Chronic Inflammation and the Onset and Pace of Reproductive Maturation in Mayan Girls

by

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Ethics Statement

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Abstract

Life History Theory proposes that, when metabolic energy availability is limited, trade-offs ensue amongst growth, maintenance, development, and reproductive tasks. Thus, we hypothesize that limited energy availability resulting from chronic inflammation should create a trade-off between immune function and reproductive maturation, leading to a delay in the maturation of the female reproductive system. We assessed reproductive hormone profiles (follicle-stimulating hormone, estrogen, and progesterone) and inflammation status (using C-reactive protein and interleukin-1β as biomarkers) of 20 Guatemalan girls in 2013 (before menarche) and in 2017 (after menarche). We observed an average delay of 15 months (95% confidence interval [5.8, 24.1]) of menarche in girls with chronic inflammation compared to girls with no inflammation. However, our results did not provide evidence that chronic inflammation affected cycle length or ovulation frequency. This study aims to contribute to filling the gap in our understanding of the biological effects of low-grade immunological challenges, such as chronic inflammation, on girls' reproductive maturation process.

Keywords: Chronic inflammation; age at menarche; life history theory; reproductive maturation; ovarian cycle length; ovulatory frequency

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Chapter 1. Introduction

Life History Theory (LHT) proposes that, when metabolic energy availability is limited, trade-offs ensue amongst growth, maintenance, development, and reproductive tasks (Coall, 2016; Ellison et al., 2012; Amir, 2016; Placek & Quinlan, 2012). Metabolic energy availability refers to the energy resulting from food intake and fat storage (Elmquist, 2006), which is paramount to maintaining cellular functions underlying said tasks. Thus, individual energy allocation priorities in response to resource availability result in characteristic patterns of growth, mortality, and reproductive function across the lifespan of the organism (Ellison, 2003). Therefore, as reproductive activities rely on metabolic energy availability, LHT can help to explain variation within and among populations in reproductive traits, such as age at menarche. For example, **the pace and timing of the reproductive maturation process can be extended or delayed if other more urgent metabolic tasks, such as immune function, are prioritized.**

Reproductive maturation in women can be observed with the advent of **menarche**, considered as the shift from childhood to adolescence. Menarche is the moment at which girls experience their first ovarian cycle (Coal, 2016), and it is the most studied event during the complex maturation process of puberty. It is important to understand which factors affect the timing of menarche because both very early and very late onsets have been linked to increased risk of developing diseases, such as type 2 diabetes (Won et al., 2016; Baek et al., 2015), obesity (Won et al., 2016; Bralić et al., 2012), cardiovascular diseases (Won et al., 2016), breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer, 2012), psychopathologies (Graber, 2013), and metabolic syndrome (Kim & Je, 2019) later in life. Besides being associated with women's health problems in adulthood, early menarche onset can also affect women's overall reproductive lifespan by altering demographic patterns via changes in the population's total fertility rate (Khan, 1995; Komura et al., 1992; Valeggia & Ellison, 2003). In that regard, it was shown that girls younger than 11 years old at menarche had lower odds of subfecundity and infertility than girls with later onset of menarche (after 15 years old) (Guldbrandsen, 2014).

Although the factors that cause variation in the timing of menarche and pace of the transition through puberty are still not fully understood, it is known that such variation

can be influenced by **genetic** heritage (Guldbrandsen, 2014; Brooks-Gunn & Warren, 1988) and by **environmental factors**. Some examples of environmental factors that can affect age at menarche are: nutrition (Khan, 1995), BMI during childhood (Lazzeri, 2018), birth weight (Terry et al., 2009), preterm birth (Hui et al., 2012), having a mother who smoked heavily during her pregnancy (Yermachenko & Dvornyk, 2014; Ferris et al., 2010), and male effect (Mendle, 2006) [a phenomenon in which girls experience high levels of stress as a result of growing up in households with an unrelated adult (Ellis & Garber, 2000)] . These studies support LHT in that, when an individual is experiencing restricted resources due to environmental challenges, energy allocation priorities are created in response to resource availability, which results in characteristic patterns of growth, development, and reproductive function across its lifespan (Ellison, 2003).

Additionally, age at menarche has been linked to female life expectancy due to variables related to socioeconomic inequalities (Thomas et al., 2001; Al-Sahab et al., 2010), which is also associated with challenging environments and, therefore, energy availability. For example, women in non-industrialized countries have shorter lifespans due to their higher risk of infectious diseases (Workowski, & Bolan, 2015; Aral & Holmes, 1991), limited food availability (Khan, 1995; Committee on Adolescent Health Care, 2015), and higher mortality rates in the population (Placek & Quinlan, 2012; Promislow & Harvey, 1990). Improvements in socioeconomic conditions, nutrition, and general health may explain the progressive decline of mean age at menarche observed in the last 150 years in Western cultures (Warren, 1983). Currently, in well-nourished populations of industrialized countries - such as Canada (Al-Sahab et al., 2010) - the average age of menarche is about 12 years old (Committee on Adolescent Health Care, 2015), while in developing countries - such as Guatemala (Khan, 1995) - average age of menarche is close to 14 years old, and can even reach up to 16 years old in countries such as Papua New Guinea (Khan, 1995).

Although the mechanisms involved in the onset of menarche are not yet entirely clear, it is currently known that reproductive maturation results from a cascade of events that starts with the maturation of eggs (gametogenesis), followed by secretion of gonadal steroids (Bordini & Rosenfield, 2011; Toufexis, 2014; Flinn, 2011), leading to the development of secondary sexual characteristics and reproductive functions (Bordini & Rosenfield, 2011). During girls' puberty, gonadotropin-releasing hormone (GnRH) is released from the hypothalamus and transported to the anterior pituitary (Long et al.,

2018). This process is key to determining the pace and timing of reproductive maturation due to its role in the release, production, and balance of reproductive hormones such as follicle-stimulating-hormone (FSH), estrogen, and progesterone (LaBarbera, 2010). GnRH release and transportation seem to be directly regulated by *kisspeptin* neurons (Long et al., 2018). *Kisspeptin* neurons create a responsivity network that integrates nutritional, endocrinal, social, environmental, and immunological cues that influence the downstream of the reproductive axis function (Long et al., 2018).

