolism Data

Participant	OCP Brand	Phase	Pill Progestin	Progestin Dose (mg)	Metabolized Progestin	Original Group Designation
1	Lessina 1/20	M	LNG	0.1	LNG	Low E, Low P, Low A
2	Tri-Lo-Sprintec	T	NGM	0.25	LNG	Low E, Low P, Low A
3	Tri-Lo-Marzia	T	NGM	0.25	LNG	Low E, Low P, Low A
4	Cryselle	M	NG	0.3	LNG	High E, Low P Low A
5	Sprintec	M	NGM	0.25	LNG	High E, Low P Low A
6	Tri-Previfem	T	NGM	0.25	LNG	High E, Low P Low A
7	Trisprintec	T	NGM	0.25	LNG	High E, Low P Low A
8	Sprintec	M	NGM	0.25	LNG	High E, Low P Low A
9	Tri-Femynor	T	NGM	0.25	LNG	High E, Low P Low A
10	Sprintec	M	NGM	0.25	LNG	High E, Low P Low A
11	Ortho-Tri-Cyclen	T	NGM	0.25	LNG	High E, Low P Low A
12	Cryselle	M	NG	0.3	LNG	High E, Low P Low A
13	Junel 1/20 Fe	M	NET-A	1	NET	Low E, High P, Low A
14	Junel 1/20 Fe	M	NET-A	1	NET	Low E, High P, Low A
15	Junel 1/20 Fe	M	NET-A	1	NET	Low E, High P, Low A
16	LoLoestrin Fe	N	NET-A	1	NET	Low E, High P, Low A

The term “pill progestin” refers to the progestin contained into the pill taken by a given subject. It does not consider the metabolism of the pill

The term “metabolized progestin” refers to an active metabolite of the progestin contained in a subject’s OCP, which was detected by UPLC-MS in Aim 1.

M= monophasic; T= Triphasic LNG= Levonorgestrel, NG= Norgestrel, NGM= Norgestimate, NET-A- Norethindrone Acetate, NET- Norgestimate

Table 12 Pill Pharmacokinetic Parameters

Participant	T_{max} (hours)	$T_{1/2}$ (hours)	K_E (1/hour)	Literature Bioavailability	Measured Concentration by UPLC-MS	$T_{measured}$ (hours)	$T_{measured}$ - T_{max} (hours)	C_{peak} (pg/mL)	PK Reference
1	1.3 ± 0.4	25.4 ± 9.7	0.0252	100%	181.79	9.75	8.45	224.852	[38]
2	1.7 ± 1.2	36.4 ± 10.2	0.0176	100%	128.49	9	7.3	146.059	[40]
3	1.7 ± 1.3	36.4 ± 10.2	0.0176	100%	127.31	12	10.3	152.541	[37]
4	1.26 ± 0.35	39.09 ± 12.39	0.0199	100%	829.16	2.5	1.24	848.922	[12]
5	1.67 ± 1.32	45 ± 20.4	0.0142	100%	208.94	9.5	7.83	233.510	[40]
6	2.58 ± 2.97	40.2 ± 15.4	0.0159	100%	85.72	13	10.42	101.164	[44]
7	2.58 ± 2.97	40.2 ± 15.4	0.0159	100%	72.97	9	6.42	80.811	[41]
8	1.67 ± 1.32	45 ± 20.4	0.0142	100%	163.96	12	10.33	189.859	[40]
9	2.58 ± 2.97	40.2 ± 15.4	0.0159	100%	222.36	9	6.42	246.254	[45]
10	1.67 ± 1.32	45 ± 20.4	0.0142	100%	194.00	11	9.33	221.484	[50]
11	2.58 ± 2.97	40.2 ± 15.4	0.0159	100%	339.74	11	8.42	388.400	[43]
12	1.26 ± 0.35	39.09 ± 12.39	0.0199	100%	208.13	9.5	8.24	243.408	[12]
13	1.3 [0.7-4.0 hours]	8.4	0.0761	64%	1432.7	19	17.7	5507.175	[52]
14	1.3 [0.7-.0 hours]	8.4	0.0761	64%	760.30	11	9.7	1590.177	[52]
15	1.3 [0.7-4.0 hours]	8.4	0.0761	64%	665.61	12	10.7	1502.184	[52]
16	1.3 [0.7 -3.0 hours]	10	0.0639	64%	255.76	10.5	9.2	460.415	[39]

T_{max}= Time to C_{max} ; $T_{1/2}$ = Elimination half-life of the progestin, K_e elimination rate constant, $T_{measured}$= time from the pill ingestion to bloo draw.
[X] Indicates reference number

Table 13 Pill and Serum Pharmacodynamic Variables for All Participants

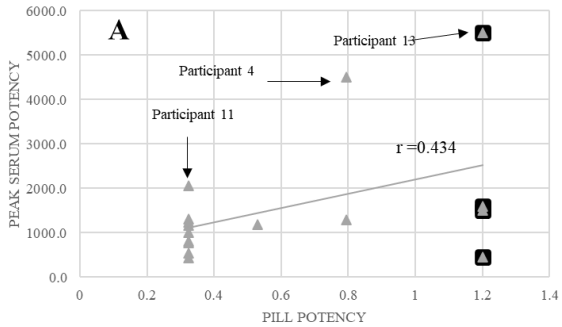
Participant	Pill Potency (mg NET)	Peak Serum Potency, Pill Week Three (pg/mL NET)	Pill Androgenicity (mg NET)	Peak Serum Androgenicity, Pill Week Three (pg/mL NET)
1	0.53	1191.72	0.83	1866.27
2	0.325	774.11	0.475	1212.29
3	0.325	808.47	0.475	1266.09
4	0.795	4499.29	1.245	7046.06
5	0.325	1237.60	0.475	1938.13
6	0.325	536.17	0.475	839.66
7	0.325	428.30	0.475	670.73
8	0.325	1006.25	0.475	1575.83
9	0.325	1305.14	0.475	2043.91
10	0.325	1173.86	0.475	1838.31
11	0.325	2058.52	0.475	3223.72
12	0.795	1290.06	1.245	2020.29
13	1.2	5507.17	0.6	5507.17
14	1.2	1590.18	0.6	1590.18
15	1.2	1502.18	0.6	1502.18
16	1.2	460.42	0.6	460.42

Table 14 Descriptive Statistics and Bivariate Correlations Between Pill and Serum Pharmacodynamic Variables

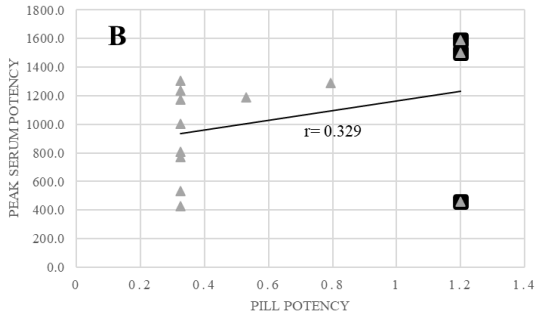
	Pill Potency (mg NET)	Peak Serum Potency (pg/mL NET)	Pearson Correlation	p-value	Pill Androgenicity (mg NET)	Peak Serum Androgenicity (pg/mL NET)	Pearson Correlation	p-value
All Participants (n=16)	0.62 ± 0.4	1585.6 ± 1414.6	0.434	0.093	0.62 ± 0.3	2162.6 ± 1753.6	**0.525***	**0.037**
LNG Group (n=12)	0.42 ± 0.18	1359.1 ± 1067.6	**0.645***	0.02	0.63 ± 0.30	2128 ± 1685.9	**0.642***	**0.02**
NET Group (n=4)*	1.20 ± 0	2264.99 ± 2221.5	N/A	N/A	0.60 ± 0	2264.99 ± 2221.5	N/A	N/A

*Correlations could not be made due to a lack of variability in the pill PDV data for this group

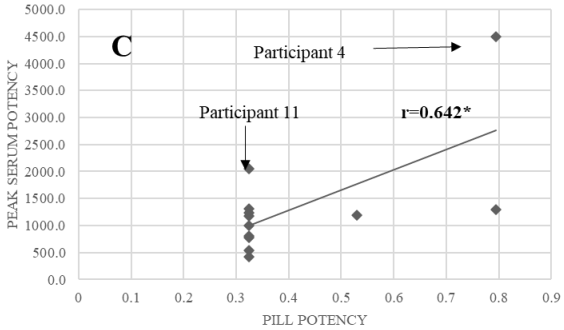
All Participants



All Participants Without Outliers



LNG Group



LNG Group Without Outliers

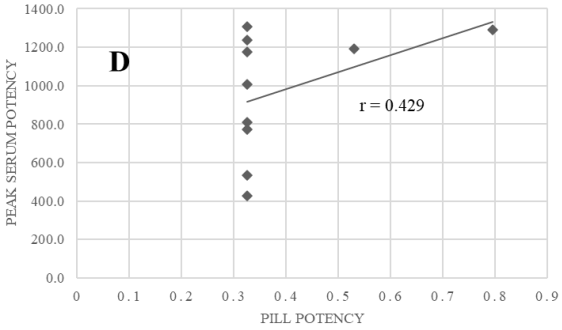


Figure 15 Scatterplots Displaying Associations Between Pill and Serum Potency

The units on pill potency are milligrams of Norethindrone (mg NET). The units on serum potency are pg/mL NET

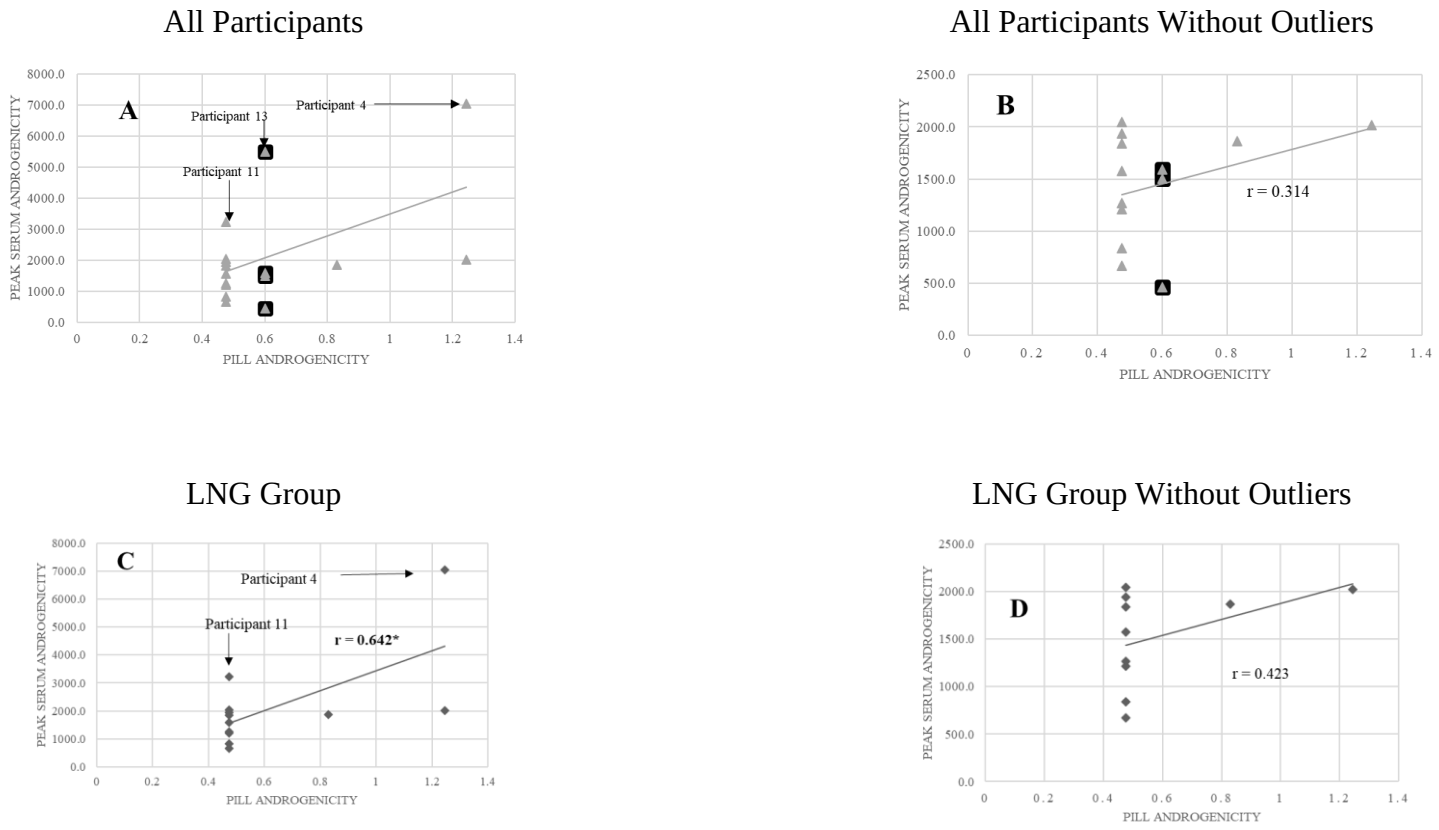


Figure 16 Scatterplots Displaying Associations Between Pill and Serum Androgenicity

The units on pill androgenicity are milligrams of Norethindrone (mg NET). The units on serum androgenicity are pg/mL NET

Aim III: Examination of the Relationship Between Pill and Serum Pharmacodynamic Variables and Anterior Knee Laxity during On Pill and Off Pill Weeks

Section I: Associations Between Pill and Serum Pharmacodynamic Variables and AKL during Pill Week Three

The following results are based on associations between the reported progestin concentrations (pill box) and AKL values when measured at week three. Due to prior history of knee injuries, there is no L AKL data for Participants 2 and 9 and no R AKL data for Participant 7 (Table 4). Therefore, results are based on a total of 14 knees for left leg analyses, and 15 knees for right leg analyses (Table 15)

Table 15 AKL Data by Participant

Participant	L AKL Pill Week Three (mm)	L AKL Pill Week Four (mm)	Δ L AKL (mm)	R AKL Pill Week Three (mm)	R AKL Pill Week Four (mm)	Δ R AKL (mm)	Injury Data
1	6.1	5.3	-0.7	3.4	2.2	-1.2	
2	No Data	No Data	No Data	1.8	2.2	0.4	Left Knee Injury
3	4.6	3.3	-1.3	3.9	3.9	0.1	
4	4.3	9.2	4.8	2.7	3.5	0.8	
5	2.7	3.2	0.5	4.9	1.9	-3.0	
6	5.4	6.1	0.7	3.3	6.2	2.9	
7	7.0	4.3	-2.7	No Data	No Data	No Data	Right ACL Injury
8	6.0	5.8	-0.2	5.0	2.6	-2.4	
9	No Data	No Data	No Data	4.1	5.0	0.9	
10	5.7	5.4	-0.3	4.1	3.6	-0.5	
11	5.9	5.5	-0.4	4.6	4.9	0.2	
12	4.7	2.7	-2.1	2.1	1.0	-1.1	
13	9.6	8.8	-0.8	7.0	8.9	1.9	
14	6.5	5.8	-0.8	4.5	5.1	0.6	
15	8.9	10.4)	1.5	5.7	8.6	3.0	
16	4.3	4.6	0.4	3.4	2.8	-0.6	

All Participants

There were no significant relationships between pill PDVs and AKL. Although not significant, pill potency tended to be positively correlated with AKL while pill androgenicity tended to be negatively correlated with AKL (Table 17 and Figure 16)

Table 16 Bivariate Correlations Between Pill Pharmacodynamic Variables and AKL for ALL PARTICIPANTS during Pill Week Three

	Pill Potency		Pill Androgenicity	
	r	**p-value**	**r**	**p-value**
L AKL (n=14)	0.439	0.12	-0.180	0.54
R AKL (n=15)	0.306	0.27	-0.437	0.27

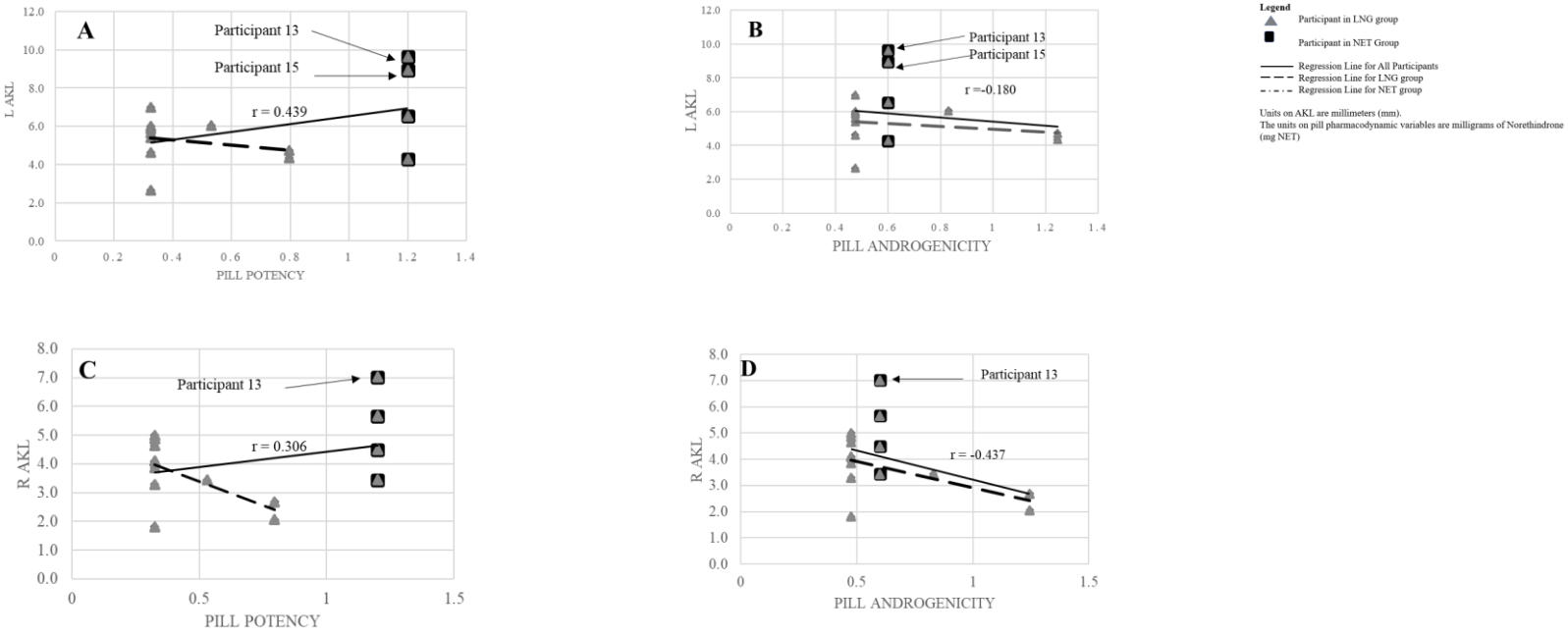


Figure 17 Scatterplots Displaying Associations Between Pill Pharmacodynamic Variables and AKL for ALL PARTICIPANTS during Pill Week Three

There were no significant correlations between serum PDVs and AKL. While not significant, both higher peak serum potency and higher peak serum androgenicity tended to be associated with greater AKL during the third pill week. (Table 17 and Figure 18)

Table 17 Bivariate Correlations Between Serum Pharmacodynamic Variables and AKL for ALL PARTICIPANTS during Pill Week Three

	Peak Serum Potency		Peak Serum Androgenicity	
	r	p-value	r	p-value
L AKL (n=14)	0.376	0.19	0.143	0.63
R AKL (n=15)	0.415	0.12	0.198	0.48

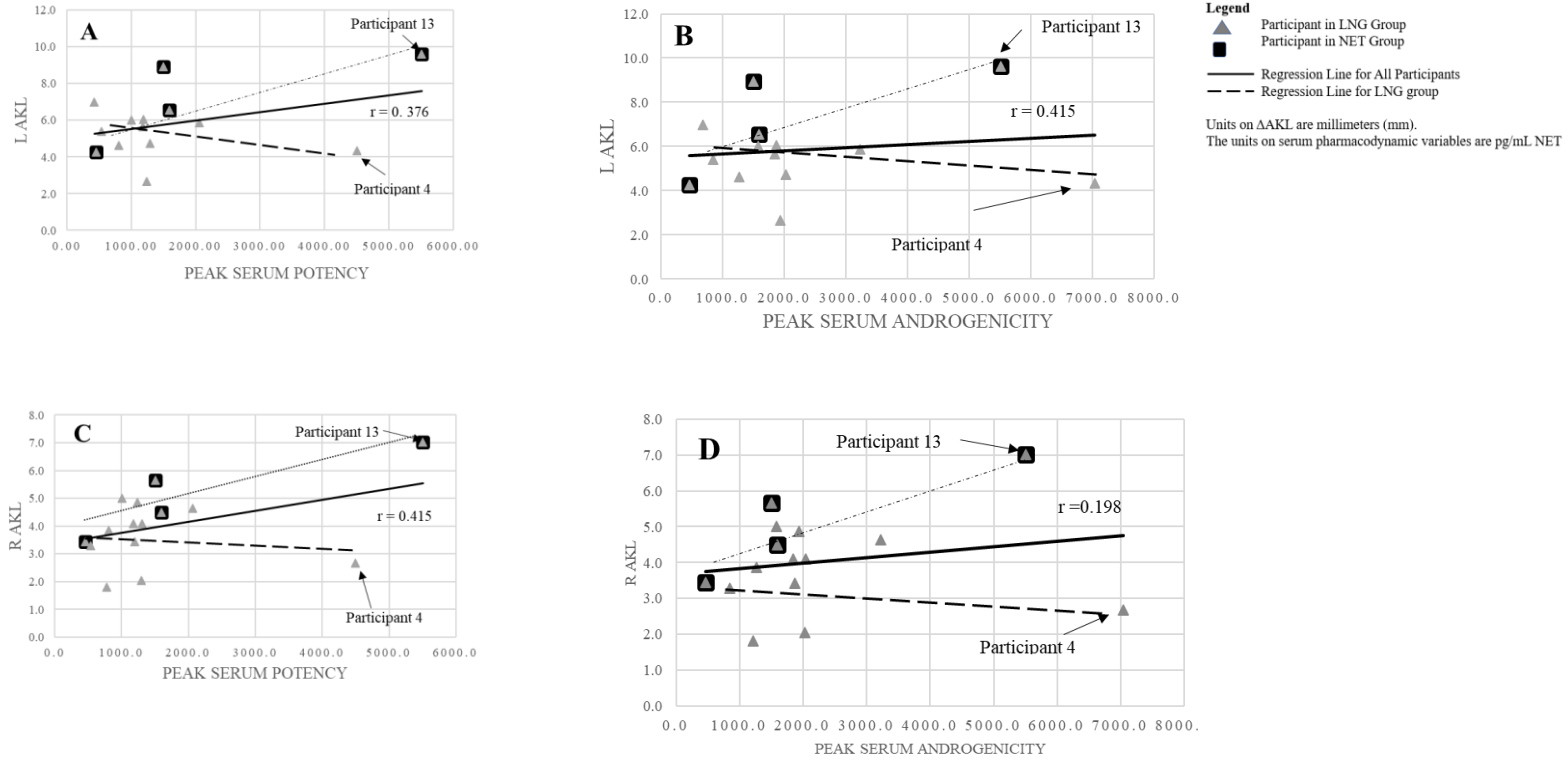


Figure 18 Scatterplots Displaying Relationships Between Serum Pharmacodynamic Variables and AKL for ALL PARTICIPANTS During Pill Week Three

LNG Group Only

In the LNG group, there were no significant relationships between greater pill potency and pill androgenicity with AKL (p-value range .057 to .586) (Table 18 and Figure 19) or with serum potency and androgenicity and AKL (p-value range .386 to .680) (Table 19 and Figure 20)

Table 18 Bivariate Correlations Between Pill Pharmacodynamic Variables and Week Three AKL Among the LNG GROUP

	Pill Potency	Pill Androgenicity
L AKL (n=9)	-0.227	-0.221
R AKL (n=11)	-0.587	-0.586

Table 19 Bivariate Correlations Between Serum Pharmacodynamic Variables and Week Three AKL Among the LNG GROUP

	Peak Serum Potency	Peak Serum Androgenicity
L AKL (n=9)	-0.309	-0.309
R AKL (n=11)	-0.141	-0.141

