ULTRASOUND CHARACTERIZATION OF ANTRAL FOLLICLE DYNAMICS ACROSS THE ADIPOSITY SPECTRUM

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Alexis Lyn Oldfield

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Obesity affects more than one-third of reproductive aged women and is associated with many adverse reproductive health outcomes. These include an increased likelihood of anovulatory cycles, longer time to pregnancy, infertility, a decreased efficacy of contraceptive methods, an increased risk of late menopause, endometrial hyperplasia, and gynecological cancers. Some women with obesity, however, maintain regular menstrual cycles. Of those that do, altered ovarian hormone production has been documented, including decreased luteal progesterone concentrations, and reduced luteinizing hormone pulse amplitude in addition to lower follicle stimulating hormone (FSH) and anti-Mullerian hormone (AMH) production, albeit controversial. These endocrine aberrations likely reflect alterations in antral ovarian follicle development, but this has never been systematically evaluated in this population. An understanding of antral follicle development in obesity may be necessary to develop effective care for the reproductive dysfunction commonly seen in this population particularly in light of our increasingly obesogenic demographic. The central objective of this dissertation was to characterize antral follicle and endocrine dynamics in women with obesity and regular ovulatory cycles. In **Chapter 1**, we use serial ultrasonography and venipuncture to characterize antral follicle development and ovarian hormone production during an inter-ovulatory interval (IOI) in women with obesity and regular ovulatory cycles and determine any differences in follicle and endocrine dynamics between obese and non-obese groups. We observed that women with obesity display fewer selectable (6-9mm) follicles across the IOI, as well as fewer recruitment events, smaller ovulatory follicle diameters at selection, and less anovulatory dominant follicles. These alterations in follicle dynamics were accompanied by decreased progesterone and AMH concentrations in women with obesity. In

Chapter 2, we demonstrate the impact of a 6-month hypocaloric dietary intervention on follicle and endocrine dynamics in women with obesity and regular ovulatory cycles. We showed that weight loss was accompanied by an increased ovulatory follicle diameter at selection, increased number anovulatory dominant follicles, as well as a decreased total antral follicle count driven by fewer recruitable (2-5mm) follicles across the IOI. We also observed increased luteal progesterone production following weight loss. Lastly, in **Chapter 3**, we investigate the impact of obesity on endometrial development across the IOI, and report on any impact of a 6-month hypocaloric dietary intervention on endometrial thickness with weight loss. We discovered that women with obesity and regular ovulatory cycles show a thicker endometrium across the IOI, with a lesser degree of change in endometrial thickness at the secretory-proliferative phase transition compared to their non-obese counterparts. These differences in endometrial development were not improved with weight loss in the short-term, despite increased progesterone production. Collectively, this dissertation provides new knowledge related to the impact of obesity on suppressed antral follicle development and ovarian hormone production, and further improvements in antral follicle development and luteinization following weight loss, albeit these changes are insufficient to improve increased endometrial thickness in the short term.

BIOGRAPHICAL SKETCH

Alexis Lyn Oldfield was born on July 27, 1996, to Aimee and Aaron Oldfield. Growing up in Hamilton, NJ alongside her siblings Austin and Alyssa, Alexis was always interested in reading and trying new things. Once she began schooling she fell in love with math, science, and medicine. In high school she focused in on biology and chemistry, but ultimately decided to pursue a Bachelor of Science in chemistry from The College of New Jersey (2014-2017). Alexis joined Dr. Danielle Guarracino's research group in the fall of 2015. The group's focus was on discovering a peptide that inhibited interactions in thrombosis. During her three years in the lab, this work enabled Alexis to explore her problem-solving skills and learn more about applying the scientific method; however, she realized that her interests lied more in the clinical research space than with basic science. This led her to apply for the Biomedical and Biological Sciences PhD program at Cornell (2018). During her first laboratory rotation in Dr. Marla Lujan's group, Alexis would ultimately find her home for the next four years. Over this time, she began to investigate antral follicle dynamics in eumenorrheic women with obesity, and ultimately her work led her to investigate the role of obesity on endometrial dynamics, as well as a role of weight loss in improving antral follicle dynamics. With her free time during her PhD, Alexis enjoyed cycling, running, yoga, reading, and spending time with her family and friends.

This dissertation is dedicated to my great-grandmother, Gigi.

Thank you for always inspiring me to be my best.

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Disclaimer: The content in this dissertation is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Diabetes and Digestive and Kidney Diseases, National Center for Advancing Translational Sciences, or the National Institutes of Health.

TABLE OF CONTENTS

LIST OF FIGURES

PREFACE

CHAPTER 1

CHAPTER 2

CHAPTER 3

LIST OF TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 3

INTRODUCTION

Obesity

Obesity is defined as excess body weight for a given height and is typically described as having a body mass index (BMI) greater than or equal to 30 kg/m^2 (Hales et al. 2020). The prevalence of obesity in the world has more than doubled since 1980 (Todd R. and Colin 2011). Obesity increases the risk of developing cardiovascular, respiratory, and metabolic diseases (Haslam and James 2022) in addition to heightening the risk for sleep apnea, arthritis, fatty liver diseases, gallbladder and kidney diseases, and cancer (NIDDK 2022a). Obesity decreases life expectancy by 7 years at the age of 40 (Haslam and James 2022). Consequently, about 300,000 deaths per year in the US, and 4 million deaths globally, can be attributed to obesity (Office of the Surgeon General, Office of Disease Prevention and Health Promotion, and National Institutes of Health 2001). Factors driving the current obesity epidemic consist of a complex combination of environmental, sociocultural, physiological, medical, behavioral, genetic, and epigenetic factors (Haslam and James 2022) and remains a high area of priority for research across the globe.

Obesity and Female Reproductive Health

Women are more likely to have obesity than men (Hales et al. 2020). Domestically, obesity affects more than two thirds of women, and over one-third of reproductive aged women (Adela Hruby, PhD and Frank B. Hu, MD, PhD 2015; Hales et al. 2020; Vaamonde and Álvarez-Món 2020). Obesity is associated with many adverse female reproductive health outcomes including an increased risk of anovulation, longer time to pregnancy, reduced efficacy of contraceptive measures, infertility, increased risk of late menopause, and a higher risk of endometrial hyperplasia and gynecological cancers (Dağ and Dilbaz 2015; Damodaran and Swaminathan

2013; Goldsammler, Merhi, and Buyuk 2018; Kyrou et al. 2000; Purcell and Moley 2011; Shaw and Edelman 2013; Silvestris et al. 2018; D. Zhu et al. 2018).

Excess adiposity has been shown to adversely impact ovarian function. Women with obesity, regardless of cycle status, have been shown to have altered reproductive hormone profiles. Increased testosterone concentrations, and decreased concentrations of sex hormone binding globulin (SHBG), compared to lean women, have been reported in those with obesity and are posited to impact ovarian follicle development and ovulation (Cooper et al. 2015; Hautanen 2000; Pasquali 2006; Stanikova et al. 2019). Altered luteinizing hormone (LH) pulse amplitude, lower concentrations of follicle stimulating hormone (FSH) and progesterone (P4), as well as increased concentrations of estradiol (E2), have also been noted in those with obesity, consistent with impairments at all levels of the hypothalamic-pituitary-ovarian (HPO) axis (Vanden Brink et al. 2015; Broughton and Moley 2017; Cortet-Rudelli et al. 2002; Jain et al. 2007a; De Pergola et al. 2006; Wang et al. 2014; Yeung et al. 2013). The alignment of ovarian follicle development with known alterations in hormone production is currently unknown, but likely underlies the unfavorable reproductive health outcomes in obesity.

Normal Ovarian Follicle Development

Ovarian follicles contain a single oocyte surrounded by various layers of somatic cells, known as granulosa and theca cells (Richards et al. 2010). Oocytes represent the germ cells, and granulosa and theca cells are responsible for ovarian steroid production. Follicle formation begins in utero, and females are born with a finite ovarian reserve consisting of approximately 400,000 primordial ovarian follicles that lay dormant until stimulated to begin growing (Cox and Takov 2018; Findlay et al. 2015). Ovarian follicle development is the process of follicular growth and atresia (Baerwald, Adams, and Pierson 2012). The proper growth and development of the follicle

depends on precise signals from the HPO axis which are necessary for successful reproductive outcomes (Baerwald et al. 2012; Gougeon 2003). Ultimately, these signals from the HPO axis are vital to ensure the proper fate of the ovarian follicles.

Hypothalamic-Pituitary Axis

Hormones produced by the hypothalamus, anterior pituitary and ovary provide the necessary physiological signals that drive folliculogenesis. First, pulsatile production of the peptide hormone gonadotropin-releasing hormone (GnRH) from GnRH neurons of the hypothalamus stimulate both the production and release of the gonadotropins, FSH and LH, from the anterior pituitary (Espey, Bellinger, and Healy 2003). The release of gonadotropins is controlled by the amplitude and frequency of the GnRH pulses (Espey et al. 2003); FSH synthesis and release is stimulated by slow GnRH pulses whereas fast GnRH pulses primarily drive LH synthesis and release (McCartney, Eagleson, and Marshall 2002; Reame et al. 1985).

LH and FSH enter the general circulation and act at the ovary. FSH receptors are located on the surface of granulosa cells. When FSH binds to its receptors on granulosa cells, the aromatase system is induced (Orlowski and Sarao 2019), which catalyzes the conversion of androgens (produced in thecal cells) into estradiol (E2) (Hillier 1994). LH binds to the LH receptors on theca cells at all stages of follicle development and receptors on granulosa cells at the later stages of follicle development – namely, after follicle selection (Gougeon 1996). The binding of LH to LH receptors on theca cells induces androgen production (Burger 2002). Ovarian estrogens are formed in the granulosa cells through androgen conversion by aromatase and androgen synthesis is under stimulatory control of LH. Granulosa cell proliferation and the growth of follicles into the mid-antral stage is also induced upon FSH binding, giving FSH a dual role (Orlowski and Sarao 2019). Granulosa cells develop LH receptors in the mid to late follicular phase, and upon

LH binding to the granulosa cells of these larger follicles, LH stimulates granulosa cell proliferation and E2 production (Gougeon 2003; Murphy 2003). At these later stages of follicle development, LH can synergize with FSH to sustain follicle development. LH can also bind to the luteinized theca and granulosa cells of the corpus luteum (CL) to stimulate progesterone (P4) production (Gougeon 1996).

E2 is a steroid hormone produced by the granulosa cells of antral follicles and synthesized from testosterone. At low concentrations E2 provides a negative feedback mechanism to the anterior pituitary suppressing follicle growth and gonadotropin secretion (Gougeon 2003; Gougeon 1996). At high concentrations, E2 provides a positive feedback mechanism that ultimately triggers the LH surge and ovulation (Espey et al. 2003). As more E2 is produced by growing antral follicles, more LH receptors are synthesized by theca cells, which increases androgens production by theca cells and E2 by granulosa cells, which causes LH to spike significantly and induce ovulation. P4 is a steroid hormone produced by the luteinized granulosa and theca cells of the pre-ovulatory follicle and the CL (Skinner et al. 2008). During the luteal phase, P4 suppresses gonadotropin secretion through a negative feedback mechanism to the anterior pituitary (Espey et al. 2003). In the follicular phase, with the demise of the CL, P4 production is suppressed by LH.

Ovarian Follicle Development

Ovarian follicle development begins in utero. At about 4 months of gestational age, primordial follicles begin to form in the fetal ovary after primordial germ cells have migrated from the yolk sac endoderm to the gonadal ridge and begin undergoing mitotic divisions (Gougeon 1998; Gougeon 1996). Follicles that contain oocytes are arrested in the dictate state of meiosis prophase I and make up the ovarian reserve that provides the reproductive potential over a

woman's lifespan (Cox and Takov 2018). This primordial reserve is established by a balance between the availability of a large number of germ cells along with programmed cell death. At 20 weeks gestational age, it is thought that there are about 7 million germ cells occupying a female's ovarian reserve (Cox and Takov 2018). Depletion of this ovarian reserve begins during fetal life and continues throughout a woman's life (Yang et al. 2022). At around 22 weeks gestation, some primordial follicles are activated to form the first growing, or primary follicles (Cox and Takov 2018). Some primordial follicles initiate this growth immediately while others remain arrested for months, or even decades (Fortune 2003; Gougeon 1979). The entire duration of human folliculogenesis spans more than 175 days (Cox and Takov 2018; Gougeon 1998). Pre-antral follicles (0.1-0.2mm) develop independent of gonadotropin support. At about 0.2-0.4mm a fluid-filled cavity, or antrum, begins to develop and follicles become responsive to gonadotropins. Follicles may develop to this early antral stage throughout childhood, but then quickly regress in the absence of mature gonadotropin signaling (Baerwald et al. 2012). Once puberty occurs and the HPO axis matures (Marques et al. 2000), pulsatile release of FSH and LH leads to the cyclic development of antral follicles and the onset of ovulation and the menstrual cycle (Lacroix et al. 2022). Figure 1 shows a timeline for ovarian follicle development and the regulation by the hypothalamic-pituitary axis in the process.

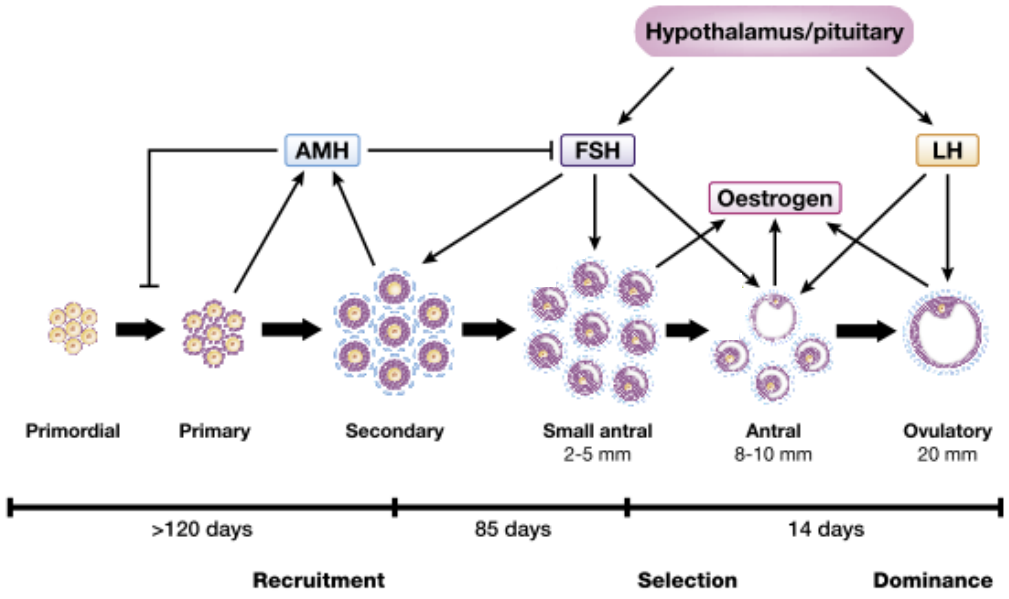

Figure P.1. Overview of folliculogenesis. Adapted from (Fleming et al. 2015)

Pre-Antral Stage

Primordial follicles form through oocyte assembly, and they are characterized by a single layer of granulosa cells (Gougeon 1986). Once activated, they enter the primary stage of development and granulosa cells begin to proliferate and differentiate (Baerwald et al. 2012; Gougeon 1996). When the follicles enter the growth phase, their size increases by the enlargement of the oocyte along with the proliferation of the granulosa cells and acquisition of theca cells (Gougeon 2003). This proliferation gives the follicles multiple layers and they are now described as secondary follicles. Once primary follicles reach the secondary stage, their vasculature develops and the granulosa and theca cells begin to express gonadotropin and steroid receptors (Gougeon 1979, 1986; Gougeon 1996). As stated previously, the early stages of follicle development are gonadotropin independent, but small preantral follicles are gonadotropin responsive. Therefore, FSH is not a survival factor, rather FSH can help to promote follicle growth, granulosa cell proliferation, and differentiation in follicles from the primary stage

and onward. The development of pre-antral follicles is primarily mediated by paracrine and autocrine factors that are secreted by the oocyte and adjacent follicles (Aerts and Bols 2010).

Antral Stage

Antral folliculogenesis occurs in waves of growth and regression (A. Baerwald, Adams, and Pierson 2003). Early on in the antral stage, a fluid filled cavity forms (Gougeon and Lefevre 1983). The follicular fluid that fills the antrum is derived from the bloodstream and contains components that are secreted by the somatic cells of the follicle. There are three morphological classes of antral follicles as they progress towards ovulation: 1) small recruitable follicles, 2-5mm in size, 2) medium selectable follicles, 6-9mm in size, and 3) large dominant follicles, greater than 10mm in size. Antral follicle growth is continuous and represents the proliferation and replication of the granulosa cells. The designation of recruitable, selectable, and dominant follicles reflects the relative abundance of granulosa cell layers present in the follicle (Gougeon 1986).

The first step in folliculogenesis is recruitment, which occurs when a group or cohort of recruitable antral follicles (2-5mm) emerges from the pre-antral pool (Angela R. Baerwald, Adams, and Pierson 2004). This occurs, at minimum, once during the menstrual cycle and typically occurs during the late-luteal or early follicular phase (Gougeon 1979). At this point, the follicles become gonadotropin dependent for growth, and in the absence of gonadotropin stimulation, undergo atresia (Gougeon 2003). This stimulation is described as an increase in FSH concentrations above a specific threshold; this threshold marks the amount of FSH necessary to support recruitable to selectable stage growth (Angela R. Baerwald et al. 2004).

In this mid-antral stage, follicles grow together until follicle selection occurs. Follicle selection is the process by which a (future) dominant follicle is chosen from the cohort for preferential growth (Baerwald et al. 2012; Gougeon 2003; Messinisi 2006). This selection occurs

in the early to mid-follicular phase leading up to ovulation. Once selected, the chosen follicle diverges from the cohort as it begins to grow, and the subordinate follicles undergo atresia. Dominance occurs when the selected follicle reaches 10mm in diameter, typically during the mid-follicular phase in women (Gougeon 2003; Messinisi 2006). The dominant follicle also begins to produce substantial amounts of estradiol (Gougeon 2003; Kishi et al. 2018; Messinisi 2006). Estradiol is an FSH suppressor. A decline of FSH is critical for the selection of the dominant follicle (Gougeon 2003; Messinisi 2006). As estradiol levels rise, FSH levels decline and the subordinate follicles regress (Hillier 1994), as they are not able to thrive in declining FSH conditions, leading them to undergo atresia. In the dominant follicle, theca cells respond to LH to produce androgens and P4. Androgens are aromatized to estradiol within this growing follicle, which further increases estradiol concentrations (Reame et al. 1985). The production of estradiol increases the frequency and amplitude of LH pulses from the pituitary to increase aromatase activity. This increased aromatase activity is critical for the transition to the LH-dependent growth as this induces expression of LH receptors on the granulosa cells (Zeleznik 2004) enabling the follicle to survive in the declining FSH conditions, as it becomes primarily LH dependent (Hillier 1994; Zeleznik 2004).

The dominant follicle grows at a rapid rate once selected (A. Baerwald et al. 2003; Angela R. Baerwald et al. 2004; Baerwald, Walker, and Pierson 2009), but whether or not the follicle reaches ovulation is dependent on the frequency and amplitude of LH pulses induced by the pituitary (Gougeon 2003). Granulosa and theca cells continually proliferate as the antral volume increases (Gougeon 1996). Estradiol synthesis increases progressively from the dominant follicle, initiating the LH surge (Gougeon 2003). There is a small increase in P4 levels before the LH surge indicative of an increase in LH amplitude and frequency (Fukushima et al. 1986; Yding Andersen et al. 2011). The LH surge typically lasts 24-36 hours and is sufficient to inhibit further cell

proliferation and trigger both the luteinization of granulosa and theca cells and ovulation (Kerin 1982).