Immune challenges appear to affect reproductive function in reproductively mature women. Recent studies have shown that women's gonadal steroids, progesterone (Gursoy et al., 2015), estrogen (Khan, Cowan & Ahmed , 2012; Khan & Ansar Ahmed, 2016), and FSH (Long et al., 2018), are linked to immune system regulation in reproductively mature women, which may help to explain why menstrual cycles seem to be underpinned by inflammatory patterns (Alvergne & Högqvist Tabor, 2018). Furthermore, it seems that the reproductive axis of this population is rapidly affected by the activation of the immune system when facing a challenge, which, in turn, modulates the secretion of gonadal steroids (Long et al., 2018).

Although there are previous studies that have linked age at menarche with several environmental factors (see Figure 1.1), to the best of our knowledge, there are no studies exploring the effects of **chronic inflammation** on the timing and pace of reproductive maturation in adolescent girls. Chronic inflammation is one type of immunological challenges that can lead to negative energy balance due to *i)* reduction in glucose availability and, thereby, a decrease in cellular energy (Lacourt, 2018); and *ii)* cell hypoxia, which causes fundamental changes to tissue metabolism (Kominsky, Campbell & Colgan, 2010). Thus, applying an LHT perspective, inflammation and immune responses increase metabolic energy demand (Kominsky, Campbell & Colgan, 2010), which can affect patterns of metabolic energy allocation among different physiological tasks such as growth, reproductive function, and immune function.

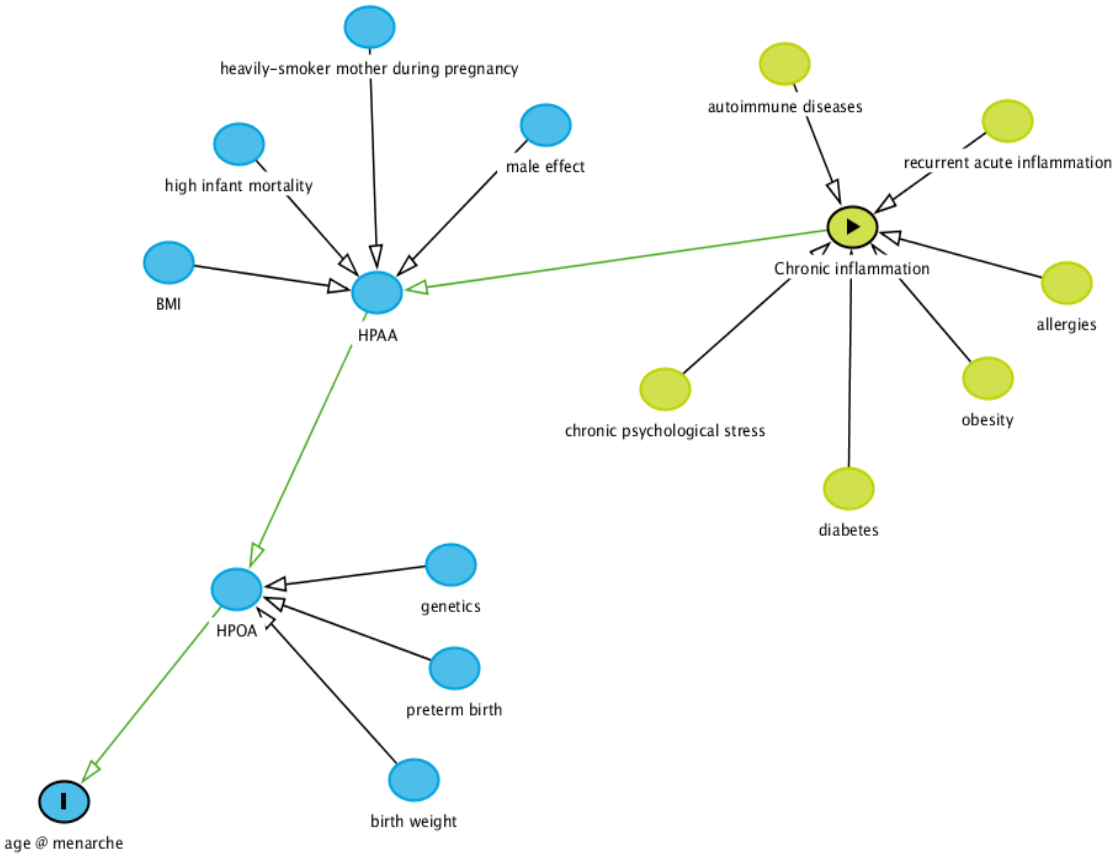

Figure 1.1. Interplay among chronic inflammation, the hypothalamic-pituitary-ovarian axis (HPOA), the hypothalamic-pituitary-adrenal axis (HPAA) and the currently known variables that can affect these relationships. Chronic inflammation is considered as the exposure and age at menarche as the outcome in this DAG.

This **exploratory and descriptive study** aims to contribute to filling the gap in our understanding of the biological effects of low-grade immunological challenges, such as chronic inflammation, on girls' reproductive maturation process. To that end, the **main objective** of this research is to examine the relationships between chronic inflammation and rate of reproductive maturation of girls living in similar geographic locations and slightly different socio-economic circumstances.

Goals: We wanted to investigate the links between **immunological challenges and the reproductive maturation transition,** in Guatemalan girls from rural Mayan

communities at two different times of their lives: in 2013, before their menarche, and in 2017, after menarche.

We **hypothesize** that limited energy availability resulting from chronic inflammation should create a trade-off between immune function and reproductive maturation, leading to a delay in the maturation of the women's reproductive system.

Our **testable predictions** are:

i) **Chronic inflammation**, measured by the biomarkers interleukin-1 β and C-reactive protein, should be associated with **later age at menarche.**

ii) **Chronic inflammation** should be associated with a **slower pace of reproductive maturation post-menarche**, which can be observed by longer cycles length and lower ovulation frequency.

Chapter 2. Literature Review

2.1. How Life History Theory Can Help to Explain the Effects of Chronic Inflammation on Reproductive Maturation in Women

2.1.1. Life History Theory

To understand the fundamentals of Life History Theory (LHT), it is essential to first understand what metabolic energy is and its biological role in an organism's physiology. Metabolic energy availability refers to the energy resulting from food intake and fat storage in the cells (Elmquist, 2006), which is paramount to maintaining cellular functions underlying growth, maintenance, development and other physiological activities, such as reproductive function. As the same metabolic energy cannot be allocated to two or more biological functions at the same time, natural selection has shaped patterns of metabolic energy allocation that favour an individual's survival and optimize reproductive efforts when under environmental energetic constraints (Ellison, 2003).