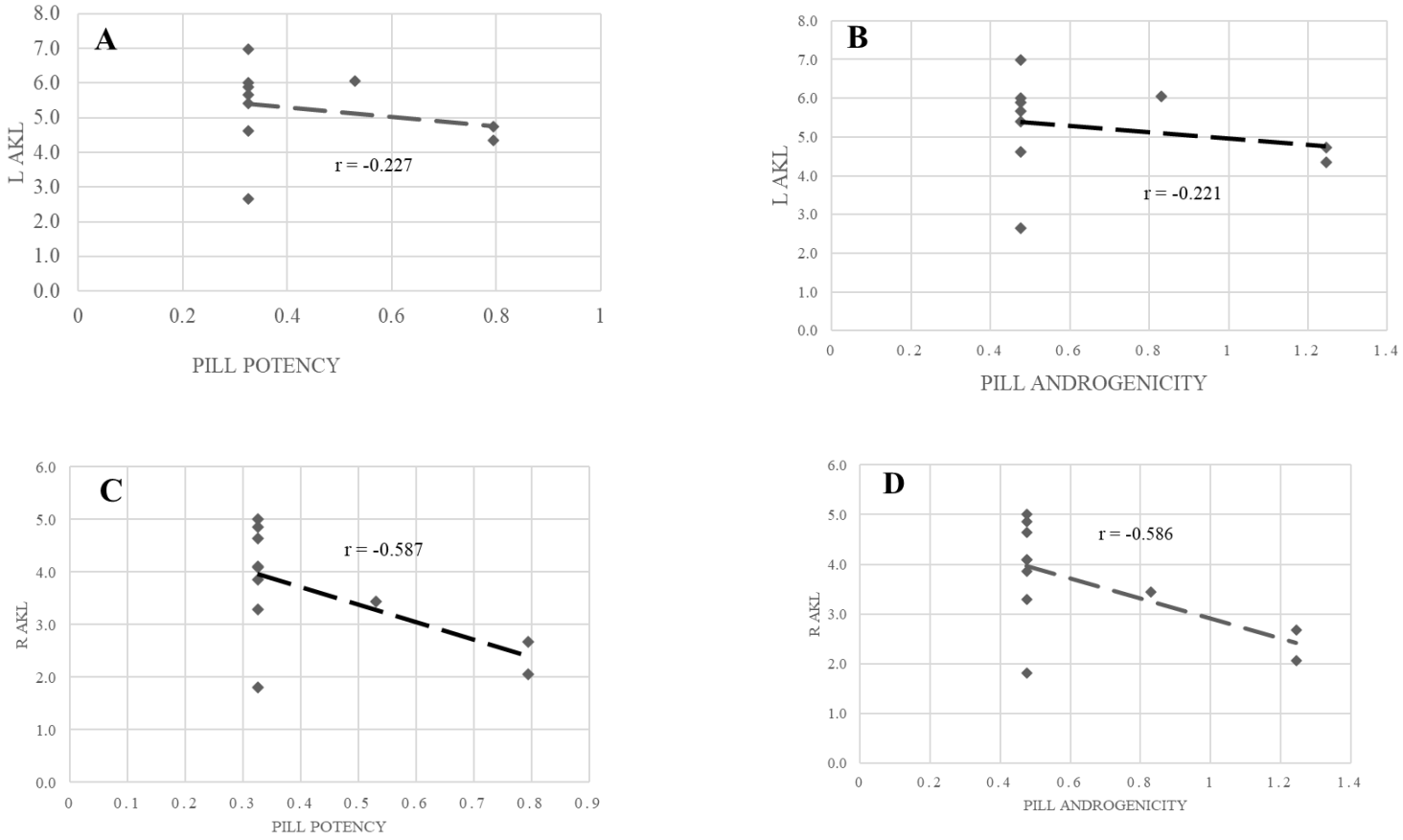


Figure 19 Scatterplots Displaying Relationships Between Pill Pharmacodynamic Variables and AKL in the LNG GROUP during Pill Week Three

The units on AKL are millimeters (mm). The units on pill pharmacodynamic are milligrams of Norethindrone (mg NET)

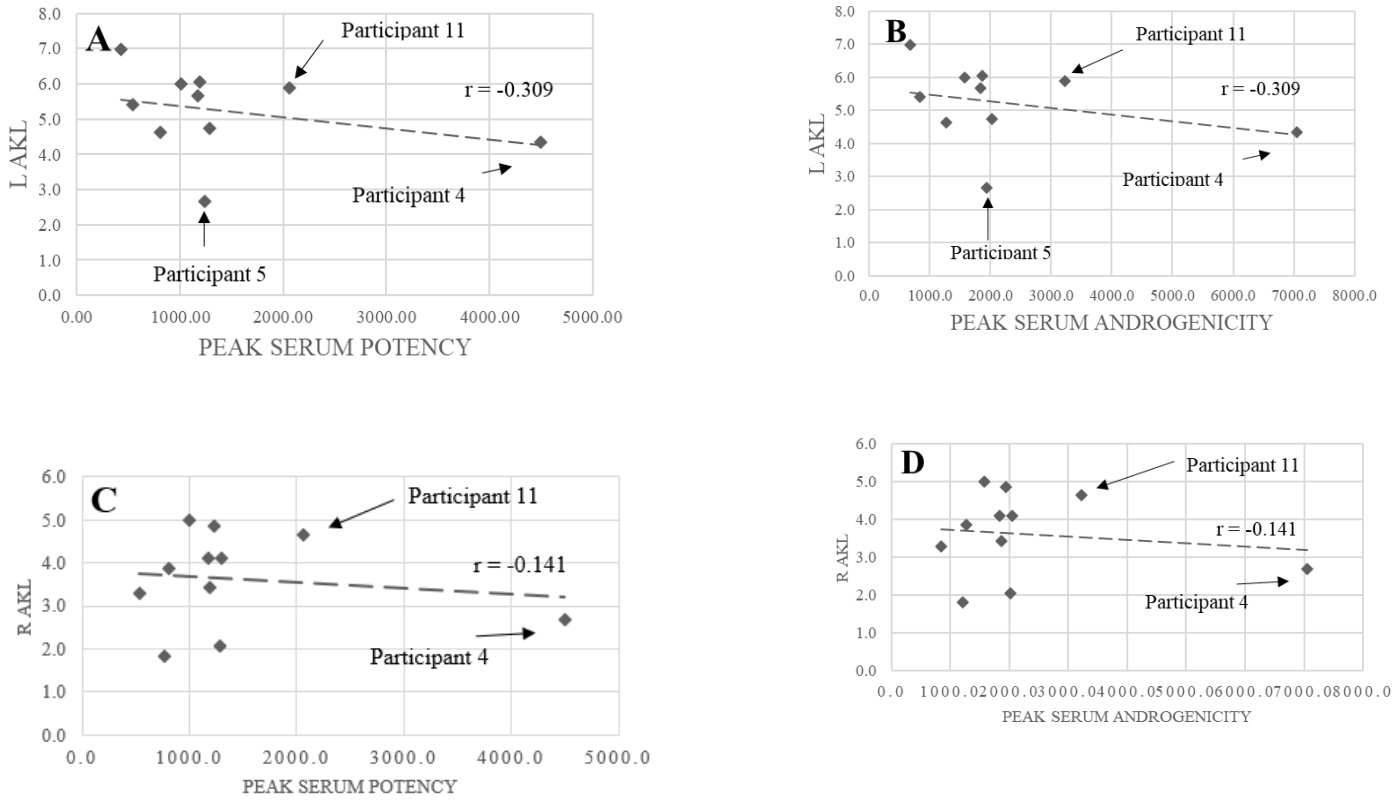


Figure 20 Scatterplots Displaying Associations Between Serum Pharmacodynamic Variables and AKL for the LNG GROUP During Pill Week Three

The units on AKL are millimeters (mm). The units on serum pharmacodynamic variables are pg/mLNET

NET Group

Among participants in this group, non-significant positive relationships were observed between peak serum potency and peak serum androgenicity with AKL on both knees (p=0.103 to 0.245) (Table 20 and Figure 21)

Table 20 Bivariate Correlations Between Serum Pharmacodynamic Variables and AKL During Pill Week Three Among the NET GROUP

	Peak Serum Potency	Peak Serum Androgenicity
L AKL (N = 4)	0.755	0.755
R AKL (N = 4)	0.897	0.897

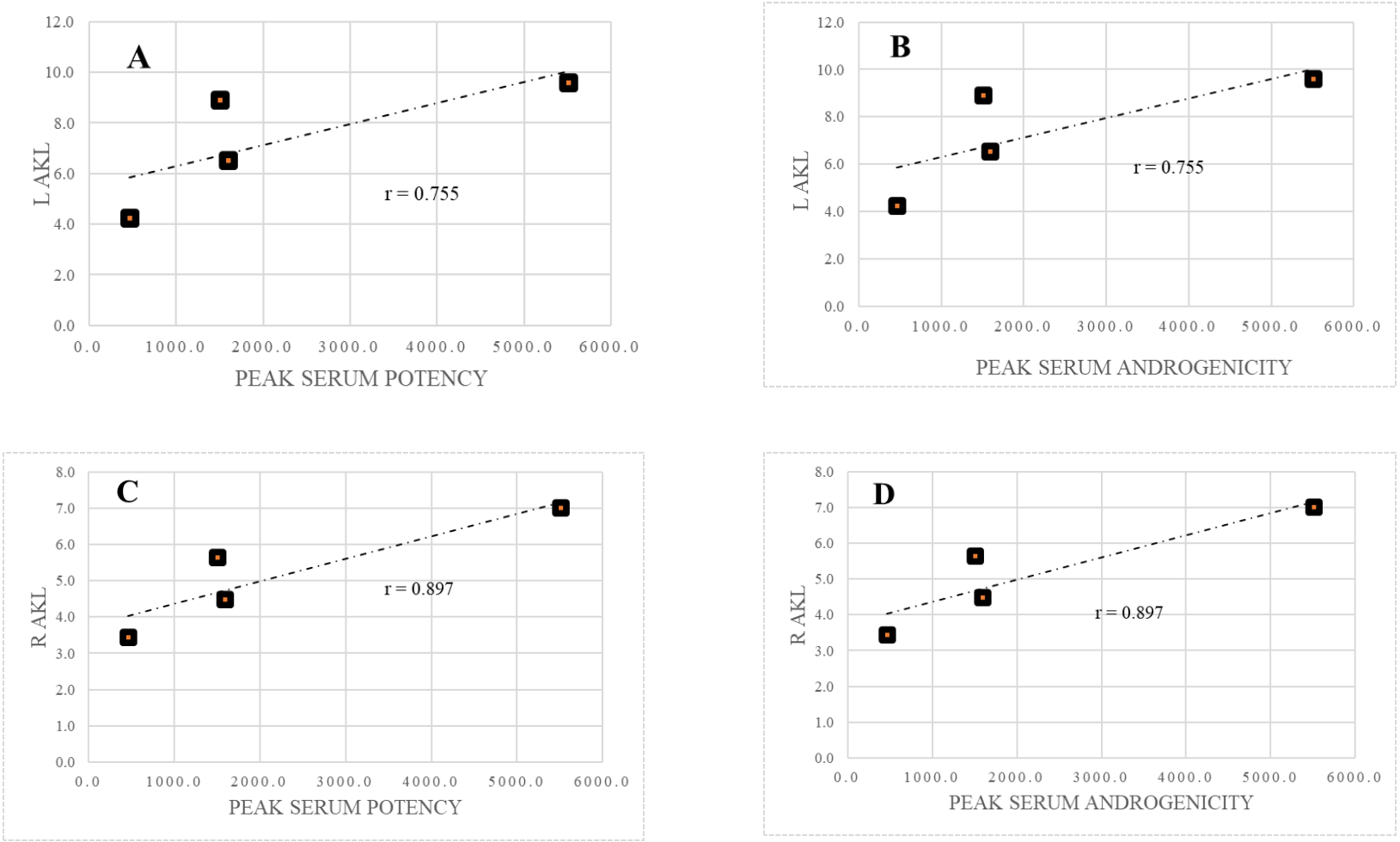


Figure 21 Scatterplots Displaying Associations Between Serum Pharmacodynamic Variables and AKL for the NET GROUP During Pill Week Three

The units on AKL are millimeters (mm). The units on serum pharmacodynamic variables are pg/mL NET

Section II: Associations between Pill and Serum Pharmacodynamic Variables and the Change in AKL from Pill Week Three to Pill Week Four

All Participants

Among all participants, there was no significant correlation between pill potency and pill androgenicity during end of the third pill week with the increase in AKL during interval of time between the third and fourth pill weeks (p-value range 0.201 to 0.850) (Table 21 and Figure 22). Findings were similar for the relationship between serum values and the change in AKL (p-value range 0.138 to 0.292), with the exception of peak serum androgenicity which was positive correlated with a greater change in AKL (P=.038) (Table 22 and Figure 23 B)

Table 21 Bivariate Correlations Between Pill Pharmacodynamic Variables and the Change in AKL from Pill Week Three to Pill Week Four

	Pill Potency	Pill Androgenicity
Δ L AKL (n=14)	0.197	0.337
Δ R AKL (n=15)	0.350	-0.054

Table 22 Bivariate Correlations Between Serum Pharmacodynamic Variables and the Change in AKL from Pill Week Three to Pill Week Four Among ALL PARTICIPANTS

	Peak Serum Potency	Peak Serum Androgenicity
Δ L AKL (n = 14)	0.417	**0.559***
Δ R AKL (n = 15)	0.292	0.177

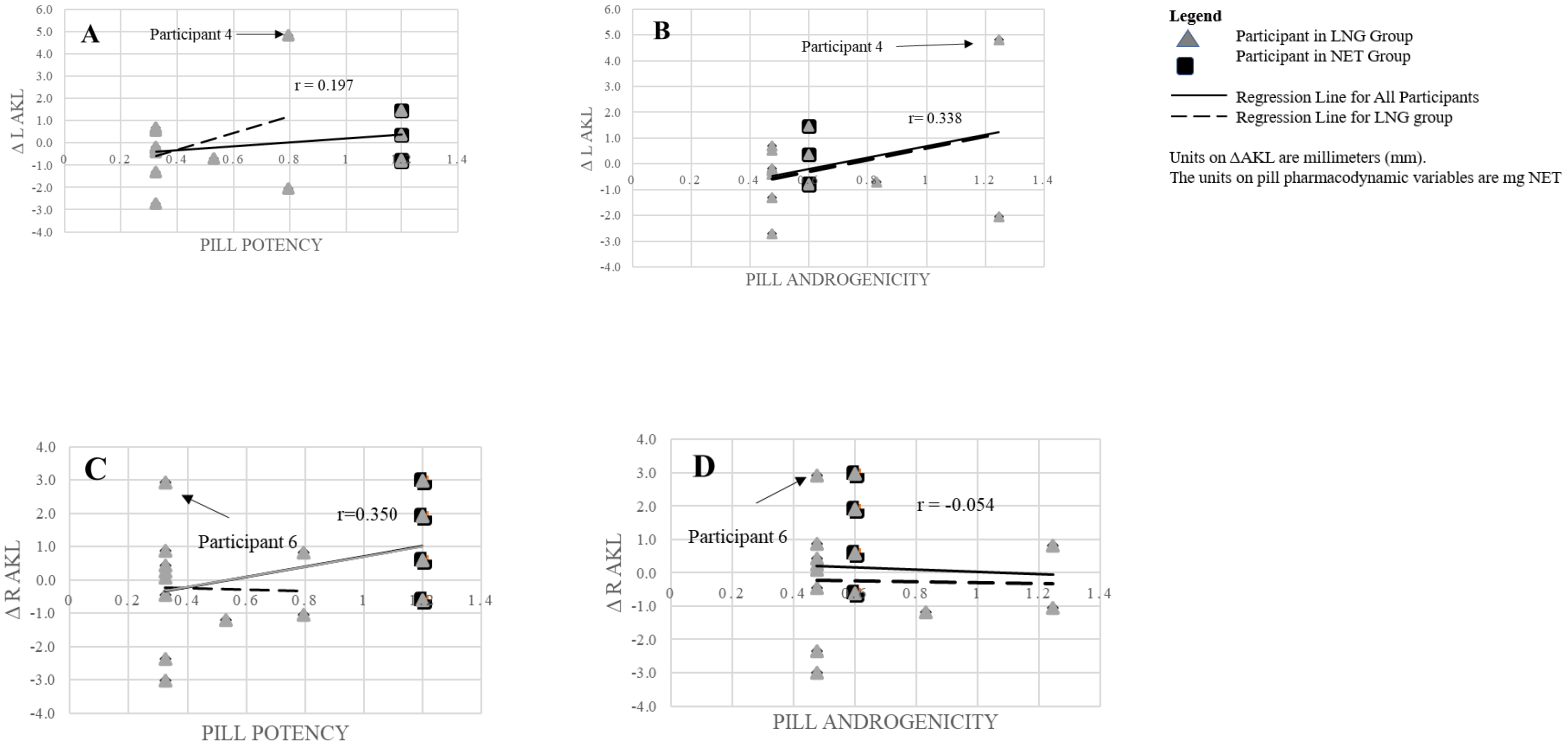


Figure 22 Scatterplots Displaying Associations Between Pill Pharmacodynamic Variables and the Change in AKL Among ALL PARTICIPANTS from Pill Week Three to Pill Week Four

AKL is measured in millimeters (mm), pill pharmacodynamic variables are measured in mg NET. The triangles (▲) signify the LNG group and the squares (■) signify the NET group

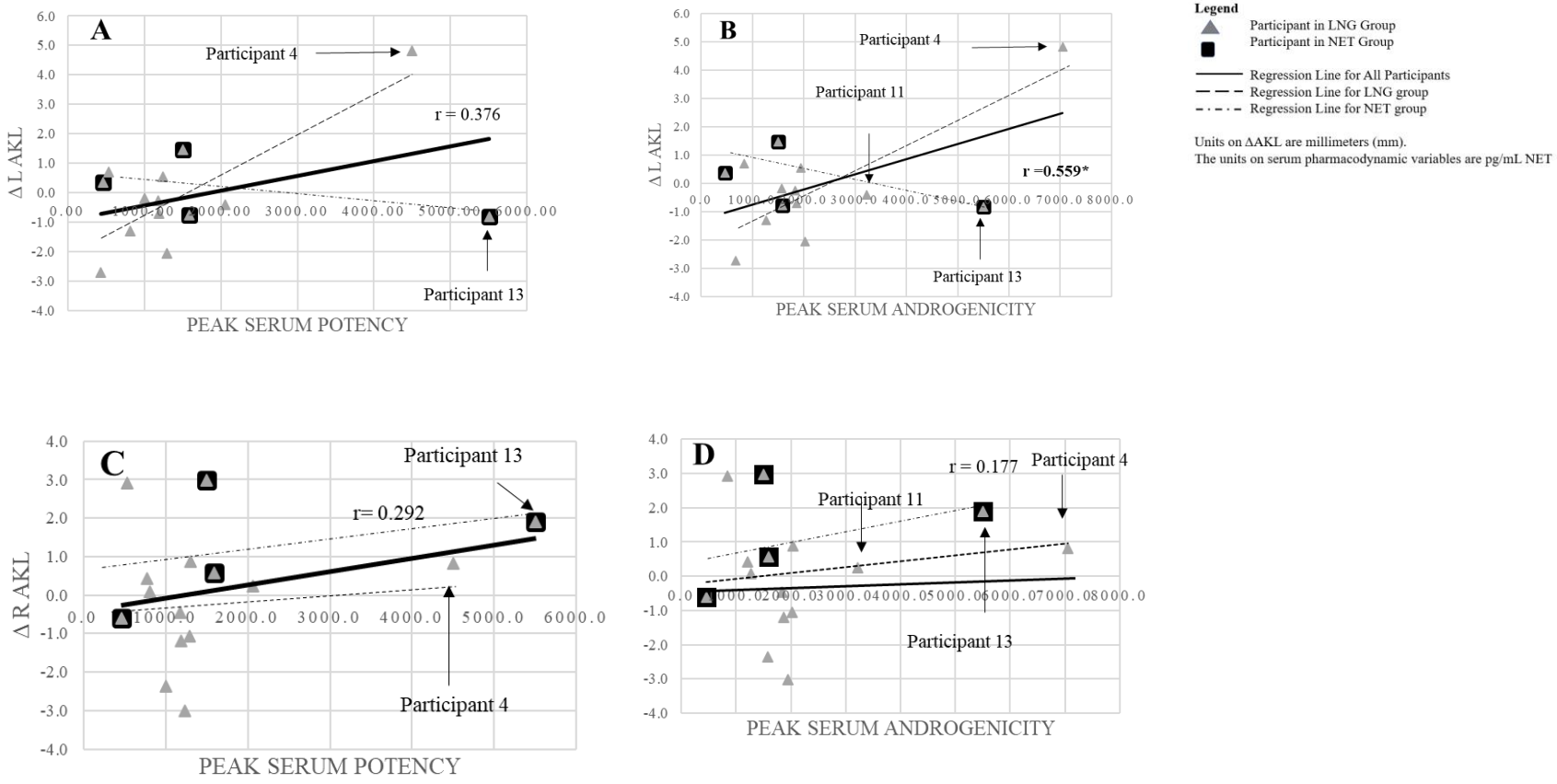


Figure 23 Scatterplots Displaying Associations Between Serum Pharmacodynamic Variables and the Change in AKL Pill Week Three to Pill Week Four Among ALL PARTICIPANTS

LNG Group

Within the LNG group, no significant correlations were observed between higher pill potency and androgenicity and greater increases in AKL from pill week three to pill week four (p-value range 0.300 to 0.945) (Table 23 and Figure 24). However, there was a significant positive correlation between higher peak serum potency and androgenicity during pill week three with a greater increase in left AKL from weeks three and four (p=.003). However, this was not observed on the right limb (p=0.768) (Table 24 and Figure 24 C and D).

Table 23 Bivariate Correlations Between Pill Pharmacodynamic Variables and the Change in AKL from Pill Week Three to Pill Week Four Among the LNG GROUP

	Pill Potency	Pill Androgenicity
Δ L AKL (n=10)	0.365	0.362
Δ R AKL (n=11)	-0.024	-0.027

Table 24 Bivariate Correlations Between Serum Pharmacodynamic Variables and the Change in from Pill Week Three to Pill Week Four Among the LNG GROUP

	Peak Serum Potency	Peak Serum Androgenicity
Δ L AKL (n = 12)	**0.829****	**0.829****
Δ R AKL (n = 11)	0.101	0.101

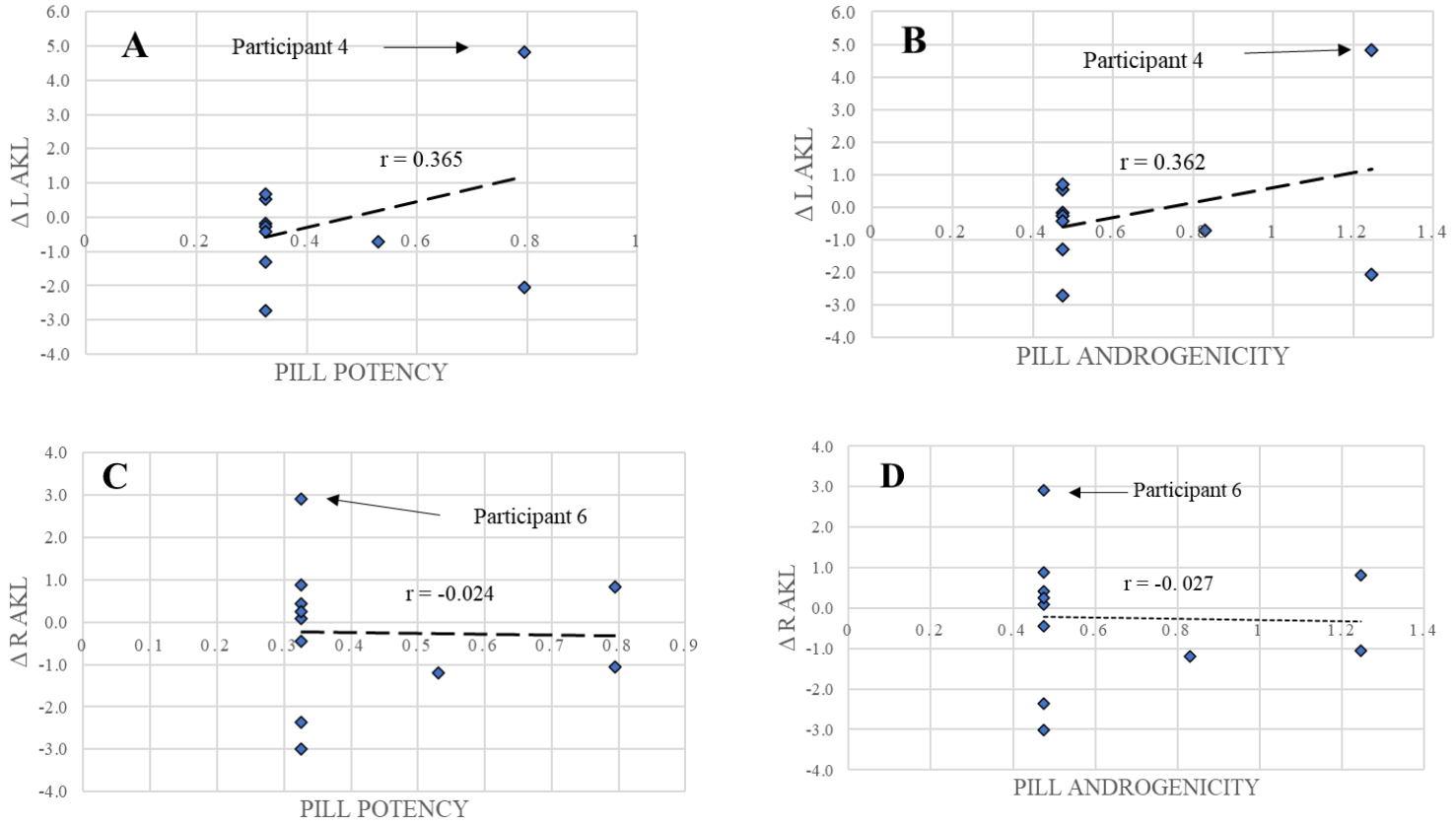


Figure 24 Scatterplots Displaying Associations Between Pill Pharmacodynamic Variables and Change in AKL from Pill Week Three to Pill Week Four Among the LNG GROUP

AKL is measured in millimeters (mm), pill pharmacodynamic variables are measured in mg NET.