Luteal Stage

Immediately after the LH surge, there is an inverse relationship between P4 and E2 production. P4 levels rise very rapidly as E2 levels decline. LH pulses, however, are still occurring and are critical to the development and function of the CL. As these hormonal changes occur, the follicular wall is degraded and transformed. The cells of the CL originate from theca and granulosa cells following the breakdown of the basal lamina of the ovulatory follicle (Stouffer et al. 1984). The luteinized theca and granulosa cells transition from producing primarily estrogen, to producing P4 (Gougeon 2003). There are two types of luteal cells, small and large. Small luteal cells are the primary source of luteal androgens, while large luteal cells are the primary site for luteal estrogen and P4 synthesis (Wiltbank et al. 2012). The CL is rapidly growing and is thought to grow to a diameter of 25-40mm during the luteal phase. The growth of the CL is associated with an increase in luteal blood flow, as well as an increase in P4 (Henríquez et al. 2016; Tamanini and De Ambrogi 2004). During the luteal phase of the menstrual cycle, P4 production by the CL acts at the endometrium to promote implantation (Gougeon 2003; Tamanini and De Ambrogi 2004).

If a non-fertilized cycle occurs, the CL undergoes regression, or luteolysis, by way of apoptosis or autophagy (Gougeon 2003). This is associated with the loss of functional and structural integrity of the gland and a rapid decline in P4 and E2 production (Gougeon 2003). As the gland loses function, menstruation begins when the endometrial blood supply is lost and the endometrial lining begins to degrade (Gougeon 2003). With a decrease in P4 and E2 negative

feedback to the hypothalamic-pituitary axis, FSH begin to increase, inducing the start of the next ovarian cycle (Gougeon 2003).

Endometrial Development

As follicle development occurs in the ovary, the endometrium of the uterus is undergoing dynamic changes in response to the steroid hormones produced by the ovary. During the follicular phase of the ovarian cycle, the proliferative phase of the menstrual cycle occurs (Monis and Tetrokalashvili 2019). In this proliferative phase, the E2 that is produced by the growing follicles stimulate the development and proliferation of the endometrial lining (Baerwald and Pierson 2004; Monis and Tetrokalashvili 2019). Endometrial thickness peaks just prior to ovulation. Endometrial development is primed by estrogen to create an environment conducive to implantation (Baerwald and Pierson 2004; Reed and Carr 2000). The luteal phase of ovarian cycle, coincides with the secretory phase of the menstrual cycle (Thiyagarajan, Basit, and Jeanmonod 2021). P4 produced by the CL is critical for increasing vascularization and blood flow within the endometrium and stimulating endometrial glands to secrete nutrients that could nourish an early embryo (Filant and Spencer 2014). During this secretory phase, endometrial thickness plateaus and then declines with menstruation and the withdraw of P4 and E2 support from the CL (Baerwald and Pierson 2004; Thiyagarajan et al. 2021). The monthly shedding of the endometrial lining is thought to enhance the likelihood of implantation of the next cycle (Holesh, Bass, and Lord 2022). Further, a proper or complete shedding of the endometrial lining avoids the development of endometrial hyperplasia, a condition of thickened endometrial tissue commonly resulting from insufficient progesterone production and unopposed estrogen effects (Singh and Puckett 2022) that can lead to uterine cancers (Singh and Puckett 2022).

RATIONALE FOR THE CURRENT STUDIES

Women with obesity and regular cycles have known disruptions in reproductive hormone production that may underlie adverse reproductive health outcomes in this population. Longitudinal studies are needed to determine the degree to which antral follicle and endocrine dynamics are impaired in those with seemingly regular ovulatory cycles and the degree to which these disruptions may align with functional changes in the female reproductive tract. To do so, in **Chapter 1**, a prospective cohort study was conducted to characterize antral follicle development and reproductive hormone production in obese women with regular menstrual cycles. Further, a subset of the eumenorrheic participants in the obese cohort in Chapter 1 partook in a 6-month hypocaloric dietary intervention in order to determine if weight loss could improve antral follicle and hormone dynamics. In **Chapter 2,** we present a comparison of antral follicle and endocrine dynamics before and after the intervention, as well as a comparison of the post-intervention changes to that of the non-obese control group from Chapter 1. Building on our findings that P4 levels were significantly decreased across the cycle in obesity, and improved with weight loss, in **Chapter 3** we sought to determine any impact of obesity on endometrial development both across the cycle and after a weight loss intervention. Together, this research promises to create a better description of antral follicle and endometrial development in the context of obesity, which can better fit our current demographic and inform on their reproductive health risks.

CHAPTER 1

OBESITY IS ASSOCIATED WITH ALTERATIONS IN ANTRAL FOLLICLE DYNAMICS IN EUMENORRHEIC WOMEN

Abstract

Study Question

Are ovarian antral follicle dynamics altered in women with obesity and regular ovulatory cycles?

Summary Answer

Eumenorrheic women with obesity displayed evidence of suppressed antral follicle dynamics as judged by fewer recruitment events, selectable follicles, and anovulatory dominant follicles, as well as lower anti-Müllerian hormone (AMH) concentrations and an increased prevalence of luteal phase defects.

What is known already?

Ovarian antral follicle development is a dynamic process involving distinct follicular and endocrine events that are critical for the occurrence of regular monthly ovulations. Follicle dynamics have not been prospectively evaluated in eumenorrheic women with obesity despite the known impact of obesity on gonadotropin production, ovarian steroid hormone concentrations, and fecundity.

Study design, size, duration

Prospective, longitudinal study of 42 women over one inter-ovulatory interval (IOI).

Participants/Materials, setting, methods

Twenty-one women with obesity (total percent body fat ≥35%) and 21 women without obesity (total percent body fat <35%) underwent transvaginal ultrasonography and venipuncture every-other-day for one IOI at an academic clinical research unit. Participants were aged 19-38 years and had a history of self-reported regular menstrual cycles (21-35 days). Follicle number and diameter (≥2 mm) were quantified at each visit. Individual growth profiles for all follicles that grew to ≥7 mm were assessed. Blood samples were assayed for gonadotropins, AMH, estradiol, and progesterone.

Main results and the role of chance

Women with obesity exhibited fewer recruitment events (mean ± standard deviation, 1 ± 1 vs. 2 ± 1 events; p=0.010) and fewer selectable follicles (4 ± 3 vs. 8 ± 6 follicles per participant; p=0.022) during an IOI compared to women without obesity. AMH levels were lower in women with obesity (4.40 ± 3.01 vs. 5.94 ± 2.49 ng/mL; p=0.023), while gonadotropin profiles were similar between groups, across the IOI. Of the individual follicles tracked, fewer follicles progressed to >10 mm in the cohort with obesity (30 vs. 40 follicles; p=0.04) and fewer anovulatory follicles achieved dominance (9 vs. 18 follicles; p=0.041). Ovulatory follicles were selected at smaller diameters in women with versus without obesity (7.5 ± 1.6 vs. 9.5 ± 1.9 mm; p=0.001). Luteal phase defects were also more common in women with versus without obesity, as defined by either integrated (76 vs. 29%, p=0.002) or maximum (71 vs. 24%, p=0.002) luteal progesterone.

Limitations, reasons for caution

This study was limited to an assessment of antral follicle dynamics and cannot inform on earlier stages of folliculogenesis. This study was observational and cannot address causation between obesity and altered antral follicle dynamics. Lastly, data cannot be extrapolated to account for reduced fecundity and fertility in obesity.

Wider implications of the findings

The increasing global prevalence of obesity necessitates an understanding of the mechanisms that underlie obesity-related adverse reproductive health outcomes. Eumenorrheic women with obesity demonstrate altered ovarian antral follicle and endocrine dynamics compared to their counterparts without obesity. The degree to which abnormal granulosa cell assembly and/or activity underlie suboptimal luteinization and subfertility requires further investigation.

Study funding/competing interest.

Cornell University, President's Council of Cornell Women, United States Department of Agriculture (Grant No. 8106), and National Institutes of Health (R01-HD0937848). BYJ and HVB were supported by doctoral training awards from the National Institutes of Health (T32-DK007158) and Canadian Institutes of Health Research (Grant No. 146182), respectively.

Trial registration number

NCT01927432, NCT01785719

Introduction

More than one-third of reproductive-aged women globally are living with obesity (Hruby et al. 2015; Hales et al. 2020; Vaamonde and Álvarez-Món 2020). Obesity is associated with adverse reproductive health outcomes, including increased likelihood of anovulation, longer time to pregnancy, reduced fertility, and an increased risk of late menopause (Dağ and Dilbaz 2015; Damodaran and Swaminathan 2013; Goldsammler et al. 2018; Kyrou et al. 2000; Purcell and Moley 2011; Shaw and Edelman 2013; Silvestris et al. 2018; Zhu et al. 2018). An increased understanding of the mechanisms by which obesity affects the hypothalamic-pituitary-ovarian axis is needed to appropriately manage these adverse reproductive health outcomes.

Despite an increased likelihood of anovulation (Dağ and Dilbaz 2015), a substantial percentage of women with obesity report regular menstrual cycles (Lasquety, Rodriguez, and Fehring 2012). However, eumenorrheic women with obesity demonstrate endocrine abnormalities, including lower luteal progesterone (P4) concentrations (Jain et al. 2007; De Pergola et al. 2006; Yeung et al. 2013), reduced luteinizing hormone (LH) pulse amplitude (Jain et al. 2007b), decreased follicle-stimulating hormone (FSH) during the follicular phase (De Pergola et al. 2006; Yeung et al. 2013), and elevated estradiol (E2) concentrations across the menstrual cycle (De Pergola et al. 2006; Yeung et al. 2013). Precise control of endocrine hormone dynamics is critical for the maintenance of ovarian folliculogenesis. The nature of antral follicle dynamics in women with obesity, and how it aligns with these known endocrine abnormalities, is unknown; yet both could underlie the adverse reproductive health outcomes that are common in this population. Effective reproductive health care should consider the mechanisms by which obesity adversely impacts ovarian antral follicle and endocrine hormone dynamics (Hurt et al. 2010).

Using transvaginal ultrasonography, antral follicle dynamics can be monitored by tracking the growth of uniquely identifiable follicles (Identity Method) (Knopf et al. 1989; Pierson and Ginther 1988) or evaluating overall changes in follicle number and diameter (Non-identity Method) (Baerwald et al. 2003; Vanden Brink et al. 2013; D Rouleau et al. 2012). These approaches have been used to describe wave-like patterns of antral follicle development in healthy, eumenorrheic women of reproductive age (Baerwald et al. 2003; Baerwald et al. 2004). Follicle dynamics is thought to be a primary factor determining menstrual cycle length (Baerwald et al. 2004) and may relate to fertility potential (Townson et al. 2002). Antral follicle dynamics have also been evaluated in those using hormonal contraceptives (Baerwald et al. 2004; Birtch et al. 2006), those of late reproductive age (Vanden Brink et al. 2015), and those with reproductive disorders including polycystic ovary syndrome (PCOS) (Jarrett et al. 2020), and luteinized unruptured follicles (Bashir et al. 2018). However, the impact of obesity on antral follicle dynamics has not been prospectively evaluated.

The objectives of this study were to contrast antral follicle growth and endocrine hormone dynamics between eumenorrheic women, with and without obesity, during an inter-ovulatory interval (IOI). We hypothesized that women with obesity would show differences in all key stages of follicle development including recruitment, selection, and ovulation, and that altered follicle dynamics would align with disruptions in endocrine hormone dynamics in both the follicular and luteal phases.

Methods

Ethical considerations

Participant data from two ongoing, prospective, observational studies were analyzed. Both protocols were approved by the Institutional Review Board at Cornell University and registered at ClinicalTrials.gov (Identifiers: NCT01927432, NCT01785719). Before procedures were performed, informed consent was obtained from all participants.

Study participants

Women of reproductive age (18-38 years) were recruited from the general population between October 2009 and September 2021. Women were eligible for the study if they had consistent and optimal visualization of both ovaries on ultrasonography. Women were excluded if they were using medications known or suspected to interfere with reproductive function in the two months prior to the study; were pregnant or lactating in the six months prior to the study; had a history of premature ovarian failure; or had any pre-existing confounding medical conditions, including but not limited to untreated thyroid abnormalities or hyperprolactinemia.

Women who completed the study were retrospectively evaluated for inclusion in the current analysis (*n*=112). Groups of interest included: (1) women with regular menstrual cycles and obesity, and (2) women with regular menstrual cycles without obesity. Obesity was defined by a total percent body fat (PFT) ≥35% using whole-body dual x-ray absorptiometry (Piqueras et al. 2021; Valdez 1991). Menstrual cycle regularity was defined by a self-reported menstrual cycle length of 21-35 days in the last year with regularity confirmed using post-hoc ultrasound monitoring of ovarian antral follicle development during an IOI (described below). All women

included were normo-androgenic, as defined by a total testosterone (T) concentration ≤61.5 ng/dl based on a threshold derived in an internal reference cohort; clinical measures of hyperandrogenism were not considered (hirsutism).

Ultrasonographic measurements

Serial transvaginal ultrasonography was used to evaluate antral follicle dynamics, as previously described (Baerwald et al. 2003; Vanden Brink et al. 2013; Jarrett et al. 2020; Rouleau et al. 2012). In all women, ultrasound scans began prior to ovulation (range: cycle day 10 to 23) and were conducted approximately every other day for one IOI. An IOI was defined as the interval from one ovulation to the next ovulation, which represented the luteal phase following ovulation, menstruation, and the follicular phase preceding the subsequent ovulation. When a follicle ≥14-to-16 mm was detected, ultrasound scans were performed daily until its fate, either regression or ovulation, was confirmed. Ovulation was defined as the sonographic detection of a corpus luteum during the IOI and was later confirmed with a rise in serum P4 of ≥1.5 ng/mL (Baerwald et al. 2005).

Scans were performed using a GE Voluson E8 Expert System or a GE Voluson E10 Expert System and 6-12 MHz 3D/4D transducer (GE Healthcare, Milwaukee, WI). Ovaries were imaged from their inner to outer margins in the longitudinal plane using the automated volume modality. Two-dimensional cineloops were archived and evaluated offline by three investigators [Sante DICOM Editor, Santesoft LTD, Athens, Greece]. For each visit during the IOI, follicle number and diameter were assessed in each ovary. In order to obtain reliable follicle counts, investigators used the grid system approach (Lujan et al. 2010) (single measures ICC=0.932). The diameter of each follicle was measured in the largest cross-sectional view and calculated as the average of

its two orthogonal dimensions (i.e., length × width). If a large follicle (i.e., ≥10 mm) was detected, then orthogonal dimensions were repeated in a second plane and the four dimensions were averaged. Follicle diameter was rounded to the nearest whole number.

Growth and regression profiles of individual follicles that grew to 7 mm or greater were identified using the Identity Method (Baerwald et al. 2003; Vanden Brink et al. 2013; Jarrett et al. 2020; Rouleau et al. 2012). Briefly, all follicles ≥4 mm were sketched on paper to generate a map of antral follicles within each ovary. Maps were completed for each ovary at each visit of the IOI. Individual follicles were mapped for their location using anatomical landmarks and positions relative to other follicles within the ovary. Each follicle that grew to ≥ 7 mm was uniquely identified and changes in diameter were tracked over time from the day of first detection (i.e., at 4-5 mm) to last detection (i.e., at 4-5 mm or ovulation). Growth and regression rates of each uniquely identified follicle were then calculated. Sonographic presence was defined as the interval of time between the first and last days of sonographic detection of a follicle (Baerwald et al. 2009; Jarrett et al. 2020). The growth phase was defined as the interval of time from the day of first detection to the day of maximal follicle diameter (Baerwald et al. 2009; Jarrett et al. 2020). The regression phase was defined as the interval of time from the day of maximal diameter to the day of last detection (Baerwald et al. 2009; Jarrett et al. 2020). A follicle was considered to be in a static phase if it was observed within 1 mm of its maximal diameter for at least three consecutive days (or two consecutive visits) (Baerwald et al. 2009; Jarrett et al. 2020). The first and last days of a static phase coincided with the end of the growth phase and beginning of the regression phase, respectively.

A recruitment event was defined as the emergence of two or more follicles ≥4 mm within a three-day (or two-visit) window, that further grew to ≥7 mm, in conjunction with an increase and subsequent decrease in the number of follicles ≥5 mm (Adapted from: (Baerwald et al. 2003; Baerwald et al. 2004)). Follicle waves were not characterized herein as described by Baerwald et al. due to our less frequent blood sampling protocol (Baerwald et al. 2003; Baerwald et al. 2004). Dominance was defined as the growth of a follicle to ≥10 mm that exceeded the next largest follicle by ≥2 mm (Baerwald et al. 2004). Selection was defined as the day when the future dominant follicle grew ≥1 mm larger than the subsequent follicles in the ovary and remained larger (Baerwald et al. 2003).

In the present analysis, no differences in the number of uniquely identified follicles between the left and right ovaries were detected (data not shown). Therefore, follicle number and diameter data from both ovaries were combined (Baerwald et al. 2003a; Vanden Brink et al. 2013; Jarrett et al. 2020). The total number and proportion of follicles detected in different diameter categories were graphed for each participant over the IOI. Diameter categories of physiologic interest (i.e., antral follicle counts [AFCs]) included: ≥2 mm, ≥5 mm, 2-5 mm, 6-9 mm, and ≥10 mm. Growth profiles of uniquely identified follicles were also graphed for each participant.

Biochemical measurements

Non-fasted blood samples were collected every other day during the IOI. Blood was collected into a clot-activated tube and allowed to sit at room temperature for 30–60 minutes. Serum was isolated by centrifugation and stored at –80°C until analysis. Chemiluminescence immunoassays (Immulite 2000, Siemens Medical Solutions Diagnostics, Deerfield, IL) were performed to measure serum concentrations of FSH, LH, E2, and P4. Luteal phase defects (LPDs) were

defined by decreased luteal phase length (<10 days) and/or biochemical measures of integrated luteal P4 <80 ng/mL or peak P4 <10 ng/mL, as per the American Society for Reproductive Medicine recommendations (Practice Committee of the American Society for Reproductive Medicine 2021). Inter- and intra-assay coefficients of variation (CV) were as follows: FSH (4.9%, 2.6%), LH (6.2%, 3.9%), E2 (9.7%, 8.6%), and P4 (11.8%, 7.2), respectively.

Fasted blood samples were also drawn on a single day of the IOI to assess androgen and glucoregulatory status. Measurements were standardized such that no dominant follicles or active corpora lutea were present at the time of the sample. Serum sex hormone binding globulin (SHBG) was measured by chemiluminescence immunoassay (inter-assay CV: 5.0%; intra-assay CV: 3.1%) and total T was measured by liquid chromatography tandem mass spectrometry (Brigham Research Assay Core, Boston, MA) [inter-assay CV: 6.4%]. Free androgen index (FAI) was calculated as: (total T [nmol/L] / SHBG [nmol/L]) × 100 (Vermeulen et al. 1999). Glucose was measured with a standard glucometer (Accu-Check Aviva, Roche Diabetes Care, Inc., Indianapolis, IN) and insulin was measured by chemiluminescence immunoassay (inter-assay CV: 6.2%; intra-assay CV: 4.8%). The homeostatic model assessment for insulin resistance (HOMA-IR) was calculated as: (fasting glucose [nmol/L] × fasting insulin [mIU/mL]) ÷ 22.5 (Wallace et al. 2004). AMH was measured by enzyme-linked immunosorbent assay (Ansh Labs, Webster, TX) [intra-assay CV:2.9%].

Anthropometry was performed on a single day of the study. Participants were weighed on a standard digital scale, BMI was calculated as kg/m^2, and waist circumference was measured with a soft tape between the lowest rib and iliac crest. Dual x-ray absorptiometry (Discovery-A, Hologic, Inc., Bedford, MA) was used to conduct total adiposity as a measure of fat versus lean mass.

Statistical analysis

All analyses were performed using JMP Pro 14.0.1 (SAS Institute, Cary, NC). Data were log-transformed if not normally distributed before analyses. Cross-sectional data were compared between groups using t-tests. Fisher's exact tests were used to compare cross-sectional categorical data between groups. Follicular and endocrine data were centralized to the day of ovulation and evaluated by: (1) normalizing the data across the IOI and (2) averaging the data across the luteal and follicular phases. Mixed-effect models evaluated longitudinal between-group differences in follicle number, follicle populations, growth parameters, and endocrine hormones (main fixed effect: obesity). Participant identifier was used as a random effect and day as a fixed effect across all models. The statistical significance threshold was set at $P<0.050$.