A consequence of these LHT principles is that, when metabolic energy is limited, energetic resources allocated towards reproductive effort imply fewer energetic resources available for other physiologic tasks such as, for example, immune function, growth, and tissue maintenance and repair (Ellison, 2003). Thus, reproduction may be impaired if not considered as a priority over the other tasks. The axis that regulates metabolic energy allocation is the hypothalamic-pituitary-adrenal axis (HPAA), also known as the "stress axis". The HPAA works as an intermediary between cues of challenging environmental conditions and the individual's physiological responses to those challenges (Herman et al., 2016). HPAA activation results in an increase in the release of glucocorticoids (cortisol in humans), which in turn allocates metabolic energy reserves towards tasks considered most urgent at the expense of those considered less urgent, depending on the individual's current circumstances (Coall, 2016; Ellison et al. 2012; Amir, 2016, Placek & Quinlan, 2012).

2.1.2. Hypothalamic-pituitary-adrenal Axis and Hypothalamic-pituitary-ovarian Axis Interplay

The HPAA is not only responsible for modulating somatic and psychosocial challenges through the regulation of cortisol levels but may also affect the cascade of hormonal changes that occur during reproductive maturation (Flinn, 2011). The HPAA interplays with the hypothalamic-pituitary-ovarian axis (HPOA) through the action of gonadal steroids (Toufexis, 2014), influencing reproductive function (Nepomnaschy, 2007) and the pace of reproductive maturation (Nepomnaschy et al., 2004; Nepomnaschy, 2012; Flinn, 2011) in reaction to environmental changes.

The HPOA is responsible for reproductive development and regulation of the reproductive function (Marques et al., 2018). During the menarche transition, HPOA function is regulated by increased and pulsatile levels of gonadotropin-releasing hormone (GnRH), which are hypothesized to result from the action of *kisspeptin* neurons (Long et al., 2018). These neurons are considered mediators of HPOA's response to physiological and environmental cues (Long et al., 2018) via the regulation of GnRH levels, which stimulate follicle-stimulating hormone (FSH) and luteinizing hormone (LH) production by the anterior pituitary. Increases in FHS and LH levels trigger menarche, the onset of ovarian cyclicity, by leading first to ovarian production of estrogen and, eventually, progesterone (Marques et al., 2018; LaBarbera, 2010; Zhang et al., 2008).

The HPOA continues to mature post-menarche. During this period of the reproductive maturation transition, reproductive hormones produced and released by the HPOA tend to present inconsistent patterns. The HPOA maturation process can take at least 3 years after menarche to be completed (Committee on Adolescent Health Care, 2015; WHO, 1986; Hickey, 2003).This maturation process is reflected in the variation observed during this period in the length of menstrual cycles and frequency of ovulation, which differ from those of reproductively mature women. For example, adolescent girls present a wider range of menstrual cycle lengths than adult women (between 20 to 45 days, and 21 to 34 days, respectively) (Hillard, 2008).

2.1.3. Chronic Inflammation: Definition and Immunology

Chronic inflammation is also known as low-grade inflammation and can last from months to years (Pahwa et al., 2020). It can result from autoimmune diseases (Khan & Ansar Ahmed, 2016), chronic psychological stress (Cohen et al., 2012), recurrent acute inflammation, long-term exposure of toxic compounds, and auto-inflammatory disorders (Pahwa et al., 2020). Chronic inflammation-mediated health conditions such as diabetes, cardiovascular diseases, allergies, strokes, chronic respiratory diseases, heart disorders, and cancer (Pahwa et al., 2020) are responsible for 3 out of 5 deaths worldwide (Tsai et al., 2019).

The pathophysiology of chronic inflammation is complex. Chronic inflammation induces a metabolic response that leads to reduced cellular energy resulting in *i)* reduced glucose availability (Lacourt, 2018); and *ii)* cell hypoxia, affecting tissue metabolism (Kominsky, Campbell & Colgan, 2010). This metabolic shift linked to immune response involves intense recruitment of immune cells (neutrophils and monocytes) and increasing lymphocyte populations (Kominsky, Campbell & Colgan, 2010), both related to aerobic glycolytic energy production (Lacourt, 2018).

Long-term reliance on aerobic glycolytic energy production in response to chronic inflammation can lead to metabolic acidosis and cell oxidation, which ultimately reduce cellular nutrient availability and insulin sensitivity (Kominsky, Campbell & Colgan, 2010). Thus, the resulting decrease in cellular energy can affect patterns of metabolic energy allocation among different physiological tasks due to fundamental changes to tissue metabolism (Lacourt, 2018).

2.1.4. Chronic Low-Grade Inflammation and Women's Reproductive Status

Women's reproductive hormones play an important role in the regulation of the immune system. Estradiol, a form of estrogen, is linked to the regulation of cells of the innate immune system [neutrophils, macrophages/monocytes, natural killer cells (NK), dendritic cells (DC)], and the adaptive immune system (T and B cells) via complex molecular mechanisms (Khan, Cowan & Ahmed , 2012; Khan & Ansar Ahmed, 2016).

Additionally, the fluctuation of hormonal levels during the menstrual cycle underlies inflammatory patterns, affecting immune system status (Alvergne & Högqvist Tabor, 2018). Previous research has suggested that increased progesterone levels in the luteal phase of the menstrual cycle seem to affect the immunological system by creating an imbalance in the abundance of cytokines. This imbalance is due to a shift in the ratio between T-helper type 1 (Th1) and 2 (Th2) T cells, favoring Th2 cells (Gursoy et al., 2015; Faas et al., 2000). Higher levels of Th2 cells in plasma stimulate interleukin-1 β (IL-1β) (Ridker, 2016) and C-reactive protein (CRP) synthesis (Gursoy et al., 2015). Both CRP and IL-1β are widely accepted as biomarkers of inflammation, and they help to characterize chronic and acute inflammatory status (Ridker, 2016; Del Giudice & Gangestad, 2018). Moderate levels of CRP (Tao et al., 2018) combined with high levels of IL-1β (Lopez-Castejon & Brough, 2011; Dinarello, 2011) provide a reliable marker of chronic inflammation (Del Giudice & Gangestad, 2018).

Progesterone and estrogen affect/modulate immune function by binding to receptors present in several types of immune cells (Oertelt-Prigione, 2012). Estrogen receptors (ER) α and β have been described in most immune cells (Couse et al., 1997), and progesterone receptors (PR) have been described in lymphocytes and T cells (Oertelt-Prigione, 2012).

Thus, as HPOA (through reproductive hormones regulation) seems to be associated with inflammation, it is expected that the pace and timing of reproductive maturation can vary in response to metabolic energy availability, a common variable that underlies all these systems.