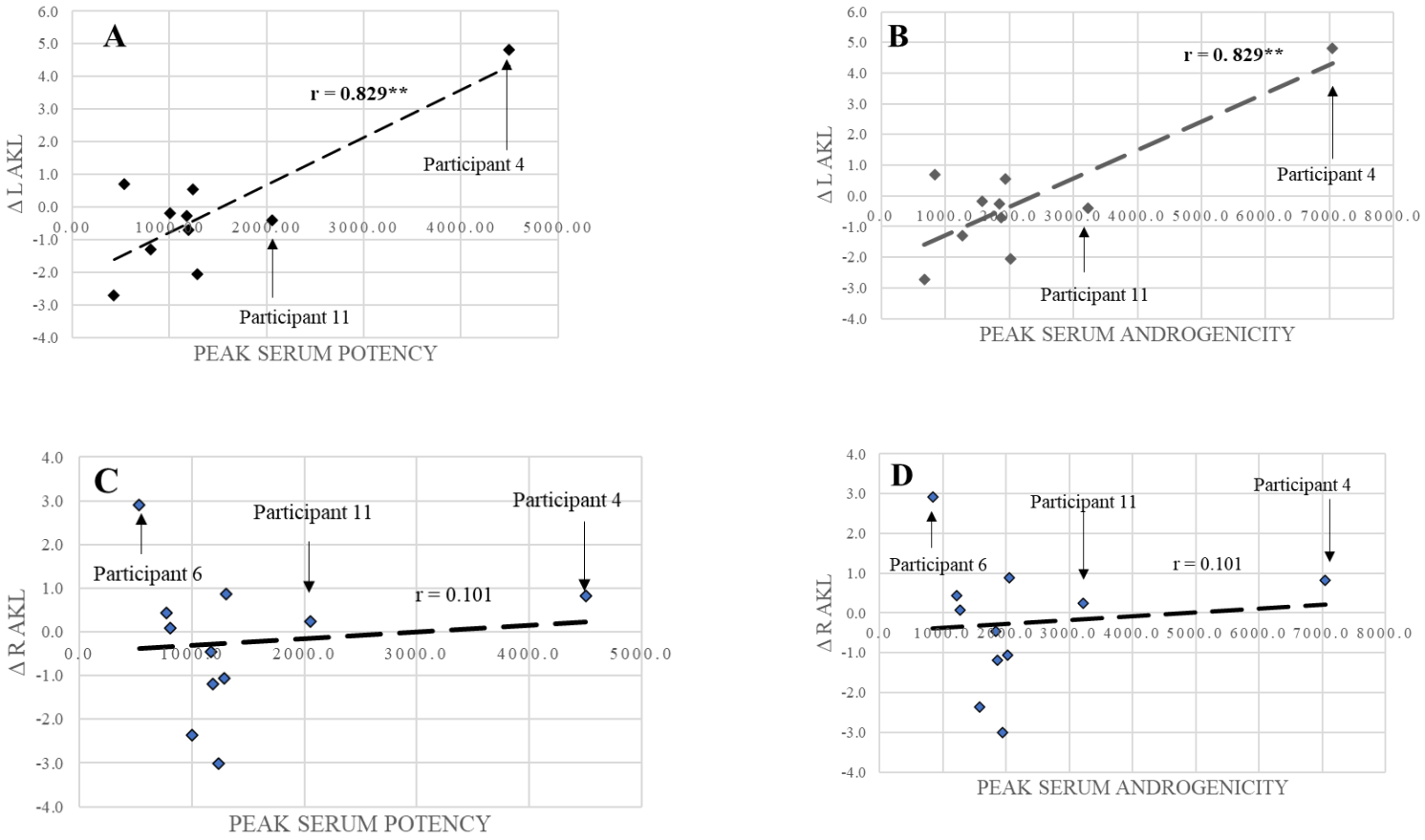


Figure 25 Scatterplots Displaying Associations Between Serum Pharmacodynamic Variables and the Change in AKL Pill Week Three to Pill Week Four Among the LNG Group

The units on ΔAKL are millimeters (mm). The units on serum PDVs are pg/mL of NET

NET Group

Correlations were not significant between greater peak serum potency and androgenicity during the third pill week and changes in AKL in the interval of time between weeks three and four (Table 25 and Figure 26). While not significant, opposite relationships were noted between the left and right sides.

Table 25 Bivariate Correlations Between Serum Pharmacodynamic Variables and the Change in AKL from Pill Week Three to Pill Week Four Among the NET GROUP

	Peak Serum Potency	Peak Serum Androgenicity
Δ L AKL	-0.541	-0.541
Δ R AKL	0.442	0.442

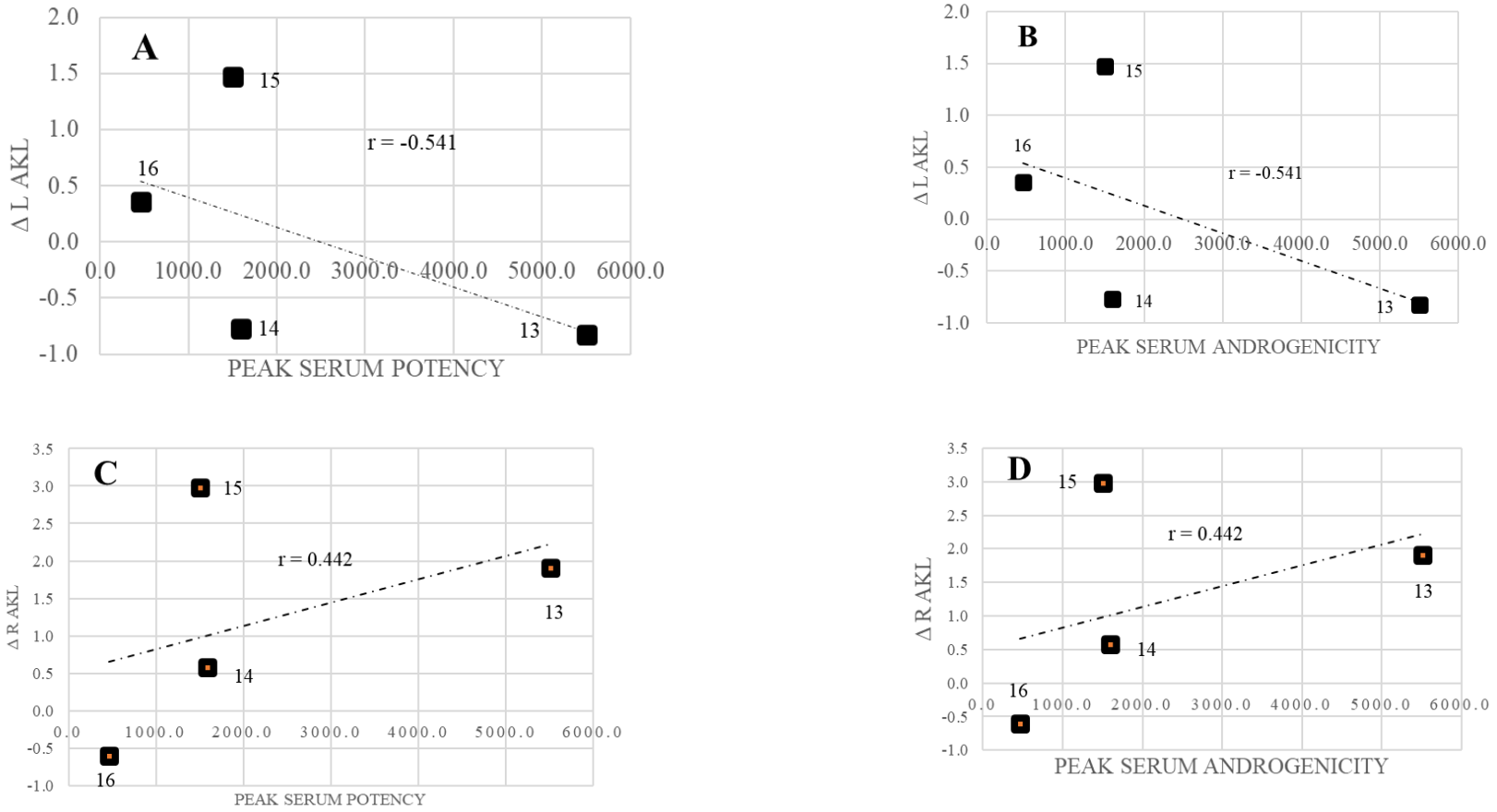


Figure 26 Scatterplots Displaying Associations Between Serum Pharmacodynamic Variables and the Change in AKL Pill Week Three to Pill Week Four Among the NET GROUP

The units on Δ AKL millimeters. The units on the serum pharmacodynamic variables are pg/mL NET

CHAPTER V: EXECUTIVE SUMMARY

Aim I Determination of UPLC-MS and Sample Preparation Method

Aim IA Validation of UPLC-MS Method for The Quantitation of Progestins

This sub-aim was successful but only after months of dealing with a variety of challenges.

Pre-Proposal Method Development - The completion of Objective I: Completion of this objective allowed for the decision to use all of the instrument settings for all assays afterward. The standard preparation method used Objective II was sufficient for use in the next objective. Objective III was partially completed. As mentioned in the results, the amount of NET recovered from the technical replicate was much greater that what was expected. The most likely cause of this may have been a miscalculation of the amount of NET that should have been spiked into the technical replicate.

The preliminary work completed was deemed sufficient to proceed with measurement of endogenous and exogenous hormones in the study's participants for the following reasons: First, the decision to use a biphenyl column was based on a previously validated LC-MS method for steroid hormones (Phenomenex). This, along with the gradient profile provided by the manufacturer of this column, produced consistent calibration curves in Objectives II and III. Next, The LC-MS methods from which this new assay was derived from assays that had was already been validated for estradiol and testosterone, it was decided that there was no need to perform any preliminary work with these hormones. Finally, it was thought that the sample preparation procedure used for this pilot work was sufficient to prepare serum samples since it had been used or the aforementioned hormones, and even though the extraction efficiency on NET did not make practical sense (% recovery of 182%), that such issues could be resolved by an additional UPLC-MS run with technical replicates prepared in duplicate or triplicate, which was done as part of the Post-Proposal UPLC-MS Assay. However, at that stage of method development, it was decided to proceed to Aim IB to conserve the limited amount of blank human serum and analytical standard that remained after the pilot work.

It is important to note that this pilot work was not as rigorous as the work performed in Aim I. For example, calibration curves run in steps two and three were always run with single injections in this aim. Moreover, the extraction experiment run in step three was not the same as later experiments. Only one technical replicate was used to assess extraction efficiency, and a methanol blank instead of blank human serum was used[17]. However, it was decided that this work was sufficient to conduct hormone assays on the study's participants.

Post-Proposal UPLC-MS Assay- This assay, as mentioned when introduced, combined the lessons learned from Objectives I-III was well as post-proposal piloting before it was completed. Modifications to Objective III were especially difficult to validate, and the results presented were from the eleventh attempt to determine the extraction efficiency of the sample preparation process. Throughout those attempts, it was assumed that analyte recovery would be greater than 50% based on the information gathered from pre-proposal pilot work. Therefore, spike solutions were prepared at such a level so that they would fall within the middle range of the calibration curve generated in Aim IB, which was ~15 ng/mL. Unfortunately, this resulted in all analytes being detected below the LLOQ of each assay's calibration curve. Once it was decided to select 75 ng/mL and 250 ng/mL as expected concentrations, the experiment was successful, though recovery % was low for all analytes.

This sub-aim included a troubleshooting step to ensure the type of glassware used in previous experiments was not the cause of the difficulty in the extraction. Specifically, glasses in which analytes were dried and had a large surface area (~1 in^2) and the small amount fluid used to reconstitute the samples, 80 µL, may have been insufficient to capture all of the dried material in each glass tube. Therefore, the extraction in this experiment made use of specialized recovery vials, which were much smaller than the tubes used in previous experiments. Moreover, the recovery vials were rinsed twice to ensure optimal recovery. The double rinse resulted in an increase in the amount of progesterone recovered but did not result in an increase in the recovery of the other analytes (Table B.8).

The most likely explanation for the low recovery for of T, NET, and LNG in the extraction solution was the solubility of each of these hormones. The sample preparation method

[17] It should be noted that peak areas of all analytes in the technical blank used in the Post-Proposal Assay were below each analytes' LLOQ (See Table)

for this assay was based on a previously published UPLC-MS method for the quantitation of testosterone and estradiol (Schofield 2017) and it was assumed that, because of the similarity of the molecular structures of P4 and the progestins to estradiol, it would be appropriate to use LLE procedure to prepare all subject samples. For example, it was assumed that all analytes would be soluble in hexane, an organic solvent, and the primary component of the extraction solution. It was discovered that is not the case. LNG is not soluble in hexane (Bayer 2017). Solubility information for NET in hexane could not be found. Other LC-MS methods for the quantitation of levonorgestrel have used different extraction solutions (hexane-dichloromethane) and different extraction methods (Edelman 2016; Gong 2012; Praditpan 2017)

Though recovery was low, results of the extraction were highly reproducible across all technical replicates and therefore, average % recoveries were used to determine the concentrations of each hormone present in participants' serum analyzed in Aim IB.

Aim IB Measurement of Serum Hormone Levels in Study Participants

The post-proposal assay performed after the assay run under this Aim were very similar. The LLOQs between both assays were very similar, though there were small differences in the LLOQ for each run. Average % CVs tended to be lower in the Aim IA-Post Proposal Assay due to the increase practice of the lab technician (Foli) in preparing calibration curves between the time experiments involved in the two sub-aims were conducted (Table 7 and Table 8). Average relative errors percentages were also very similar between assays.

There were two main differences between the two sub-aims. First, the assay performed under this aim had 10 calibrators instead of the 14 used in the IA-Post-Proposal Assay. Moreover, the upper limit of quantitation was set at 250 ng/mL for the progestins LNG and NET and 50 ng/mL for P_4 and T since progestin concentrations were expected to be higher than the concentrations of the endogenous hormones. However, it was later realized this overcomplicated the preparation process, and simplifications were made. The second difference was the retention times for each analyte. As was mentioned in a previous footnote, the LC column that had been used for all UPLC-MS runs up to the experiment under Aim IB was rendered unusable. Therefore, another biphenyl column made by the same manufacturer was used in the Post-Proposal Assay, and in this assay, retention times for all analytes were shifted down by an average of 0.27 seconds.

In short, the goals of Aim IA and IB were accomplished, producing a valid and accurate method for the determination of NET, LNG, T, and P_4.

Aim IC Qualitative Analysis of Participant Samples Containing 17β-Estradiol

The presence of estradiol in participant samples 10-16 was confirmed by this experiment through a discovery of estradiol's retention time (adjusted), source fragment and fragmentation pattern in both a standard and participant sample, as well as the presence of a 255.18 *m/z* source fragment that appeared around 4.02-4.03 minutes in the Aim IB XICs of Participants 10-15.

Because estradiol appeared far below the LLOQ of the calibration curve generated in Aim IA, it was decided not to run a calibration curve for this experiment. Peak areas, instead of concentrations, were thus used to make comparisons of estradiol levels among the subjects in the second assay that was run. Because the peak areas of the estradiol amounts are on the order of 1 x 10^6, and depended on the initial volume of participants' samples, peak areas were normalized to 1 mL of serum, then divided by 10000 to make the number more readable. Unfortunately, the hypothesis of this thesis, which eventually ended up in the splitting of the sample into LNG and NET groups, made use of this data in the final analysis very difficult to interpret. Specifically, there was no pharmacological or hormonal explanation for the presence of estradiol in some participants, but not in others. Therefore, it was not included in any of the analyses that involved AKL.

Aim II: Examination of the Relationship Between Pill and Serum Pharmacological Parameters

The results from the analyses in this aim suggest, that, within the boundaries of this thesis, there are positive, but non-significant associations between pill and serum PDVs among all participants and among participants in the LNG group. The reasons for this include 1) the differences in the pharmacological parameters of the LNG and NET groups and 2) measurement error.

Differences in progestin pharmacological parameters likely impacted the associations between pill and serum PDV for all subjects. Specifically, differences in measured and peak serum levels, and pill dosages. The average bioavailability of NET is 64% but without any subjects who took NET at a different dosage, it would be difficult make a correct association between pill and serum PDVs, especially when the bioavailability of LNG is accepted to be 100% (Back 1987). Therefore, a comparison of serum levels across all of these study's subjects,

given the different pharmacokinetics of LNG and NET, may have confounded our results, and is what led to the secondary analyses in the LNG group only.

Possible measurement error within the LNG group. One participant, participant 4, heavily influenced the association between pill and serum PDVs within this group. Her progestin levels were very high, when compared to the rest of the participants in this group. At first, it was thought that her progestin measured levels were high because she had taken her pill about 2.5 hours before her week three laboratory visit. However, when calculations were made to estimate peak serum potency and androgenicity among all participants to account for the metabolism of the progestins over time, her peak serum levels were still much higher than the other participants. Therefore, a secondary analysis was conducted that removed Participant 4. After this was done, the significant association between pill and serum pharmacodynamic variables disappeared. This greatly reduced the variability in the pill data, as 9 of the remaining 11 participants had pill potency and androgenicity data fixed at 0.325 mg NET and 0.475 mg NET respectively.

The next possible explanation for the lack of a statistically significant association between pill and serum potency, was the different pharmacodynamics of the pill progestins of the participants within the LNG group. There are two biologically active metabolites of norgestimate, namely LNG and LNG-3-oxime (also known as norelgestromine) (Kuhl 2005).

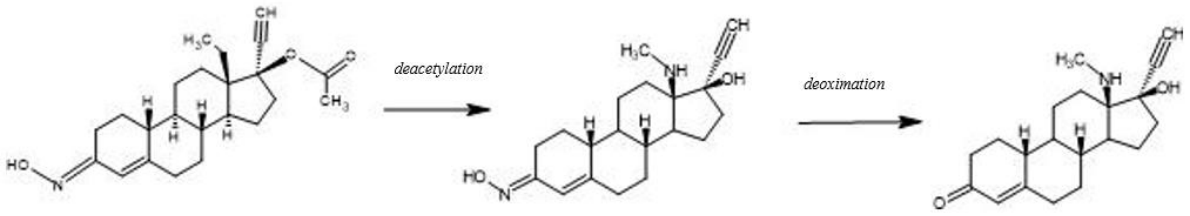


Figure 27 Metabolism of LNG-3-Oxime

LNG-3-oxime is a progestin that has little androgenic activity, but unlike LNG, it does not travel thorough the blood bound to sex hormone binding globulin (SHBG). Therefore, the amount of free LNG-3-oxime is greater than that of LNG, though it has a lower relative binding affinity (RBA) to the progesterone receptor than LNG (Stancyck 1997, Kuhl 2005).

The contribution of LNG-3-oxime to peak serum potency was not factored into this thesis. Interestingly though, the UPLC-MS method used in this study was able to detect. molecular ion of an *m/z* value that matched that of LNG-3-oxime in the serum of participants who took an OCP containing norgestimate (Participants 2-3, 5-11) at a retention time of 4.85-4.86 (Figure 28). However, confirmation of the identity of this compound was LNG-3 oxime would require an UPLC-MS run with an analytical standard of the compound.

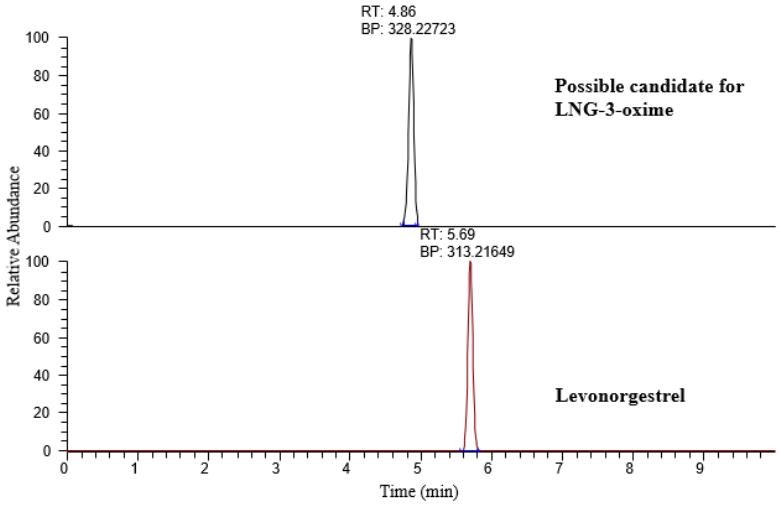


Figure 28 XIC Displaying a Candidate Molecular Ion for LNG-3-oxime and LNG for Participant 2, Pill Week Three

Sample preparation concerns and the measurement of free progestins instead of total progestin levels. The serum sample preparation method used exhibited low recoveries for both LNG and NET. This likely reduced the ability to make strong associations between pill and serum potency and androgenicity. Moreover, the methods did not measure progestins that may have been bound to SHBG or albumin. At least one reference points to 98% of LNG and 94% of NET being bound to either SHBG or albumin respectively (Kuhl 2005).

Error in measurement of pharmacokinetic parameters. Peak serum progestin levels were not measured directly since participants had their blood drawn early in the morning, when serum levels of their endogenous hormones were expected to be at their nadirs. However, most participants took their week three pill the night before their lab visits, as instructed, generally between the hours of 8:00 and 11:30 PM. Therefore, C_{peak} estimates were made on based averages of the pharmacokinetic parameters (T_{max}, $t_{1/2}$) listed on a government database

containing these data for each OCP brand (https://dailymed.nlm.nih.gov). However, as shown in Table 11 and in the literature (Jusko 2017) there is some inter-subject variability in all of these parameters, and therefore, the estimated C_{peak} used in this study may not represent the true C_{peak} for each individual. Despite the possible error in C_{peak} estimates, these estimates provided better associations between pill and serum PDVs than the associations between pill PDVs and the progestin levels that were measured directly ($C_{measured}$).

Future studies examining the effects of pill and serum PDVs on anterior knee laxity would benefit by increasing total sample size, but more specifically, increasing sample size among participants among the different OCP groups that active women may fit into. The data presented in Table 11 shows that the progestin dose, even among subjects within the same group based on this thesis' original group stratification, are metabolized at different rates, which may affect participants' daily exposure to exogenous hormones. A more robust and comprehensive assessment of individual pharmacokinetic analysis would capture these differences as well. This assessment would involve sampling participants at multiple time points within one "on-pill" day, thus providing researchers with specific pharmacodynamic parameters for each participant, including T_{max}, C_{peak}, $t_{1/2}$ and k_e. Conducting a study in this manner would also make calculations of the area under time x progestin concentration curve possible, which would give researchers a better picture of the duration of progestin exposure throughout the course of a day. All of this information would no doubt result in a better understanding of the pharmacodynamic effects of progestins on AKL.

Aim III: Examination of the Relationship Between Pill and Serum Pharmacodynamic Variables and Anterior Knee Laxity During On- and Off Pill Weeks

Associations Between Pill and Serum Pharmacodynamic Variables and AKL during Pill Week Three

Eight analyses and twelve secondary analyses were performed to examine the relationship between the relevant pharmacodynamic variables and AKL during the third pill week (Table 26). However, the lack of power in this study prevented any of the results from reaching statistical significance.

Table 26 Result Summary- Analyses Examining the Association between Pill and Serum PDVs on AKL During Pill Week Three

	Pill Potency	Pill Androgenicity	Peak Serum Potency	Peak Serum Androgenicity
		Primary Analysis		
L AKL	+	-	+	+
R AKL	+	-	+	+
		LNG Group		
L AKL	-	-	-	-
R AKL	-	-	-	-
		NET Group		
L AKL	No Variability in Pill Data	No Variability in Pill Data	+	+
R AKL	No Variability in Pill Data	No Variability in Pill Data	+	+

The (+) signifies a positive association between the relevant PDV and AKL.
The (–) signifies a negative association between the relevant PDV and AKL

Among all participants, pill potency and AKL were positively correlated, but pill androgenicity was negatively associated with AKL. Additionally, six out of eight of the associations between peak serum PDVs and AKL were positive, which is contrary to the hypothesis of this study. Examination of the scatterplots of the peak serum PDV/AKL associations among all participants suggested participants in the NET group likely influenced this finding (Figure 18). When groups were stratified, analyses run on participants in the NET group revealed greater peak serum potency and androgenicity were associated with greater AKL during the third pill week. These data showed that the positive association between serum PDVs and AKL among all participants, was indeed influenced by the presence of the NET group. One possible explanation for the positive correlations between peak serum PDVs and AKL among this group may be an indirect influence of NET on estradiol levels. Specifically, estradiol, which may result in an increase AK, was present in greater amounts in the NET group during pill week three, when compared to most subjects in the LNG group Table 10) This is only speculation though and may require a larger sample size before conclusions can be drawn.