Results

Participant characteristics

Forty-two women were eligible for inclusion in the present analysis (with obesity: n=21; without obesity: n=21). Reproductive, anthropometric, and metabolic features are compared between groups in Table 1. By design, women with obesity had a higher PFT ($P<0.0001$), but similar menstrual cycle lengths ($P=0.582$), compared to their counterparts without obesity. Groups did not differ in terms of age, total T, FAI, or early follicular LH, FSH, and LH:FSH (All: $P\geq0.050$). However, AMH levels were significantly decreased in women with obesity ($P=0.007$). As expected, women with obesity also had an increased adiposity and impaired insulin sensitivity compared to their counterparts without obesity (Table 1).

Table 1.1. Baseline characteristics of the study participants.

	Non-Obese	Obese
Participant (N)	21	21
Age (years)	29 ± 6	29 ± 4
Reproductive Markers		
Menstrual Cycle Length (days)	29 ± 3	30 ± 2
Luteal Phase (days)	16 ± 4	17 ± 4
Follicular Phase (days)	13 ± 2	12 ± 2
Total Testosterone (ng/dl)	21.9 ± 12.7	21.1 ± 11.3
Free Androgen Index	1.28 ± 0.74	1.93 ± 1.34
LH:FSH	0.73 ± 0.32	0.75 ± 0.47
Anti-Müllerian Hormone (pg/mL)	5.94 ± 2.49	4.40 ± 3.01*
Anthropometric Markers		
Percent Total Fat (%)	27.5 ± 3.7	43.6 ± 4.9****
BMI (kg/m^2)	22.9 ± 3.2	34.4 ± 5.1****
Trunk Fat Percentage (%)	23.8 ± 4.7	43.0 ± 6.1****
Waist Circumference (cm)	79 ± 8	104 ± 18****
Waist:Hip Ratio	0.80 ± 0.05	0.85 ± 0.08
Metabolic Markers		
Systolic Blood Pressure (mmhg)	111 ± 10	115 ± 10
Diastolic Blood Pressure (mmhg)	68 ± 7	71 ± 9
Fasting Glucose (mg/dl)	93.6 ± 12.2	92.1 ± 6.3
Fasting Insulin (miu/L)	4.29 ± 2.22	9.96 ± 5.60****
HOMA-IR	1.00 ± 0.56	2.27 ± 1.31**

Data are presented as mean ± standard deviation. Within rows, * denote significant differences between groups, adjusted values *P < 0.05, **P<0.01, *** P<0.001, **** P<0.0001. Reproductive, anthropometric, and metabolic endpoints were evaluated on a standardized day of the inter-ovulatory interval during the early follicular phase of the menstrual cycle.

Abbreviations: BMI, body mass index;; HOMA-IR, homeostatic model assessment of insulin resistance LH:FSH, luteinizing hormone: follicle stimulating hormone.

Overall, all women demonstrated normal IOI (mean ± standard deviation [SD], 28 ± 6 days), follicular phase (16 ± 5 days), and luteal phase lengths (12 ± 3 days) (Bull et al. 2019), regardless of obesity status. Mean IOI, follicular phase, and luteal phase lengths did not differ between women with and without obesity (P≥0.100). Ovulation of a dominant follicle was observed at least twice in all women (i.e., at the beginning and end of the IOI). One woman without obesity experienced a double ovulation at the end of the IOI.

AFC across an IOI

Mean profiles of AFC ≥2 mm (Figure 1A), AFC 2-5 mm (Figure 1B), AFC 6-9 mm (Figure 1C) and AFC ≥10 mm (Figure 1D) are shown for both groups in Figure 1. AFC ≥2 mm did not differ between groups on any given day of the IOI ($P_{OBESITY}$≥0.100). Likewise, there were no differences in AFC 2-5 mm or AFC ≥10 mm between groups across the IOI ($P_{OBESITY}$≥0.100). By contrast, on any given day of the IOI, women with obesity displayed fewer 6-9 mm follicles than women without obesity ($P_{OBESITY}$=0.040, $P_{DAY*OBESITY}$=0.002).

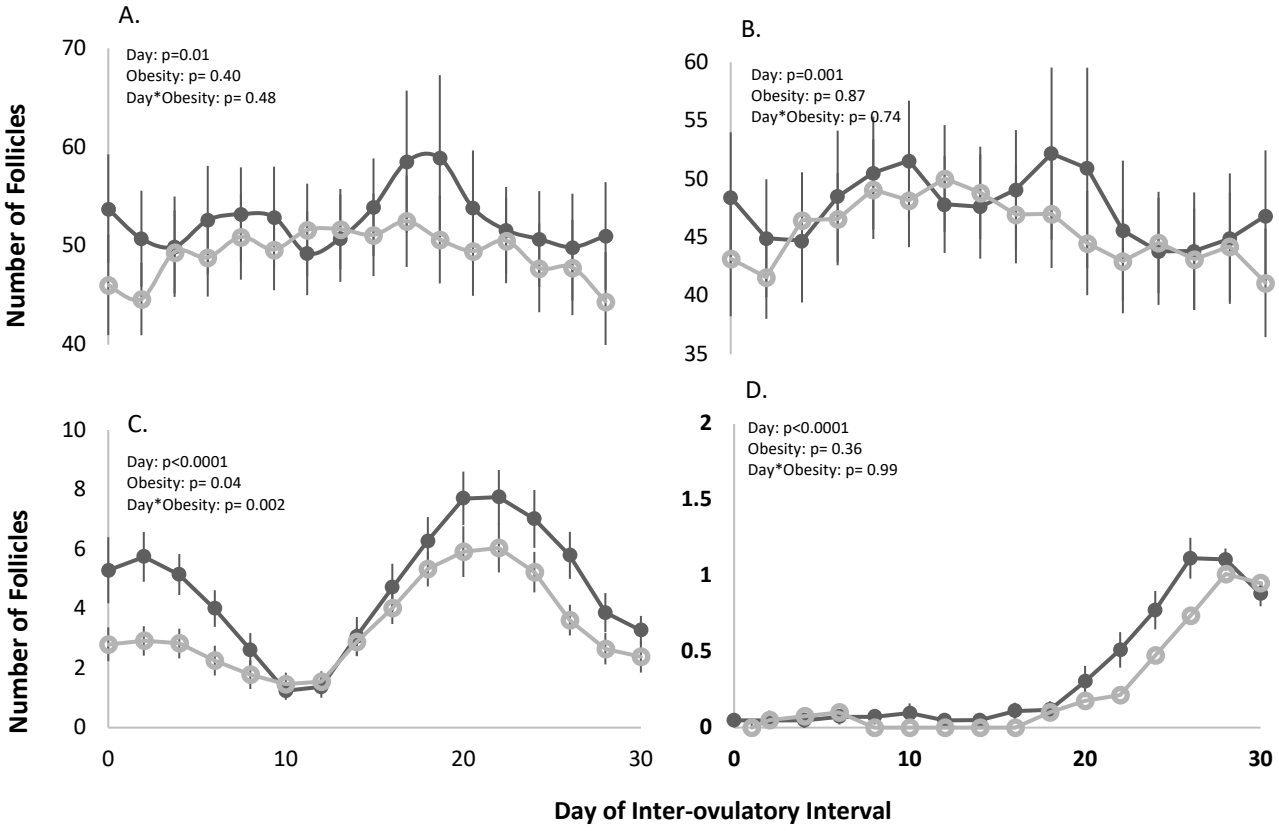

Figure 1.1. ***Longitudinal profiles of total (A), 2-5 mm (B), 6-9 mm (C) and ≥10 mm (D) antral follicle counts across an inter-ovulatory interval (IOI) in non-obese (●) and obese women (○).*** Day to day changes in total follicle counts per follicle size category were monitored using the Non-Identity Method. Mixed models showed a day effect for total, 2-5 mm, 6-9 mm and ≥10 mm follicles, and an obesity effect for 6-9 mm follicles. Day-by-obesity effects were noted for 6-9 mm follicles.

Reproductive hormones during an IOI

Mean profiles of endocrine hormones during an IOI are depicted for both groups in Figure 2. There were no differences in LH and FSH on any given day between non-obese and obese groups (Figure 2A and 2B, respectively; Both: $P_{OBESITY}$>0.050). By contrast, changes in E2 concentrations differed across the IOI by obesity status (Figure 2C; $P_{DAY*OBESITY}$=0.001) and P4 concentrations were lower on any day of the IOI in women with obesity (Figure 2D; $P_{OBESITY}$=0.001, $P_{DAY*OBESITY}$<0.001).

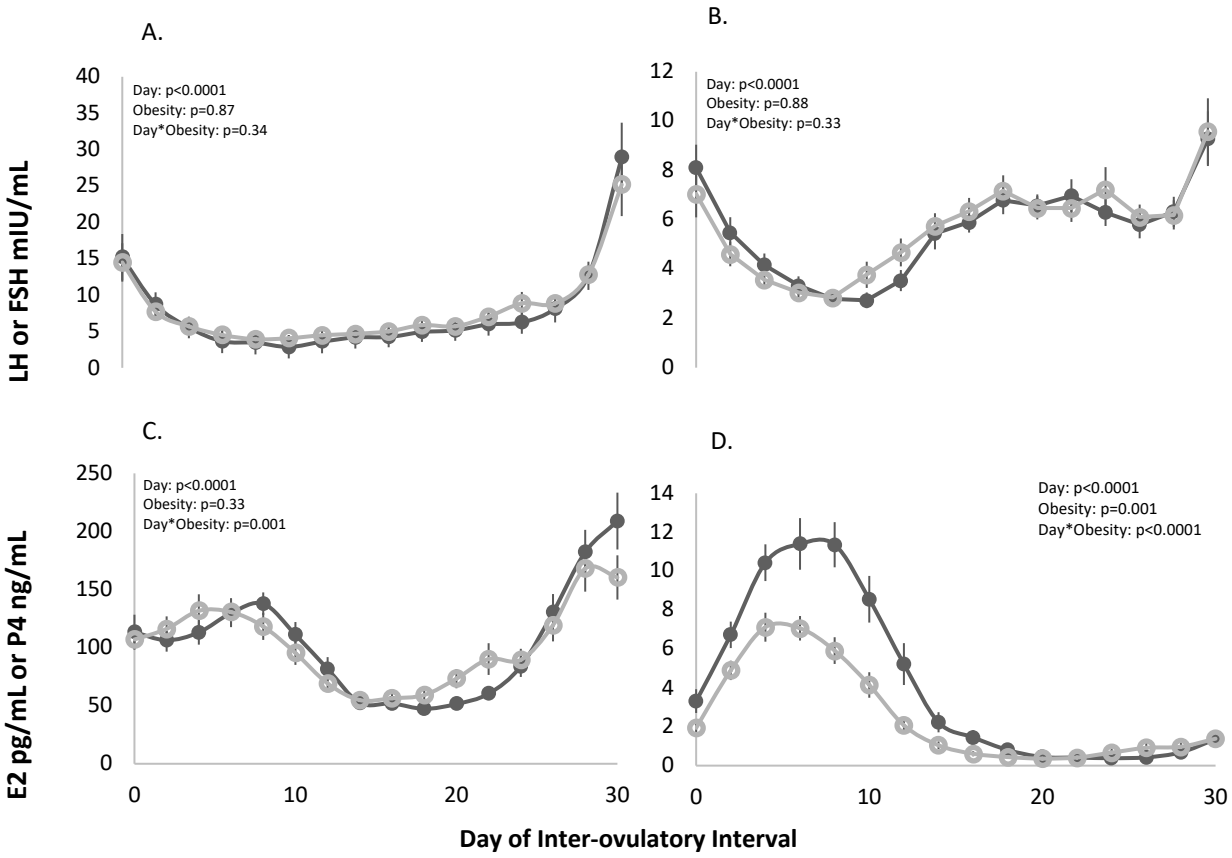

Figure 1.2. ***Longitudinal profiles of luteinizing hormone (LH) (A), follicle stimulating hormone (FSH) (B), estradiol (E2) (C) and progesterone (P4) (D) across an inter-ovulatory interval (IOI) in non-obese (●) and obese women (○).*** Day to day changes in hormone concentrations were monitored by serial venipuncture. Mixed models showed a day effect for LH, FSH, E2, and P4. An obesity effect was noted for P4, and a day-by-obesity effect was noted for E2 and P4.

Follicle counts and hormones by menstrual cycle phase

Mean follicle populations and endocrine hormone concentrations are presented for the follicular and luteal phases in Table 2. During the follicular phase, women with obesity displayed similar follicle counts and follicle size populations, as well as LH, FSH, and E2 concentrations compared to women without obesity (All: $P_{OBESITY} \geq 0.050$). By contrast, P4 concentrations were significantly lower in women with obesity during the follicular phase compared women without obesity ($P_{OBESITY}=0.027$). In the luteal phase, there were no differences in AFC, AFC 2-5 mm, LH, FSH, or E2 levels (All: $P_{OBESITY}>0.05$). The proportion of 2-5 mm follicles was significantly increased in women with obesity ($P_{OBESITY}=0.008$). By contrast, AFC 6-9 mm and the proportion of 6-9 mm follicles were decreased in the luteal phases of women with obesity compared to women without obesity (Both: $P_{OBESITY}<0.050$). Lastly, P4 concentrations were significantly lower across the luteal phase in the women with obesity ($P_{OBESITY}=0.001$).

Table 1.2. Impact of day and obesity on average number of follicles per diameter category and hormone concentrations during the follicular and luteal phase.

	Non-Obese (n=21)	Obese (n=21)	Day Fixed Effect	Obesity Fixed Effect
Follicular Phase:				
AFC	54 ± 28	50 ± 20	P<0.0001	P=0.671
AFC 2-5 mm	48 ± 30	46 ± 20	P<0.0001	P=0.854
AFC 6-9 mm	6 ± 4	4 ± 3	P<0.0001	P=0.056
Proportion 2-5mm (%)	86.0 ± 9.6	89.5 ± 8.5	P<0.0001	P=0.162
Proportion 6-9mm (%)	12.5 ± 9.4	9.0 ± 8.2	P<0.0001	P=0.139
Mean LH (miu/ml)	9.57 ± 12.66	9.68 ± 11.69	P<0.0001	P=0.727
Mean FSH (miu/ml)	6.76 ± 3.64	6.97 ± 3.50	P=0.158	P=0.845
Mean E2 (pg/ml)	100.39 ± 9.75	103.73 ± 88.45	P<0.0001	P=0.887
Mean P4 (ng/ml)	0.85 ± 1.41	0.41 ± 0.32	P<0.0001	P=0.027
Luteal Phase:				
AFC	59 ± 21	47 ± 19	P=0.974	P=0.559
AFC 2-5 mm	45 ± 22	45 ± 19	P=0.274	P=0.764
AFC 6-9 mm	4 ± 4	2 ± 2	P<0.0001	P=0.006
Proportion 2-5mm (%)	90.9 ± 8.5	95.0 ± 5.0	P<0.0001	P=0.008
Proportion 6-9mm (%)	8.9 ± 8.5	5.0 ± 5.0	P<0.0001	P=0.010
Mean LH (miu/ml)	5.84 ± 6.44	6.27 ± 6.02	P<0.0001	P=0.770
Mean FSH (miu/ml)	4.11 ± 2.61	3.93 ± 2.56	P<0.0001	P=0.637
Mean E2 (pg/ml)	115.59 ± 57.51	116.33 ± 5.00	P<0.0001	P=0.727
Mean P4 (ng/ml)	7.85 ± 5.74	4.95 ± 3.34	P<0.0001	P=0.001

Data are presented as mean ± standard deviation. Groups were contrasted using generalized linear mixed models.

Abbreviations: AFC, antral follicle count; E2, estradiol; FSH, follicle stimulating hormone; LH, luteinizing hormone; P4, progesterone.

The prevalence rates of LPDs are presented according to three definitions (Practice Committee Reproductive 2021) (Figure 3). Based on biochemical measures, women with obesity had a greater incidence of LPDs compared to women without obesity. Namely, 16 women with obesity (76%) and six women without obesity (29%) displayed LPD by integrated luteal P4, while 15 women with obesity (71%) and five women without obesity (24%) had LPDs based on peak luteal P4 (Both: P<0.010). The incidence of LPDs defined by luteal phase length did not differ between groups (P=0.067).

Recruitment, selection, and ovulation

In women without obesity, two or three recruitment events were commonly observed during the IOI (Table 3). In contrast, only one or two recruitment events were observed during the IOI in women with obesity (Table 3). Ultimately, 91% of women with obesity and 95% of women without obesity exhibited at least one recruitment event across the IOI. There was a significant difference in the number of recruitment events between groups, with women with obesity displaying fewer events (P=0.007) (Table 3).

Table 1.3. Recruitment events in non-obese and obese women during natural cycles.

	Non-Obese (n=21)	Obese (n=21)
Recruitment		
Number of Recruitment Events	2 ± 1	1 ± 1***
Distribution of Events (N, %)		
0	1 / 21 (4.8)	2 / 21 (9.5)**
1	2 / 21 (9.5)	13 / 21 (61.9)**
2	9 / 21 (42.9)	5 / 21 (23.8)**
3	9 / 21 (42.9)	1 / 21 (4.8)**

Data are presented as mean ± standard deviation or proportion (%). Within rows, * denote significant differences between groups, adjusted values *P < 0.05, **P<0.01, *** P<0.001, ****P<0.0001.

In women with obesity, 6.5% of all 2-5 mm antral follicles grew to ≥7 mm, compared to 9.4% in women without obesity. As a result, women with obesity displayed fewer selectable follicles (6-9 mm) compared to women without obesity (P<0.001). Of those follicles that progressed from the selectable pool to dominance, there were no differences in the maximum diameter of anovulatory follicles at selection (P=0.323). However, ovulatory follicles were selected at significantly smaller diameters in women with obesity compared to those without obesity (P<0.010) (Table 4), although the day of selection did not differ between groups (P=0.810). Overall, women with obesity displayed fewer dominant follicles (P=0.041) manifesting as fewer anovulatory follicles (P=0.04), with six women with obesity (28.6%) and 12 women without obesity (57.1%) demonstrating anovulatory dominant follicles (P<0.010) (Table 4). However, a similar relative proportion of

selectable follicles achieved dominance (30 of 121 (25%) vs 40 of 197 follicles (20%) follicles in the group with versus without obesity, respectively, P=0.348). Of the anovulatory dominant follicles, maximal diameters did not differ between groups (P=0.763). By design, all women experienced ovulatory dominant follicles. There was no difference in maximal diameters achieved by the ovulatory follicles between groups (P=0.628) (Table 4).

Table 1.4. Follicle kinetics of dominant follicles in non-obese and obese women.

	Non-Obese (n=21)	Obese (n=21)
Characteristics of Anovulatory dfs:		
Total number over the IOI (N)	18	9*
Prevalence (% of participants)	12/21 (57.1)	6/21 (28.6)**
Prevalence in the Follicular phase (N participants, %)	10/21 (47.6)	4/21 (19.0)*
Prevalence in the Luteal phase (N participants, %)	5/21 (23.8)	2/21 (9.5)
Diameter at selection (mm)	7.4 ± 1.0	6.6 ± 1.1
Sonographic Presence (days)	16.31 ± 3.90	15.83 ± 2.32
Growth Phase (days)	7.38 ± 2.36	7.50 ± 2.88
Growth Rate (mm/day)	0.94 ± 0.24	0.96 ± 0.35
Static Phase (days)	1.31 ± 0.75	1.50 ± 1.22
Regression Phase (days)	7.62 ± 3.59	6.83 ± 2.64
Regression Rate (mm/day)	0.75 ± 0.26	0.82 ± 0.15
Maximum diameter (mm)	10.7 ± 1.0	10.5 ± 0.8
Characteristics of Ovulatory dfs:		
Total number over the IOI (N)	22	21
Prevalence (% of participants)	21/21 (100)	21/21 (100)
Emergence to Ovulation		
Day of emergence (day)	13.8 ± 4.1	14.5 ± 3.9
Growth phase (days)	15.4 ± 3.1	14.8 ± 2.7
Growth rate (mm/day)	1.03 ± 0.22	1.05 ± 0.23
Selection to Ovulation		
Diameter at selection (mm)	9.5 ± 1.9	7.5 ± 1.7**
Day of selection (day)	21.0 ± 3.9	20.0 ± 3.0
Growth phase (days)	8.9 ± 1.9	9.0 ± 2.4
Growth rate (mm/day)	1.22 ± 0.27	1.26 ± 0.24
Maximum diameter of ovulatory dominant follicles (mm)	19.8 ± 2.9	19.5 ± 2.8

Data are presented as mean ± standard deviation or proportion (%). Within rows, * denote significant differences between groups, adjusted values *P < 0.05, **P<0.01, *** P<0.001, ****P<0.0001.