Chapter 3. Methods

3.1. Population and Biospecimens collection

3.1.1. Study Population

Fieldwork was conducted in 2013 and 2017 in two rural Kaqchikel Mayan communities in highlands of southwestern Guatemala. Over 99% of the inhabitants of these towns at the time of data collection were of Kaqchikel Mayan ancestry. Socioeconomic structure and traits such as diet, genetics, daily activities, and everyday environmental and psychological stressors are similar within and between those communities and more homogeneous than those to be found in most urban, industrialized populations.

3.1.2. Data and Specimens Collection

The data collection protocol was approved by Simon Fraser University's Research Ethics Board (2013: study# 2012s0668 and 2017: study# 2016s0576). To test my hypotheses, I analyzed urine and saliva specimens collected from 20 girls who participated in the study in 2013 and 2017 (age range: 10-11 in 2013; 14-15 in 2017).

In 2013, first morning urine (FMU) specimens were collected every day (except on Sundays) for 3 weeks. In 2017, FMU specimens were collected every other day (except on Sundays) for 4 months from the same participants as in 2013. Participants collected their first urinary voids of each day in clean, dry, inert plastic containers provided by our research team the previous night. Local female research assistants went every morning to the participants' homes to collect said samples and transported them in coolers filled with blue-ice packs to our field station within 4h of urinary void. FMU samples were aliquoted into 2 mL cryo-vials and stored at -10°C at the station until the end of each field season. Samples were then transported on dry ice from the field to our laboratory at Simon Fraser University, where we stored them at -80°C until analysis.

Salivary specimens were collected on one day in both 2013 and 2017. Saliva was collected by asking the participants to chew a piece of sugarless gum for 30 seconds and then spit the gum into a cup. After a few seconds, each participant was asked to spit saliva into a labelled container. After collection, samples were stored at -10°C in the field until the end of each field season, then transported on dry ice to the laboratory at Simon Fraser University, where they were stored at -80°C until analysis.

We used immune markers **interleukin-1 β (IL-1 β)** and **C-reactive protein (CRP)** to estimate chronic inflammation; and urinary metabolites of the reproductive hormones **follicle-stimulating hormone (FSH)**, **estrogen (E)** and **progesterone (P)** to estimate girls' reproductive maturation status and quality of ovarian cycles by assessing ovulation frequency and ovarian cycle length.

3.2. Immunomarkers measurement – Saliva

3.2.1. Salimetrics® CRP assay

The salivary CRP indirect sandwich immunoassay kit from Salimetrics® (#1-3302; State College, PA, USA) is an enzyme-linked immunosorbent assay (ELISA) specifically designed and validated for the quantitative measurement of salivary CRP.

On the day of assay, the saliva samples were completely thawed, vortexed, and centrifuged at 1500 x g for 15 minutes. Thawed samples, all reagents, and Microtitre Plates were brought to room temperature before use.

Wash buffer was prepared by diluting 100 mL of wash buffer concentrate (10X) with 900 mL of room-temperature deionized water. Each CRP control vial was reconstituted with 0.5 mL of deionized water and let sit for 20 minutes at room temperature and mixed well before use.

Serial dilutions of the CRP Standard were performed (Salimetrics #1-3002; State College, PA, USA) by pipetting 150 µL of CRP sample diluent into each of five polypropylene microcentrifuge tubes (Tubes 2-6). An amount of 150 µL of the 3,000 pg/mL standard was added to each of Tube 1 and 2. To serially dilute the standard two-

fold, Tube 2 was vortexed and then 150 uL of its contents was added into Tube 3. This process was repeated with the contents of Tubes 3 through 5. The final concentrations of standards for tubes 1 through 6 were, respectively, 3000 pg/mL, 1500 pg/mL, 750 pg/mL, 375 pg/mL, 187.5 pg/mL, and 93.75 pg/mL. Standard concentrations in pmol/L were 130.43, 65.22, 32.61, 16.30, 8.15 and 4.08 pmol/L, respectively. CRP sample diluent was used as the zero standard.

An amount of 15 µL of each saliva sample was diluted 10X using 135 µL of CRP sample diluent. Finally, 50 µL of standards, controls, and diluted saliva samples were pipetted into their corresponding wells in duplicate.

An amount of 80 uL of CRP antibody enzyme conjugate was mixed with 20 mL of assay diluent, and 150 µL of the solution was added to each well using a multichannel pipette. The plates were covered and placed on a plate rotator set at 500 rpm for 2 hours at room temperature, and then washed 4 times with 1X wash buffer.

Using a multichannel pipette, 200 µL of TMB substrate solution were added into each well, covered, and incubated in the dark at room temperature for 30 minutes, mixing constantly on a plate rotator at 500 rpm. Then, 50 µL of stop solution was added to each well, and the plates were placed back on the plate rotator for 3 minutes at 500 rpm until the green color turned to yellow in all of the wells. The plates were read at 450 nm in a Victor X5 plate reader. The standard curve was fit using a 4-parameter non-linear curve.

3.2.2. Salimetrics® IL 1- β assay

All reagents and microplates were brought to room temperature before use. wash buffer concentrate (10X) 10-fold was diluted with room-temperature deionized water (100 mL of wash buffer concentrate (10X) to 900 mL of deionized water) to prepare 1X wash buffer. Each control vial was reconstituted with 1.0 mL of deionized water, and the IL-1β standard vial was reconstituted with 500 µL of deionized water.

Two-fold serial dilutions of the IL-1β standard were performed (Salimetrisc #1-3302; State College, PA, USA) by pipetting 300 µL of IL-1β Assay Diluent into each of five polypropylene microcentrifuge tubes (Tubes 2-6). Then, 300 µL of the 200 pg/mL

standard was added to each of Tube 1 and 2. To serially dilute the standard two-fold, Tube 2 was vortexed and 300 uL of its contents was added into Tube 3. This process was repeated with the contents of Tubes 3 through 7. The final concentrations of standards for Tubes 1 through 7 were, respectively, 200 pg/mL, 100 pg/mL, 50 pg/mL, 25 pg/mL, 12.5 pg/mL, 6.25 pg/mL and 3.13 pg/mL. Standard concentrations in pmol/L were 11.8, 6.0, 3.0, 1.5, 0.7, 0.4 and 0.2 respectively. IL-1β assay diluent was used as the zero standard.