When examining only the LNG group, consistent negative associations were observed between both pill/serum PDVs and AKL during the third pill week, a finding that *is* consistent with the hypothesis of this thesis.

Associations Between Pill and Serum Pharmacodynamic variables and the Change in AKL from Pill Week Three to Pill Week Four

Eight primary analyses and twelve secondary analyses were performed to examine the relationships between the relevant pharmacodynamic variables and the change in AKL from the third to the fourth pill weeks.

Table 27 Result Summary - Analyses Examining the Association between Pill and Serum PDV and the Change in AKL From Pill Week Three to Pill Week Four

	Pill Potency	Pill Androgenicity	Peak Serum Potency	Peak Serum Androgenicity
		Primary Analysis		
ΔL AKL	+	+	+	+
ΔR AKL	+	None	+	+
		Secondary (Group) Analysis -LNG Group		
ΔL AKL	+	+	+ (sig)	+ (sig)
ΔR AKL	None	None	+	+
ΔL AKL	Could Not Be Performed	Could Not Be Performed	-	-
ΔR AKL	Could Not Be Performed	Could Not Be Performed	+	+

Seven of the eight primary analyses were consistent, and suggested that among all participants, greater potency and androgenicity during the third pill weeks results in a greater increase AKL during the interval of time between pill weeks 3 and week 4. This finding also appeared in the LNG group. These findings would be in agreement with the hypothesis for this study. Specifically, as progestin values decrease from week 3 (pill week) to week 4 (non-pill week), one would expect an increase in AKL if progestins are responsible for reducing AKL values.

The inconsistencies in the results observed within the NET group might be explained partially by the low sample size, but also by the inconsistent changes in AKL that occurred within this group. For example, some participants' laxity increased from week three to week four on one leg but decreased on the other. These inconsistencies have no known hormonal explanation, and therefore, the relationship between serum PDVs and AKL within the NET group is unclear.

For all analyses, it is also important to note that while hormone assays were reproducible, and precise, the sample preparation procedure, which resulted in very low hormone recoveries make the results listed in both Section I and II far from conclusive.

Future Directions

In conclusion, though the hypotheses of this thesis could not be properly supported or refuted, the lessons learned may support future research in this area of study.

Improvements to Recruitment and Hypothesis Testing

It is the opinion of this author that future recruiting efforts should be based on *progestin type* instead of the pill PDVs due to several difficulties that arose from recruiting this way:

1. Many women who took pills that contained the progestins of interest had to be turned away
2. Pills are made in such a way that parametric regression analysis examining the effects of potency and androgenicity on AKL would face the issue of high multicollinearity between the two variables.
3. This study provides preliminary evidence that the hormonal and pharmacological profiles of women on different progestins and different doses differed greatly. Hormonal concentrations of progesterone, testosterone, and estradiol differed between subjects, even when comparing the small number of subjects who would have been given the same group designation based on the potency and androgenicity of their progestins. (Table 11 and Table 12). Pharmacokinetic parameters, such peak progestin levels, time to peak, elimination half-life and elimination half-life are all sensitive to progestin dose, and the OCP phase, even for participants, who would fall within the same group (Table 11). Moreover, the metabolism of these pills, as well as their pharmacokinetic parameters may be affected by the phase of the pill. Specifically, OCP users in the available sample took both monophasic and triphasic pills. Differences in hormone exposure over an extended period could impact AKL but these differences were not accounted for in this study. Therefore, it may be more reasonable to group study participants based on the progestin type before comparisons are made across progestin types.

Moreover, future research may be more fruitful if hypotheses are directed at an examination on serum PDVs *within* each progestin group. To highlight this point, LNG is only used in OCPs that

would be considered low potency, low androgenicity, and low estrogenicity (Table A.1). Conversely, OCPs that contain norethindrone acetate (NET-A) are not made in such a way that would fit into a low potency, low androgenicity, and low estrogenicity group. Testing a hypothesis examining the effects of serum or pill PDVs is such a way might be better suited to consider the inter and inter-participant pharmacokinetic and pharmacodynamics that are unique to the progestins within each group.

Finally, one factor that was not considered in the examination of the differences in pill and serum PDVs was the presence of mono and triphasic hormones.

Improvements to Data Collection

Though the way data was collected was appropriate for a first attempt to examine the relationship between potency/androgenicity and AKL, future studies may benefit from taking multiple serum measurements within one day so as to get a better distribution of the time x concentration curve for each subject. This would provide a closer estimate of the concentrations.

Changes to Sample Preparation and UPLC-MS Assays

Future research would also benefit from a few additions to the sample preparation procedure and methods used to examine exogenous hormone levels in participants. The addition of a sample preparation step that involves separation the separation of progestins from their binding proteins would provide information about total and free hormone levels. It would also be useful to obtain information on the changes in binding protein levels, which progestins, along with ethinyl estradiol, are known to influence (Kuhl 2005). Lastly, due to the presence of two metabolically active metabolites from norgestimate, future research should include LNG-3-oxime as an analyte of interest in hormone analysis.

An optimized version of this UPLC-MS method for the determination of progestins and endogenous hormones in human serum might involve the use of a different sample preparation process, perhaps using other organic solvents, such as acetone, methanol, ethanol, or dimethyl sulfoxide (DMSO) if LLE was the sample preparation method of choice. Alternatively, because of the solid-phase extraction (SPE), though much more costly, may be another option. Regardless of which type of procedure is chosen, sample preparation is a very labor-intensive process and would best be carried out with a team of technicians who have experience with bench top chemistry.

UPLC-MS methods for the quantitation of, NET, LNG, LNG-3-oxime, and other progestins have existed for at least forty years and have been used in studies to examine the pharmacodynamics of progestins by themselves, or to examine their interactions with other drugs (Boyd 2001; Hammond 2003; Jusko 2017) Moreover, there are a number of studies that have examined some of these hormones in combination with endogenous hormones and T, *in combination* with other hormones, or by themselves, are in existence (Blue 2018; Gong 2012; Huang 2016; Matějíček 2007; Saxena 2015; Theron 2004; Wong 1999; Yuan 2019). Therefore, development of a mass spectrometry method for the simultaneous quantitation of E2, EE, P4, NET, LNG-3-oxime, and T in active women on hormonal oral contraceptives is certainly feasible.

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APPENDIX A: PARTICIPANT VISIT MATERIALS

Physical Activity and Health History

1. Do you have any General Health Problems or Illnesses? (e.g. diabetes, respiratory disease) Yes____ No____

2. Do you have any vestibular (inner ear) or balance disorders? Yes____ No____

3. Do you smoke? Yes____ No____

4. Do you drink alcohol? Yes____ No____ If yes, how often?_____

5. Have you donated blood or blood products in the past month? Yes____ No____

6. Do you plan to donate blood or blood products in the next month? Yes____ No____

7. Do you have any history of anemia? Yes____ No____
8. If yes, when were you diagnosed? _____

9. Do you have any history of connective tissue disease or disorders? (e.g. Ehlers-Danlos, Marfan's Syndrome, Rheumatoid Arthritis) Yes____ No____

10. Please list any medications you take regularly: _____

Please list any previous injuries to your lower extremities. Please include a description of the injury (e.g. ligament sprain, muscle strain), severity of the injury, date of the injury, and whether it was on the left or right side.

Body Part	Description	Severity	Date of Injury	L or R
Hip				
Thigh				
Knee				
Lower Leg				
Ankle				
Foot				

Please list any previous surgery to your lower extremities (e.g. hip, thigh, knee, leg, ankle, foot) Include a description of the surgery, the date of the surgery, and whether it was on the left or right side)

Body Part	Description	Date of Surgery	L or R

Please list all physical activities that you are currently engaged in. For each activity, please indicate how much time you spend each week in this activity, the intensity of the activity (i.e. competitive or recreational) and for how long you have been regularly participating in the activity.

Activity	#Days/week	#Minutes/Day	Intensity	Activity Began When?

What time of day do you generally engage in the above activities?

Please list other conditions / concerns that you feel we should be aware of:

The Activity Rating Scale

Please indicate how often you performed each activity in your healthiest and most active state, **in the past year.**

	Less than one time in a month	**One time in a month**	**One time in a week**	**2 or 3 times in a week**	**4 or more times in a week**
Running: running while playing a sport or jogging					
Cutting: Changing directions while running					
Decelerating: coming to a quick stop while running					
Pivoting: turning your body with your foot planted while playing a sport; For example: skiing, skating, kicking, throwing, hitting a ball (golf, tennis, squash), etc.					

Investigator Comments:

Female Hormonal History

This questionnaire asks questions about your menstrual cycle. As a reminder, this information is strictly confidential. None of this information will be shared with anyone besides the study investigators. Your survey uses a coded identification number in substitution for your name. If you have any questions, or do not understand any of the questions, please let us know.

Subject Code:_____ Date:_____ Date of Birth:_____

_____

Menstrual Cycle Questions

1. How old were you when you started your menstrual periods (Age in years + months)? _____

2. On average, how many days were there between your menstrual periods (from Day 1 of your period to Day 1 of the next period)? _____

3. How many menstrual periods have you had in the last 12 months? _____

4. Does your cycle length vary more than 1-2 days per month? YES NO

5. Have you missed any menstrual periods within the last 12 months (Please Circle)? YES NO

6. Since starting your menstrual periods, has there ever been an extended time where you did not have a menstrual period (Please Circle)? YES NO

7. If YES, when was the most recent time that you missed a period(s) and how long did that last? _____

Hormonal Contraceptive Use

1. Are you *currently* taking hormones (e.g. birth control pills, estrogen therapy, etc) for any reason (Please Circle)? YES NO

2. If YES, please list the brand name(s) and dosage (please also take a picture of the pill and/or record the exact brand, generic names as well as the dosage):
   _____

3. When did you start using this type? (date/year) _____

4. How old were you when you started taking birth control pills (Age in years + months)?_____

5. Have you *previously* taken any other form of hormones (e.g. birth control pills, estrogen therapy, etc) for any reason? (Please Circle) YES NO

6. If yes, please list the brand name(s) and date(s) of use_____
   _____
   _____

7. What date did you take the first pill in your current pill pack (month/day)?_____
8. What date will you take the last pill in your current pill pack (month/day)?_____

Pregnancy

1. Have you ever been pregnant? YES NO

2. Do you have plans to become pregnant in the near future? YES NO

Table A.1 List of Acceptable OCP Brands for Participant Recruitment

Brand Name	Progestin	Progestin dose (mg)	EE dose (mg)	Phase [1]	Days	Pill Potency	Pill Androgenicity
Group One: Low Estrogenicity, Low Potency, Low Androgenicity:							
Alesse 28	LNG	0.1	0.02	M	28	0.53	0.83
Aubra,	LNG	0.1	0.02	M	28	0.53	0.83
Aviane	LNG	0.1	0.02	M	28	0.53	0.83
Larissa	LNG	0.1	0.02	M	28	0.53	0.83
Lutera	LNG	0.1	0.02	M	28	0.53	0.83
Levlite	LNG	0.1	0.02	M	28	0.53	0.83
Orysthia	LNG	0.1	0.02	M	28	0.53	0.83
Sronyx	LNG	0.1	0.02	M	28	0.53	0.83
Vienva	LNG	0.1	0.02	M	28	0.53	0.83
Lessina 1/20	LN G	0.1	0.02	M	28	0.53	0.83
Tri-Lo- Sprintec	Norgestimate	0.25	0.025	T	28	0.325	0.475
Tri-VyLibra Lo	Norgestimate	0.25	0.025	T	28	0.325	0.475
Ortho Tri-Cyclen Lo	Norgestimate	0.25	0.025	T	28	0.325	0.475
Trinessa Lo	Norgestimate	0.25	0.025	T	28	0.325	0.475
Tri Lo Marzia	Norgestimate	0.25	0.025	T	28	0.325	0.475
Kaitlib FE	NET	0.08	0.025	M	24 (on)- (4 off)	0.8	0.8
Group Two: High Estrogenicity, Low Potency, Low Androgenicity							
Balziva	NET	0.4	0.035	M	28	0.4	0.4
Loestrin 1.5/30	NET-A	1.5	0.030	M	28	0.18	0.09
Junel 1.5/30	NET-A	1.5	0.030	M	28	0.18	0.09

Brand Name	Progestin	Progestin dose (mg)	EE dose (mg)	Phase [1]	Days	Pill Potency	Pill Androgenicity
Blisovi Fe 1.5-30	NET-A	1.5	0.030	M	28	0.18	0.09
Cilest	Norgestimate	0.25	0.035	M	21	0.325	0.475
Previfem	Norgestimate	0.25	0.035	M	28	0.325	0.475
Mononessa	Norgestimate	0.25	0.035	M	28	0.325	0.475
Ortho-Cyclen	Norgestimate	0.25	0.035	M	28	0.325	0.475
Orthro Tri-Cyclen	Norgestimate	0.25	0.035	T	28	0.325	0.475
Tri Femynor	Norgestimate	0.25	0.035	T	28	0.325	0.475
Sprintec	Norgestimate	0.25	0.035	M	28	0.325	0.475
Tri Sprintec	Norgestimate	0.25	0.035	T	28	0.325	0.475
Tri-Estarylla	Norgestimate	0.25	0.035	T	28	0.325	0.475
Tri-Linyah	Norgestimate	0.25	0.035	T	28	0.325	0.475
Trinessa	Norgestimate	0.25	0.035	T	28	0.325	0.475
Tri-Previfem	Norgestimate	0.25	0.035	T	28	0.325	0.475
MonoLinya	Norgestimate	0.25	0.035	M	28	0.325	0.475
Brevicon	NET	0.5	0.035	M	28	0.5	0.5
ModiCon	NET	0.5	0.035	M	28	0.5	0.5
Necon 0.5/35	NET	0.5	0.035	M	28	0.5	0.5
Nortrel 0.5/35	NET	0.5	0.035	M	28	0.5	0.5
Group Three: High Estrogenicity, High Potency, High Androgenicity							
Brevinor 1	NET	1	0.035	M	28	1	1
CyclaFem	NET	1	0.035	M	28	1	1
Dasetta 1/35	NET	1	0.035	M	20	1	1
Norinyl 1/35	NET	1	0.035	M	28	1	1

Brand Name	Progestin	Progestin dose (mg)	EE dose (mg)	Phase [1]	Days	Pill Potency	Pill Androgenicity
Necon 1/35	NET	1	0.035	M	28	1	1
Nortrel 1/35	NET	1	0.035	M	28	1	1
Ortho-Novum 1/35	NET	1	0.035	M	28	1	1
Blisovi Fe 1.5- 30	NET	1	0.035	M	28	1	1
Normin-1	NET	1	0.035	M	28	1	1
Nortrel 7/7/7	NET	1	0.035	T	28	1	1
Orthro Novum 7/7/7	NET	1	0.035	T	28	1	1
Group Four: Low Estrogenicity, High Potency, Low Androgenicity							
LoLoestrin Fe	NET-A	1	0.01	M	28	1.2	0.6
Junel 1/20 Fe	NET-A	1	0.02	M	28	1.2	0.6
Loestrin Fe 1/20	NET-A	1	0.02	M	28	1.2	0.6
Microgestin 1/20 Fe	NET-A	1	0.02	M	28	1.2	0.6
Blisovi 24 Fe	NET-A	1	0.02	M	24 (on)- (4 off)	1.2	0.6

M- monophasic, T- Triphasic

Subject Intake Script for Subject Familiarization Session

Subject ID:_____ Date of Visit _____

Subject Intake Script for Subject Familiarization Session

Summary Checklist

	1. Explanation of Study		**3. Eligibility Determination**
	2. Completion of Relevant Forms		**4. Relevant Questions**
	A. Informed Consent		*General Questions*
	B. PA and Health History		*OCP Compliance*
	C. Marx Activity Rating Scale		**5. Assignment of Laboratory Visit Dates**
	D. Female Hormonal History		**6. Read Reminders to Subject**

3. Eligibility Determination

Oral Contraceptive Requirement Met (YES NO) Injury History Requirement Met (YES NO)

Physical Activity Requirement Met (YES NO) Injuries if such Exist? _____

4. Questions

Important note: At this point, inform the subject that you will be touching her leg. Also inform there will always be a female research assistant present during all sessions

General Questions

Have you had your blood taken before? (YES NO) Did you have any negative reactions?

_____

What is the date of the first day of your pill pack? _____

Questions about OCP Compliance

How consistent would you say you are with your pill ingestions? Do you take your pill at the same time every day? At what time do you take them?_____

5. Assignment of first and second laboratory visits

First Visit (Circle One: Pill Week III Pill Week IV) Date/Time for 1st visit_____

Second Visit (Circle One: Pill Week III Pill Week IV) Date/Time for 2nd visit_____

Important Notes for PI: (a) Make sure you know whether subjects take a 28-pill (with off week placebo pills) or 21-day OCP. b. Consult the Excel document "Subject Group Assignments" to determine the order of laboratory visits based on Subject ID.

Age, Weight, and Height

Age (years + months)_____

Body Mass (kg/lbs)_____

Baseline Laxity Measures

Day of Pill Cycle: _____

Height (in cm) _____

General Joint Laxity

	5th	1st	Elb	Kn.	Hip
Right					
Left					

4C. Knee Laxity

	R			L		
Anterior						
Posterior						

Subject ID:_____

6. Subject Reminders

I will send a reminder for your visit at least three days before your visit date and time. In the meantime, please make sure you maintain your normal schedule of physical activity. Also, please be sure to take your birth control pill at the same time every day.

Reminder for date of week three laboratory visit: If you happen to miss taking your pill that day, please inform me, (the primary investigator), before your scheduled visit, so that I can reschedule your visit for the following day.

On the day of your laboratory visit: Please wear comfortable athletic clothing. Please do not perform any physical activity before arriving at the Applied Neuromechanics Laboratory. If you happen to be running late, please continue to walk at the pace you would normally walk. Please do not rush, as doing so may temporarily change your joint laxity, which can interfere with readings during data collection.

Laboratory Visit Data Collection Sheet

Subject ID: _____

Laboratory Visit Data Collection Sheet

Date of Visit _____ Time of Visit _____

Laboratory Visit Checklist (for PI use only)

Pill Cycle Week III IV *Pill Cycle Day* _____

☐ *3A. Intake questions* ☐☐ *3B. Blood Draws (by Technician)* ☐ *3C.Serum Processing by Technician*

☐ *3D. Sample Labeling and Storage* ☐☐ *4A. General Joint Laxity* ☐ *4B. AKL* ☐

3A. Subject Intake Questions

1 How did you get to the laboratory today? _____

2 How regularly have you taken your birth control pill since your last laboratory visit/orientation session ?(YES NO)

3 Have you taken your birth control pill at the same time every day during this pill cycle? (YES NO)

Did you take your birth control pill yesterday? (YES NO) At what time?_____(AM/PM)

4 At what time? _____

5 General Comments:

3B. Blood Draws

Technician Name _____

Volume of Blood Drawn _____ mL

4. Laxity Measurements

4A.General Joint Laxity

	5th	1st	Elb	Kn.	Hip	
Right						
Left						

4C. Anterior Knee Laxity

	R			L		
Posterior						
Anterior						

APPENDIX B: MASS SPECTROMETRY DEFINITIONS, TABLES, AND FIGURES

Mass Spectrometry Terminology

Base Peak/ Most Abundant Ion – The molecular of ion of greatest abundance within a chromatogram

Chromatogram- A visual display of the analytes eluted from the LC column during the entire LC run.

Extracted Ion Chromatogram (XIC)- The chromatogram of molecular ions of a specific *m/z* value

Injections: The method by which each standard is sampled using UPLC-MS. A needle is placed into each UPLC vial, which then takes up a small sample of the liquid contained in the UPLC vial, after which the fluid is injected into the UPLC.

LC column- A cylindrical column used to separate analytes based on their interaction with the stationary phase of the column. The stationary phase of this column was biphenyl

Mass Accuracy- The difference between the theoretical m/z value and the value measured by the mass spectrometer. This is typically measured in parts per million (ppm)

Mass-to-charge ratio (m/z) – The measurement for the mass of a molecular ion. This ratio can be equated to an analytes molecular mass in Daltons (Da) by adding or subtracting the charges depending on the ionization mode of the mass spectrometer. In this thesis, all ions are in positive mode. To find the molecular mass of a given analyte in Da, simply subtract its *m/z* by 1.

Molecular ion/Precursor ion – a charged analyte particle. In this thesis, all molecular ions carried a positive charge. In the case of an MS^2 experiment, which was performed in

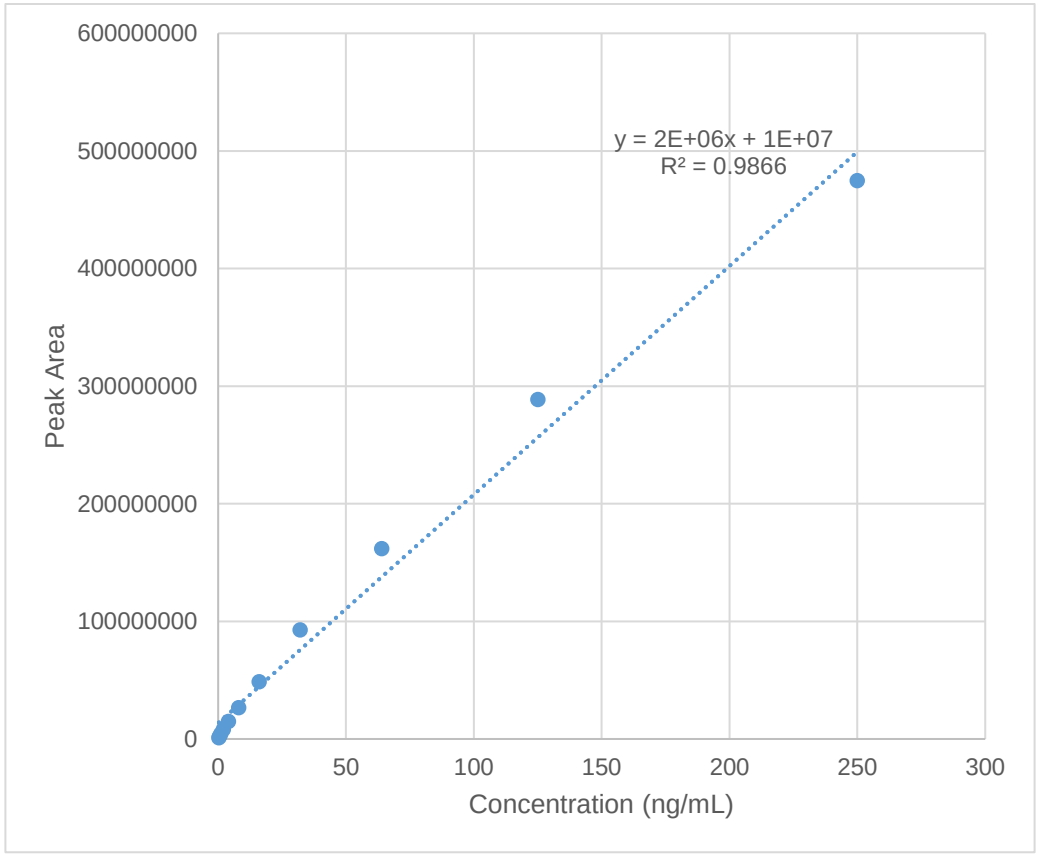
Experiment 3, the precursor ion is the original analyte particle that is fragmented in the mass spectrometer.

Peak Area- The integral of the retention time x relative abundance curve of an analytes XIC. This represents the amount of a given analyte relative to other analytes in each sample. This value can be used for inter-assay comparisons between different subjects, though an external calibration curve would be required in to compare to of another assay or sample analysis technique.

Relative abundance/Signal Intensity – A representation of the percentage of a molecular ion present within each sample, relative to the most abundant ion at a given *m/z* value.