Follicle kinetics

Complete growth and regression profiles were available for 121 uniquely identifiable follicles in the group with obesity and 197 follicles in the group without obesity. Of those uniquely identified follicles, 30 follicles progressed to dominance in women with obesity, compared to 40 follicles in women without obesity. The kinetics of anovulatory dominant follicles did not differ between groups. The length of the growth, static, and regression phases, as well as the growth and regression rates of the anovulatory dominant follicles, were all similar between groups (All: $P>0.050$). Full growth profiles of ovulatory follicles were available for all women (Table 4). Across groups, ovulatory follicles emerged on similar days of the menstrual cycle and displayed similar growth phases and growth rates from emergence to ovulation and selection to ovulation (All: $P>0.050$) (Table 4).

Discussion

To our knowledge, this study provides the most comprehensive evaluation of ovarian antral follicle and endocrine dynamics in women with obesity and regular menstrual cycles. To date, most longitudinal data characterizing reproductive function in this population have focused on endocrine assessments alone (Jain et al. 2007b; De Pergola et al. 2006; Roth et al. 2014; Yeung et al. 2013). Our findings are consistent with evidence of suppressed antral follicle development in women with obesity. Namely, fewer selectable-sized (6-9 mm) follicles were detected in women with obesity despite similar numbers of follicles in the recruitable size pool compared to their non-obese counterparts. Recruitment events occurred less often during IOIs in women with obesity and fewer dominant follicles emerged per participant. Further, ovulatory follicles were selected at smaller diameters in those with obesity. The timing and growth kinetics of ovulatory follicles did not differ between women with and without obesity albeit luteal phase P4 production was

substantially lower in those with obesity post-ovulation. Together, this new knowledge suggests that despite regular, ovulatory menstrual cycles, women with obesity display differences in antral follicle development alongside alterations in endocrine hormone production compared to their non-obese counterparts that may underlie the suboptimal reproductive health outcomes common in this population.

Previous research in primarily non-obese women with regular cycles has shown that follicular recruitment occurs in two or three waves throughout an IOI (Baerwald et al. 2003). The number of follicular waves is posited to reflect fertility potential in bovine models wherein animals with more follicular waves exhibit higher fertilization and pregnancy rates than those with fewer follicular waves (Ahmad et al. 1997; Townson et al. 2002). In our cohorts, women with obesity commonly exhibited one recruitment event. This finding contrasted those in women without obesity, who displayed primarily two or three recruitment events. Therefore, these data suggest that a decreased number of recruitment events may underlie the decreased fertility and fecundity that is commonly observed in women with obesity (Broughton and Moley 2017; Silvestris et al. 2018; Yilmaz et al. 2009). However, because fertility was not an endpoint in the present study, we are unable to test this hypothesis and it should be pursued in future studies.

We also acknowledge that our definition of recruitment events differs from that of follicle waves. By definition, follicle waves include concomitant rises and falls in FSH, reflecting the gonadotropin-dependence of antral follicles of the recruited cohort (Ginther et al. 2000, 2001). Our definition was strictly morphologic due to our relatively infrequent blood sampling methods. However, the definition of recruitment events used herein is similar to previous reports of follicle waves (Baerwald et al. 2003), which were later corroborated to align with fluctuations in FSH

(Baerwald et al. 2004). That said, FSH concentrations did not differ between cohorts across the IOI – albeit FSH tended to be lower in those with obesity during the luteal phase. Reduced FSH production has been reported in women with obesity, regular cycles, and presumptive fertility in the follicular phase (De Pergola et al. 2006) and across the menstrual cycle (Yeung et al. 2013). However, others have shown no obesity-related differences in FSH across the menstrual cycle using serial daily sampling of urinary FSH metabolites (Jain et al. 2007b). It is difficult to explain the discrepancies between studies. Degree of adiposity does not appear to be a factor as our study participants had a similar BMI to those enrolled in the studies reporting lower FSH levels (De Pergola et al. 2006; Yeung et al. 2013). Likewise, our groups were comparable in age and had similar exclusion criteria. Whether there are relevant metabolically related mechanisms, not captured by BMI or body fat percent, that underlie suppression of FSH should be pursued in future research.

Women with obesity in our study had a decreased pool of selectable-sized follicles (6-9 mm) across the IOI, despite similar numbers of recruitable-sized (2-5 mm) follicles. Growth from the recruitable cohort to the selectable stage is FSH-dependent (Macklon and Fauser 2001), and AMH is thought to exert a paracrine effect on follicles, thereby inhibiting their transition to the growing phase at the earliest stages (Chen et al. 2020; Nilsson et al. 2011; Themmen 2005; Weenen et al. 2004). While FSH concentrations were comparable in women with and without obesity, AMH levels were significantly depressed in women with obesity. AMH is known to regulate the transition of primordial follicles into the growing follicle pool, and at later stages becomes a brake in follicle development with the transition to dominance (Weenen et al. 2004). Therefore, lack of AMH may underly the decreased pool of 6-9 mm follicles. That said, our study cannot address causation and we cannot rule out the possibility that lower AMH in obesity simply reflects the smaller 6-9 mm pool, as antral follicles sized 5-8 mm have been shown to produce

the most AMH (Jeppesen et al. 2013). Future research should investigate the transition of follicles from the recruitable to selectable stage in obesity and AMH's impact.

Follicles were selected at smaller diameters in women with compared to without obesity (7.5 vs. 9.5 mm, respectively). In previous reports of antral follicle dynamics in healthy women of reproductive age, selection typically occurred in the range of 9.2 to 10.4 mm (Baerwald, Adams, and Pierson 2003; Baerwald et al. 2004; Bashir et al. 2018; Vanden Brink et al. 2013), with one report of selection occurring closer to 12.0 mm (Jarrett et al. 2020). Selection was defined by functional evidence of a future dominant follicle that grew ≥1 mm larger than the subsequent subordinate follicles. This preferential growth is associated with the acquisition of LH receptors and transition to LH-dependent growth (Zeleznik 2004). There are some physiological explanations for a smaller diameter at selection. First, a smaller size at selection may reflect earlier responsiveness to LH which has been shown in other anovulatory conditions associated with obesity, such as polycystic ovary syndrome (PCOS) (Hillier 1994; Willis et al. 1998). Increased insulin signaling in granulosa and theca cells has been posited as a potential mechanism promoting premature acquisition of LH receptors in antral follicles of women with PCOS (Poretsky et al. 1999; Wang et al. 2017). The women with obesity in our study had higher levels of fasting insulin compared to their non-obese counterparts as well as evidence of insulin resistance based on HOMA-IR. As such, it is possible that insulin concentrations in obesity may have been sufficient to alter the timing of granulosa cell LH receptor acquisition. Second, a smaller diameter at selection could relate to the decreased AMH levels detected in the women with obesity in our study. AMH inhibits the induction of LH receptor expression on granulosa cells by FSH (Di Clemente et al. 1994). Therefore, AMH levels in obesity may be insufficient to negatively modulate the FSH-dependent induction of LH receptor acquisition leading to their earlier expression. It is important to note that selection occurred at the same time point in women with and without

obesity, although the diameter of the follicle at selection differed. This finding could be attributed to the lack of differences in LH and FSH between groups given that the follicles transitioned from FSH-dependence to LH-dependence at the expected time. The lack of a detectable change in gonadotropin production suggests that metabolic factors may converge directly on the ovary to modulate follicular transitions in the context of obesity.

By design, all women in our study exhibited at least one dominant ovulatory follicle. However, anovulatory dominant follicles are capable of emerging at multiple time points during the IOI, which is consistent with our findings (Baerwald et al. 2004). To that point, we found that 85% of participants with obesity exhibited ovulatory dominant follicles that emerged within a recruitment event and 78% of anovulatory follicles emerged within a recruitment event. Similarly, 90% of non-obese participants displayed emergence of an ovulatory dominant follicle that was associated with a recruitment event and 78% of anovulatory follicles emerged within a recruitment event. Together, these observations are consistent with previous evidence of coordinated follicular growth throughout the IOI (Baerwald et al. 2003) which appears to be maintained in the context of obesity and regular menstrual cycles. That said, we detected evidence of overall suppression of 6-9 mm follicles and subsequently proportionally fewer anovulatory dominant follicles in the participants with obesity. It is important to note that once a follicle reached dominance, as defined by a diameter ≥10 mm, whether ovulatory or anovulatory, we noted no differences in the growth kinetics or maximum diameters achieved by the dominant follicles of the obese versus non-obese groups. There were also no differences in LH, FSH, or E2 levels between the groups once dominance was achieved (data not shown). Therefore, our data are consistent with suppression, and not disruption, in morphologic or endocrinologic dominant follicle development in eumenorrheic women with obesity.

Our data support the well-described findings of reductions in luteal P4 in eumenorrheic women with obesity (Carlson et al. 2012; Jain et al. 2007b; Yeung et al. 2013) and provide a physiological basis underlying the need for increased P4 supplementation in patients with obesity undergoing in-vitro fertilization to improve treatment success versus women without obesity (Whynott et al. 2021). Using American Society for Reproductive Medicine definitions for luteal phase defects (Practice Committee of the American Society for Reproductive Medicine 2021), we found evidence for LPDs in participants with obesity based on integrated and peak luteal P4 levels. Causes of LPDs in women with obesity are uncertain. Defects could be a result of abnormally functioning granulosa cells of the pre-ovulatory follicle and/or reduced number of luteinized granulosa cells in the corpus luteum (Terranova 2020). Evidence in obese, non-human primates consuming a western-style diet showed impaired granulosa cell function in the peri-ovulatory follicle (Bishop et al. 2019), as well as reduced luteal P4 production that was associated with decreased vascularization within the corpus luteum (Bishop et al. 2018, 2021). This study did not assess the vascularity of corpora lutea. Therefore, we cannot draw conclusions on how reduced luteal P4 production in our obese population may or may not associate with decreased corpora lutea vascularization. Rather, our data suggest the potential for premature follicle selection in women with obesity which could result in insufficient FSH stimulation of the ovulatory follicle (Terranova 2020), leading to abnormal luteinization after ovulation (Rice et al. 1998). Inadequate FSH stimulation of the ovulatory follicle is known to contribute to impaired corpus luteum function and LPDs in primates (Wilks et al. 1976) – albeit any compounding impact of obesity has not been directly addressed.

This study had several strengths, which included an investigation of a well characterized cohort of women recruited consecutively from the general population. In using PFT measured by dual-energy x-ray absorptiometry to define obesity, we used a more precise and direct marker of excess adiposity than BMI (De Lorenzo et al. 2016). BMI is the most commonly used marker of adiposity; however, it has proven to be an inaccurate proxy for body composition (Rothman 2008). Using PFT instead of BMI allowed for a more accurate depiction of the effect of obesity on follicle development. We excluded for androgen excess in this study to eliminate factors that could confound antral follicle dynamics in the context of obesity. Androgen excess is common in menstrual disorders such as PCOS and is known to alter follicle dynamics (Jarrett et al. 2020). Using total T levels, we were able to confirm the normo-androgenic status of all participants. Hirsutism was not used as a marker of hyperandrogenism since it has not been shown to consistently reflect current androgen levels (Vanden Brink et al. 2017; Ewing and Rouse 1978; Legro et al. 2010). This study was not without limitations. The study population was homogeneous, with 81% of participants identifying as Caucasian and 90% identifying as not Hispanic or Latino. This limits the generalization of our findings to other races and ethnicities, as both obesity rates (Petersen et al. 2019) and ovarian reserve (Mhatre V. Ho and Kelsey C. Martin 2012) are known to differ by race. We appreciate that additional research should be performed in larger, more diverse populations before large-scale conclusions be made about antral follicle and endocrine dynamics in obesity. Lastly, our data were collected at every-other-day intervals, whereas previous studies on follicle wave classification have been performed daily enabling the characterization of follicle waves (Baerwald et al. 2003; Baerwald et al. 2004). Our reduced sampling frequency limits our comparability to other studies, as we could not assess minor or major waves in our population.

In summary, our data are consistent with suppressed follicle dynamics in obesity and their alignment with reductions in AMH and luteal phase dysfunction. Given the rise of obesity globally, an understanding of antral follicle development in the context of obesity is critical for improving women's reproductive health. Further research should elaborate on mechanisms driving earlier selection and insufficient luteinization post-ovulation in obesity. This knowledge may be used to inform improvements in contraception and infertility treatments, both of which are known to be suboptimal in women with obesity (Dağ and Dilbaz 2015; Silvestris et al. 2018).

CHAPTER 2

A HYPOCALORIC DIETARY INTERVENTION IMPROVES ANTRAL FOLLICLE DYNAMICS IN WOMEN WITH OBESITY AND REGULAR OVULATORY CYCLES

Abstract

Study Question

Do antral follicle dynamics change in participants with obesity and regular ovulatory cycles after a six-month hypocaloric dietary intervention?

Summary Answer

After a six-month hypocaloric dietary intervention, eumenorrheic participants with obesity displayed evidence of improved antral follicle dynamics, defined as fewer total antral follicles, more dominant follicles, larger ovulatory follicle diameters at selection, and increased luteal progesterone production as compared to the participants pre-intervention follicle dynamics.

What is known already?

Precise events in antral folliculogenesis must occur in order for natural and regular monthly ovulations to occur. In eumenorrheic women with obesity, follicle development is suppressed as evidenced by alterations in key events of folliculogenesis. Follicle dynamics have not been evaluated in eumenorrheic participants undergoing dietary interventions despite known improvements in gonadotropin and ovarian steroid hormone concentrations after weight loss.

Study design, size, duration

Pre-and end-of intervention study of 12 participants who participated in a six-month hypocaloric dietary intervention.

Participants/Materials, setting, methods

Twelve participants living with obesity (percent body fat (PFT) ≥35%) underwent transvaginal ultrasonography and venipuncture every-other-day for one inter-ovulatory interval (IOI) before and during the final month of a six-month hypocaloric dietary intervention. Participants were aged 24-34 years and had a history of self-reported regular menstrual cycles (25-35 days). Follicle number and diameter (≥2 mm) were quantified at each study visit. Individual growth profiles for all follicles ≥7 mm were determined. Blood samples were assayed for reproductive hormones.

Main results and the role of chance

During the final month of a six-month hypocaloric dietary intervention, participants had lost an average of 10.6% body weight in kg and total antral follicle count was significantly reduced across the IOI compared to before the intervention (pre-intervention vs. post-intervention: mean ± standard deviation, 48 ± 3 vs. 44 ± 3 follicles, Mixed Model: $P_{intervention}$=0.002), which was driven by fewer 2-5 mm follicles across the IOI (44 ± 3 vs. 40 ± 4 follicles, $P_{intervention}$ =0.001). Post-intervention, participants also displayed an increased number of dominant follicles (≥10 mm) (0.3 ± 0.4 vs. 0.4 ± 0.5 follicles, p=0.001), experienced selection of ovulatory follicles at larger diameters (7.3 ± 2.0 vs. 10.9 ± 2.6 mm, p=0.007), and had increased progesterone concentrations across the luteal phase (5.29 ± 3.65 vs. 6.27 ± 4.74 ng/mL, $P_{intervention}$ <0.0001).

Limitations, reasons for caution

This study does not inform on the earliest stages of follicle development and is limited to provide knowledge on the later stages of antral follicle dynamics. This study cannot fully address causation between weight loss and sustained improvements in antral follicle dynamics. Lastly, data cannot be extrapolated to comment on potential improvements in fertility and fecundity with weight loss.

Wider implications of the findings

The increasing prevalence of obesity necessitates an understanding of the mechanisms that underlie potential improvements in reproductive health outcomes with weight loss. Eumenorrheic participants with obesity, that undergo a six-month hypocaloric dietary intervention, demonstrate improvements in antral follicle dynamics align with improvements in luteal progesterone dynamics. Potential improvements in the cellular makeup of follicles underlying a restoration of proper follicle development and amelioration of subfertility requires investigation.

Study funding/competing interest.

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Trial registration number

NCT01927432, NCT01785719

Introduction

Obesity is associated with several unfavorable reproductive health outcomes including anovulation, infertility, and endometrial dysfunction (Dağ and Dilbaz 2015; Fedorcsák et al. 2004; Goldsammler et al. 2018; Kyrou et al. 2000; Silvestris et al. 2018). Eumenorrheic females with obesity display aberrations in reproductive hormone dynamics, including decreased luteal progesterone (P4) production (Jain et al. 2007a; De Pergola et al. 2006; Yeung et al. 2013), luteinizing hormone (LH) pulse amplitude (Jain et al. 2007) as well as reduced anti-Mullerian hormone (AMH) concentrations (Chapter 1) (Chiofalo et al. 2017; Olszanecka-Glinianowicz et al. 2015; Peigné et al. 2020; Su et al. 2008) and follicle-stimulating hormone (FSH) production (De Pergola et al. 2006; Yeung et al. 2013), albeit controversial (Chapter 1) (Jain et al. 2007a; Oldfield, Kazemi, and Lujan 2021). We recently showed that participants with obesity who report regular menstrual cycles experience suppressed antral follicle dynamics, evidenced by fewer recruitment events, fewer selectable and dominant follicles, smaller diameter of the ovulatory follicle at selection, and a higher prevalence of luteal phase defects (Chapter 1). These alterations in follicle dynamics were accompanied by reduced AMH and P4 concentrations, particularly in the luteal phase. This new evidence supports a global downregulation of ovarian antral follicle development and an increased likelihood of luteal phase defects in eumenorrheic females with obesity that might underlie the subfertility (Dağ and Dilbaz 2015; Silvestris et al. 2018) and risk for endometrial hyperplasia (Epplein et al. 2008) reported in this population. Determining whether this suppression of antral follicle development in obesity is reversible with weight loss is a key next step in reproductive care.

Weight loss is currently advised as first-line therapy for women with obesity to improve overall health and wellbeing (NIDDK 2022b). Weight loss has been shown to aid in the resumption of

spontaneous ovulation in individuals with obesity and anovulatory infertility (Clark et al. 1998; Crosignani et al. 2003; Jarrett and Lujan 2017). Indeed, one study showed that a 9.8% reduction in weight led to 90% of participants experiencing spontaneous ovulations and 78% of participants experiencing pregnancy (Clark et al. 1998). Women with obesity are often advised to undergo weight loss prior to conception (Stang and Huffman 2016). Pre-conception weight loss can reduce the risk of gestational diabetes and peri-natal hypertensive disorders (Cha et al. 2021; Forsum et al. 2013) and there is some support for the benefits of pre-conception weight loss on fertility outcomes in those with subfertility– albeit controversial (Practice Committee of the American Society for Reproductive Medicine 2015). Accordingly, some studies have reported improvements in spontaneous and assisted conception, as well as live birth rates with weight loss (Best, Avenell, and Bhattacharya 2017; Kort et al. 2014), while others did not show a similar impact with weight loss (Einarsson et al. 2017; Kluge et al. 2019; Wang et al. 2021). Similarly, the effect of weight loss on reproductive outcomes in those with eumenorrhea is controversial. One study showed that even a small reduction in weight (i.e. 3.8% loss) induced by aerobic training improved FSH and estradiol (E2) concentrations (Al-Eisa, Gabr, and Alghadir 2017). By contrast, reductions in weight induced by low energy diets showed no improvements in FSH or E2 concentrations (Grenman et al. 1986; Panidis et al. 2008; Pasquali et al. 2000; Turcato et al. 1997). Type of intervention and/or degree of weight loss may have contributed to these discrepancies across studies, as weight loss via bariatric surgery in participants with regular menstrual cycles and obesity, who lost an average of 32% of their presurgical weight, showed increased LH and P4 concentrations, leading to a partial restoration of luteal function (Rochester et al. 2009). Improved luteal function with weight loss may increase the likelihood of conception for those desiring pregnancy and ensure adequate opposition of estrogen throughout the menstrual cycle, which is needed to offset risks for abnormal vaginal bleeding, hysterectomy, and endometrial hyperplasia (Chlebowski et al. 2007; Ettinger, Golditch, and Friedman 1988). Determining the impact of moderate weight loss on reproductive function via lifestyle intervention represents a feasible first

line option to better understand how weight loss may improve the reproductive axis in eumenorrheic females with obesity.