A 15-fold sample dilution was created with 20 uL of each saliva sample in 280 µL of IL-1β sample diluent. Then, 100 µL of standards, controls, and diluted saliva samples were pipetted into appropriate wells in duplicate. Each plate was covered and mixed on a plate rotator continuously at 500 rpm for 1 hour at room temperature, and then washed 4 times with 1X wash buffer.

An amount of 24 uL of antibody conjugate was diluted 1:500 with 12mL of IL-1β assay diluent, and 100 µL of this solution was added to each well using a multichannel pipette. The plate was covered and mixed on a plate rotator continuously at 500 rpm for 2 hours at room temperature, then washed 4 times with 1X wash buffer.

An amount of 60 uL of streptavidin-HRP was diluted 1:200 with 12 mL of IL-1β assay diluent, and 100 µL of this solution was added to each well using a multichannel pipette. The plate was covered and mixed on a plate rotator continuously at 500 rpm for 20 minutes at room temperature, then washed 4 times with 1X wash buffer.

Using a multichannel pipette, we added 100 µL of TMB substrate solution to each well, and the plate was covered with foil and mixed on a plate rotator continuously at 500 rpm for 20 minutes at room temperature. After that, 50 µL of stop solution was added to each well, and plate was mixed on a plate rotator for 3 minutes at 500 rpm until green color turned to yellow in all wells. The plate was read at 450 nm in a Victor X5 plate reader. The standard curve was fit using a 4-parameter non-linear curve.

3.3. Reproductive hormones measurement – Urine

3.3.1. Quansys Multiplex Assay – Estrogen, FSH

Q-Plex™ Custom Array 107749GR (Salvante et al., 2012) (Quansys Biosciences, Logan, UT, USA) is a chemiluminescent ELISA multiplex assay used to measure urinary estrogen (E), and follicular stimulating hormone (FSH) levels in the same plate, at the same time.

Sample diluent was prepared by mixing 6.75µL of competitor to 10 mL of sample diluent. Calibrator (standard) was mixed with 550µL of sample diluent, and 200µL of this solution was pipetted into the first tube out of eight, used for the serial dilutions. Then, 120µL of prepared sample diluent was added into the other seven tubes. Serial dilution was performed by transferring 60µL of the undiluted prepared calibrator from the first tube to the second, and so on, vortexing in between each transfer.

Unknown and control samples were diluted in sample diluent at 1:2, and 50µL of each diluted sample, control and standard was added into their corresponding wells in duplicate within 10 minutes. The plate was covered and placed on a plate shaker set to 500 RPM for 90 minutes at room temperature, then washed 3 times with wash buffer prepared by mixing 50 mL of the 20X concentrate with 950 mL of deionized water. Then, 50µL of detection mix was added into each well, and the plate was covered and placed on plate shaker set to 500 RPM for one hour at room temperature, and then washed three times.

An amount of 50uL of Streptavidin-HRP was pipetted into each well, and the plate was covered and returned to the plate shaker at 500 RPM for 15 minutes at room temperature, and then washed six times. Then, 50µL of previously prepared substrate (3 mL of substrate A mixed with 3 mL of substrate B and kept in the dark) was added to each well and imaging was performed using a Quansys® Q-Viewer™ immediately afterwards. The standard curve was fit using a 4-parameter logistic regression model.

3.3.2. Quansys Assay – Progesterone

Q-Plex™ Custom Array 107749GR (Quansys Biosciences) is a chemiluminescent ELISA assay used to measure urinary progesterone (PdG).

Sample diluent was prepared by mixing 5µL of competitor to 10 mL of sample diluent. Calibrator (standard) was prepared by adding 500µL sample diluent, letting the mixture sit for 5 min, and pipetting 200µL of this solution into the first tube out of 8 for serial dilution. Then, 120uL of sample diluent was pipetted into each of the other seven tubes. For the three-fold serial dilution, 60µL from the first tube was transferred to the second, and so on, vortexing in between each transfer.

Unknown and control samples were diluted in sample diluent at 1:2, and 50µL of each diluted sample, control and standard was added into their corresponding wells in duplicate within 10 minutes. The plate was covered and placed on a plate shaker set to 500 RPM for one hour at room temperature. The plate was washed 3 times with wash buffer prepared by mixing 50mL of the 20X concentrate with 950mL of deionized water. Then, 50µL of Detection Mix was added into the wells, and the plate was covered and placed on plate shaker set to 500 RPM for one hour at room temperature, and then washed three times

An amount of 50uL of Streptavidin-HRP 1X was pipetted into each well, and the plate was covered and returned to plate shaker at 500 RPM for 15 minutes at room temperature, and then washed six times. Then, 50uL of previously prepared substrate (3mL of substrate A mixed with 3 mL of substrate B and kept in the dark) was added to each well, and imaging was performed using a Quansys® Q-Viewer™ immediately afterwards. The standard curve was fit using a 4-parameter logistic regression model.

3.4. Characterization of Hormonal Profiles

Ovarian function was monitored via the quantification of reproductive hormones' urinary metabolites [pregnandiol glucuronide – PdG for progesterone, estrone glucuronide - E1G for estradiol, and the alpha sub-unit of FHS (FSHα)]. Each hormone concentration was corrected for specific gravity using refractometry to adjust for variation in hydration state of each urine specimen (Miller et al., 2004).

According to their self-reports, none of the participants had experienced menarche in 2013, which was confirmed by the low levels of both E1G in their 2013 FMU samples. In 2017, all the participant girls had already experienced their menarche, so we were able to monitor the quality of their ovarian function in terms of ovulation frequencies and cycles' lengths during the 4 months of sample collection that year. We considered day 1 as the first day of menstrual bleeding, and we scanned a 5-day sequence of E1G/PdG ratio values starting with day 7. A cycle was considered to be ovulatory cycle only if it contained a PdG peak > 2,000 ng/mL during the luteal phase (Venturoli, 1987; Apter, 1978). The algorithm to identify the day of ovulation in urine was created based on previous methodologic studies (Baird et al., 1991; O'Connor et al., 2006), and follows the steps below.

1. We looked for the highest E1G/PdG ratio value within the 3-sample (5-6 days) sequence
2. The ratio values for the last sample of the 3-sample sequence should be </= 40% of the first sample value (40% = descent criterion) to meet the criteria. We considered the second day in this sequence as the day of luteal transition (ovulation) for that cycle
3. If there were overlapping 5-day (3 sample) sequences meeting the criteria, the earliest was selected
4. If there were 2 or more non-overlapping 3-sample sequences within a cycle, and if the mean of the 2 values on either side of sample 1 in one 3-sample sequence is more than twice the mean of those values in the other 3-sample sequence, then the former 3-sample sequence is selected.
5. Cycles were judged as indeterminate if the following patterns of missing data were found:
    a. Ratio value for the sample immediately prior to the 3-sample series is missing
    b. Value for the second sample of the 3-sample series is missing
    c. Value for the third sample of the 3-sample series is missing.