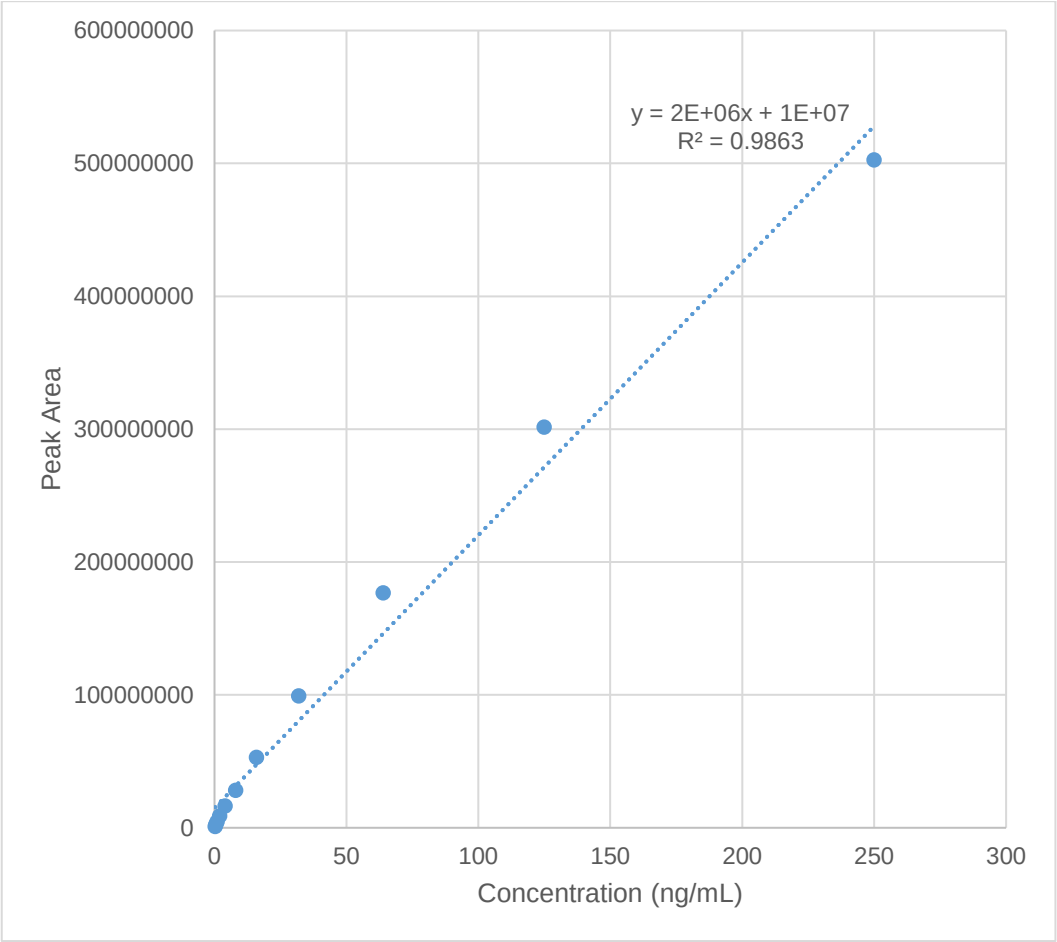
Retention Time- time taken for an analyte to elute from the LC column.

Preliminary UPLC-MS STUDY: Additional Figures



Concentration (ng/mL)	Peak Area
0.25	1080887
0.5	2201277
1	4343536
2	8301360
4	15091806
8	26862472
16	48765055
32	92856037
64	162066677
125	288799262
250	474766192

Figure B.1 Norethindrone Calibration Curve- Preliminary UPLC-MS Study (Step 3 Recovery Experiment)



Concentration (ng/mL)	Peak Area
0.25	1187631
0.5	2437072
1	4827465
2	8930127
4	16600235
8	28371413
16	53008978
32	99346210
64	176877864
125	301690547
250	502765795

Figure B.2 Levonorgestrel Calibration Curve- Preliminary UPLC-MS Study (Step 3 Recovery Experiment)

Aim I: Aim IA Post-Proposal Assay Additional Figures

Standard Preparation, Sample Preparation, UPLC Sequence

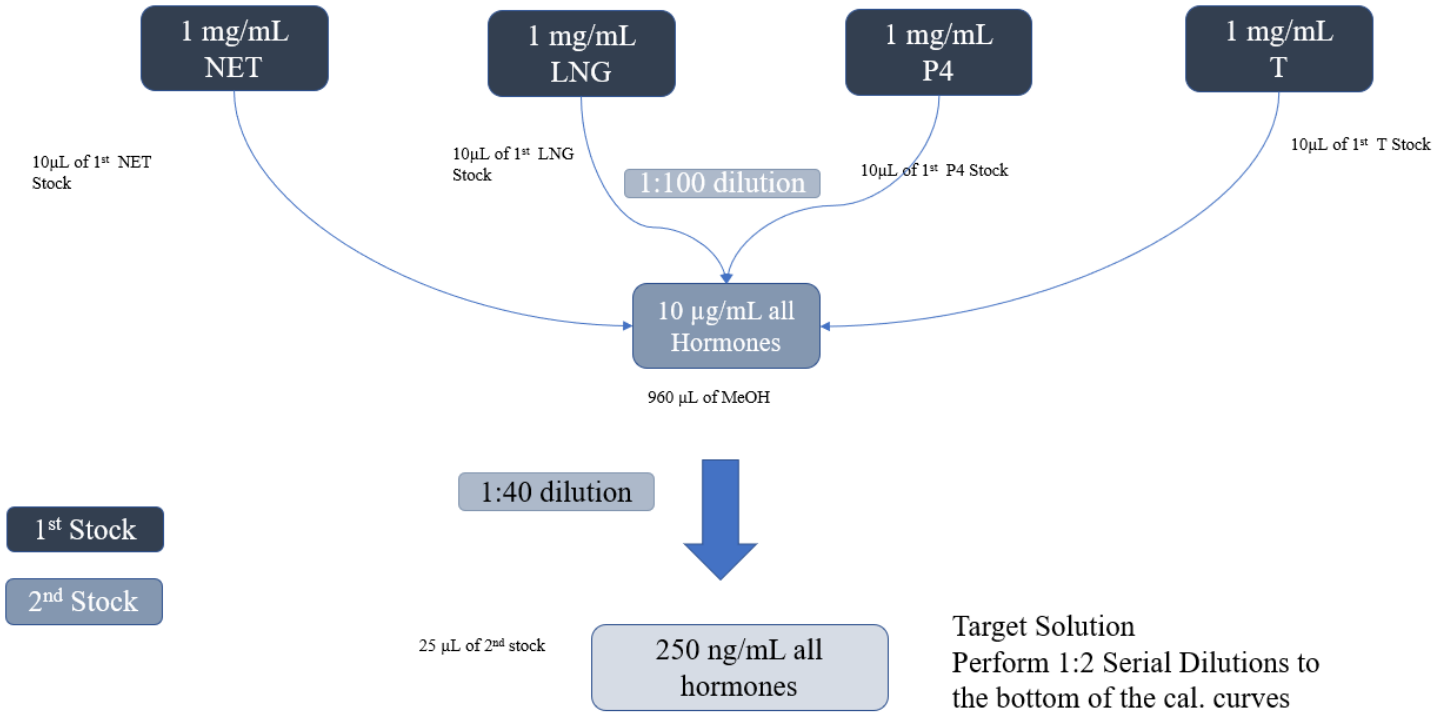


Figure B.3 Dilution Scheme for Preparation of Calibrators for External Calibration Curve (Aim IA- Post-Proposal Assay)

Table B.1 UPLC-MS Run Sequence (Aim IA Post Proposal UPLC-MS Assay)

Injection #	Sample	Autosampler Position	Injection #	Sample	Autosampler Position	Injection #	Sample	Autosampler Position	Injection #	Sample	Autosampler Position
1	BLANK1	1:A,1	28	CC7pt81-3	1:B,2	55	BLANK9	1:A,1	82	EESampleIAtt2-1	1:C,7
2	CC0pt03-1	1:A,2	29	CC15pt62-1	1:B,3	56	BLANK10	1:A,1	83	EESampleIAtt2-2	1:C,7
3	CC0pt03-2	1:A,2	30	CC15pt62-2	1:B,3	57	EESample2Att1-1	1:C,2	84	BLANK26	1:A,1
4	CC0pt03-3	1:A,2	31	CC15pt62-3	1:B,3	58	EESample2Att1-2	1:C,2	85	BLANK27	1:A,1
5	CC0pt06-1	1:A,3	32	CC31pt25-1	1:B,4	59	BLANK11	1:A,1	86	BLANK28	1:A,1
6	CC0pt06-2	1:A,3	33	CC31pt25-2	1:B,4	60	BLANK12	1:A,1	87	EESample2Att2-1	1:C,8
7	CC0pt06-3	1:A,3	34	CC31pt25-3	1:B,4	61	BLANK13	1:A,1	88	EESample2Att2-2	1:C,8
8	CC0pt12-1	1:A,4	35	CC62pt5-1	1:B,5	62	EESample3Att1-1	1:C,3	89	BLANK29	1:A,1
9	CC0pt12-2	1:A,4	36	CC62pt5-2	1:B,5	63	EESample3Att1-2	1:C,3	90	BLANK30	1:A,1
10	CC0pt12-3	1:A,4	37	CC62pt5-3	1:B,5	64	BLANK14	1:A,1	91	BLANK31	1:A,1
11	CC0pt24-1	1:A,5	38	CC125-1	1:B,6	65	BLANK15	1:A,1	92	EESample3Att2-1	1:D,1
12	CC0pt24-2	1:A,5	39	CC125-2	1:B,6	66	BLANK16	1:A,1	93	EESample3Att2-2	1:D,1
13	CC0pt24-3	1:A,5	40	CC125-3	1:B,6	67	EESample4Att1-1	1:C,4	94	BLANK32	1:A,1
14	CC0pt49-1	1:A,6	41	CC250-1	1:B,7	68	EESample4Att1-2	1:C,4	95	BLANK33	1:A,1
15	CC0pt49-2	1:A,6	42	CC250-2	1:B,7	69	BLANK17	1:A,1	96	BLANK34	1:A,1
16	CC0pt49-3	1:A,6	43	CC250-3	1:B,7	70	BLANK18	1:A,1	97	EESample4Att2-1	1:D,2
17	CC0pt98-1	1:A,7	44	BLANK2	1:A,1	71	BLANK19	1:A,1	98	EESample4Att2-2	1:D,2
18	CC0pt98-2	1:A,7	45	BLANK3	1:A,1	72	EESample5Att1-1	1:C,5	99	BLANK35	1:A,1
19	CC0pt98-3	1:A,7	46	BLANK4	1:A,1	73	EESample5Att1-2	1:C,5	100	BLANK36	1:A,1
20	CC1pt95-1	1:A,8	47	BlankSerumAtt1-1	1:B,8	74	BLANK20	1:A,1	101	BLANK37	1:A,1
21	CC1pt95-2	1:A,8	48	BlankSerumAtt1-2	1:B,8	75	BLANK21	1:A,1	102	EESample5Att2-1	1:D,3

22	CC1pt95-3	1:A,8	49	BLANK5	1:A,1	76	BLANK22	1:A,1	103	EESample5Att2-2	1:D,3
Injection #	Sample	Autosampler Position	Injection #	Sample	Autosampler Position	Injection #	Sample	Autosampler Position	Injection #	Sample	Autosampler Position
23	CC3pt91-1	1:B,1	50	BLANK6	1:A,1	77	BlankSerumAtt2-1	1:C,6	104	blank38	1:A,1
24	CC3pt91-2	1:B,1	51	BLANK7	1:A,1	78	BlankSerumAtt2-2	1:C,6	105	blank39	1:A,1
25	CC3pt91-3	1:B,1	52	EESampleIAtt1-1	1:C,1	79	BLANK23	1:A,1	106	blank40	1:A,1
26	CC7pt81-1	1:B,2	53	EESampleIAtt1-2	1:C,1	80	BLANK24	1:A,1			
27	CC7pt81-2	1:B,2	54	BLANK8	1:A,1	81	BLANK25	1:A,1			

CC- Calibration Curve; EE- Extraction Efficiency

Extracted Ion Chromatograms

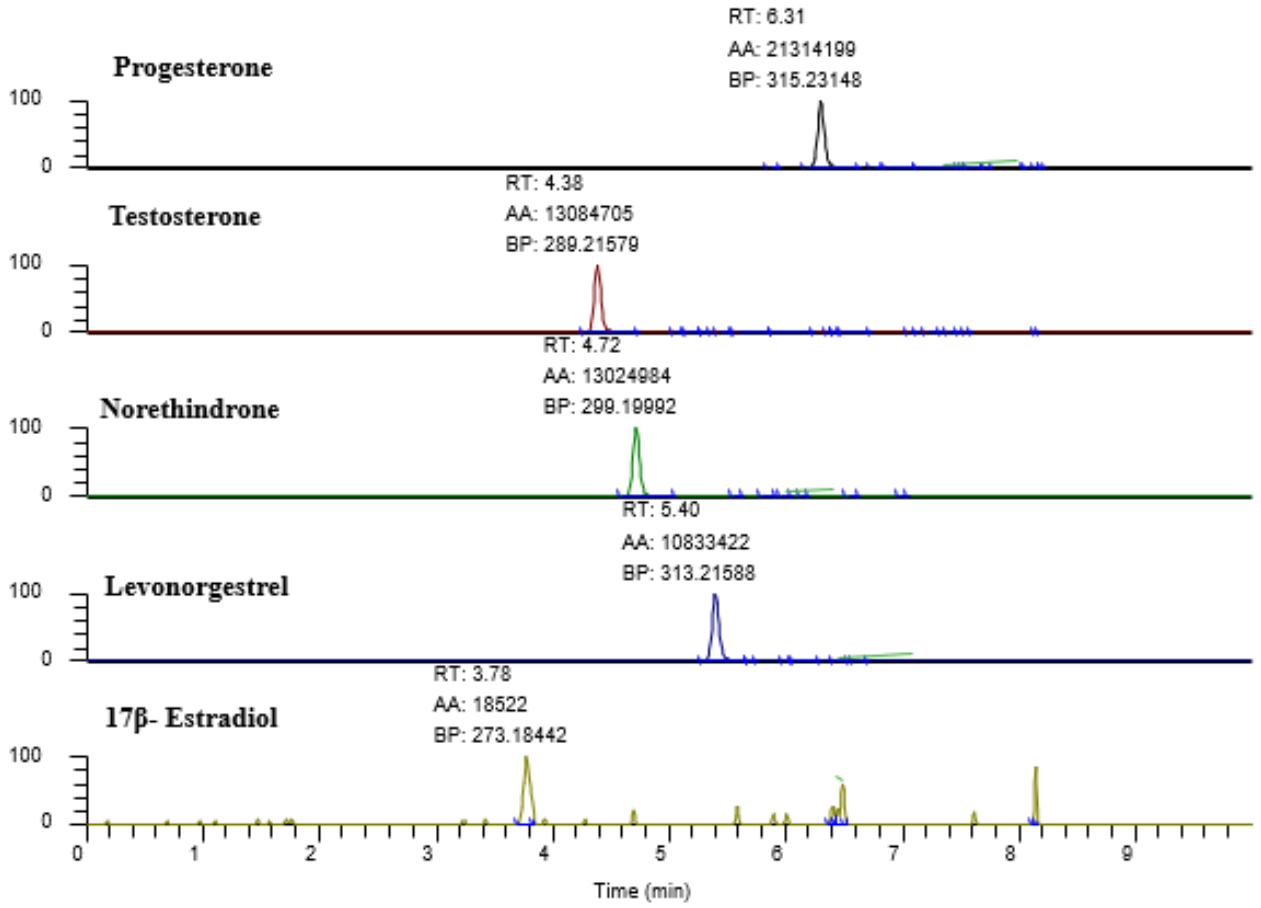


Figure B.4 Example XIC for 250 ug/mL calibrator, Replicate I (Aim IA-Post Proposal Assay)

RT: Retention Time, AA: Peak Area, BP: m/z value of base peak

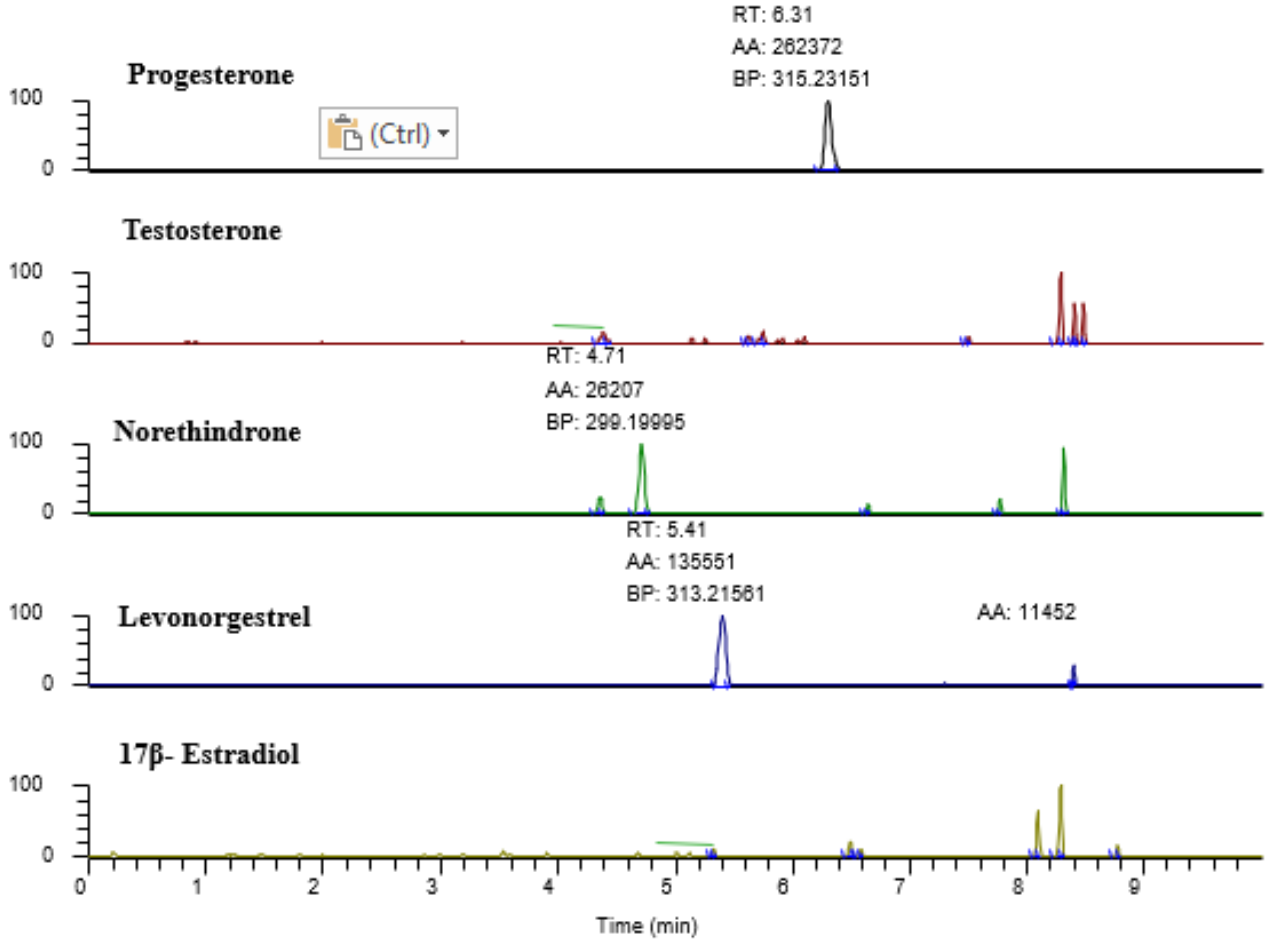


Figure B.5 Serum Blank (From Participant Who Was not on OCP)

RT: Retention Time, AA: Peak Area, BP: m/z value of base peak

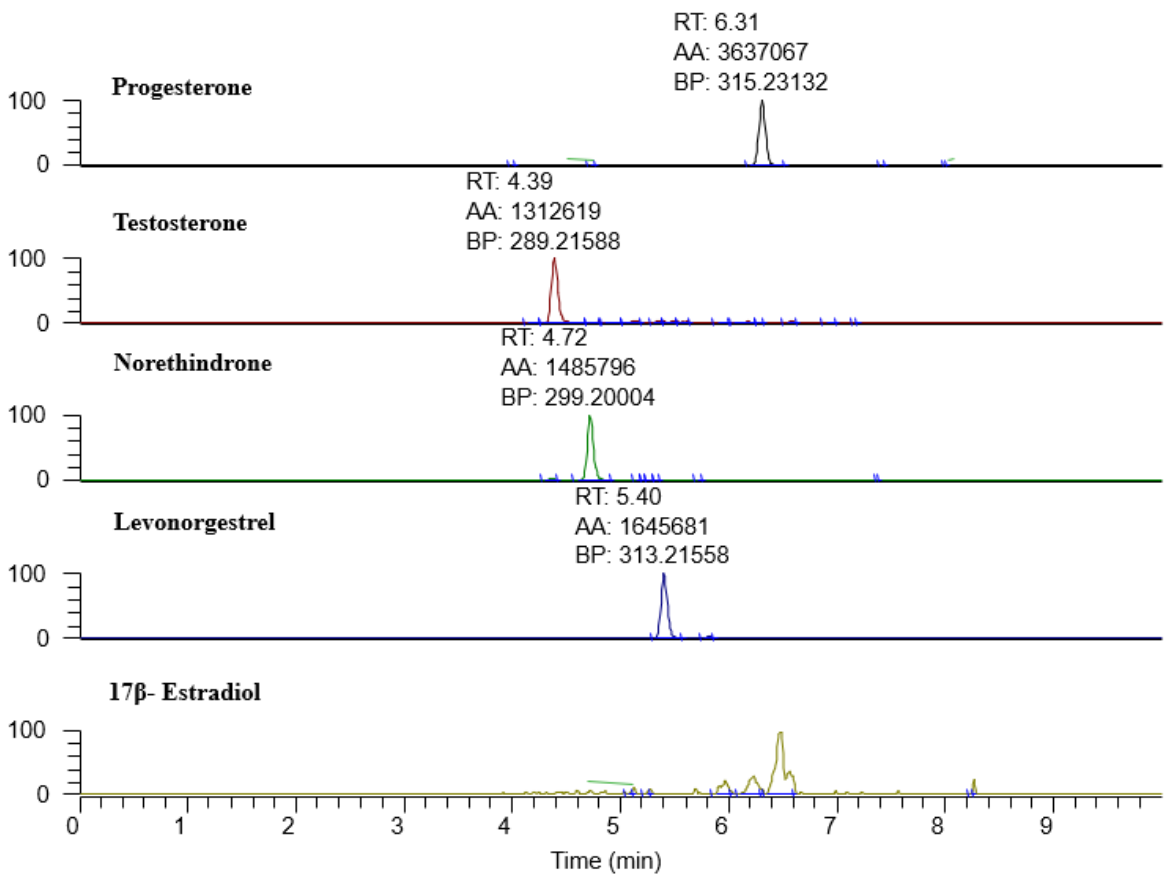


Figure B.6- Example XIC for Technical Replicate #1 Aim IA Post Proposal Assay

RT: Retention Time, AA: Peak Area, BP: m/z value of base peak

External Calibration Curves

Progesterone

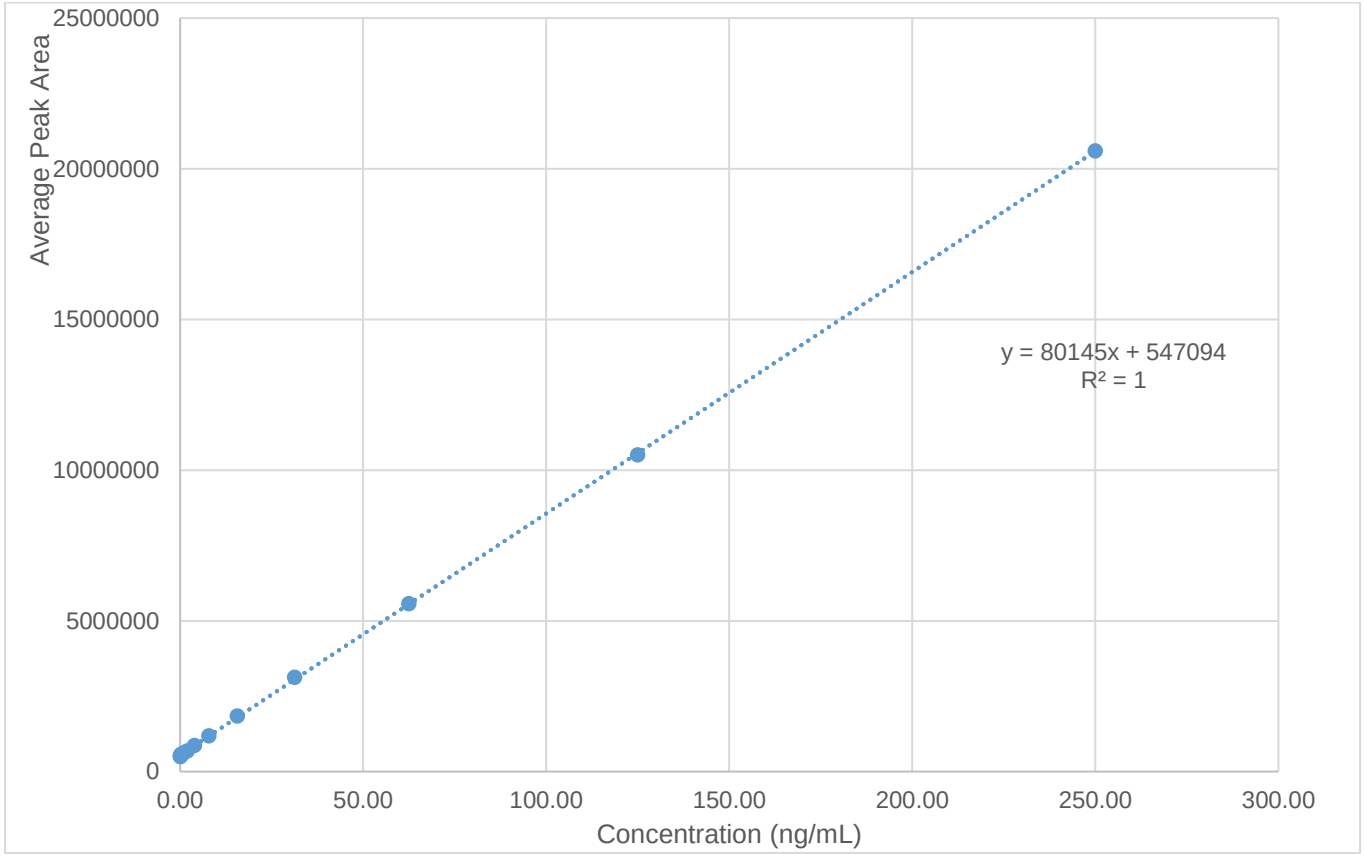


Table B.2 Progesterone Calibration Curve (Aim IA-Post Proposal Assay)