The objective of the current study was to contrast ovarian antral follicular growth and endocrine dynamics in eumenorrheic participants with obesity before and after a six-month hypocaloric dietary intervention. We hypothesized that weight loss induced by a hypocaloric dietary intervention would improve all stages of antral follicle development including an increased number of recruitment events, a larger size of the ovulatory follicle at selection, and improved luteal function following ovulation, and that these alterations in antral follicle dynamics would align with improvements in reproductive hormone concentrations across the menstrual cycle.

Methods

Study participants

This study represents a pre- and end-of-intervention analysis of 12 participants of reproductive age (18-38 years) living with obesity and regular menstrual cycles that completed a single arm, six-month hypocaloric dietary intervention as part of an ongoing registered clinical trial (NCT01785719). Participants were included in the analysis if they retrospectively met the following criteria: obesity habitus, regular menstrual cycle length, and normal androgen levels. Obesity was defined by a percent total body fat ≥35% using a dual x-ray absorptiometry (DEXA). Menstrual cycle regularity was defined as a self-reported menstrual cycle length between 21 and 35 days in the last year and confirmed post-hoc using ultrasound monitoring of ovarian antral follicle development during an inter-ovulatory interval (IOI; described below). All participants included were confirmed to be normoandrogenic as defined by a total testosterone (T) of <61.5

ng/dl based on a threshold derived using an internal reference cohort. Clinical measures of hyperandrogenism were not considered (hirsutism). In order to be eligible for inclusion, participants must have had consistent and optimal visualization of both ovaries on ultrasonography. Participants were excluded if they were using medications known or suspected to interfere with reproductive function in the two months prior to the study; pregnant or lactating in the six months prior to the study; had a history of premature ovarian failure; or had any pre-existing confounding medical conditions. All participants were also required to be at the action stage of readiness to lose weight (Kristal et al. 1999), as judged by a validated questionnaire (Duffy 2006) and willingness to adhere to a prescribed commercial diet for six months.

Ethical Considerations

This study was approved by the Institutional Review Board at Cornell University and registered at ClinicalTrials.gov (NCT01785719). Informed consent was obtained from all participants before study procedures were performed. Participants were recruited from the general Ithaca, New York population using advertisements. Data from the reference cohort were collected as part of NCT01927432 and have been reported elsewhere (Chapter 1).

Study Design

Participants were evaluated for a total of seven months. During Month 1, participants visited the Human Metabolic Research Unit at Cornell University every-other day for transvaginal ultrasonography and venipuncture. Every-other day visits spanned one IOI (i.e., Baseline IOI), which represented the time from one ovulation to the subsequent ovulation. During the mid-follicular phase (Day 7-11) of the Baseline IOI, participants also participated in a fasting metabolic

study visit following an overnight fast which consisted of an oral glucose tolerance test and physical examination. During the 5th week of the study, participants began a six-month commercial hypocaloric dietary intervention (described below). During month six of the hypocaloric dietary intervention, corresponding with Month 7 of the study period, participants resumed every-other-day visits to capture another IOI (i.e., Month 7 IOI). A fasting metabolic study visit was repeated during the mid-follicular phase of this final Month 7 IOI.

Reference Cohort

Twenty-one participants with regular menstrual cycles, normal androgen concentrations, and no evidence of obesity (i.e., total percent body fat <35%), who had completed a clinical trial (NCT01927432) with similar baseline assessments to those reported herein, served as a reference cohort (Chapter 1).

Hypocaloric Dietary Intervention

Participants were assigned to Nutrisystem® D (Nutrisystem, Inc., Fort Washington, PA), a portion-controlled, hypocaloric, and low glycemic index meal delivery system designed to help participants consume 1250-1500 calories per day (Nutrisystem 2022). A physical activity goal of 30 minutes per day was also encouraged. A nutritionist met with each participant weekly to customize meal plans, manage roadblocks to weight loss as they emerged, and counsel participants on behavioral strategies for weight loss, including self-monitoring and goal setting. Changes in anthropometry (weight, waist and hips circumference) were assessed during twice-weekly visits to the research unit. Additional body composition measures were evaluated in Month

1 (Baseline), after the achievement of weight loss benchmarks (5% and 10% initial body weight lost), and after the intervention (Month 7) using DEXA.

Ultrasonographic Measurements

Antral follicle dynamics were evaluated using serial transvaginal ultrasonography in Month 1 and Month 7 using methods previously described (Chapter 1) (A. Baerwald et al. 2003; Jarrett et al. 2020; D Rouleau et al. 2012). Scanning intervals began before ovulation (range: cycle day 8 to 15) and ultrasound scans were conducted approximately every-other day for one IOI, both before (Baseline IOI) and during the last month of the six-month hypocaloric dietary intervention (Month 7 IOI). As defined in Chapter 1, an IOI was defined as the interval from one ovulation to the next ovulation to capture the luteal and follicular phases. When a large antral follicle ≥16 mm was detected, ultrasound examinations were performed daily until the large antral follicle fate was confirmed (i.e., ovulation, regression). Ovulation was defined as the sonographic detection of a corpus luteum during the IOI and was confirmed post-hoc with a rise in serum progesterone concentrations of ≥1.5 ng/mL (Baerwald et al. 2005).

Scans were performed using a GE Voluson E8 Expert System or a GE Voluson E10 Expert System and 6-12 MHz 3D/4D transducer (GE Healthcare, Milwaukee, WI) as described in Chapter 1. Briefly, ovaries were imaged from their inner to outer margins in the longitudinal plane using the automated volume modality and evaluated offline by three investigators [Sante DICOM Editor, Santesoft LTD, Athens, Greece]. Follicles were counted and measured in each ovary at each visit using the grid system approach (Lujan et al. 2010) (Single Measures ICC=0.902). Follicle diameter was measured in the largest cross-sectional view.

As described in Chapter 1, growth and regression profiles of individual follicles ≥7 mm were tracked using the Identity Method (Baerwald et al. 2003; Vanden Brink et al. 2013; Jarrett et al. 2020; Rouleau et al. 2012). Briefly, follicles with a diameter ≥4 mm were sketched in their relative position within the ovary to generate a map of antral follicles within each ovary. Maps were completed for each ovary at each visit of the IOI. All follicles that grew to ≥7 mm were uniquely identified, and changes in diameter were tracked from day of first detection (i.e., at 4-5 mm) to last detection (i.e., at 4-5 mm or ovulation). Growth and regression rates of each uniquely identified follicle were then calculated. Sonographic presence was defined as the interval of time between the first and last days of sonographic detection of a follicle (Baerwald, Walker, and Pierson 2009; Jarrett et al. 2020). The growth phase was defined as the interval of time from first day of detection to the day of maximal follicle diameter (Baerwald et al. 2009), regression phase was defined as the interval of time from the day of maximal diameter to the day of last detection (Baerwald et al. 2009; Jarrett et al. 2020). Growth and regression rates were defined as previously described (Baerwald et al. 2009; Jarrett et al. 2020).

Utilization of every-other-day transvaginal ultrasonography allowed for the characterization of the main events in antral follicle dynamics. A recruitment event was defined when two or more follicles ≥4 mm emerged within a three-day (or two-visit) window and went on to grow to ≥7 mm and the follicular growth occurred alongside an increase and subsequent decrease in the number of follicles ≥5mm. In contrast to previous reports of antral follicle dynamics (Baerwald et al. 2003; Baerwald et al. 2004), we did not characterize follicle waves in this study due to our less frequent sampling protocol. Dominance was morphologically defined as the growth of a follicle to ≥10 (Baerwald et al. 2004). Last, selection was defined by the occurrence of a (future) dominant follicle

that grew ≥1 mm larger than subordinate follicles in the ovary and remained larger until its fate (Baerwald et al. 2003).

Because there were no differences in the number of uniquely identified follicles between the left and right ovaries (data not shown), follicle number and diameter data from both ovaries were combined as general convention (Chapter 1) (Baerwald, Adams, and Pierson 2004; Vanden Brink et al. 2013; Jarrett et al. 2020). The total number and proportion of follicles detected in different diameter categories were graphed for each participant over the IOI. Diameter categories of physiologic interest (i.e., antral follicle counts [AFCs]) included: ≥2 mm (total follicle count), 2-5 mm (recruitable follicles), 6-9 mm (selectable follicles), and ≥10 mm (dominant follicles).

Biochemical measurements

Non-fasted blood samples were collected every other day during the IOI. Blood was collected into a clot-activated tube and allowed to sit at room temperature for 30–60 minutes. Serum was isolated by centrifugation and stored at –80°C until analysis. Chemiluminescence immunoassays (Immulite 2000, Siemens Medical Solutions Diagnostics, Deerfield, IL) were used to measure serum concentrations of FSH, LH, E2, and P4. Inter- and intra-assay coefficients of variation (CV) were as follows: FSH (4.9%, 2.6%), LH (6.2%, 3.9%), E2 (9.7%, 8.6%), and P4 (11.8%, 7.2), respectively. Luteal phase defects (LPDs) were defined as per the American Society of Reproductive Medicine (ASRM) recommendations (Practice Committee of the American Society for Reproductive Medicine 2021) of a decreased luteal phase length (<10 days) and/or biochemical measures of integrated luteal P4 <80 ng/mL or peak P4<10 ng/mL

To assess androgen and glucoregulatory status, fasted blood samples were drawn on a single day of each IOI at a standardized time such that no dominant follicles or active corpora lutea were present. Serum sex hormone binding globulin (SHBG) was measured by chemiluminescence immunoassay (inter-assay CV: 5.0%; intra-assay CV: 3.1%) and total T was measured by liquid chromatography tandem mass spectrometry (inter-assay CV: 6.4%) as previously described (Vanden Brink et al. 2014; Snyder et al. 2016). The free androgen index (FAI) was calculated as: (total T [nmol/L] / SHBG [nmol/L]) × 100 (Vermeulen et al. 1999). Glucose was measured with a standard glucometer (Accu-Check Aviva, Roche Diabetes Care, Inc., Indianapolis, IN) and insulin was measured by chemiluminescence immunoassay (inter-assay CV: 6.2%; intra-assay CV: 4.8%;). Homeostatic model assessment for insulin resistance (HOMA-IR) was calculated as: (fasting glucose [mmol/L] × fasting insulin [mIU/mL]) ÷ 22.5 (Wallace et al. 2004). AMH was measured by enzyme-linked immunosorbent assay at commercial facility (Ansh Labs, Webster, TX) (inter-assay CV: 5.7%; intra-assay CV: 2.9%).

Statistical analysis

All analyses were performed using JMP Pro 14.0.1 (SAS Institute, Cary, NC). Data were log-transformed if not normally distributed before analyses. Continuous cross-sectional data, obtained before and after the intervention, were compared using matched-pairs t-tests. Fisher's exact tests were used to compare categorical variables across time points and between groups (i.e., LPDs). Longitudinal follicular and endocrine data were centralized to the day of ovulation and evaluated by: (1) normalizing the data across the IOI and (2) separately averaging the data across the luteal and follicular phases. Mixed-effect models evaluated the impact of the intervention on follicle number, follicle size populations, growth parameters, and endocrine hormones (main fixed effect: intervention status). Participant identifier was used as a random

effect and day as a fixed effect across all models. The statistical significance threshold was set at $P<0.05$.

Results

Participant Characteristics

Reproductive, anthropometric, and metabolic features of participants, before and after the six-month hypocaloric dietary intervention are contrasted in Table 1. On average, participants lost 10.6 ± 3.8% (mean ± standard deviation) of their initial body weight (Range: 4.2-15.4%) during the six-month intervention. Participants also experienced significant reductions in body mass index (BMI), waist circumference, and total and truncal adiposity after the hypocaloric dietary intervention (All; $P<0.05$). Forty-two percent of participants experienced a shift in BMI category from "obese" (BMI$\geq$30 kg/m^2) to "overweight" (BMI 25.0-29.9 kg/m^2), 33% shifted from "Class 2" obesity (BMI 35.0-39.9 kg/m^2) to "Class 1" obesity (BMI 30.0-34.9 kg/m^2), and 25% did not show a change in designation. During the Baseline period, participants displayed a 29 ± 4 day IOI with a 17 ± 3 day follicular phase and 12 ± 2 day luteal phase length. By contrast, during the Month 7 of the intervention, participants displayed a 27 ± 4 day IOI (14 ± 3 day follicular phase and 13 ± 4 day luteal phase) resulting in a 2-day shorter cycle in the Month 7 IOI. No changes were observed in the other reproductive characteristics during the intervention. However, participants experienced improvements in markers of glucose tolerance and insulin resistance, including reduced fasting insulin and HOMA-IR (Table 1).

Table 2.1. Characteristics of the study participants.

	Baseline IOI (N=12)	Month 7 IOI (N=12)	Reference Cohort (N=21)	Baseline vs. Month 7 p-value	Month 7 vs. Reference p-value
Age (years)	31 ± 3	31 ± 3	29 ± 6	0.006	0.299
Reproductive Markers					
Menstrual Cycle Length (days)	29 ± 4	27 ± 4	29 ± 3	0.029	0.164
Hirsutism Score	4 ± 4	4 ± 4	4 ± 5	1.000	0.148
Total Testosterone (ng/dL)	14.7 ± 7.7	20.3 ± 9.4	21.9 ± 12.7	0.071	0.684
Free Androgen Index	1.50 ± 1.10	1.58 ± 1.00	1.28 ± 0.74	0.599	0.224
LH: FSH	0.71 ± 0.24	0.78 ± 0.31	0.73 ± 0.32	0.350	0.701
Anti-Müllerian Hormone (pg/mL)	4.86 ± 3.22	5.52 ± 3.94	5.94 ± 2.49	0.299	0.745
Anthropometric Markers					
Weight (kg)	96.7.2 ± 18.4	87.2 ± 15.1	62.7 ± 11.2	0.001	<0.0001
BMI (kg/m^2)	35.3 ± 5.8	31.7 ± 4.7	22.9 ± 3.2	<0.0001	<0.0001
Percent Total Fat (%)	45.2 ± 4.2	43.2 ± 4.5	27.5 ± 3.7	0.0183	<0.0001
Truncal Fat Percentage (%)	45.1 ± 4.2	42.6 ± 4.1	23.8 ± 4.7	0.006	<0.0001
Waist Circumference (cm)	104 ± 15	95 ± 8	79 ± 8	0.003	<0.0001
Waist: Hips Ratio	0.84 ± 0.06	0.82 ± 0.04	0.80 ± 0.05	0.126	0.412
Metabolic Markers					
Systolic Blood Pressure (mmHg)	112 ± 9	118 ± 19	111 ± 10	0.383	0.215
Diastolic Blood Pressure (mmHg)	71 ± 10	78 ± 22	68 ± 7	0.381	0.161
Fasting Glucose (mg/dL)	89.8 ± 6.1	92.4 ± 5.5	93.6 ± 12.2	0.218	0.909
Fasting Insulin (mIU/L)	8.32 ± 3.89	6.00 ± 3.49	4.29 ± 2.22	0.028	0.152
HOMA-IR	1.81 ± 0.82	1.36 ± 0.79	1.00 ± 0.56	0.043	0.185

Data are presented as mean ± standard deviation.
Abbreviations: BMI, body mass index; HOMA-IR, homeostatic model assessment of insulin resistance; LH:FSH, luteinizing hormone: follicle stimulating hormone.

AFC across an IOI

Mean profiles of AFC ≥2 mm (Figure 1A), AFC 2-5 mm (Figure 1B), AFC 6-9 mm (Figure 1C) and AFC ≥10 mm (Figure 1D) are shown for participants before and after the intervention in Figure 1. Total AFC ≥2 mm was significantly decreased across Month 7 IOI compared to Baseline IOI (Baseline vs. Month 7 IOI: 48 ± 3 vs. 44 ± 3 follicles, $P_{Intervention}$=0.002), manifesting as fewer 2-5 mm follicles (44 ± 3 vs. 40 ± 4 follicles, $P_{Intervention}$=0.001). On average, an increased number of dominant follicles was also detected (≥10 mm; 0.3 ± 0.4 vs. 0.4 ± 0.5 follicles, p=0.001) across the IOI. There were no differences in the 6-9 mm follicle population across the IOI after the intervention ($P_{Intervention}$=0.816).

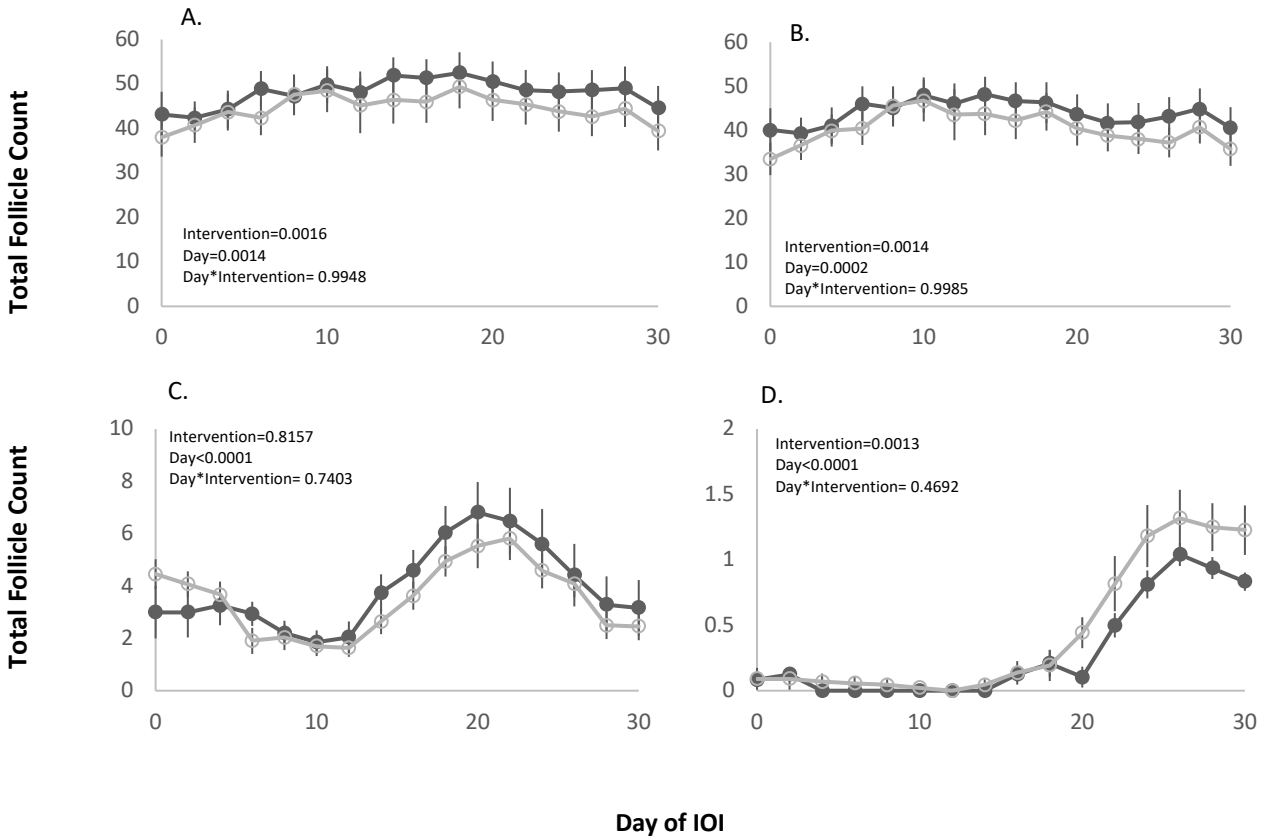

Figure 2.1. ***Longitudinal profiles of total (A), 2-5mm (B), 6-9mm (C) and ≥10mm (D) antral follicle counts across an inter-ovulatory interval (IOI) in pre-weight loss (●) and post-weight loss women (○).*** Day to day changes in total follicle counts per follicle size category were monitored using the Non-Identity Method. Mixed models showed a Day effect for total, 2-5mm, 6-9mm and ≥ 10mm follicles, and an Obesity effect for total, 2-5mm, and ≥ 10mm follicles.