3.5. Methods of Statistical Analysis

3.5.1. Data Description

Participants' age at menarche (in months), time from menarche to the time of data collection (in months), and cycle length (number of days between ovulations) were all treated as continuous variables, while ovulatory function frequency was treated as a (discrete) count variable. To estimate the effect of chronic inflammation on cycle length and ovulatory function frequency, we adjusted our statistical models for age at menarche. This adjustment was necessary because ovarian cycles tend to regulate over the first 3 years post-menarche. Thus, in the first 2 years after menarche, the normal cycle length ranges from 21 to 45 days. From the third year on, the normal range is 21 to 34 days, which is similar to that of adult women (Hickey 2003).

Chronic inflammation was treated as a binary variable (presence or absence). Chronic inflammation was considered present if an individual exhibited a moderate level of CRP (600-3000pg/mL) combined with high level of IL-1β (equal or over 200pg/mL). CRP levels to evaluate inflammatory status to determine the basal levels of girls aged 10–16 years are based on previous studies of Gursoy et al. (2015), Cullen et al. (2017), Wener et al. (2000), and Woloshin & Schwartz (2005). These levels were adjusted from serum levels to salivary levels by using the regression equation $y = 1553.15\ x - 1413.19$; where y = serum CRP and x = salivary CRP (Cullen et al., 2017). No association has been reported between IL-1β and age and sex (Di Iorio, 2003), and the definition follows that of Salimetrics® (#1-3302; State College, PA, USA).

Cycle lengths were determined as the intervals between ovulations over the 4-month study period. The data included a total of 31 right-censored cycle lengths, 14 at the beginning of the study period and 17 at the end of the study period in 2017.

3.5.2. Statistical Analysis

Through statistical analysis of the data, we evaluated the influence of chronic inflammation on age at menarche, ovarian cycle length, and ovarian function frequency. We performed a Welch's 2-sample t-test to assess the **relationship between age at**

menarche and presence or absence of chronic inflammation in 2013. Welch's 2-sample t-test allows for the variance of age at menarche to differ depending on the presence/absence of chronic inflammation. To analyze the **relationship between cycle length (number of days between ovulations) and presence/absence of chronic inflammation in 2017**, we fit a Cox model with an individual-specific random effect (adjusting for number of months from menarche) to the data from 2017. This model accounts for correlation among cycle lengths observed on the same girl and for right-censoring of some of the cycle lengths. To analyze the **relationship between ovulation frequency and chronic inflammation in 2017** (adjusting for time from menarche), a Poisson generalized linear model was used. The response variable was the number of ovulations and the predictor variables were the chronic inflammation indicator and the number of months from menarche.

R software (version 3.6.2) and the packages survival (version 3.1-8), coxme (version 2.2-16), and ggplot2 (version 3.2.1) were used to perform the statistical analyses in this study.

Chapter 4. Results

4.1. Age at Menarche and Chronic Inflammation

In 2013, before the 20 participants reached their menarche, 9 girls showed presence of chronic inflammation and 11 did not. According to the self-reported data collected in 2017, girls who in 2013 showed signs of **chronic inflammation**, **experienced menarche an average of 15 months later** than their counterparts who did not show signs of chronic inflammation. The estimated mean age at menarche for girls with chronic inflammation was 173.8 months (14.5 years) compared to an estimated mean of 158.9 months (13.2 years) for girls with no chronic inflammation (p=0.003, 2-sample t-test allowing for unequal variances) (Figure 4.1.1). The 95% CI for the difference in mean age at menarche is (5.8, 24.1). I.e., comparing girls with and without chronic inflammation, differences in mean age at menarche ranging from 5.8 to 24.1 months were compatible with our data given our statistical assumptions.

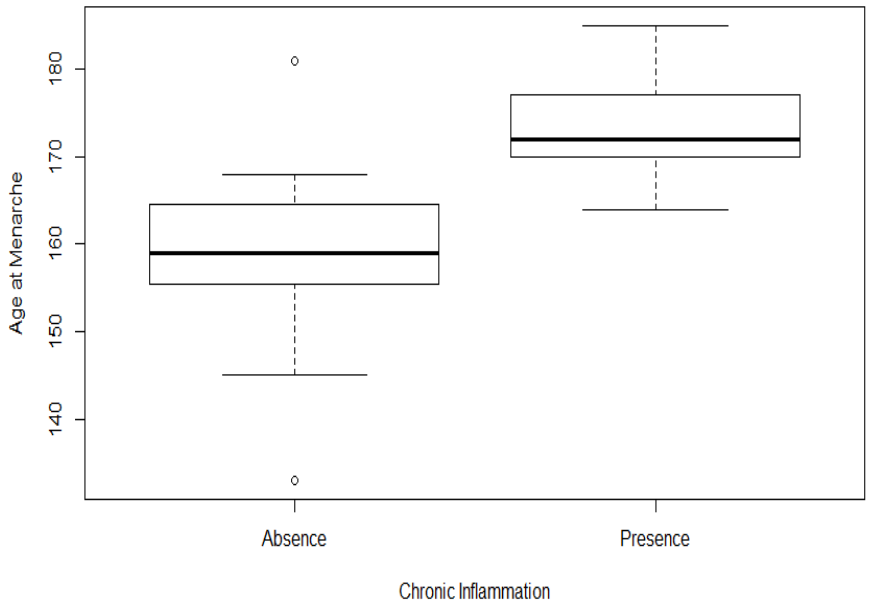

Figure 4.1.1. Girls showing symptoms of chronic inflammation in 2013 had an estimated mean age of menarche of 173.8 months, which is statistically significantly higher than 158.9 months, the estimated mean age at menarche of girls not showing symptoms of chronic inflammation in 2013.

The relationship between age at menarche and levels of the immune markers CRP and IL 1-β in 2013 can be observed in Figure 4.1.2.