Standard Concentration (ng/mL)	Injection 1 Peak Area	Injection 2 Peak Area	Injection 3 Peak Area	Average Peak Area	St. Dev	% CV	Predicted Concentration (ng/mL)	RE%
0.03 (LLOD)	**488491**	**510770**	**493719**	**497660**	**15754**	3%	**-0.62**	**-2121%**
0.06	507086	536227	540659	527991	20606	4%	-0.24	-491%
0.12	506740	554154	562605	541166	33527	6%	-0.07	-161%
0.24	564644	579214	573974	572611	10303	2%	0.32	30%
0.49 (LLOQ)	**570750**	**564644**	**620732**	**585375**	**4318**	1%	**0.48**	**-2%**
0.98	621219	620700	622586	621502	367	0%	0.93	-5%
1.95	691617	694957	681697	689424	2362	0%	1.78	-9%
3.91	855860	860138	885556	867185	3025	0%	3.99	2%
7.81	1157050	1184672	1211072	1184265	19532	2%	7.95	2%
15.63	1823811	1836238	1859601	1839883	8787	0%	16.13	3%
31.25	3105495	3151275	3132353	3129708	32371	1%	32.22	3%
62.50	5619131	5563774	5526133	5569679	39143	1%	62.67	0%
125.00	10481359	10461557	10575294	10506070	14002	0%	124.26	-1%
250.00	20128759	20333160	21329354	20597091	642341	3%	250.17	0%
					Average CV%	**2%**	**Average Residual %**	**-1%**

Testosterone

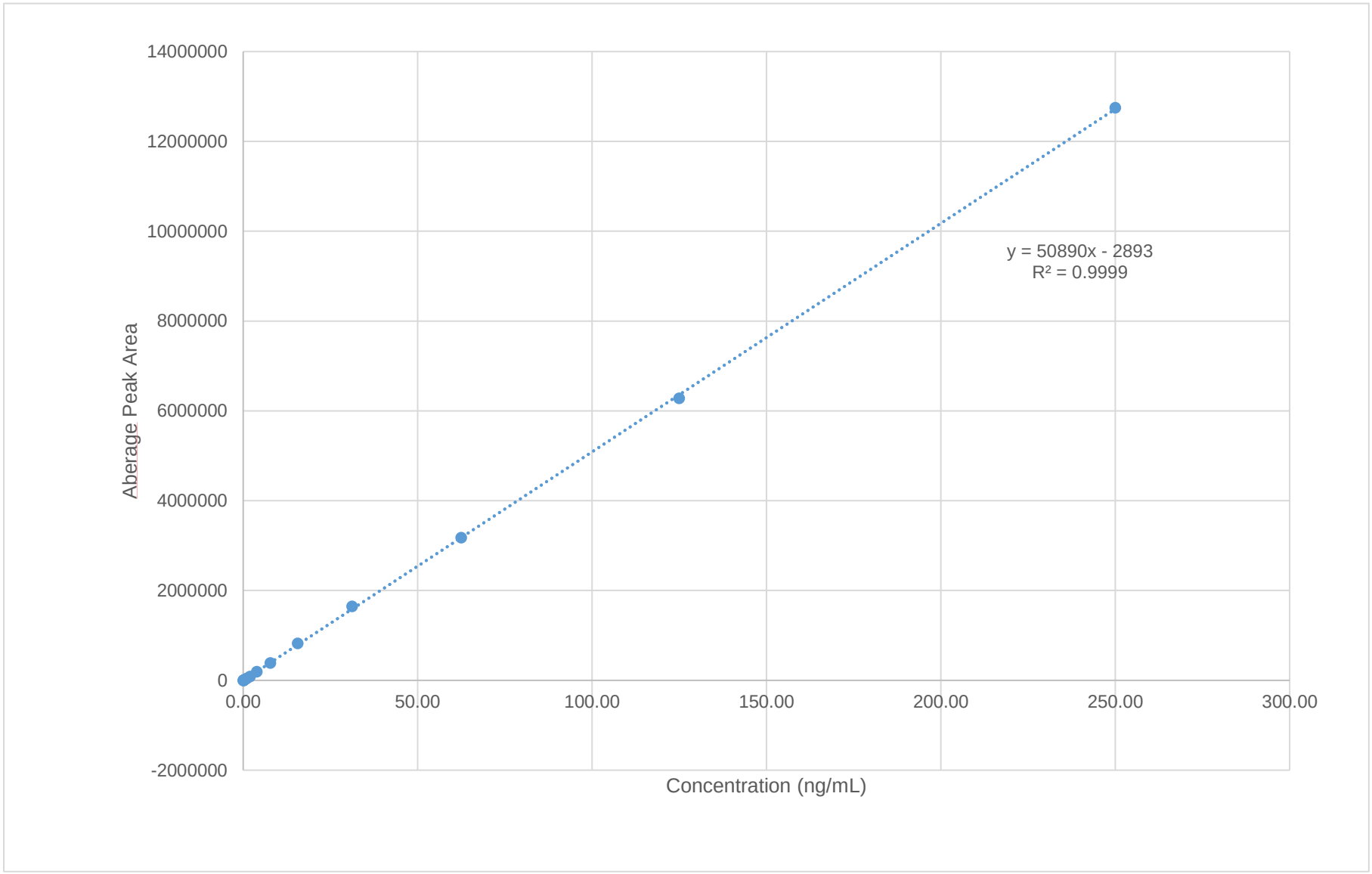


Table B.3 Testosterone Calibration Curve (Aim IA)

Standard Concentration (ng/mL)	Injection 1 Peak Area	Injection 2 Peak Area	Injection 3 Peak Area	Average Peak Area	St. Dev	% CV	Predicted Concentration (ng/mL)	RE%
0.03	0	0	0	0	0	0	**N/A**	**N/A**
0.06	0	0	0	0	0	0	**N/A**	**N/A**
0.12	0	0	0	0	0	0	**N/A**	**N/A**
0.24 (LLOD/LLOQ)	**6319**	**9773**	**7898**	**7997**	**2442**	31%	**0.21**	**-12%**
0.49	17944	18642	21051	19212	494	3%	0.43	-11%
0.98	46500	40480	44655	43878	4257	10%	0.92	-6%
1.95	80306	88113	82296	83572	5520	7%	1.70	-13%
3.91	192649	185776	191927	190117	4860	3%	3.79	-3%
7.81	384187	382746	389265	385399	1019	0%	7.63	-2%
15.63	807246	809831	843615	820231	1828	0%	16.17	4%
31.25	1629763	1628271	1677251	1645095	1055	0%	32.38	4%
62.50	3156497	3198407	3178710	3177871	29635	1%	62.50	0%
125.00	6196846	6242015	6397035	6278632	31939	1%	123.43	-1%
250.00	12558772	12543124	13151428	12751108	346776	3%	250.62	0%
					Average CV%	**5%**	Average Residual %	**-4%**

Norethindrone

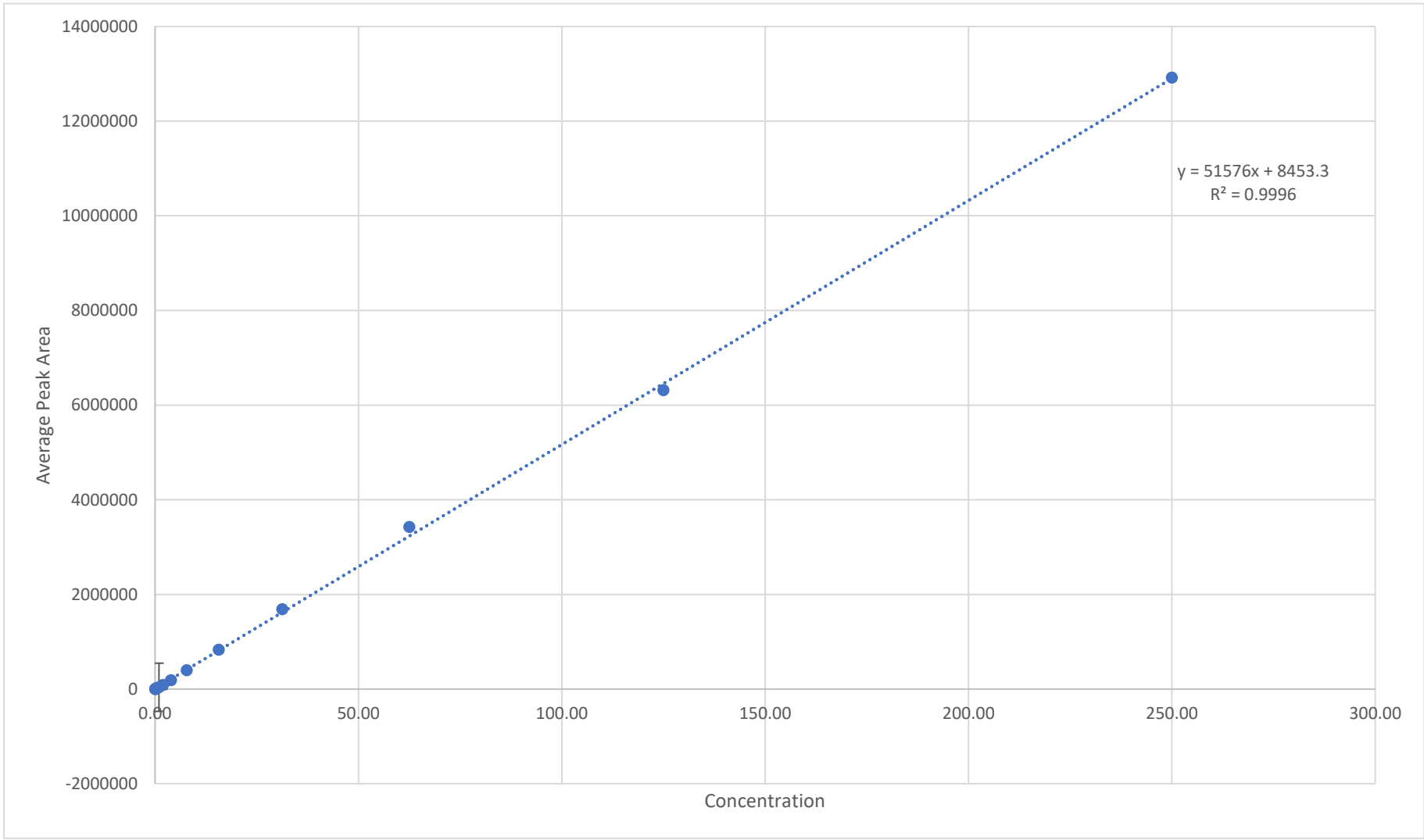


Table B.4 Norethindrone Calibration Curve (Aim IA)

Standard Concentration (ng/mL)	Injection 1 Peak Area	Injection 2 Peak Area	Injection 3 Peak Area	Average Peak Area	St. Dev	% CV	Predicted Concentration (ng/mL)	RE%
0.03	0	0	0	0	0	0	-0.16	-637%
0.06	0	0	0	0	0	0	-0.16	-369%
0.12	0	0	0	0	0	0	-0.16	-234%
0.24 (LLOD)	**3070**	**10008**	**9612**	**7563**	**4906**	65%	**-0.02**	**-107%**
0.49	24348	18091	17687	20042	4424	22%	0.22	-54%
0.98	35059	37769	39691	37506	1916	5%	0.56	-42%
1.95	83391	88650	85028	85690	3719	4%	1.50	-23%
3.91 (LLOQ)	**180798**	**195981**	**183563**	**186781**	**10736**	6%	**3.46**	**-11%**
7.81	399533	390982	402017	397511	6046	2%	7.54	-3%
15.63	829514	824062	831961	828512	3855	0%	15.90	2%
31.25	1669265	1702910	1687208	1686461	23791	1%	32.53	4%
62.50	5619131	5563774	5526133	5569679	39143	15%	62.67	0%
125.00	10481359	10461557	10575294	10506070	14002	1%	124.26	-1%
250.00	20128759	20333160	21329354	20597091	642341	1%	250.17	0%
					Average CV%	**11%**	**Average Residual %**	**-4%**

Levonorgestrel

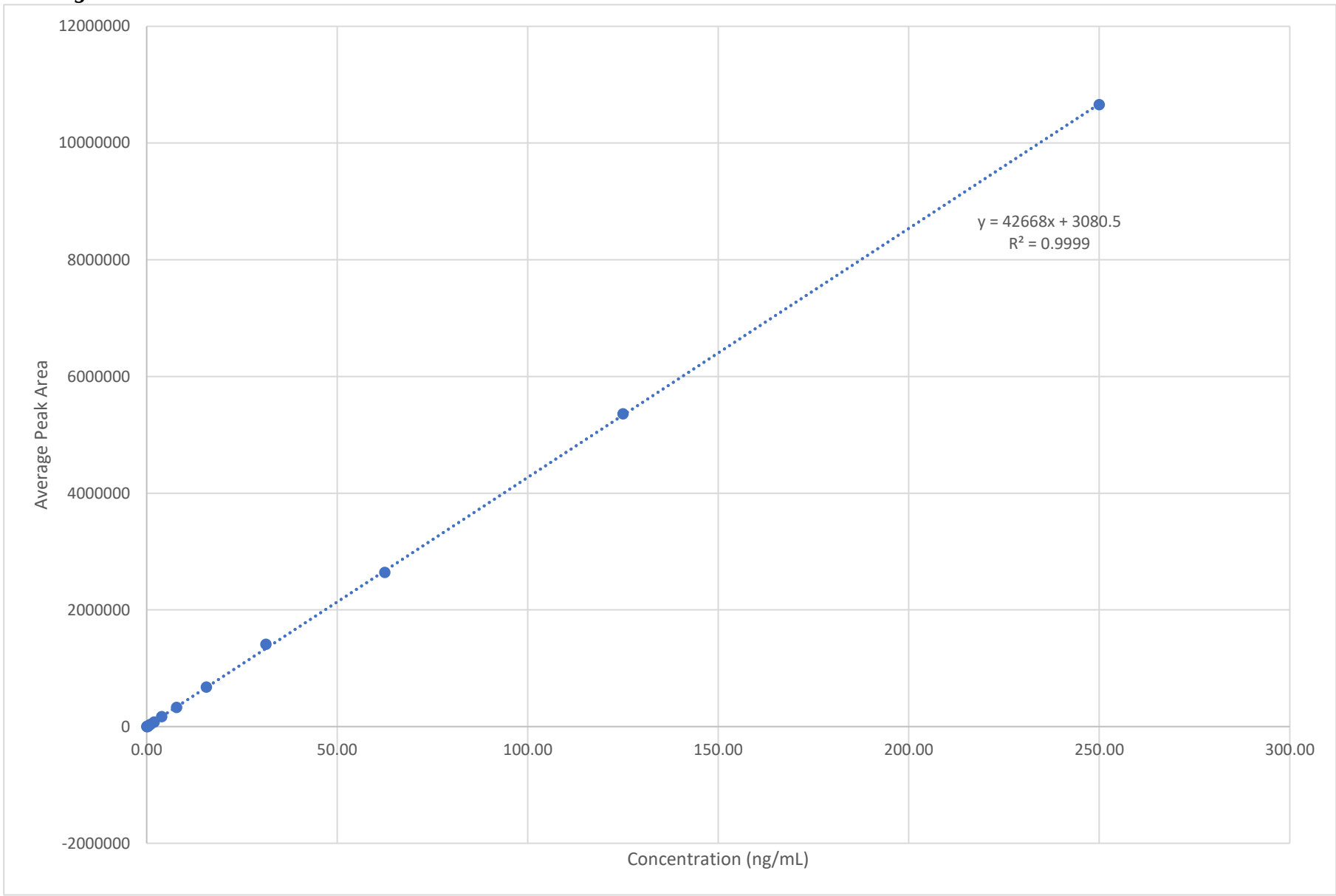


Table B.5 Levonorgestrel Calibration Curve (Aim IA)

Standard Concentration (ng/mL)	Injection 1 Peak Area	Injection 2 Peak Area	Injection 3 Peak Area	Average Peak Area	St. Dev	% CV	Predicted Concentration (ng/mL)	RE%
0.03	0	0	0	0	0	N/A	-0.07	-637%
0.06	0	0	0	0	0	N/A	-0.07	-369%
0.12 (LLOD)	**0**	**0**	**4301**	**4301**	**0**	**N/A**	**0.03**	**-234%**
0.24	0	0	0	0	0	N/A	-0.07	**-107%**
0.49	14439	Bad Injection	17611	16025	2243	14%	0.30	-54%
0.98	38206	38424	37708	38113	154	0%	0.82	-42%
1.95	71409	76244	79041	75565	3419	5%	1.70	-23%
3.91 (LLOQ)	**167652**	**178796**	**162635**	**169694**	**7880**	5%	**3.90**	**-11%**
7.81	321785	324387	344207	330126	1840	1%	7.66	-3%
15.63	676210	665594	691307	677704	7507	1%	15.81	2%
31.25	1429895	1401450	1394023	1408456	20114	1%	32.94	4%
62.50	2647149	2671311	2603433	2640631	17085	1%	61.82	0%
125.00	5294677	5304091	5473142	5357303	6657	0%	125.49	-1%
250	10596711	10551944	10824680	10657778	146264	1%	249.71	0%
					Average CV%	**3%**	**verage Residual %**	**-3%**

Estradiol

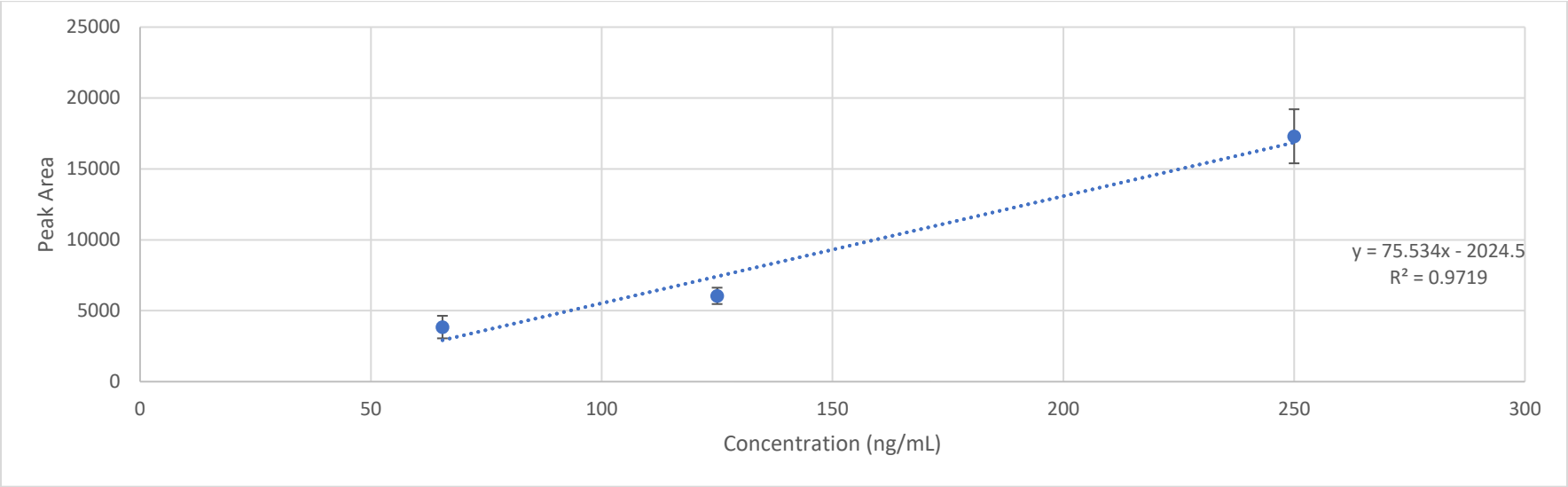


Table B.6 Estradiol Calibration Curve Raw Data (Aim IA-Post Proposal Assay)

Standard Concentration (ng/mL)	Injection 1 Peak Area	Injection 2 Peak Area	Injection 3 Peak Area	Average Peak Area	St. Dev	% CV	Predicted Concentration (ng/mL)	RE%
65.5	3288	4411	0	3849.5	794.1	21%	77.8	-19%
125	5724	6715	5710	6049.7	576.2	10%	106.9	14%
250	18522	15105	18273	17300.0	1905.0	11%	255.8	-2%
					Average %CV	**14%**	**Average Residual %**	**-2%**

Recovery Experiment Expanded Tables

Table B.7 Recovery Experiment Expanded Table (First Solvent Rinse)

Analyte	Sample Designation	Expected Concentration (ng/mL)	Injection 1 Peak Area	Injection 2 Peak Area	Average	St. Dev	Difference from Blank	Measured Concentration (ng/mL)	% Recovery
Progesterone	Blank	0	263846	279527	271686.5	11088	0	< LOQ	
	Technical Replicate 1	75	3644581	2745227	3194904	635939	2923217.5	29.6	39.53%
	Technical Replicate 2	75	3290962	2595261	2943111.5	23977	2671425	26.5	35.34%
	Technical Replicate 3	250	9695570	7011174	8353372	491935	8081685.5	94	37.60%
	Technical Replicate 4	250	9298357	7023792	8161074.5	1898155	7889388	91.6	36.64%
							Total % Recovery		**37.28 ± 1.8%**
Testosterone	Blank	0	6650	8126	7388	1044	0	< LOQ	
	Technical Replicate 1	75	1324920	988648	1156784	237780	1155740	22.8	30.40%
	Technical Replicate 2	75	1244134	1024542	1134338	155275	1133294	22.3	29.80%
	Technical Replicate 3	250	3181399	2326292	2753845.5	604652	2752802	54.1	21.70%
	Technical Replicate 4	250	2509686	2054583	2282134.5	321806	2281091	44.9	18.00%
							Total % Recovery		**24.9 ± 6.1%**

Analyte	Sample Designation	Expected Concentration (ng/mL)	Injection 1 Peak Area	Injection 2 Peak Area	Average	St. Dev	Difference from Blank	Measured Concentration (ng/mL)	% Recovery
Norethindrone	Blank	0	26688	33589	30138.5	4879.7	0	< LOQ	
	Technical Replicate 1	75	1485894	1110391	1298143	265520.7	1026456	29.6	39.50%
	Technical Replicate 2	75	1470999	1212440	1341720	182828.8	693734.5	26.5	35.30%
	Technical Replicate 3	250	4365841	3109980	3737911	888027.8	1070033	94	37.60%
	Technical Replicate 4	250	3845806	2997239	3421523	600027.5	3466224	91.6	36.60%
							Total % Recovery		**26.4 ± 1.3%**
Levonorgestrel	Blank	0	135551	142215	140220.5	2821	0	<LOQ	
	Technical Replicate 1	75	1646508	1185889	1416198.5	325707	2923217.5	26.8	35.67%
	Technical Replicate 2	75	1508920	1189256	1349088	226037	2671425	25.2	33.57%
	Technical Replicate 3	250	4815146	3567823	4191484.5	881991	8081685.5	91.8	36.72%
	Technical Replicate 4	250	4681135	3647014	4164074.5	731234	7889388	91.2	36.46%
						1044	**Total % Recovery**		**35.61 ±1.4%**

Table B.8 Recovery Experiment (Second Solvent Rinse)