Reproductive Hormones during an IOI

Mean profiles of reproductive hormones before and after the intervention are shown in Figure 2. There were no changes in LH or E2 during Month 7 IOI compared to Baseline IOI (Figure 2A and 2C, respectively; both, $P_{Intervention}>0.05$). Changes in FSH concentrations across the IOI, differed in Month 7 IOI compared to Baseline IOI, as shown in Figure 2B ($P_{Day*Intervention}=0.009$). P4 levels were increased across in Month 7 IOI compared to Baseline IOI (Figure 2D; $P_{Intervention}=0.013$).

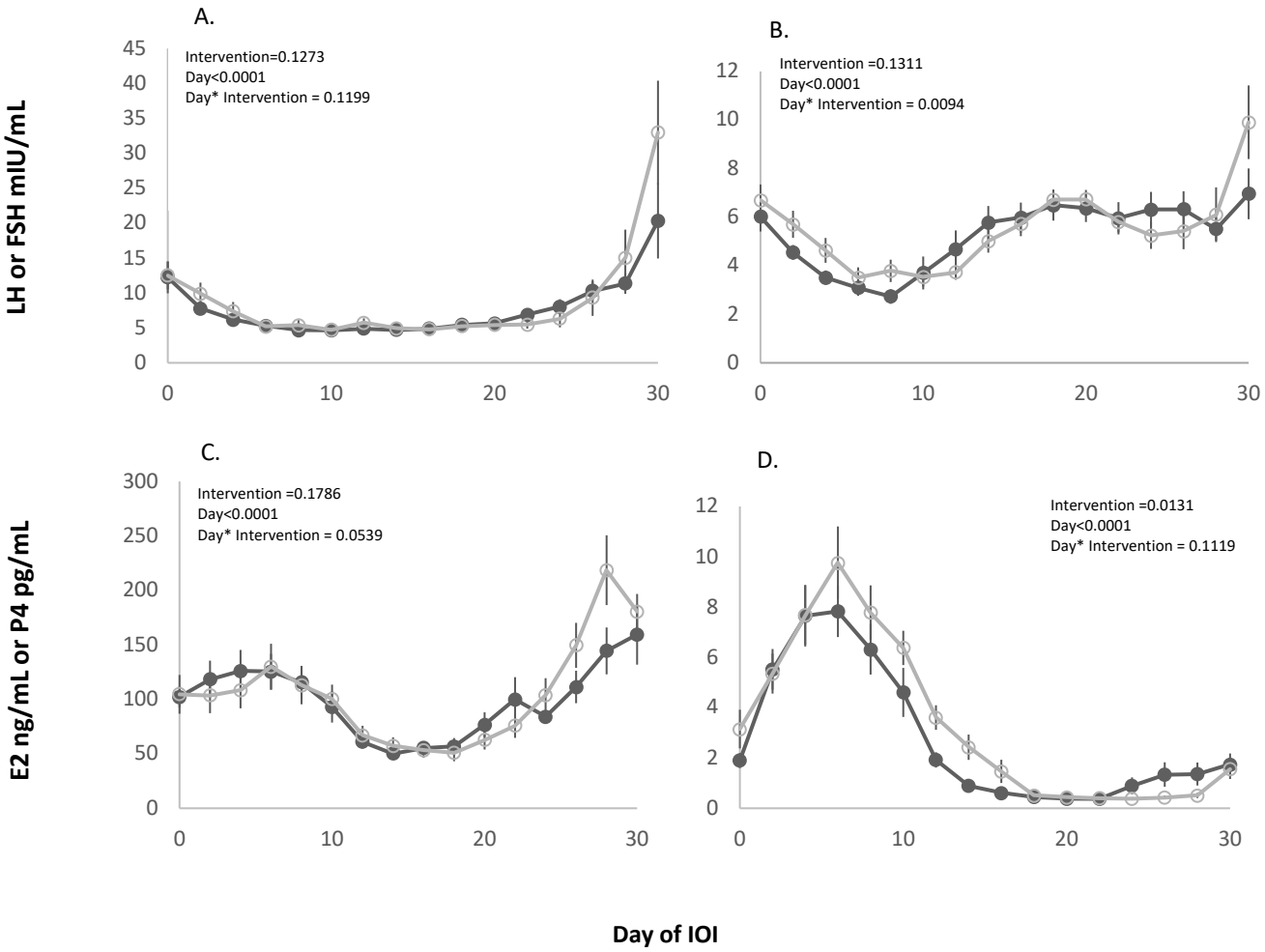

Figure 2.2. ***Longitudinal profiles of LH (A), FSH (B), estradiol (C) and progesterone (D) across an inter-ovulatory interval (IOI) in pre-weight loss (●) and post-weight loss women (○).*** Day to day changes in hormone concentrations were monitored by serial venipuncture. Mixed models showed a Day effect for LH, FSH, estradiol (E2) and progesterone (P4). An Obesity effect was noted for P4 and a Day by Obesity effect noted FSH.

Follicle diameter and endocrine hormones by menstrual cycle phase

Mean follicle populations and hormone concentrations are presented for the follicular and luteal phases of the menstrual cycle in Table 2. No changes were observed in follicular phase numbers of selectable follicles, or concentrations of LH, FSH, E2, and P4 before versus after the intervention (All; $P \geq 0.05$). By contrast, in Month 7 IOI, AFC ≥2 mm, 2-5 mm follicle counts and the proportion of 6-9 mm follicles were decreased relative to total follicle count, while the proportion of 2-5 mm follicles and the growth of dominant follicles were increased compared to Baseline IOI (All; $P<0.05$). P4 levels were significantly increased across the luteal phase in the participants after the intervention ($P<0.0001$), but there were no differences in luteal AFC ≥2 mm, recruitable follicles, selectable follicles, the proportion of follicles, or LH, FSH, and E2 levels (All; $P>0.05$) (Table 2).

Table 2.2. Impact of day and intervention status on follicle number and diameter, as well as endocrine hormones, during the follicular and luteal phase.

	Baseline IOI (n=12)	Month 7 IOI (n=12)	Day Effect	Intervention Effect
Follicular Phase:				
AFC	49 ± 22	45 ± 16	P=0.014	P=0.003
AFC 2-5 mm	44 ± 22	40 ± 13	P=0.019	P=0.011
AFC 6-9 mm	4 ± 3	4 ± 4	P=0.020	P=0.166
Proportion 2-5mm (%)	87.8 ± 9.2	89.7 ± 6.2	P=0.010	P=0.011
Proportion 6-9mm (%)	10.7 ± 8.7	8.2 ± 6.2	P=0.071	P=0.005
Proportion ≥10mm (%)	1.5 ± 2.1	2.3 ± 2.3	P<0.0001	P=0.014
Mean LH	9.54 ± 10.69	11.38 ± 16.47	P<0.0001	P=0.492
Mean FSH	6.17 ± 2.56	6.51 ± 3.36	P=0.029	P=0.358
Mean E2	109.38 ± 97.60	114.63 ± 92.25	P<0.0001	P=0.096
Mean P4	0.47 ± 0.38	0.75 ± 1.36	P<0.0001	P=0.299
Luteal Phase:				
AFC	45 ± 16	44 ± 17	P=0.569	P=0.285
AFC 2-5 mm	43 ± 16	41 ± 16	P=0.097	P=0.186
AFC 6-9 mm	2 ± 2	3 ± 3	P=0.132	P=0.581
Proportion 2-5mm (%)	94.0 ± 5.3	93.8 ± 5.8	P<0.001	P=0.776
Proportion 6-9mm (%)	5.9 ± 5.3	6.0 ± 5.5	P<0.001	P=0.943
Proportion ≥10mm (%)	0.1 ± 0.3	0.2 ± 0.9	P=0.698	P=0.131
Mean LH	6.65 ± 4.62	6.17 ± 5.07	P<0.0001	P=0.920
Mean FSH	3.83 ± 1.85	4.16 ± 2.23	P<0.0001	P=0.380
Mean E2	114.03 ± 56.24	103.34 ± 64.34	P<0.0001	P=0.918
Mean P4	5.29 ± 3.65	6.27 ± 4.74	P<0.0001	P<0.0001

Data are presented as mean ± standard deviation. Mixed model results for day effect and intervention effect are shown.
Abbreviations: AFC, antral follicle count; FSH, follicle stimulating hormone; E2, estradiol; LH, luteinizing hormone; P4, progesterone.

The prevalence of LPDs are presented according to the three definitions set forth by the ASRM (Practice Committee of the American Society for Reproductive Medicine 2021). Figure 3 shows changes in LPD classification before and after the intervention for individual participants. Broadly, there were no differences in the prevalence rates of LPDs before versus after the intervention (All; P>0.05). Fifty-eight percent of participants met the criteria for LPDs, based on integrated luteal P4, in Baseline IOI (N=7) and 50% met the criteria in Month 7 IOI (N=6), with only two participants showing improvements in LPD. Based on peak luteal P4, 50% of participants in Baseline IOI (N=6) and 67% of participants in Month 7 IOI (N=8) had LPDs, with only one

participant showing an improvement. Using luteal phase length, 25% of Baseline IOI participants (N=3) and 33% of Month 7 IOI participants (N=4) displayed LPDs, with only three participants showing improvements in LPDs after the intervention by this metric.

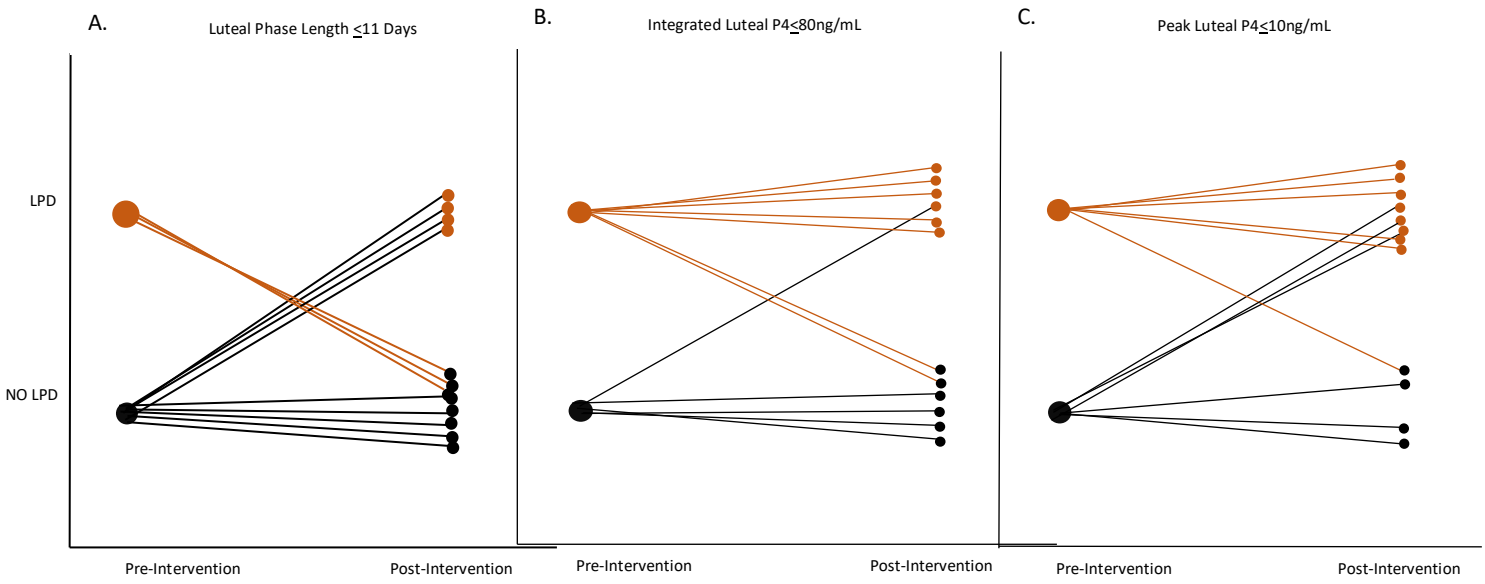

Figure 2.3. ***Changes in Luteal Phase Defects in women pre-post intervention. Changes in luteal phase length (A), integrated progesterone (B), and peak progesterone (C) defects shown.*** Fisher's Exact tests show no differences in the prevalence of luteal phase defects before and after the intervention.

Recruitment, Selection, and Ovulation

Table 3 summarizes the number and distribution of recruitment events experienced by the participants before and after the intervention. During Baseline IOI, 6 of 12 participants demonstrated one recruitment event and 5 of 12 participants exhibited two recruitment events. In Month 7 IOI, 6 of 12 participants experienced two recruitment events and 2 of 12 participants displayed three recruitment events. Ultimately, this shift in the number of recruitment events in Month 7 IOI did not reach statistical significance (P=0.068) (Table 3).

Table 2.3. Recruitment events in the Baseline IOI, Month 7 IOI, and a reference cohort during natural cycles.

	Baseline IOI (n=12)	Month 7 IOI (n=12)	Reference Cohort (n=21)	Baseline vs. Month 7 P-value	Month 7 vs. Reference P-value
Recruitment					
Number of recruitment events	1 ± 0.7	2 ± 0.9	2 ± 0.9	0.068	0.448
Distribution of events (N, %)					
0	1 / 12 (8.3%)	1 / 12 (8.3%)	1 / 21 (4.8%)		
1	6 / 12 (50.0%)	3 / 12 (25.0%)	2 / 21 (9.5%)		
2	5 / 12 (41.7%)	6 / 12 (50.0%)	9 / 21 (42.9%)		
3	0 / 12 (0.0%)	2 / 12 (16.7%)	9 / 21 (42.9%)		

Data are presented as mean ± standard deviation or proportion (%).

The characteristics of dominant follicles before and after the intervention are presented in Table 4. In both Baseline and Month 7 IOIs, 42% of participants experienced anovulatory follicles and incidence rates did not differ (P=1.000). Of those follicles that progressed from the selectable pool to dominance, there were no differences in the maximum diameter achieved or the diameter of anovulatory follicles at selection before or after the intervention (P>0.05). However, ovulatory follicles in Month 7 IOI were selected at a significantly larger diameter than those in Baseline IOI (P=0.007) (Table 4), although the day of selection did not differ after the intervention (P=0.281). By design, all of the study participants experienced ovulatory dominant follicles, and there was no difference in the maximum diameter of the ovulatory follicles in Baseline versus Month 7 IOI (P=0.134) (Table 4).

Table 2.4. Follicle kinetics of dominant follicles the Baseline IOI, Month 7 IOI, and a reference cohort during natural cycles.

	Baseline IOI (n=12)	Month 7 IOI (n=12)	Reference Cohort (n=21)	Baseline vs. Month 7 P-value	Month 7 vs. Reference P-value
Characteristics of Anovulatory DFs:					
Total number over the IOI (N)	7	8	18	0.723	0.656
Prevalence (% of participants)	5/12 (47.1%)	5/12 (41.7%)	12/21 (57.1%)	1.000	0.656
Prevalence in the Follicular phase (N, %)	4/12 (33.3%)	5/12 (41.7%)	10/21 (47.6%)	0.104	0.506
Prevalence in the Luteal phase (N, %)	2/12 (16.7%)	0/12 (0.0%)	5/21 (23.8%)	0.166	0.021
Maximum diameter (mm)	10.5 ± 0.8	11.6 ± 1.7	10.7 ± 1.0	0.175	0.186
Characteristics of Ovulatory DFs:					
Total number over the IOI (N)	12	12	22	1.000	1.000
Prevalence (N, %)	12/12 (100%)	12/12 (100%)	21/21 (100%)	1.000	1.000
Emergence to Ovulation					
Growth phase (days)	14.8 ± 2.6	14.8 ± 4.1	15.4 ± 3.1	0.855	0.552
Growth rate (mm/day)	1.02 ± 0.23	1.18 ± 0.32	1.03 ± 0.22	0.213	0.363
Selection to Ovulation					
Diameter at selection (mm)	7.3 ± 2.0	10.9 ± 2.6	9.5 ± 1.9	0.007	0.125
Day of selection (day)	21.1 ± 2.9	20.6 ± 3.1	21.0 ± 3.9	0.281	0.859
Growth phase (days)	8.8 ± 3.2	7.5 ± 2.2	8.9 ± 1.9	0.101	0.081
Growth rate (mm/day)	1.17 ± 0.36	1.28 ± 0.34	1.22 ± 0.27	0.296	0.500
Maximum diameter of ovulatory dominant follicles (mm)	19.1 ± 2.2	20.3 ± 3.3	19.8 ± 2.9	0.134	0.675

Data are presented as mean ± standard deviation or proportion (%).

Follicle Kinetics

Complete growth and regression profiles were available for 73 uniquely identifiable follicles in Baseline IOI and 59 follicles in Month 7 IOI. Of the uniquely identified follicles, 19 follicles and 20 follicles progressed to dominance in Baseline IOI and Month 7 IOI periods, respectively. The kinetics of the anovulatory dominant follicles did not differ before and after the intervention. Namely, the length of the growth, static, and regression phases, as well as the growth and regression rates were all similar before and after the intervention (Data not shown: All; $P>0.05$). Full growth profiles of ovulatory follicles were available for 100% of participants before and after the intervention and are summarized in Table 4. The ovulatory follicles during Baseline and Month 7 IOIs had similar growth phases and growth rates from emergence to ovulation and from selection to ovulation (All: $P>0.05$) (Table 4).

Comparison with a non-obese reference cohort

Month 7 IOI data were compared to an age-matched reference cohort of participants with regular menstrual cycles but no evidence of obesity (n=21) (Chapter 1). As shown in Table 1, characteristics during Month 7 IOI did not differ from the reference cohort for any reproductive marker. By contrast, the groups differed in weight, BMI, percent total fat, trunk fat percentage, and waist circumference, as expected (All; $P<0.001$). After the intervention, the metabolic markers at Month 7 IOI were comparable to the reference cohort, including blood pressure, fasting glucose, fasting insulin, and HOMA-IR levels (All; $P>0.05$). Additionally, key events of follicle development are contrasted between groups in Tables 3 and 4. There were no differences in any element of antral follicle development (i.e., number of recruitment events, diameter at selection, number of dominant follicles) when comparing Month 7 IOI to the reference cohort (All; $P>0.05$).

Discussion

Provided in this study is an evaluation of ovarian antral follicle dynamics in participants with obesity and regular menstrual cycles in the final month of a 6-month hypocaloric dietary intervention. Our findings are consistent with evidence of improved antral follicle development in participants following a short-term hypocaloric dietary intervention aimed at weight loss. Most notably, after the intervention participants had lower total antral follicle counts driven by a decrease in the recruitable size (2-5 mm) follicle pool, as well as an increased number of dominant follicles (≥10 mm) across the IOI. Recruitment events tended to occur more often during the IOI after the intervention and selection of the ovulatory follicles occurred at larger diameters following the intervention, more in line with the reference cohort. The timing and growth kinetics of ovulatory follicles did not differ before and after the intervention. While luteal P4 levels were significantly increased in Month 7 IOI, this increase in P4 production did not result in any overall improvement in the prevalence of LPDs. Collectively, these findings suggest that following 6-month hypocaloric dietary intervention participants with obesity and regular ovulatory cycles demonstrate follicle development similar to those documented in non-obese participants with regular cycles.

To our knowledge, these data represent the first comprehensive assessment of endocrine and ovarian morphological changes following a hypocaloric dietary intervention in eumenorrheic women with obesity. These data are unique as most data characterizing menstrual cycle function with weight loss in women with regular cycles have been limited to endocrine and metabolic assessments (Al-Eisa et al. 2017; Grenman et al. 1986; Harrison et al. 2012; Kogure et al. 2016; MacKintosh et al. 2019; Moran et al. 2007; Nikokavoura et al. 2015; Panidis et al. 2008; Pasquali et al. 2000; Rochester et al. 2009; Turcato et al. 1997). In the present study, we utilized intensive serial transvaginal ultrasonography to characterize follicle dynamics which allowed for a more

comprehensive evaluation of ovarian function after a hypocaloric dietary intervention confirming improvements in folliculogenesis that were presumed due to previous endocrine and metabolic assessments.