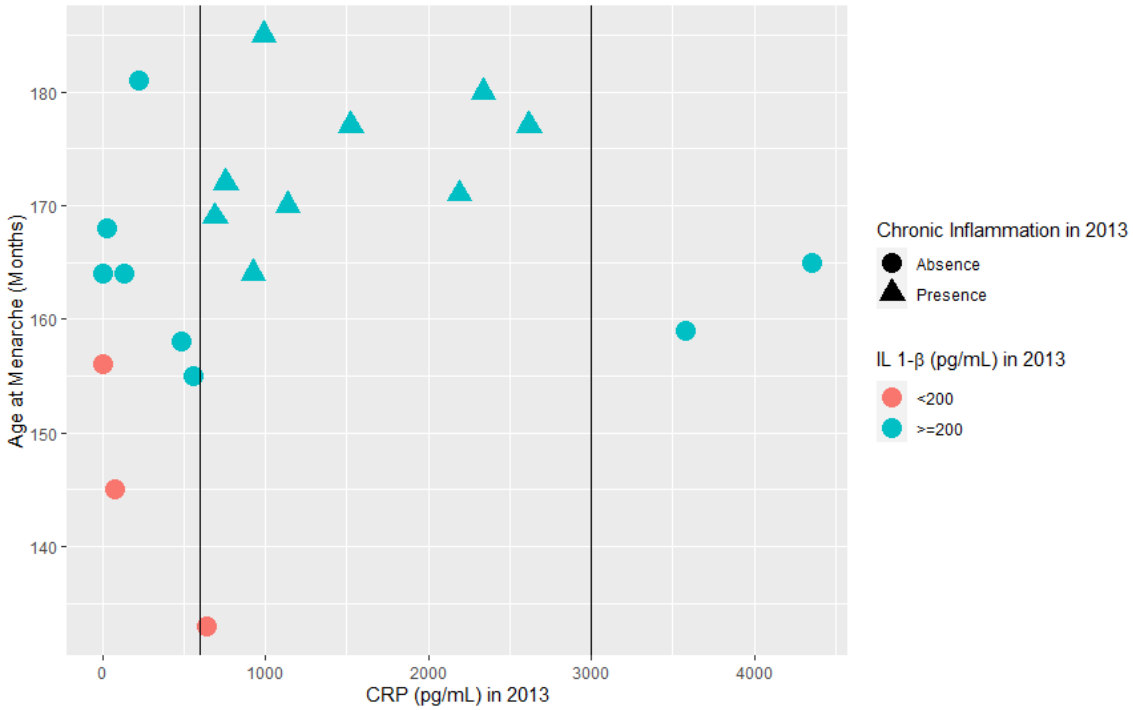

Figure 4.1.2. Relationship between age at menarche (in months) and CRP and IL 1-β in 2013. IL 1-β levels of higher than 200 pg/mL combined with CRP levels from 600 to 3,000 pg/mL indicate chronic inflammation.

4.2. Cycle length and chronic inflammation

In 2017, all 20 participating girls had already had their menarche. Of these 20 participants only 5 showed presence of chronic inflammation. During the 4-month 2017 study period, 17 of the 20 participants had at least one ovulation and a maximum of 4 ovulations. For a given number of months from menarche, the ratio of the hazard rate (HR) of ovulation for girls with chronic inflammation to that for girls without chronic inflammation was estimated as 1.03 (95% CI [0.118, 3.61], p=0.62, SD= 1.1, Cox model with a girl-specific random effect), after adjusting for time from menarche (Table 4.2.1)). Our results were inconclusive regarding a possible effect of **chronic inflammation** on **cycle length** among girls who ovulated during the study period. Specifically, our 95% CI indicates that the data are consistent with a hazard ratio in the range of 0.118 to 3.61,

given our statistical assumptions. Some associations in this range are meaningfully different than 1 (both larger and smaller), and some are not.

Table 4.2.1. Estimate of the effect of chronic inflammation on age at menarche (in months) based on the Cox mixed-effects model. The Cox model with girl-specific random effect treats cycle lengths observed on different girls as independent but on the same girl as correlated.

Effect	Coefficient (β)	se(coef)	Z-score	P-values
Months since menarche	0.029	0.028	1.05	0.29
Presence of chronic inflammation-2017	-0.427	0.873	-0.49	0.62

4.3. Ovulatory Frequency and Chronic Inflammation

We used a Poisson generalized linear model to evaluate a possible association between participants experiencing **chronic inflammation** in 2017, after menarche, and their **ovulatory frequency** during the study period, after adjusting for the time from menarche (p=0.481) (Table 4.3.1 and Figure 4.3.1). For a given time since menarche, we found that the estimated mean number of ovulations of girls with chronic inflammation is 0.755 times that of girls without chronic inflammation (95% CI [0.346, 1.65]. Our 95% CI indicates that the data are consistent with differences in mean number of ovulations (adjusting for time from menarche) in the range of 0.346 to 1.65, given our statistical assumptions. Some associations in this range are meaningfully different than 1 (both larger and smaller) and some are not.

Table 4.3.1. Estimate of the effect of chronic inflammation on age at menarche.

Effect	Estimate	Std. Error	Z-score	Pr(>|z|)
(Intercept)	0.52	0.28	1.85	0.06
Months from menarche	0.012	0.012	0.98	0.32
Presence of chronic inflammation-2017	-0.28	0.39	-0.7	0.48

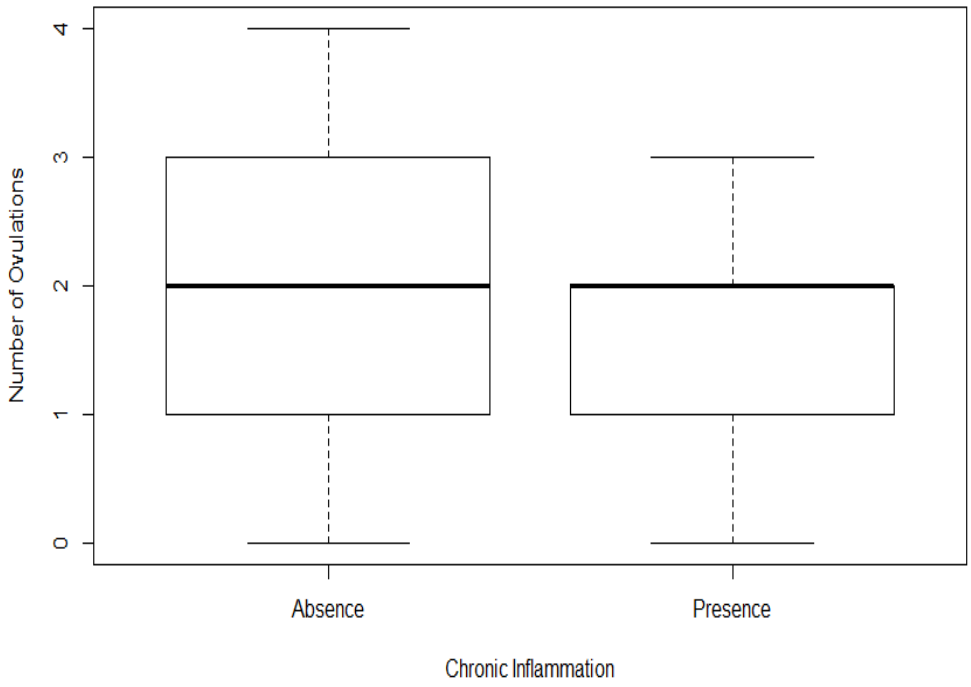

Figure 4.3.1. Distribution of the number of ovulations during the 4-month study period in 2017 by chronic inflammation status. Mean number of ovulations did not differ significantly across groups.