Analyte	Sample Designation	Expected Concentration (ng/mL)	Injection 1 Peak Area	Injection 2 Peak Area	Average	St. Dev	Peak Area Difference from Blank	Measured Concentration (ng/mL)	% Recovery
Progesterone	Blank	0	458505	289915	374210	119211.1322		-6.83	
	Technical Replicate 1	75	5457457	5579297	5518377	86153.89022	5246690.5	58.64	59%
	Technical Replicate 2	75	5463213	6096129	5779671	447539.1955	5507984.5	61.90	62%
	Technical Replicate 3	250	8415592	7496636	7956114	649800.0192	7684427.5	89.05	36%
	Technical Replicate 4	250	8362571	901589	4632080	5275710.967	4360393.5	47.58	19%
Testosterone	Blank	0	257968	201612	229790	39849.70976	228746.3104	4.6	
	Technical Replicate 1	75	105734	107914	106824	1541.492783	105780.3104	2.1	Out of CC range
	Technical Replicate 2	75	156742	169149	162945.5	8773.073834	161901.8104	3.2	Out of CC range
	Technical Replicate 3	250	359119	327580	343349.5	22301.44077	342305.8104	6.8	3%
	Technical Replicate 4	250	529347	597429	563388	48141.24388	562344.3104	11.1	4%
Norethindrone	Blank	0	664014	554516	609265	77426.77833		-16%	
	Technical Replicate 1	75	109900	110920	110410	721.2489168	-161276.5	-3.3	-4%
	Technical Replicate 2	75	163537	170758	167147.5	5106.018067	-104539	9.7	-3%
	Technical Replicate 3	250	421821	384017	402919	26731.46476	131232.5	-2.2	1%
	Technical Replicate 4	250	677197	749270	713233.5	50963.30704	441547	2.4	3%
Levonorgestrel	Blank	0	2988302	2131333	2559818	605968.5912		-0.16	
	Technical Replicate 1	75	129563	127112	128337.5	1733.118721	-143349	-3.4	-1%
	Technical Replicate 2	75	201404	189716	195560	8264.664059	-76126.5	-1.9	-1%
	Technical Replicate 3	250	606609	531714	569161.5	52958.76238	297475	6.9	3%
	Technical Replicate 4	250	794049	934725	864387	99472.95355	592700.5	13.8	6%

Calculations

Equation 8 Expected Recoveries for Technical Replicates 1-4

Replicates 1 and 2

$$(24\ \mu L\ of\ 4\ hormone\ mixture)\left(\frac{250\ ng}{1000\ \mu L}\right)\left(\frac{1}{0.080\ mL\ of\ 1{:}1\ MeOH\ solution}\right)$$

$$=\ 75\frac{ng}{mL} expected\ recovery\ \ of\ all\ hormones$$

Replicates 3 and 4

$$(80\ \mu L\ of\ 4\ hormone\ mixture)\left(\frac{250\ ng}{1000\ \mu L}\right)\left(\frac{1}{0.080\ mL\ of\ 1{:}1\ MeOH\ solution}\right)$$

$$=\ 250\frac{ng}{mL} expected\ recovery\ \ of\ all\ hormones$$

Equation 9 Determination of Progesterone in Serum (Sample Recovery Experiment Calculation)

1. Calculated Difference from Blank (Peak Areas)

$$3194904\ -271686.5=\ 2923217.5$$

2. Measured Concentration by using regression line from Progesterone Calibration Curve

$$Concentration = \left(\frac{2923217.5 - 547094.008}{80145.48}\right) = 26.5\frac{ng}{mL}$$

3. % Recovery

$$\%\ Analyte\ Recovered\ \frac{26.5\frac{ng}{mL} measured}{75\frac{ng}{ml}\ expected} = 39.53\%$$

Technical Replicate 1, Used as an example

Aim I: Aim IB Additional Figures

Standard Preparation, Sample Preparation, UPLC Sequence

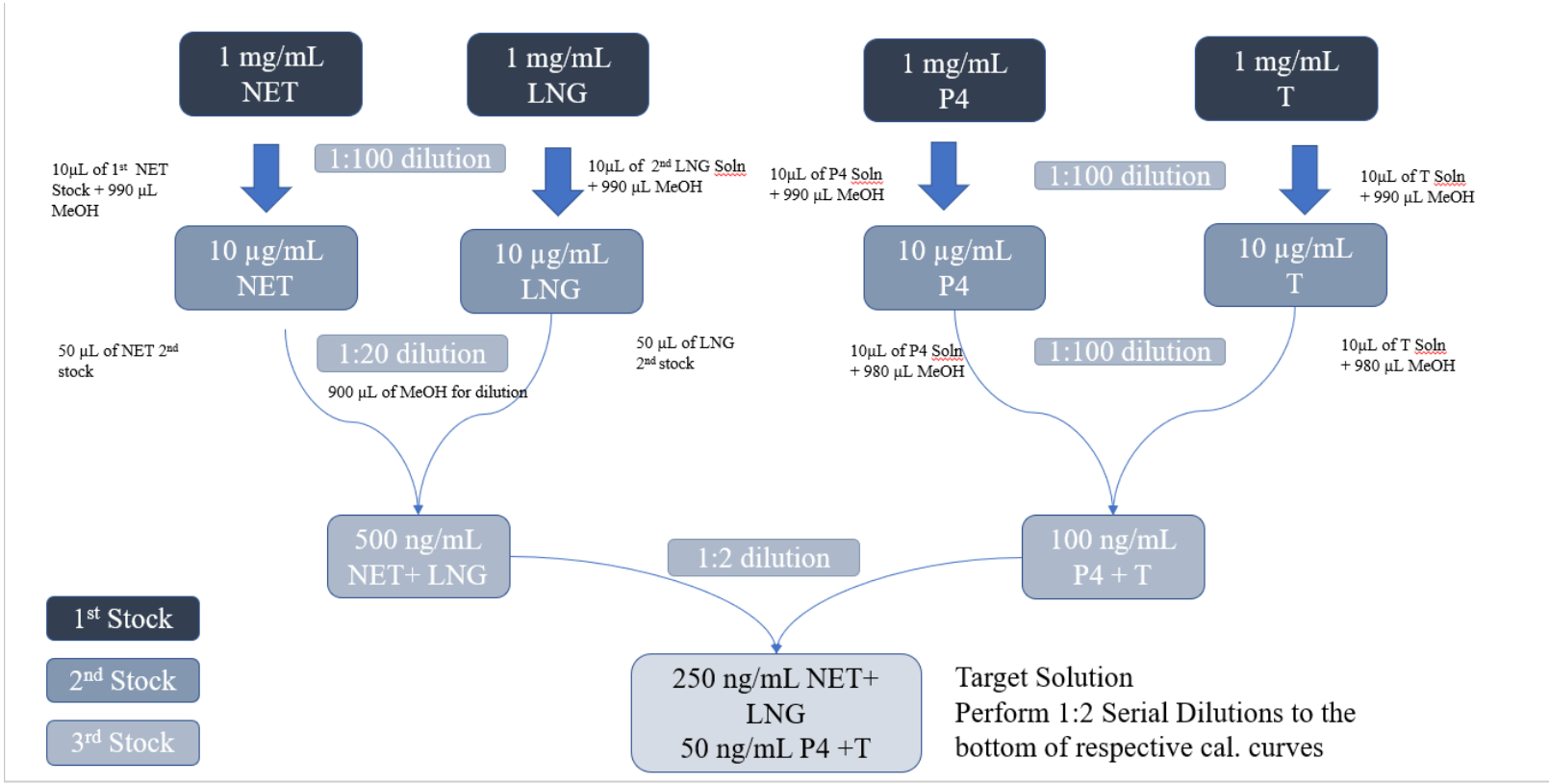


Figure B.7 Dilution Scheme for Preparation of External Calibration Curves (Aim IB)

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Table B.9 UPLC-MS Run Sequence (Aim IB)

Injection #	Sample	Autosampler Position	Injection #	Sample	Autosampler Position
1	BLANK1	1:A,1	46	BLANK8	1:A,1
2	CC0pt24-1	1:A,2	47	BLANK9	1:A,1
3	CC0pt49-1	1:A,3	48	BLANK10	1:A,1
4	CC0pt98-1	1:A,4	49	EXOAKL12B	1:F,5
5	CC1pt95-1	1:A,5	50	BLANK11	1:F,5
6	CC3pt91-1	1:A,6	51	BLANK12	1:F,5
7	CC7pt81-1	1:A,7	52	EXOAKLextraction	1:F,6
8	CC15pt63-1	1:A,8	53	BLANK13	1:F,6
9	CC31pt25-1	1:B,1	54	BLANK14	1:F,6
10	CC62p5-1	1:B,2	55	BLANK15	1:F,6
11	CC125-1	1:B,3	56	EXOAKL01wk4	1:D,5
12	CC250-1	1:B,4	57	EXOAKL02wk4	1:D,6
13	BLANK2	1:A,1	58	EXOAKL03wk4	1:D,7
14	BLANK3	1:A,1	59	EXOAKL04wk4	1:D,8
15	BLANK4	1:A,1	60	EXOAKL05wk4	1:E,1
16	EXOAKL01wk3	1:B,5	61	EXOAKL06wk4	1:E,2
17	EXOAKL02wk3	1:B,6	62	EXOAKL07wk4	1:E,3
18	EXOAKL03wk3	1:B,7	63	EXOAKL08wk4	1:E,4
19	EXOAKL04wk3	1:B,8	64	EXOAKL09wk4	1:E,5
20	EXOAKL05wk3	1:C,1	65	EXOAKL10wk4	1:E,6
21	EXOAKL06wk3	1:C,2	66	EXOAKL11wk4	1:E,7
22	EXOAKL07wk3	1:C,3	67	EXOAKL12wk4	1:E,8
23	EXOAKL08wk3	1:C,4	68	EXOAKL13wk4	1:F,1
24	EXOAKL09wk3	1:C,5	69	EXOAKL14wk4	1:F,2
25	EXOAKL10wk3	1:C,6	70	EXOAKL15wk4	1:F,3
26	EXOAKL11wk3	1:C,7	71	EXOAKL16wk4	1:F,4
27	EXOAKL12wk3	1:C,8	72	EXOAKL12B	1:F,5
28	EXOAKL13wk3	1:D,1	73	BLANK16	1:A,1
29	EXOAKL14wk3	1:D,2	74	BLANK17	1:A,1
30	EXOAKL15wk3	1:D,3	75	EXOAKLextraction	1:F,6
31	EXOAKL16wk3	1:D,4	76	BLANK18	1:A,1
32	BLANK5	1:A,1	77	BLANK19	1:A,1
33	BLANK6	1:A,1	78	BLANK20	1:A,1
34	BLANK7	1:A,1	79	CC0pt24-3	1:A,2
35	CC0pt24-2	1:A,2	80	CC0pt49-3	1:A,3
36	CC0pt49-2	1:A,3	81	CC0pt98-3	1:A,4
37	CC0pt98-2	1:A,4	82	CC1pt95-3	1:A,5
38	CC1pt95-2	1:A,5	83	CC3pt91-3	1:A,6
39	CC3pt91-2	1:A,6	84	CC7pt81-3	1:A,7
40	CC7pt81-2	1:A,7	85	CC15pt63-3	1:A,8
41	CC15pt63-2	1:A,8	86	CC31pt25-3	1:B,1
42	CC31pt25-2	1:B,1	87	CC62pt5-3	1:B,2
43	CC62pt5-2	1:B,2	88	CC125-3	1:B,3
44	CC125-2	1:B,3	89	CC250-3	1:B,4
45	CC250-2	1:B,4	90	BLANK21	1:A,1

Injection #	Sample	Autosampler Position
91	BLANK22	1:A,1

Extracted Ion Chromatograms

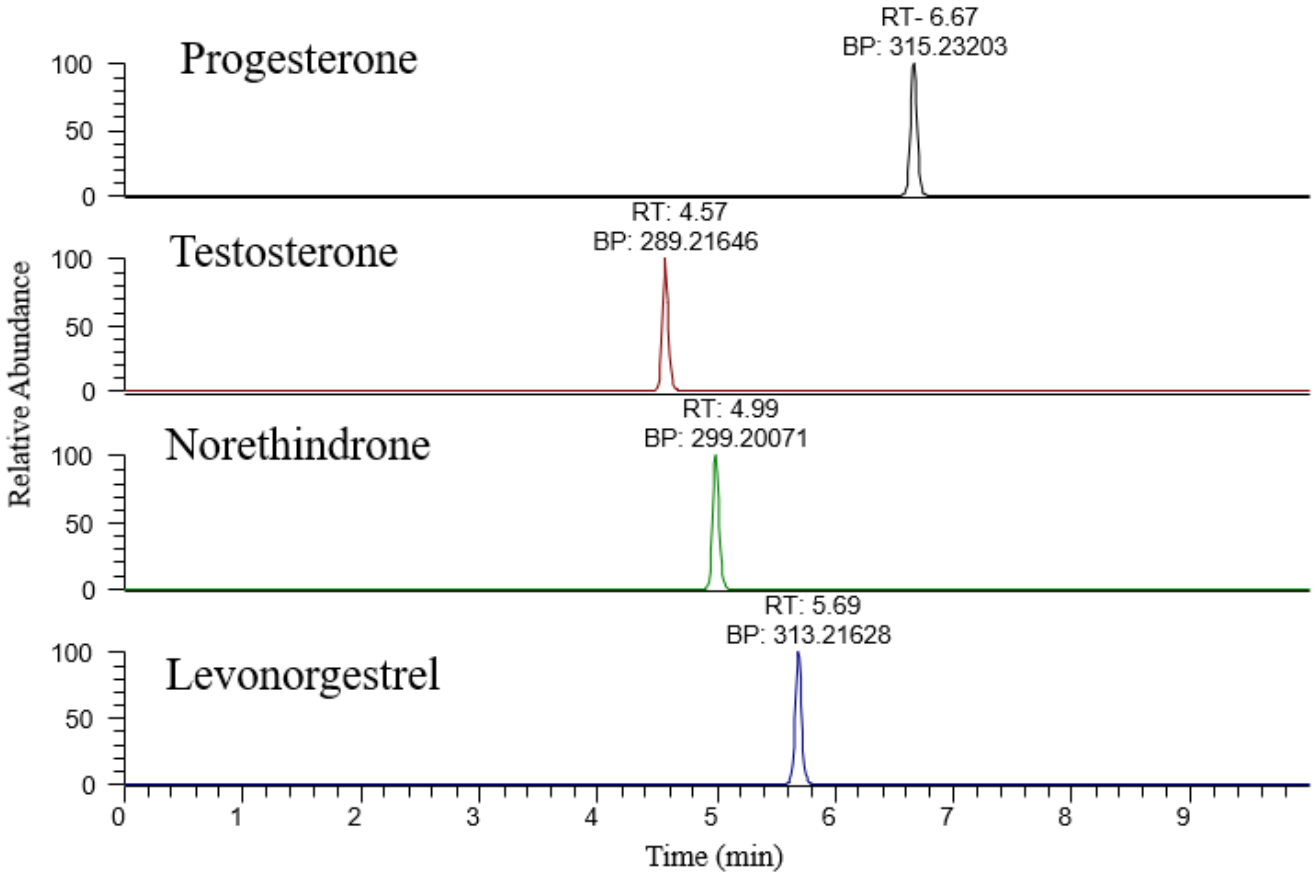


Figure B.8 XIC for 250 ug/mL Calibrator, Replicate 1, Aim IB

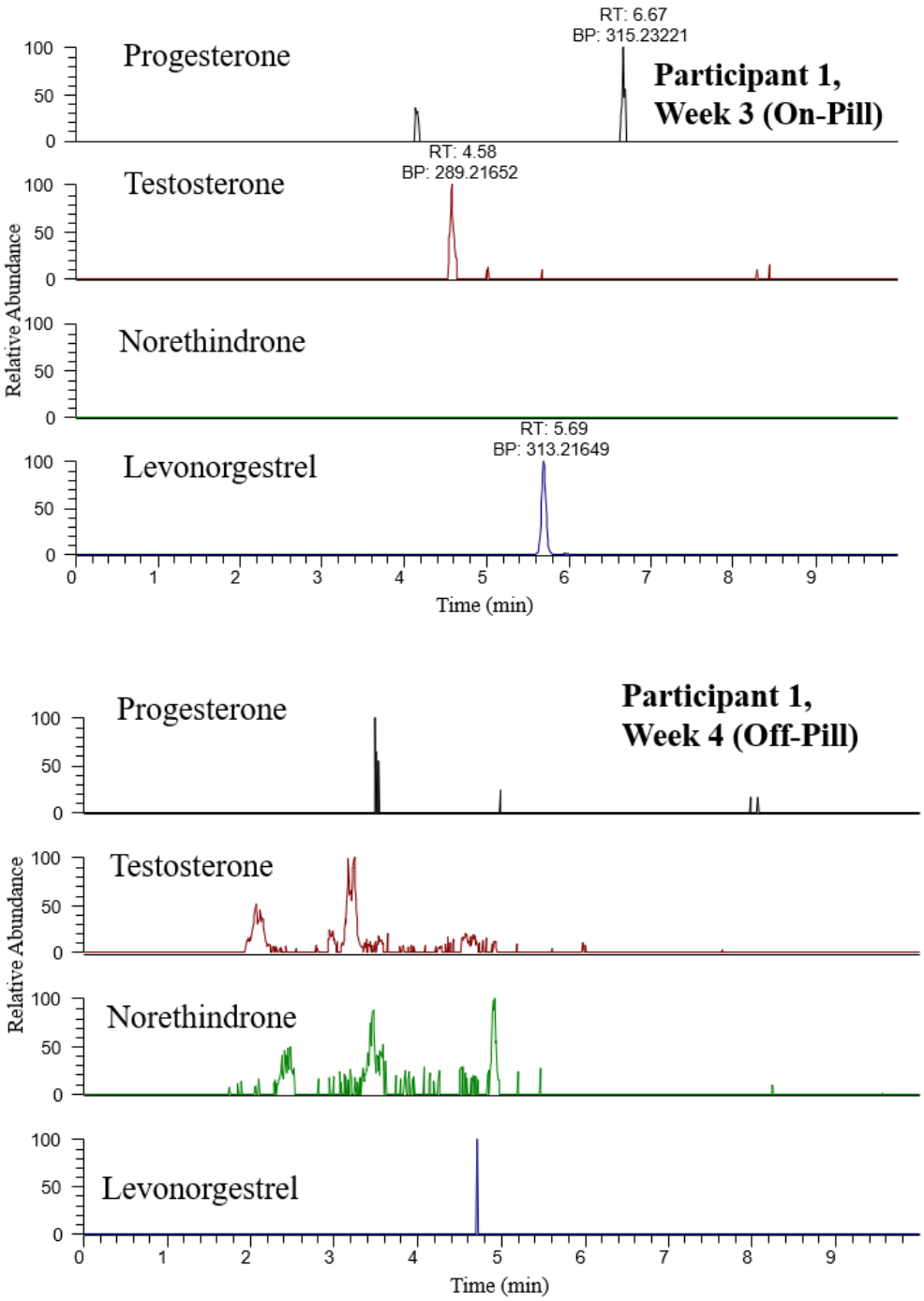
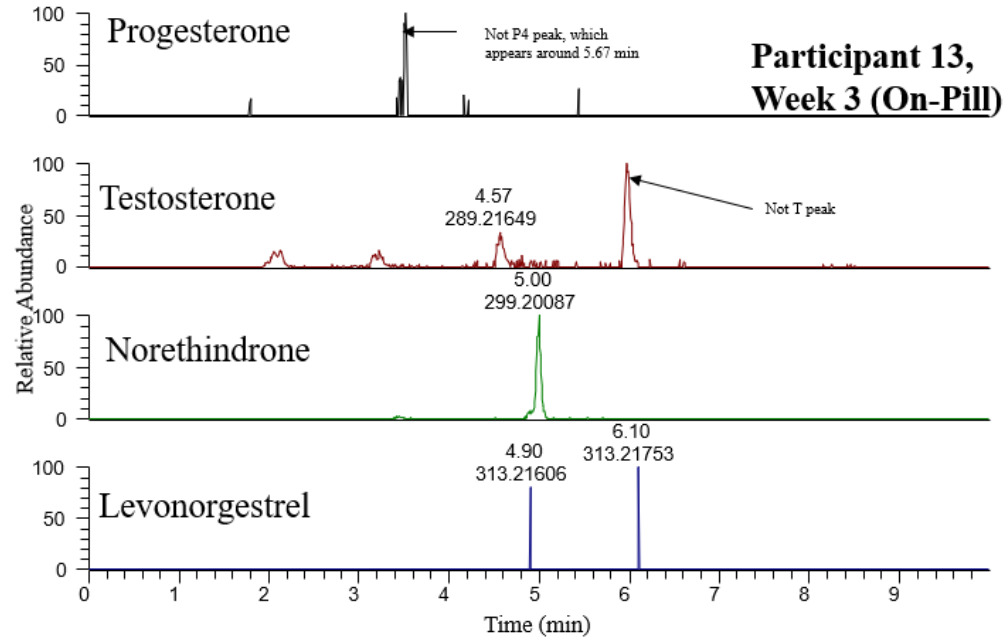


Figure B.9 XICs for Participant 1 (LNG Group)

RT: Retention Time, Base Peak



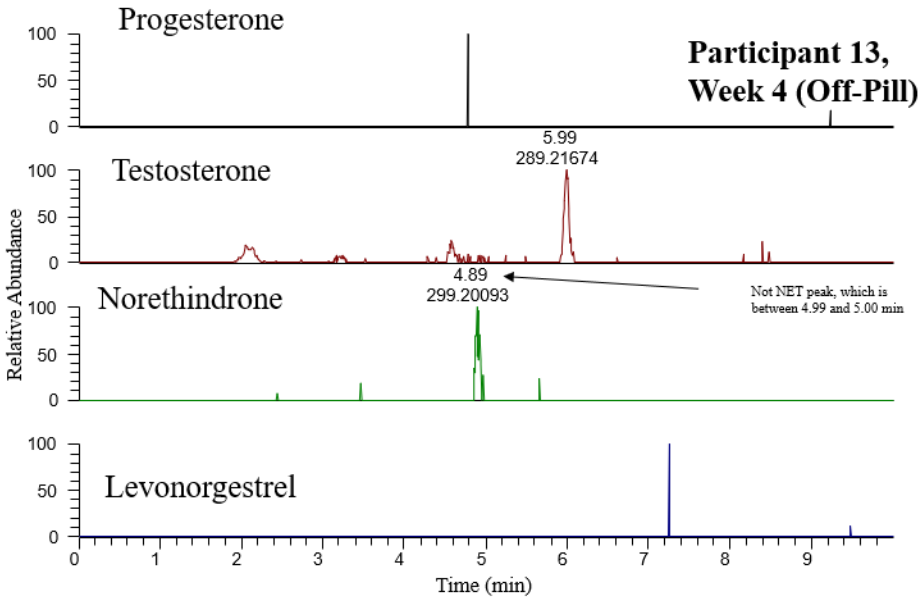


Figure B.10 XICs for Participant 13 (LNG Group)

RT: Retention Time, Base Peak

External Calibration Curves

Progesterone

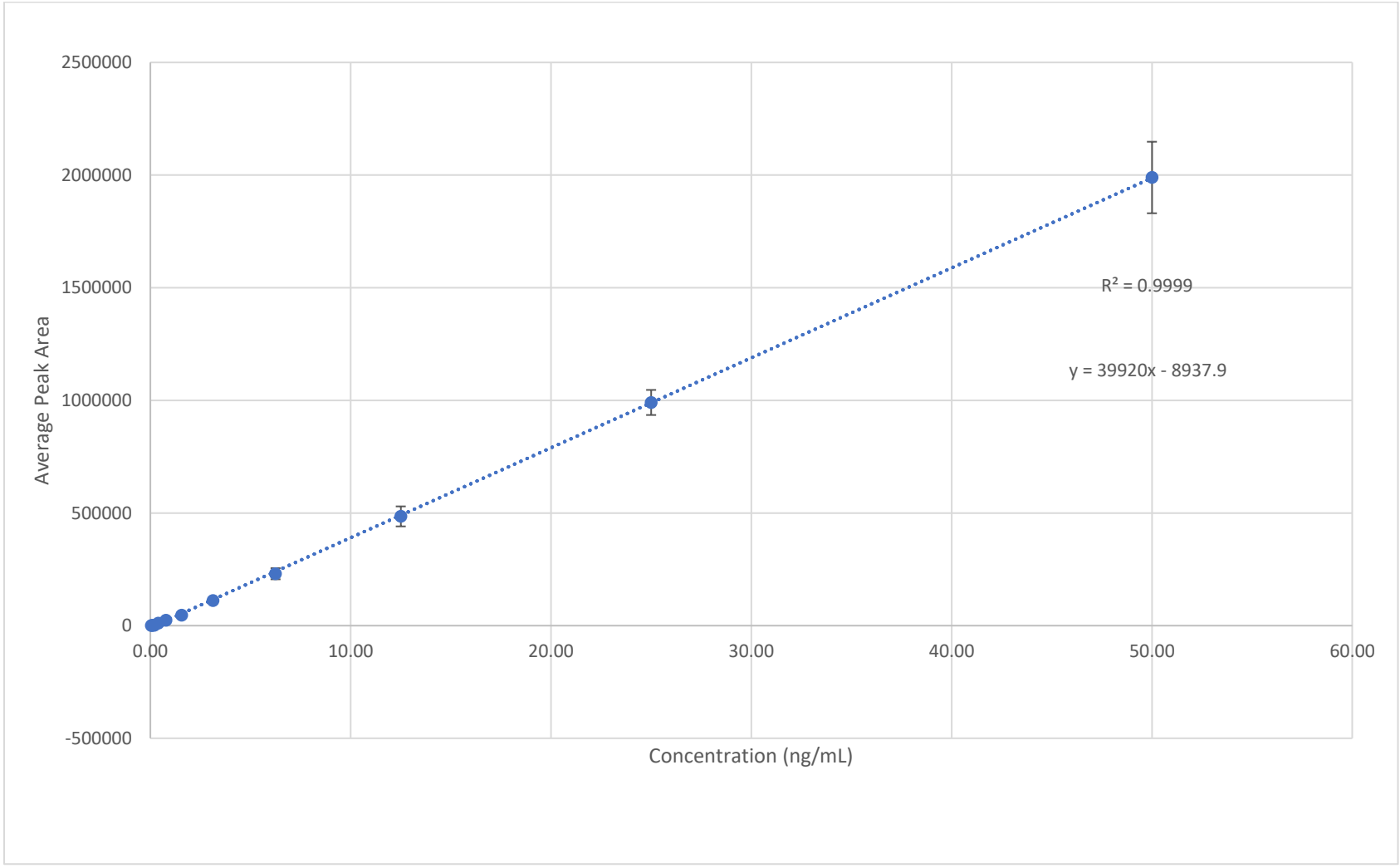


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Table B.10 Progesterone Calibration Curve (Aim IB)