After the intervention, participants exhibited a decreased antral follicle pool which was driven by fewer recruitable sized (2-5 mm) follicles across the IOI, while maintaining similar numbers of selectable (6-9 mm) follicles. The average number of 2-5mm follicles was 5 follicles less with a more substantial reduction in the range of follicles noted post intervention (15-74 vs. 27-69 follicles). While it has been postulated that obesity suppresses gonadotropin production which could lead to alterations in follicle dynamics (Rosenfield and Bordini 2010), our data are more consistent with obesity affecting follicle transitions via other factors, possibly metabolic because androgen and gonadotropin concentrations remained unchanged, whereas fasting insulin and HOMA-IR levels improved after the intervention. Our data align with obesity contributing to the manifestation of an emerging polycystic ovary syndrome (PCOS) phenotype of metabolic origins. In PCOS, follicular excess represents one of the cardinal features, specifically those 2-5 mm in diameter (Christ et al. 2015; Jarrett et al. 2020; Jonard et al. 2003). PCOS is intimately associated with hyperinsulinemia and insulin resistance (DeUgarte, Bartolucci, and Azziz 2005), which are posited to contribute to excess androgen production and follicle arrest (Dewailly et al. 2007). Accordingly, some PCOS studies have shown a positive correlation of the number of 2-5mm antral follicles with fasting insulin concentrations and other markers of insulin resistance (Dewailly et al. 2007; Franks et al. 1998)– albeit controversial (Christ et al. 2015). Likewise a study of non-PCOS participants with unexplained fertility showed that a higher degree of insulin resistance was associated with elevated total antral follicle count (Dickerson et al. 2010). Ultimately, alterations in glucoregulation could underlie the changes in follicle populations noted in our study. The mechanisms by which insulin contributes to follicular excess are not well described. Insulin can

act as a co-gonadotropin with LH on theca cells to produce more local androgens which are known to be necessary for follicle activation (Franks and Hardy 2018; Gervásio et al. 2014). Increased follicle activation could conceivably lead to a larger recruitable follicle pool. It is possible that the improvements in fasting insulin and HOMA-IR after the dietary intervention in our study dampened the paracrine actions of ovarian androgens leading to fewer total and 2-5 mm antral follicle count across the IOI.

We noted a trend towards increased recruitment events after a hypocaloric dietary intervention. Given that the number of recruitment events in Month 7 IOI was comparable to that of the reference cohort, this change in the number of events may be viewed as beneficial. Although not statistically significant, an increased number of recruitment events may suggest improved fertility potential after weight loss as a higher number of waves (or recruitment events) has been shown to reflect fertility potential in bovine models (Ahmad et al. 1997; Townson et al. 2002). Follicle waves are known to be FSH-dependent (Angela R. Baerwald et al. 2004). We showed that changes in FSH across the IOI differed by intervention status, most notably with participants in Month 7 IOI exhibiting increased pre-ovulatory FSH production. After the intervention participants also demonstrated a clear increase in FSH levels on the day of emergence of the ovulatory follicle cohort. This was in contrast to Baseline IOI in which the increase in FSH occurred earlier (Baseline IOI vs. Month 7 IOI: 11 ± 1 vs. 13 ± 1 day, respectively; P=0.045) and did not coincide with the day of emergence of the ovulatory follicle (Baseline IOI vs. Month 7 IOI: 15 ± 1 vs. 13 ± 1 day, respectively; P=0.039). This lag between the rise in FSH and emergence of the ovulatory follicle during Baseline IOI could possibly reflect a reduced receptivity of mid-antral follicles to FSH. Given that FSH upregulates FSH receptor expression within the ovary, and that some report decreased FSH concentrations with obesity (De Pergola et al. 2006; Yeung et al. 2013), a decreased number of FSH receptors could be the reason for this delay; although we did not show

a decrease in FSH concentrations with obesity, per se. We did not measure levels of activins and inhibins in our study, however, their regulation by metabolic factors could have downstream consequences for FSH receptor activity following a hypocaloric dietary intervention. Activins and inhibins aid in the regulation of FSH receptors (Lu et al. 2009; Nakamura 1993) and their concentrations are known to be impacted by obesity status (Eldar-Geva et al. 2001; De Pergola et al. 2006; Zaragosi et al. 2010) and insulin concentrations in vitro (Gonzales, Risbridger, and de Kretser 1989; Kuo et al. 2018). Ultimately, the effects of weight loss on FSH production in eumenorrheic women is controversial. Many studies showed no effect on FSH levels post weight loss induced by dietary (Grenman et al. 1986; Turcato et al. 1997), pharmacologic (Pasquali et al. 2000), or surgical (Rochester et al. 2009) interventions. In contrast, one study showed increased FSH following bariatric surgery (MacKintosh et al. 2019) and one study showed increased FSH following 12 weeks of aerobic training (Al-Eisa et al. 2017). Our data do not support a global increase of FSH concentrations after weight loss, rather a better alignment between FSH and follicle development. These alterations in FSH dynamics may not have been captured by earlier studies that did not employ more frequent sampling.

Follicle selection occurred at larger diameters in Month 7 IOI compared to Baseline IOI, and the increased diameter at selection was comparable to that of the reference cohort. We previously showed that the follicles of participants with obesity were selected at a smaller diameter compared to non-obese participants (Chapter 1), which we posited might reflect earlier acquisition of LH receptors and an untimely transition to LH-dependent growth versus the those without obesity (Zeleznik 2004). In anovulatory conditions, such as PCOS, premature acquisition of LH receptors in antral follicles is posited to occur owing to increased insulin signaling in both granulosa and theca cells (Poretsky et al. 1999; Wang et al. 2017). Follicles from ovulatory women typically respond to LH at 9.5-10 mm, however, those from anovulatory women with PCOS were shown to

respond to LH as early as 4 mm (Willis et al. 1998). Improving the metabolic state of participants with weight loss may have been sufficient to alter the size at selection (7.3 mm in Baseline IOI versus 10.9 mm in Month 7 IOI) suggesting insulin may be the main factor regulating premature follicle selection in those with obesity. After the intervention, participants had 28% lower concentrations of fasting insulin and demonstrated an improvement in HOMA-IR levels, with both levels comparable to that of the reference cohort (Chapter 1). Together, our data provides evidence that metabolic factors may play a substantial role in follicle selection and LH receptor acquisition even in the context of eumenorrhea.

By design, all of the participants in our study exhibited at least one instance of dominance during each IOI as per the manifestation of a dominant ovulatory follicle, but multiple dominant follicles can emerge during an IOI (Angela R. Baerwald et al. 2004). Across Month 7 IOI, participants exhibited an increased number of dominant follicles, specifically in the follicular phase. This increased propensity for morphologic dominance may have occurred due to the tendency for the increased number of recruitment events seen in Month 7, or improvements in the size of the ovulatory follicle at selection which could have downstream benefits for future follicle transitions. However, it is important to note that once a follicle reached dominance, as defined by a diameter **≥**10 mm, whether ovulatory or anovulatory, we noted no differences in the growth kinetics or maximum diameters achieved by the dominant follicles during Baseline and Month 7 IOIs. There were also no differences in reproductive hormone concentrations before and after the intervention once dominance was achieved (data not shown). Collectively, the emergence of more dominant follicles from the selectable pool suggests that the suppression of morphologically dominant follicle development previously reported in eumenorrheic participants with obesity may be reversible with weight loss.

Weight loss has been shown to improve luteal function as judged by increased P4 concentrations, specifically in the context of bariatric surgery (Rochester et al. 2009). In this earlier study, P4 levels increased by 55% in those that had lost 32% of their initial presurgical body weight (Rochester et al. 2009). Our study is consistent with the conclusion that more modest reductions in body weight (10.6%) also result in significant increases in P4. Indeed, P4 levels in our study increased approximately 16%, suggesting a dose-response effect of weight loss on luteal function. This notion of a dose-response effect on luteal function is supported by other studies as a report showing a 5.6%, and another showing a 15.3% reduction in weight did not result in increased P4 levels (MacKintosh et al. 2019; Pasquali et al. 2000). Further, we previously reported an increased prevalence of LPDs in participants with obesity and regular cycles compared to their non-obese counterparts (Chapter 1) (Practice Committee of the American Society for Reproductive Medicine 2021). In the current study, there were no differences in the rates of LPDs before and after the intervention and rates of LPDs in Month 7 IOI remained higher compared to the reference cohort. Therefore, despite the increase in luteal P4 after the intervention, the degree of weight loss induced by the 6-month hypocaloric intervention used in this study was not sufficient to reduce the risk of LPDs.

This prospective study had several strengths which included a well characterized study population recruited from the general population. Twelve participants who had been previously determined to be at a stage of weight loss readiness successfully completed a 6-month hypocaloric dietary invention that induced on average a 10.6% reduction in total body weight with the vast majority transitioning to a more favorable BMI class. In general, 5% is considered a clinically meaningful reduction in weight and has been shown to be associated with improvements in hyperinsulinemia

and insulin sensitivity (Galan 2019), as well as improved pregnancy outcomes (Sigal Klipstein; Ginny Ryan 2019; Stang and Huffman 2016). This minimum amount of weight loss was achieved by all but one participant who only showed a 4.2% peak reduction in weight while on the intervention. Consistent with a beneficial impact on health, the participants in our study had lower levels of fasting insulin after the intervention compared to baseline, and Month 7 IOI levels were comparable to the reference cohort. Additionally, during Month 7 IOI, participants showed an improvement in HOMA-IR levels transitioning from “pre-diabetic” to “optimal” levels (Gayoso-Diz et al. 2013), which were comparable to the reference cohort. Collectively, our intervention was sufficiently successful to induce metabolic changes that could conceivably intersect with positive changes in reproductive health outcomes. To eliminate factors that could confound follicle dynamics in the context of obesity, we excluded for hyperandrogenism in this study. Hyperandrogenism is common in reproductive disorders such as PCOS and alters follicle dynamics (Jarrett et al. 2020). Our use of total testosterone levels to confirm the normo-androgenic status of all of the participants, rather than hirsutism, was important as hirsutism has not been shown to be a proxy for current androgen levels (Vanden Brink et al. 2017; Ewing and Rouse 1978; Legro et al. 2010). This study also had limitations. With 100% of participants identifying as Caucasian and 92% identifying as not Hispanic or Latino, our findings cannot be generalized to other races and ethnicities, as both obesity rates (Petersen et al. 2019) and ovarian reserve (Ho and Martin 2012) are known to differ by race and ethnicity. Further research should be performed in larger, more diverse populations before more robust conclusions can be made about follicle dynamics improving with weight loss. Last, our intervention should be considered short-term and we are unable to comment on whether any improvements in follicle and endocrine dynamics are sustained following weight loss in the long-term.

In summary, our data are consistent with improved follicle dynamics after a hypocaloric dietary intervention and their alignment with improvements in FSH, insulin and P4 concentrations – albeit improvements were insufficient to offset rates of LPD. Given our previous reports of suppressed follicle dynamics and luteal phase dysfunction in participants with obesity and regular cycles, understanding changes in follicle development with a hypocaloric dietary intervention is critical for informing the degree to which lifestyle intervention and weight loss may effectively restore and/or promote reproductive health. Further research is needed to elucidate on the specific metabolic factors necessary to drive favorable reproductive outcomes in eumenorrheic participants with obesity following a hypocaloric dietary intervention.

CHAPTER 3

OBESITY ALTERS ENDOMETRIAL THICKNESS DURING NATURAL OVULATORY CYCLES: IMPACT OF A HYPOCALORIC DIETARY INTERVENTION

Abstract

Study Question

Does obesity impact changes in endometrial thickness (ET) during the menstrual cycle in women with regular ovulatory cycles?

Summary Answer

Eumenorrheic women with obesity displayed a thicker endometrial lining compared to eumenorrheic women without obesity on any given day across an interovulatory interval (IOI). Changes in ET were less pronounced with obesity as judged by an increased minimum ET and decreased percent change in ET overall. Progesterone (P4) concentrations were lower during the IOI in those with obesity and a decreased change in P4 concentrations was noted at the secretory-proliferative phase transition. Following weight loss induced by a hypocaloric dietary intervention, a thicker endometrium persisted compared to baseline, with a higher minimum ET and lower percent change in ET noted post-intervention, despite increased progesterone concentrations across the IOI.

What is known already?

Endometrial development across the menstrual cycle is a dynamic process driven by fluctuations in ovarian steroid hormone concentrations that are critical for the occurrence of regular monthly menstruation and optimal uterine health. The impact of obesity on endometrial development in

the context of regular ovulatory cycles has not been prospectively evaluated, despite the increased risk of endometrial hyperplasia in those with obesity.

Study design, size, duration

Prospective, longitudinal study of 38 women over one IOI, including a subgroup analysis of 11 women before and after a hypocaloric dietary intervention.

Participants/Materials, setting, methods

Twenty women with obesity (total percent body fat ≥35%) and 18 women without obesity (total percent body fat <35%) underwent transvaginal ultrasonography and venipuncture every-other-day for one IOI at an academic clinical research unit. Participants were aged 19-38 years and had a history of self-reported regular menstrual cycles (21-35 days). Eleven of the 20 women with obesity partook in a 6-month hypocaloric dietary intervention and ultrasound scans and venipuncture were repeated every-other-day during the final month of the intervention (Month 7 IOI). For all participants, ET was quantified at each visit. Blood samples were assayed for estradiol (E2) and P4 and plotted across the IOI.

Main results and the role of chance

Women with obesity had a thicker endometrium on any given day across the IOI (mean ± standard deviation, Obese vs. Non-Obese: 8.4 ± 1.4 vs. 7.2 ± 1.4 mm; P=0.013) and demonstrated a decreased percent change in ET (69.0 ± 10.4 vs. 77.4 ± 8.6%; P=0.011) across the IOI compared to women without obesity. Differences between groups were most pronounced in the proliferative

phase, with women with obesity also demonstrating an increased minimum ET (4.1 ± 1.3 vs. 2.9 ± 1.4 mm; P=0.008) post-menses. P4 concentrations across the IOI were lower in women with obesity (2.52 ± 3.23 vs. 3.99 ± 5.41 ng/mL; P=0.0003) and a smaller change in P4 was noted during the secretory to proliferative phase transition (90.2 ± 7.4 vs. 95.1 ± 1.6%; P=0.013) compared to their non-obese counterparts. E2 profiles did not differ between groups (108.34 ± 74.56 vs. 110.11 ± 76.47 pg/mL; P=0.7182). Following a hypocaloric dietary intervention, participants continued to have a thicker endometrium across the IOI (Month 7 vs. Baseline: 9.4 ± 1.3 vs. 8.2 ± 1.2 mm; P=0.0005) and experienced a decreased percent change in ET (59.8 ± 1.3 vs. 71 ± 1.2%; P=0.014) compared to Baseline IOI. In Month 7, the minimum ET in the proliferative phase was increased compared to Baseline IOI (5.5 ± 1.9 vs. 3.8 ± 1.1 mm; P=0.006) despite an overall increase in P4 concentrations following the intervention (3.24 ± 3.85 vs. 2.78 ± 3.43 ng/mL; P=0.013). E2 concentrations during Month 7 were similar to concentrations during Baseline IOI (109.23 ± 71.34 vs. 100.61 ± 61.64 pg/mL; P=0.2263).

Limitations, reasons for caution

This study was limited to an assessment of ET and cannot inform on vascularization, cellular makeup, or other factors related to the risk of endometrial hyperplasia. These data cannot be generalized to inform on reduced fertility potential in obesity and/or any possible improvements with weight loss as fertility outcomes were not assessed in this study. The weight loss intervention employed was short-term and findings cannot be extended to inform on sustained changes in endometrial development with weight loss.

Wider implications of the findings

Women with obesity experience incomplete shedding of the endometrial lining even in the context of regular ovulatory cycles and may benefit from routine monitoring of ET to gauge the risk of endometrial hyperplasia. P4 supplementation may be needed in this population to curb the risks of endometrial hyperplasia and uterine cancer. Further, improvements in P4 concentrations following weight loss induced by short-term hypocaloric dieting should not be considered sufficient to enable proper endometrial shedding.

Study funding/competing interest.

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Trial registration number

NCT01927432, NCT01785719

Introduction

Endometrial development during the menstrual cycle is a dynamic process closely associated with antral follicle development and ovarian steroid hormone production (Baerwald and Pierson 2004). The proliferative phase of the menstrual cycle coincides with the follicular phase of the ovarian cycle wherein growing antral follicles, particularly the dominant ovulatory follicle, produce estradiol (E2) that prompts the growth and proliferation of the endometrial lining (Monis and Tetrokalashvili 2019). In the days leading up to ovulation and during the luteal phase of the ovarian cycle (or the secretory phase of the menstrual cycle) progesterone (P4) concentrations rise to maintain the estrogen-primed endometrium (Young 2013). In particular, P4 produced by the corpus luteum post-ovulation promotes an environment that is favorable for implantation by increasing vascularization and blood flow within the endometrium and stimulating endometrial glands to secrete nutrients that could nourish an early embryo (Filant and Spencer 2014). However, if implantation does not occur, the corpus luteum regresses causing a sharp withdrawal of P4 concentrations which in turn leads to degradation of the endometrial lining and the onset of menstruation (Reed and Carr 2000). Regular and complete shedding of endometrial lining is critical for reducing the risk of endometrial hyperplasia and endometrial carcinoma (Singh and Puckett 2022).

Endometrial hyperplasia is a pathological condition characterized by changes in the endometrial glands and stromal structures that line the uterine cavity, leading to a persistently thickened endometrium (Sobczuk and Sobczuk 2017). Hyperplasia most commonly results from chronic unopposed estrogen exposure which results in perturbed structural endometrial remodeling characterized by an abnormal ratio of glands to stroma (Singh and Puckett 2022; Sobczuk and Sobczuk 2017). If left untreated, endometrial hyperplasia can progress to cancer (Siegel, Miller,

and Jemal 2018). Known risk factors of endometrial hyperplasia include anovulation, age, hormone replacement therapy, ovarian tumors, familial history (Farquhar et al. 1999; Singh and Puckett 2022; Wise, Jordan, et al. 2016), as well as metabolic derangements such as type II diabetes and obesity (Epplein et al. 2008; Farquhar et al. 1999; Singh and Puckett 2022; Wise, Jordan, et al. 2016). Indeed, women with obesity were found to be four times more likely to develop endometrial hyperplasia or uterine cancer compared to those without obesity (Wise, Gill, et al. 2016). However, reports of risk for endometrial hyperplasia in obesity are confounded by the concurrent presence of anovulation (Epplein et al. 2008; Wise, Jordan, et al. 2016) which makes the risk of obesity alone unclear.

A direct correlation between endometrial thickness (ET) and obesity has been documented in anovulatory women (Hsu et al. 2011; Liao et al. 2013; Souter et al. 2011). By contrast, endometrial dynamics have not been prospectively evaluated in eumenorrheic women with obesity. It has been well-described that women with regular cycles and obesity have decreased P4 production (Chapter 1) (Jain et al. 2007a; De Pergola et al. 2006; Yeung et al. 2013) suggesting a possible impact on endometrial development in this population. Likewise, those with regular cycles and insulin resistance have been shown to have a thicker endometrium compared to age and BMI-matched individuals with normal glucoregulatory status (Iatrakis et al. 2006). A demonstration that endometrial growth is impacted despite the regular occurrence of menses could have implications for the regular screening of endometrial hyperplasia even in those reporting regular menstrual cyclicity.

Weight loss has been shown to improve reproductive health outcomes in women with data largely geared toward pregnancy outcomes (Best et al. 2017; Cha et al. 2021; Forsum et al. 2013; Kort

et al. 2014) and the resumption of ovulation in anovulatory women (Clark et al. 1998; Crosignani et al. 2003; Jarrett and Lujan 2017). Studies conducted specifically in eumenorrheic women with obesity undergoing weight loss show alterations in gonadotropins and ovarian steroid hormone concentrations (Chapter 2) (Al-Eisa et al. 2017; Grenman et al. 1986; Panidis et al. 2008; Pasquali et al. 2000; Rochester et al. 2009; Turcato et al. 1997). However, to our knowledge no data exists on the impact of weight loss on endometrial growth in eumenorrheic women with obesity. We recently showed that weight loss improves follicle dynamics and P4 production across the menstrual cycle (Chapter 2). Increases in P4 concentrations following a hypocaloric dietary intervention could have a positive impact on endometrial dynamics and may offset any risk for endometrial hyperplasia even in those with regular ovulatory cycles.

The objective of this study was to contrast ET and ovarian steroid dynamics between eumenorrheic women with and without obesity during an inter-ovulatory interval (IOI). We hypothesized that women with obesity would display a thicker endometrium across the IOI alongside decreased P4 production compared to their non-obese counterparts. Further, any impact of a 6-month hypocaloric dietary intervention on endometrial development was determined. We hypothesized that after the dietary intervention those experiencing weight loss would show a thinner endometrium across the IOI accompanied by increased P4 concentrations.