Chapter 5. Discussion and Conclusion

Female ovarian function has been shown to be sensitive to metabolic energy balance and energy flux (Ellison, 2003). Metabolic energy availability in cells decreases when an individual is experiencing immunological responses due to long-term inflammation (Kominsky, Campbell & Colgan, 2010). Thus, there is an energetic association between immune function and female's reproductive function. For example, recent studies have shown that gonadal steroids – such as estrogen and progesterone - can affect women's immune function, and this dynamic is directly linked to metabolic energy availability and inflammatory patterns (Khan, Cowan & Ahmed, 2012; Khan & Ansar Ahmed, 2016; Gursoy et al., 2015; Faas et al., 2000; Alvergne & Högqvist Tabor, 2018). The menarche transition imposes significant metabolic energetic demands on girls. Chronic inflammation is an immunological challenge and, as such, energetically demanding (Lacourt, 2018; Kominsky, Campbell & Colgan, 2010). Consequently, chronic inflammation should also affect the timing and pace of reproductive maturation (Ellison, 1981). Yet, as far as we know, no studies have explored the link between chronic inflammation and the timing and pace of menarche.

Our results are consistent with previous studies that showing that chronic inflammation can affect women's reproductive function. They are also consistent with our hypothesis that this immune challenge can delay the timing of menarche in girls. Indeed, we observed an average delay of 15 months (95% confidence interval [5.8, 24.1]) of menarche in girls with chronic inflammation compared to girls with no inflammation. It is important to emphasize that we had a relatively small sample size, which led to a relatively wide confidence interval. Our data are consistent with the lower bound of this confidence interval, 5.8 months. This estimated delay is comparable to those found by studies investigating the influence that other variables have on menarche timing. For instance, studies of Guatemalan girls (Khan, 1995) and Italian girls (Lazzeri, 2018) showed that menarche onset was an average of 6 months earlier for girls with higher calorie intake. Growing up in households with an unrelated adult male (male effect) was also shown to be associated with earlier age at menarche by 4 to 6 months when compared to girls who did not have the same experience (Mendle, 2006).

Importantly, exposures taking place earlier during development also appear to affect girls' age at menarche. For example, it was found that girls who had mothers who smoked heavily during pregnancy reached menarche an average of 3.7 (Yermachenko & Dvornyk, 2014) and 5.3 (Ferris et al., 2010) months later than girls who did not. Terry et al. (2009) presented evidence that girls born with low birth weight reached menarche an average of 7.2 months earlier than girls born with normal birth weight. Finally, a study conducted by Hui et al. (2012) found that girls who were born preterm reached menarche an average of 4 months earlier than girls born at term, even after adjusting for birth weight (Hui et al., 2012). All these studies, including the present research, are consistent with the idea that stringent energetic conditions during development can shape an individual's life history trajectory and, therefore, alter the timing of reproductive maturation, as stated by Life History Theory (Ellison, 2003).

While our results suggest that chronic inflammation is associated with delayed age of menarche, the estimate of the parameter of interest has a large standard error, so our estimate of the magnitude of the delay is imprecise. They are inconclusive regarding the effects of chronic inflammation on cycle length and ovulation frequency. Regarding cycle length, in our sample, the estimated hazard rate associated with cycle length of girls with chronic inflammation is 1.03 times that of girls without. Nonetheless, our data are also compatible with factors ranging from 0.118, which represents a substantial negative association, to 3.61, which represents a substantial positive association. Regarding ovulation frequency, in our sample, the estimated mean number of ovulations of girls with chronic inflammation was 0.755 times that of girls without chronic inflammation. However, our data are also consistent with factors ranging from 0.346, which represents a substantial negative association, to 1.65, which represents a substantial positive association. Future studies with larger sample sizes and longer data collection periods will be necessary to investigate the potential link between chronic inflammation and ovarian function quality post-menarche.

Although our study was not conclusive in providing evidence that chronic inflammation does or does not affect cycle length and ovulation frequency, previous studies have indicated that there is a relationship between immune function and women's gonadal steroids levels. Faas et al. (2000), for example, have found that higher progesterone levels during the luteal phase of the menstrual cycle induces a shift towards a Th2-type immune response in women. Gursoy et al. (2015), found evidence

that CRP levels are inversely correlated to estrogen levels and that higher CRP levels during the follicular phase of the menstrual cycle indicated inflammatory status. It has also been consistently demonstrated that estrogen levels have regulatory action over innate and adaptive immune cells (Khan, Cowan & Ahmed, 2012; Khan & Ansar Ahmed, 2016). Such regulatory action of estrogen and progesterone over the immune system is due to their ability to bind to specific receptors present on several immune cells (Oertelt-Prigione, 2012). Better understanding of the mechanisms involved in these relationships and the effects of women's reproductive hormones over immune function would help to understand the timing of reproductive maturation in girls.

Although it is currently accepted that ovarian function is associated with immune function patterns, the effects of chronic inflammation on variability in age and pace of menarche transition are just beginning to be investigated. Although recent publications demonstrate an increasing interest in the effects of immune challenges on ovarian function, reports on associations between immunological activity and the menstrual cycle of young women are still scarce. Further research is needed to confirm our findings regarding a possible delay in the age of menarche as a result of chronic inflammation, as well as to investigate its possible effects on the quality and regularity of menstrual cycles in adolescent girls immediately after menarche. To that end, our study should be replicated with *i)* a larger number of participants; *ii)* more frequent collection of salivary samples to assess inflammatory status; and *iii)* investigation of genetic factors by comparing girls' age at menarche to their mom's.

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