Standard Concentration (ng/mL)	Injection 1 Peak Area	Injection 2 Peak Area	Injection 3 Peak Area	Average Peak Area	St. Dev	% CV	Predicted Concentration (ng/mL)	RE%
0.05	0	0	0	0	0	**N/A**	**N/A**	**N/A**
0.10 (LLOD)	**0**	**1558**	**3629**	**1729**	**1821**	105%	**0.29**	**199%**
0.20	2145	1743	2392	2093	328	16%	0.30	54%
0.39	8889	12026	12876	11264	2100	19%	0.53	36%
0.78 (LLOQ)	**21494**	**25301**	**24662**	**23819**	**2039**	**9%**	**0.84**	**8%**
1.56	42819	44404	50350	45858	3970	9%	1.40	-11%
3.13	99242	116001	120030	111758	11025	10%	3.05	-3%
6.25	209514	224102	257554	230390	24630	11%	6.02	-4%
12.50	443877	478958	531990	484942	44360	9%	12.39	-1%
25.00	932466	996287	1043346	990700	55651	6%	25.05	0%
50.00	1817102	2020842	2129691	1989212	158677	8%	50.04	0%
					Average CV%	**20%**	Average Residual %	**-1%**

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Testosterone

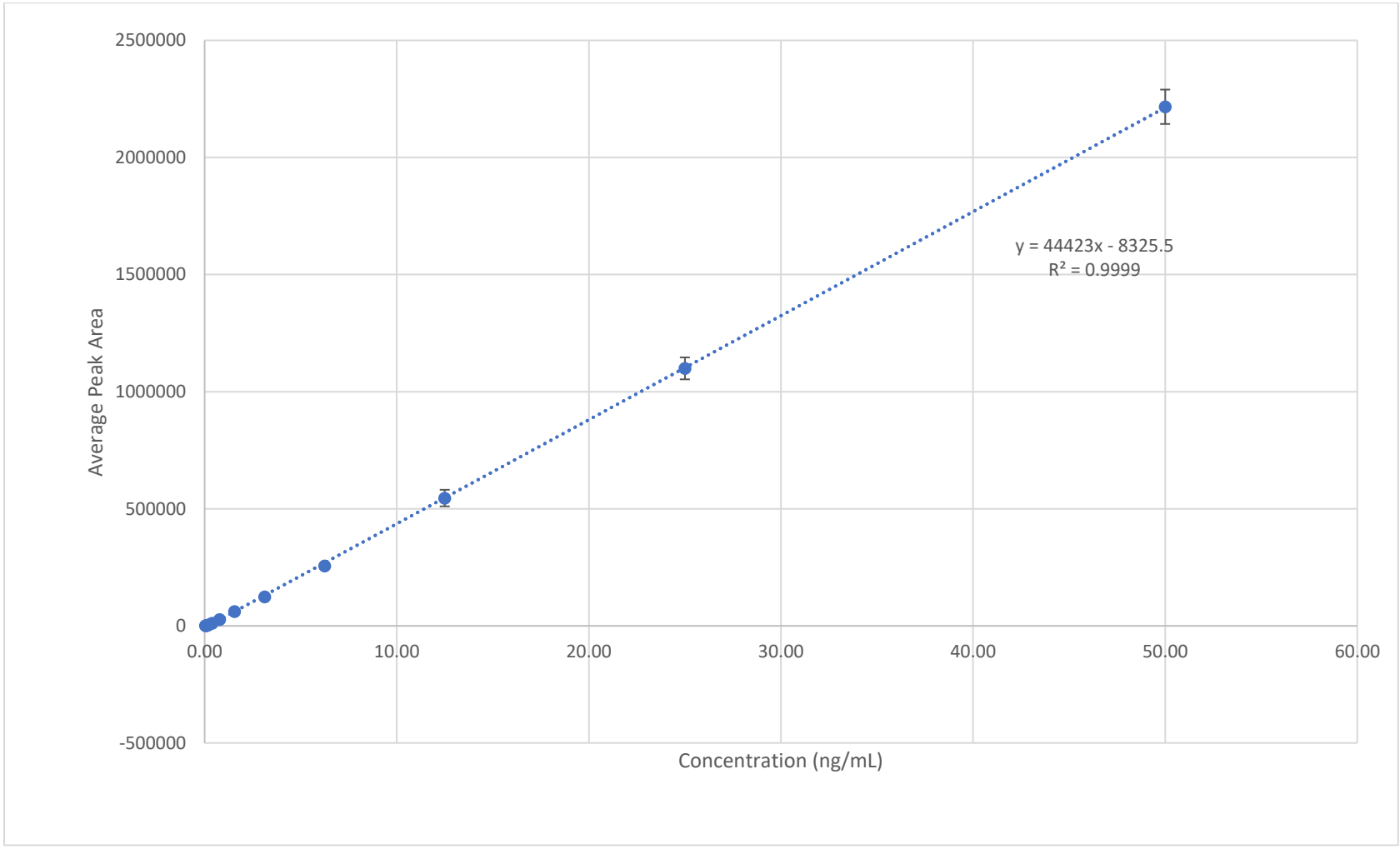

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Table B.11 Testosterone Calibration Curve (Aim IB)

Standard Concentration (ng/mL)	Injection 1 Peak Area	Injection 2 Peak Area	Injection 3 Peak Area	Average Peak Area	St. Dev	% CV	Predicted Concentration (ng/mL)	RE%
0.05 (LLOD)	**1206**	**0**	**0**	**1206**	**0**	N/A	**0.21**	**339%**
0.10	0	1247	0	1247	0	N/A	0.22	121%
0.20	1807	2881	7832	4173	2624	63%	0.28	44%
0.39 (LLOQ)	**8502**	**11766**	**12203**	**10824**	**1651**	15%	**0.43**	**10%**
0.78	29954	23650	28847	27484	2748	10%	0.81	3%
1.56	63820	55868	65774	61821	4284	7%	1.58	1%
3.13	112237	125652	133992	123960	8962	7%	2.98	-5%
6.25	244861	255911	267650	256141	9305	4%	5.95	-5%
12.50	507068	537605	592428	545700	35315	6%	12.47	0%
25.00	1074571	1058811	1165098	1099493	46834	4%	24.94	0%
50.00	2139982	2193819	2315573	2216458	73450	3%	50.08	0%
					Average CV%	**14%**	**Average Residual %**	**1%**

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Norethindrone

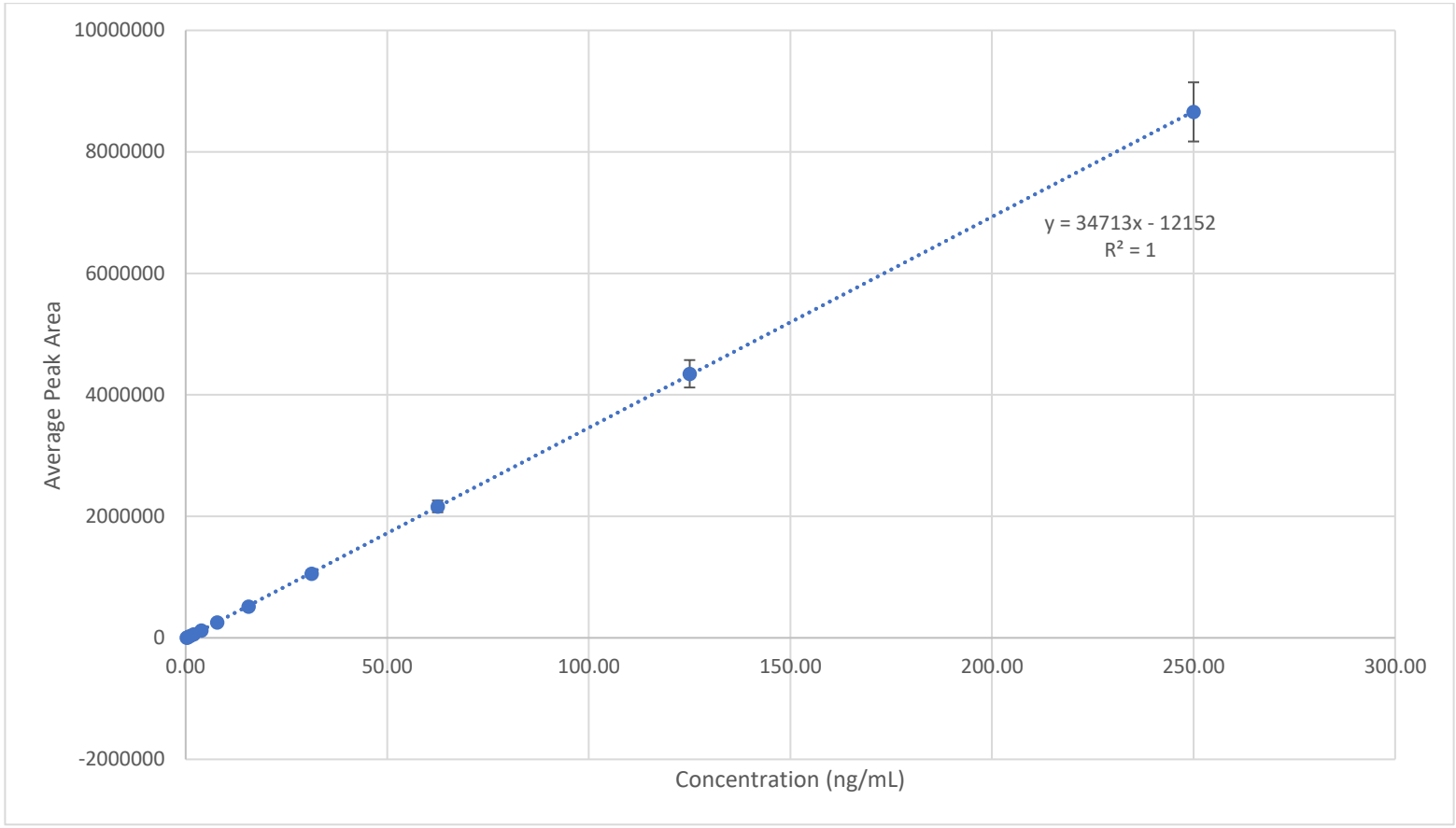

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Table B.12 Norethindrone Calibration Curve (Aim IB)

Standard Concentration (ng/mL)	Injection 1 Peak Area	Injection 2 Peak Area	Injection 3 Peak Area	Average Peak Area	St. Dev	% CV	Predicted Concentration (ng/mL)	RE%
0.24 (LLOD)	2172	2262	3762	2732	893	33%	0.43	76%
0.49	9504	10064	10011	9860	309	3%	0.63	30%
0.98 (LLOQ)	**24198**	**23923**	**31911**	**26677**	**4535**	17%	**1.12**	**15%**
1.95	54510	60076	60199	58262	3250	6%	2.03	4%
3.91	113412	121164	127570	120715	7090	6%	3.83	-2%
7.81	246484	252890	260425	253266	6978	3%	7.65	-2%
15.63	489128	508637	555124	517630	33905	7%	15.26	-2%
31.25	1022415	1029332	1120464	1057404	54721	5%	30.81	-1%
62.50	2064619	2168057	2257426	2163367	96489	4%	62.67	0%
125.00	4134941	4322278	4583996	4347072	225552	5%	125.58	0%
250.00	8234593	8548082	9189890	8657522	486961	6%	249.75	0%
					Average CV%	**6%**	**Average Residual %**	**1%**

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Levonorgestrel

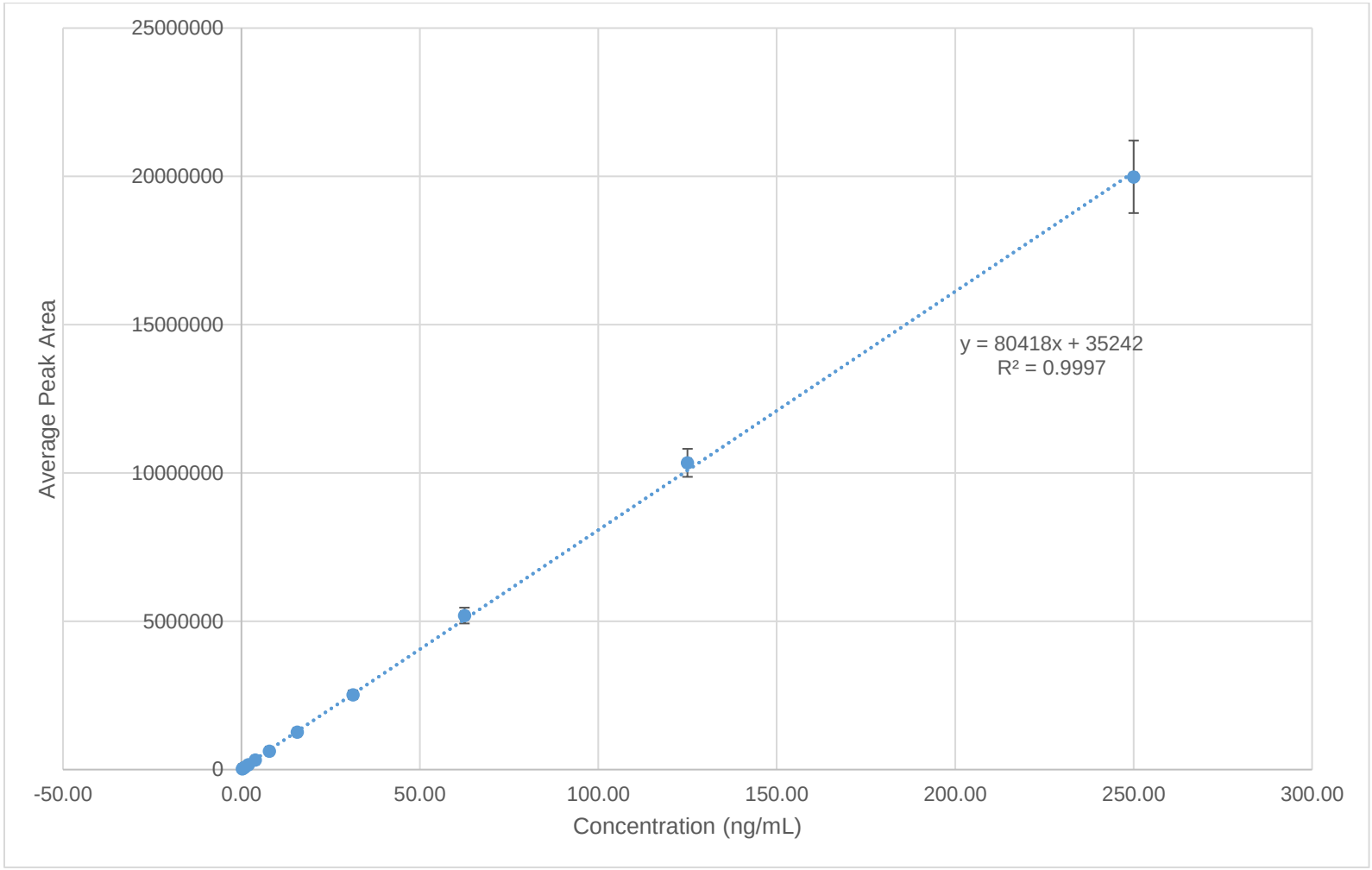

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Table B.13 Levonorgestrel Calibration Curve (Aim IB)

Standard Concentration (ng/mL)	Injection 1 Peak Area	Injection 2 Peak Area	Injection 3 Peak Area	Average Peak Area	St. Dev	% CV	Predicted Concentration (ng/mL)	RE%
0.24 (LLOD)	**22654**	**26443**	**35999**	**28365**	**6877**	24%	**-0.09**	**-135%**
0.49	40291	43007	55710	46336	8231	18%	0.14	-72%
0.98	74419	77674	91973	81355	9338	11%	0.57	-41%
1.95 (LLOQ)	**153866**	**165585**	**178685**	**166045**	**12416**	7%	**1.63**	**-17%**
3.91	304221	326278	335481	321993	16064	5%	3.57	-9%
7.81	581958	613833	674131	623307	46811	8%	7.31	-6%
15.63	1178189	1296380	1318142	1264237	75310	6%	15.28	-2%
31.25	2375742	2556983	2641906	2524877	135956	5%	30.96	-1%
62.50	4913601	5217755	5445088	5192148	266667	5%	64.13	3%
125.00	9839038	10409844	10775905	10341596	472148	5%	128.16	3%
250.00	18814996	19888834	21256253	19986694	1223567	6%	248.10	-1%
							Average Residual	
					Average CV%	**8%**	**%**	**-4%**

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Calculations

Equation 10 Sample Calculation of Serum Levonorgestrel Concentration in Participant 1, Pill Week Three

1. Measured Concentration by using regression line from Levonorgestrel Calibration Curve *Peak Area= 1821821*

$$Concentration = \left(\frac{1831821 - 35242}{80418}\right) = 22.3\,\frac{ng}{mL}\ measured\ in\ 80\ \mu L\ serum$$

2. Correction for Concentration Factor from LLE

$$22.3\,\frac{ng}{ml} * \frac{80\ \mu L\ processed\ serum}{3500\ \mu L\ collected\ serum} = 0.51\,\frac{ng}{mL}$$

3. Correction for % Recovery

$$0.51\,\frac{ng}{mL} * 0.365\ [\%\ recovery] = 0.18179\,\frac{ng}{mL}\ or\ 181.79\,\frac{pg}{mL}$$

Table B.14: Participant Hormone Concentrations Expanded Table (Pill Week Three: On Pill)

		Estradiol		Progesterone			Testosterone		
Participant Number	Serum Volume (mL)	Peak Area	Peak Area (Normalized to 1 mL serum)	Peak Area	Measured concentration (ng/mL)	Concentration in serum (pg/mL)	Peak Area	Measured concentration (ng/mL)	Concentration in serum (pg/mL)
1	3.5	ND	ND	36568	1.14	2.37	96867	2.37	16.29
2	2.4	ND	ND	11242	<LOQ	<LOQ	ND	ND	ND
3	3.8	ND	ND	ND	ND	ND	141550	3.37	21.38
4	3.2	ND	ND	42548	1.29	1.72	68039	1.72	12.93
5	3.7	ND	ND	16489	<LOQ	<LOQ	147204	3.50	22.78
6	3.8	ND	ND	ND	ND	ND	ND	ND	ND
7	3.9	ND	ND	ND	ND	ND	ND	ND	ND
8	3.7	ND	ND	13486	<LOQ	<LOQ	ND	ND	ND
9	2.5	ND	ND	ND	ND	1.98	79830	1.98	19.11
10	3.7	546920	147816	59881	1.72	18.90	831220	18.90	122.99
11	3.7	479015	129464	21162	<LOQ	<LOQ	1146132	25.99	169.13
12	3.7	411625	111250	ND	ND	12.59	551070	12.59	81.95
13	2.2	330636	150289	ND	ND	20.47	900989	20.47	224.04
14	2.5	330636	132254	5809	<LOQ	<LOQ	473917	10.86	104.56
15	2.6	226870	87258	17908	<LOQ	<LOQ	ND	ND	ND
16	2.6	311264	119717	8986	<LOQ	<LOQ	ND	ND	ND

	Norethindrone			Levonorgestrel		
Participant Number	Peak Area	Measured concentration (ng/m)	Concentration in serum (pg/mL)	Peak Area	Measured concentration (ng/m)	Concentration in serum (pg/mL)
1	ND	ND	ND	1831821	22.34	181.79
2	ND	ND	ND	905962	10.83	128.49
3	ND	ND	ND	1401222	16.99	127.31
4	ND	ND	ND	7527230	93.16	829.16
5	ND	ND	ND	2218101	27.14	208.94
6	ND	ND	ND	954993	11.44	85.72
7	ND	ND	ND	838786	9.99	72.97
8	ND	ND	ND	1748139	21.30	163.96
9	ND	ND	ND	1604904	19.52	222.36
10	ND	ND	ND	2062041	25.20	194.00
11	ND	ND	ND	3584708	44.14	339.74
12	ND	ND	ND	2209694	27.04	208.13
13	5208071	150.38	1432.74	ND	ND	ND
14	3135751	90.68	760.30	ND	ND	ND
15	2853971	82.57	665.61	ND	ND	ND
16	1089145	31.73	255.76	ND	ND	ND

Table B.15 Participant Hormone Concentrations Expanded Table (Pill Week Four: Off Pill)

		Estradiol		Progesterone			Testosterone			
Participant	Serum Volume (mL)	Peak Area	Peak Area (Normalized to 1 mL serum)	Peak Area	Measured concentration (ng/mL)	Concentration in serum (pg/mL)	Peak Area	Measured concentration (ng/mL)	Concentration in serum (pg/mL)	Concentration in serum (pg/mL)
1	3.7	490665	132612	ND	ND	ND	106121	2.58	16.76	ND
2	3.7	439588	118808	ND	ND	ND	480493	11.00	71.61	18.21
3	2.6	361156	138906	ND	ND	ND	761437	17.33	160.48	99.52
4	2.1	400305	190621	ND	ND	ND	461169	10.57	121.18	ND
5	2.7	ND	ND	8780	<LOQ	<LOQ	282225	6.54	58.33	79.71
6	2.7	117038	43347	ND	ND	ND	90743	2.23	19.88	<LOQ
7	3.7	324546	87715	ND	ND	ND	344551	7.94	51.69	<LOQ
8	3.7	147216	39788	ND	ND	ND	416736	9.57	62.27	15.68
9	2	513656	256828	ND	ND	ND	705498	16.07	193.46	ND
10	3.7	260587	70429	ND	ND	ND	335868	7.75	50.42	<LOQ
11	2.4	320215	133423	ND	ND	ND	383617	8.82	88.52	ND
12	3.7	637925	172412	ND	ND	ND	ND	ND	ND	ND
13	2.4	133141	55475	ND	ND	ND	149351	3.55	35.61	ND
14	3.7	ND	ND	ND	ND	ND	241983	5.63	50.25	ND
15	2.5	319863	127945	15136	<LOQ	<LOQ	431920	9.91	72.31	ND
16	2.4	792370	330154	ND	ND	ND	ND	ND	ND	ND

	Norethindrone			Levonorgestrel		
Participant	Peak Area	Measured concentration (ng/mL)	Concentration in serum (pg/mL)	Peak Area	Measured concentration (ng/mL)	Concentration in serum (pg/mL)
1	ND	ND	ND	ND	ND	ND
2	ND	ND	ND	225430	2.37	18.21
3	ND	ND	ND	765813	9.09	99.52
4	ND	ND	ND	ND	ND	ND
5	ND	ND	ND	642898	7.56	79.71
6	ND	ND	ND	187442	<LOQ	<LOQ
7	ND	ND	ND	45697	<LOQ	<LOQ
8	ND	ND	ND	198971	2.04	15.68
9	ND	ND	ND	ND	ND	ND
10	ND	ND	ND	25645	<LOQ	<LOQ
11	ND	ND	ND	ND	ND	ND
12	ND	ND	ND	ND	ND	ND
13	ND	ND	ND	ND	ND	ND
14	ND	ND	ND	ND	ND	ND
15	ND	ND	ND	ND	ND	ND
16	ND	ND	ND	ND	ND	ND