Methods

Study participants

This study represents an analysis of a subset of participants who completed one of two study protocols (n=112) (Identifiers: NCT01927432, NCT01785719) aimed at evaluating the impact of

adiposity and/or weight loss on follicle dynamics. Participants were retrospectively evaluated for inclusion in the current analysis based on the following criteria: 1) Non-Obese Group: women with regular menstrual cycles without obesity and an intrauterine device (IUD); 2) Obese Group: women with obesity and regular menstrual cycles without an IUD; and 3) Hypocaloric Intervention Group: those with obesity, regular cycles and without an IUD that completed a 6-month dietary intervention. Obesity was defined by a total percent body fat (PFT) ≥35% using whole-body dual x-ray absorptiometry (DEXA). Menstrual cycle regularity was defined by a self-reported history of cycles between 21 and 35 days in the last year with cycle regularity confirmed post-hoc using ultrasound monitoring of ovarian follicular development during an inter-ovulatory interval (IOI; described below). All women included were confirmed to be normo-androgenic as defined by total testosterone (T) <61.5 ng/dl based on a threshold derived in an internal reference cohort. Further, a participant was deemed eligible if they had consistent and optimal visualization of the uterus on ultrasonography. Women were excluded if they were using medications known or suspected to interfere with reproductive function in the two months prior to the study; pregnant or lactating in the six months prior to the study; had a history of premature ovarian failure; or had any pre-existing confounding medical conditions.

Ultrasonographic measurements

Serial transvaginal ultrasonography was used to evaluate endometrial development every-other day for one IOI in the Non-Obese and Obese groups. Additionally, the Hypocaloric Dietary Intervention group had endometrial development assessed every-other day for one IOI at baseline (Baseline IOI) and during the final month of a 6-month hypocaloric dietary intervention (Month 7 IOI; details of the dietary intervention are provided in Chapter 2). An IOI was defined as the time from one ovulation to the next in order to represent the secretory phase of one cycle followed by

the proliferative phase of the next cycle, ending at the subsequent ovulation. Scans were performed using a GE Voluson E8 Expert System or a GE Voluson E10 Expert System and 6–12 MHz 3D/4D transducer (GE Healthcare, Milwaukee, WI). Images were saved for off-line evaluation by two observers with excellent agreement (ICC= 0.871) across 30 images of the endometrium. The thickness of the endometrium was measured for each ultrasound examination. Thickness was measured as the distance from the anterior stratum basalis-myometrial junction to the posterior stratum basalis-myometrial junction with a 5-10 mm window from the fundus, in the mid-sagittal plane (Baerwald and Pierson 2004).

Biochemical measurements

Non-fasted blood samples were collected every other day during the scanning interval. Blood was collected into a clot-activated tube and allowed to sit at room temperature for 30–60 minutes. Serum was isolated by centrifugation and stored at –80°C until analysis. Estradiol (E2), progesterone (P4), serum sex hormone binding globulin (SHBG), total testosterone (T), glucose and fasting insulin were measured as described in Chapter 1. Free androgen index (FAI), luteinizing hormone to follicle stimulating hormone ratio (LH:FSH), and the homeostatic model for insulin resistance (HOMA-IR) were calculated as defined in Chapter 1.

Definitions

The secretory phase was defined as the day immediately following ovulation to the day preceding menses. The proliferative phase was defined as the day that menses began to the day preceding ovulation, this includes the menstrual phase. The secretory-proliferative phase transition was defined as the window between the maximum thickness at the end of the secretory phase to the

minimum thickness at the beginning of the proliferative phase, with menses. Ovulation was defined as the observation of a corpus luteum, and later confirmed with a rise in serum progesterone concentrations (Baerwald et al. 2005). Menses was also documented as a bleeding episode of ≥2 days in a 3-day bleeding interval preceded by at least 2 bleed-free days (Dasharathy et al. 2012; Harlow, Lin, and Ho 2000). The bleeding episode must have also lasted <10 days to ensure abnormal uterine bleeding was not included (Tam 2018). Percent change of the endometrium was calculated as: ((ET max – ET min)/(ET max))*100.

Statistical analysis

All analyses were performed using JMP Pro 14.0.1 (SAS Institute, Cary, NC). Cross-sectional data across groups were compared using paired and unpaired t-tests where appropriate. Endometrial and endocrine data were centralized to the day of ovulation and evaluated by: (1) normalization across the mean scanning interval and (2) averaged across secretory and proliferative phases. Mixed-effect models evaluated between-group differences in endometrial thickness and reproductive hormones (Main effects: obesity and intervention). Participant number was used as random effect in all models. Day was used as a fixed effect in all models. The statistical significance threshold was set at $P<0.05$.

Results

Comparison of endometrial development in Non-obese vs. Obese groups

Eighteen women without obesity met the criteria for inclusion in the Non-Obese group and 20 women with obesity met the criteria for inclusion in the Obese group. Characteristics of the groups are summarized in Table 1. By design, women with obesity had a higher PFT ($P<0.0001$), but

similar menstrual cycle lengths (P=0.582), compared to their counterparts without obesity. Groups did not differ in terms of age, total T, FAI, or early follicular LH:FSH (All: P≥0.050). As expected, women with obesity also had increased measures of adiposity and impaired insulin sensitivity compared to their counterparts without obesity.

Table 3.1. Baseline characteristics of the study participants.

	Non-Obese (N=18)	Obese (N=20)
Reproductive Markers		
Cycle Length (days)	30 ± 2	30 ± 2
Total Testosterone (ng/dL)	22.8 ± 10.9	21.0 ± 11.4
Free Androgen Index	1.38 ± 0.69	1.97 ± 1.31
LH:FSH	0.68 ± 0.29	0.78 ± 0.46
Anthropometric Markers		
Percent Total Fat (%)	27.0 ± 3.4	43.8 ± 5.0****
BMI (kg/m^2)	23.1 ± 3.4	34.3 ± 5.1****
Trunk Fat Percentage (%)	23.4 ± 4.6	43.2 ± 6.1****
Waist Circumference (cm)	80 ± 9	103 ± 18****
Metabolic Markers		
Systolic Blood Pressure (mmHg)	112 ± 10	115 ± 9
Diastolic Blood Pressure (mmHg)	68 ± 8	72 ± 9
Fasting Glucose (mg/dL)	94.6 ± 11.8	92.3 ± 6.6
Fasting Insulin (mIU/L)	4.44 ± 2.35	9.79 ± 5.71****
HOMA-IR	1.00 ± 0.56	2.27 ± 1.31**

Data are presented as mean ± standard deviation. Within rows, * denote significant differences between groups, adjusted values *P<0.05, **P<0.01, *** P<0.001, **** P<0.0001. Reproductive, anthropometric, and metabolic endpoints were evaluated on a standardized day of the scanning interval during the early follicular phase the menstrual cycle.

Abbreviations: BMI, body mass index; HOMA-IR, homeostatic model assessment of insulin resistance; LH:FSH, luteinizing hormone: follicle stimulating hormone.

Representative images depicting changes in endometrial development during the secretory, menstrual, and proliferative phases of an IOI for one participant without obesity and one participant with obesity are provided in Figure 1 (Panel A and B, respectively). Longitudinal changes in ET are plotted across a normalized IOI and contrasted in non-obese and obese groups in Figure 2. ET changed day-to-day across the entire IOI (P_{DAY}<0.0001). Women with obesity had a significantly thicker endometrium across the IOI ($P_{OBESITY}$<0.0001). Mixed models also

demonstrate a significant negative effect of obesity on P4 ($P_{OBESITY}$=0.002), but not E2 concentrations across the IOI ($P_{OBESITY}$=0.540).

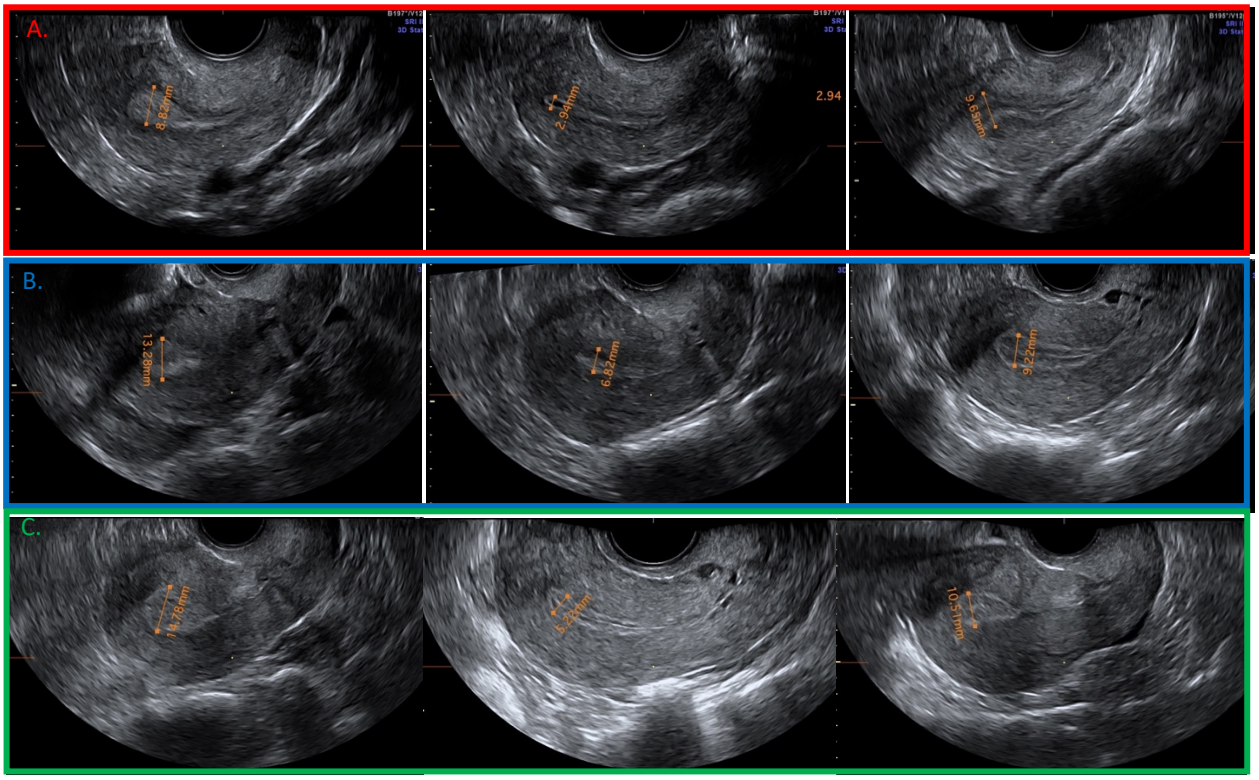

Figure 3.1. ***Ultrasonographic images of endometrial thickness in the secretory, menstrual, and proliferative phases for a non-obese participant (A) and for a single participant during the Baseline (B) and Month 7 IOI (C).***

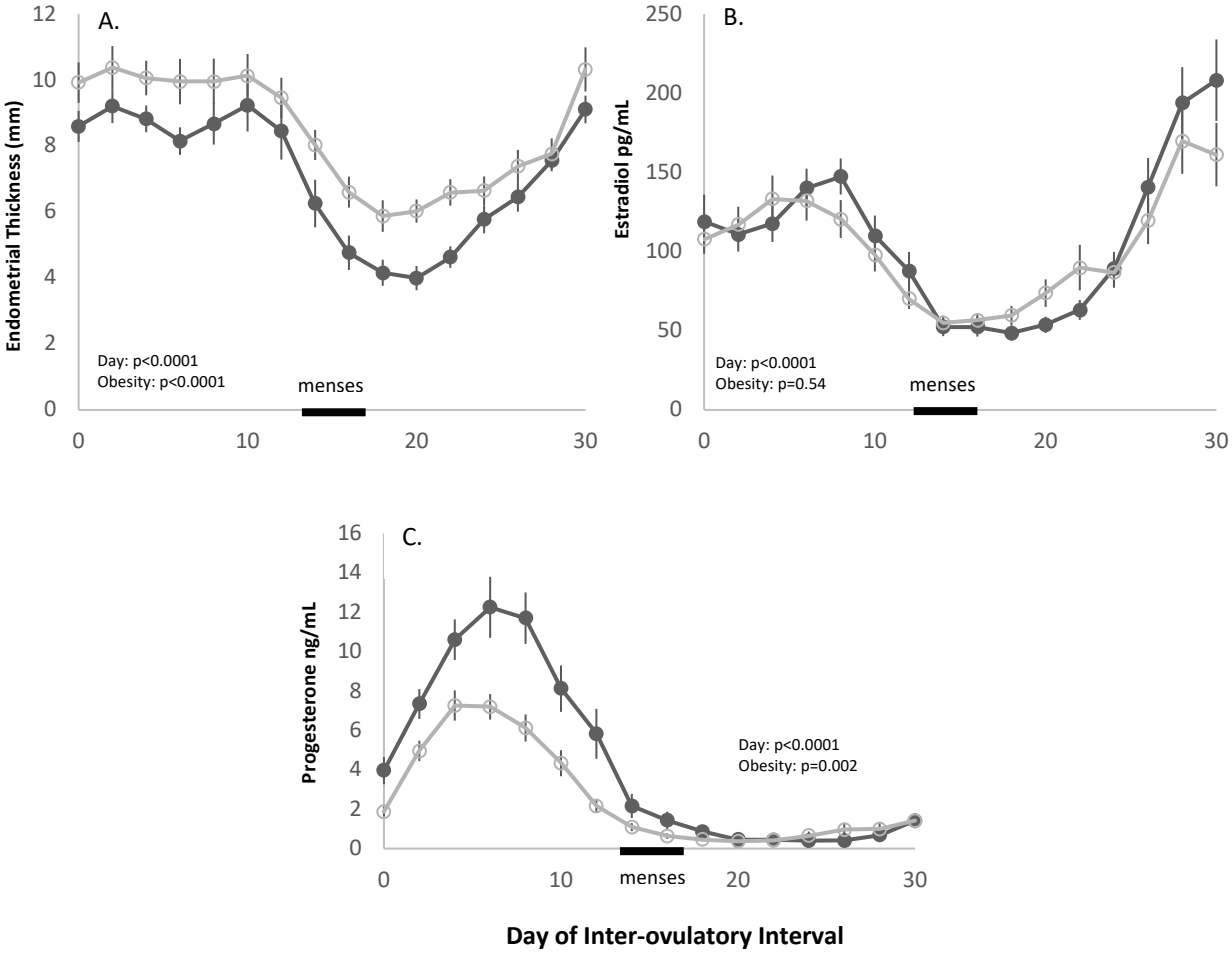

Figure 3.2. ***Longitudinal profiles of endometrial thickness (A) and estradiol (B) and progesterone (C) concentrations over an inter-ovulatory interval (IOI) in non-obese (●) and obese groups (○).*** Mixed models showed a day effect for thickness, estradiol, and progesterone, and an obesity effect for endometrial thickness and progesterone concentrations.

Differences in non-normalized endometrial data between non-obese and obese participants during the proliferative and secretory phases are presented in Table 2. The endometrial lining was thicker across the entire IOI in the obese group (Obese vs. Non-Obese: 8.4 ± 1.4 vs. 7.2 ± 1.4 mm; P=0.013) and the overall percent change in thickness across the IOI from its thinness point to its thickest point was lower in the obese group (69.0 ± 10.4 vs. 77.4 ± 8.6%; P=0.011). Differences in endometrial and endocrine dynamics were also noted by phase. During the secretory-menstrual transition, the decline in P4 concentrations was less pronounced in the obese

group (90.2 ± 7.4 vs. 95.1 ± 1.6%; P=0.013) defined as peak to minimum. In the proliferative phase, both the minimal and mean ET was thicker in women with obesity (P=0.008, P=0.002, respectively). Accordingly, the overall percent change between minimum and maximal ET across the proliferative phase was lower in the obese group versus the control group (P=0.050). No differences in endometrial or hormonal dynamics were detected in the secretory phase between the obese and non-obese groups were noted (Table 2).

Table 3.2. Endometrial characteristics across the proliferative and secretory phases.

	Non-Obese (N=18)	Obese (N=20)
Proliferative Phase		
Proliferative phase length (day)	15.8 ± 4.4	16.3 ± 3.4
ET Mean (mm)	5.8 ± 1.4	7.2 ± 1.3**
% ET change	70.8 ± 13.6	62.0 ± 13.1*
ET Min (mm)	2.9 ± 1.4	4.1 ± 1.3**
Day of ET Min	4.9 ± 2.7	7.0 ± 3.5
ET Max (mm)	9.8 ± 1.9	11.0 ± 2.5
Day of ET Max	12.7 ± 5.3	11.3 ± 7.3
Secretory Phase		
Secretory phase length (day)	12.4 ± 2.4	12.0 ± 1.7
ET Mean (mm)	8.9 ± 1.9	10.0 ± 2.1
% ET change	43.6 ± 9.3	43.5 ± 12.4
ET Min (mm)	6.5 ± 1.6	7.1 ± 1.8
Day of ET Min	8.8 ± 4.1	6.3 ± 4.7
ET Max (mm)	11.6 ± 2.7	12.8 ± 2.9
Day of ET Max	7.3 ± 3.8	5.3 ± 3.2

Data are presented as mean ± standard deviation. Within rows, * denote significant differences between groups, *P<0.05, **P<0.01, *** P<0.0001.

Abbreviations: ET, endometrial thickness.

Comparison of endometrial development in pre- and post-weight loss intervention

Eleven women met the criteria for inclusion in the Hypocaloric Dietary Intervention group and represented a subset of the data included in the Obese group (11 of the 20 participants). Characteristics of the 11 participants during Baseline IOI and Month 7 IOI are summarized in Table 3. Following the intervention during Month 7 IOI, participants had significantly lower

measures of adiposity including reduced PFT, BMI, trunk fat percentage, and waist circumference. In addition, during Month 7 IOI women showed improved insulin sensitivity compared to measures during the Baseline IOI as judged by fasting insulin and HOMA-IR. By contrast, measures of total T, FAI, and early follicular LH:FSH (All: P≥0.050) were unchanged during Month 7 IOI compared to Baseline IOI.

Table 3.3. Baseline characteristics of the study participants.

	Baseline IOI (n=11)	Month 7 IOI (n=11)
Reproductive Markers		
Cycle Length (days)	29 ± 4	27 ± 4*
Total Testosterone (ng/dL)	14.7 ± 7.7	20.3 ± 9.4
Free Androgen Index	1.50 ± 1.10	1.62 ± 1.00
LH:FSH	0.71 ± 0.24	0.78 ± 0.31
Anthropometric Markers		
Percent Total Fat (%)	45.2 ± 4.2	43.5 ± 4.1*
BMI (kg/m^2)	35.2 ± 5.8	31.7 ± 4.7***
Trunk Fat Percentage (%)	45.0 ± 4.2	42.6 ± 4.7**
Waist Circumference (cm)	104 ± 15	95 ± 8**
Metabolic Markers		
Systolic Blood Pressure (mmHg)	112 ± 9	118 ± 19
Diastolic Blood Pressure (mmHg)	71 ± 10	78 ± 22
Fasting Glucose (mg/dL)	89.8 ± 6.1	92.4 ± 5.5
Fasting Insulin (mIU/L)	8.32 ± 3.89	5.97 ± 3.49*
HOMA-IR	1.81 ± 0.82	1.36 ± 0.79*

Data are presented as mean ± standard deviation. Within rows, * denote significant differences between groups, *P<0.05, **P<0.01, ***, P<0.001 **** P<0.0001 compared to the post-WLS group.

Abbreviations: BMI, body mass index; HOMA-IR, homeostatic model assessment of insulin resistance: LH:FSH, luteinizing hormone: follicle stimulating hormone.

Representative images depicting changes in endometrial development during the secretory, menstrual, and proliferative phases for one participant during both Baseline IOI and Month 7 IOI are provided in Figure 1 (Panel B and C, respectively). Longitudinal changes of ET are plotted across an IOI and contrasted in Baseline and Month 7 IOIs in Figure 3. Overall changes in ET were detected across the IOI (P_{DAY}<0.0001). During Month 7, the ET was also thicker on any given day compared to Baseline IOI ($P_{INTERVENTION}$<0.0001). Mixed model results also showed a

significant positive effect of the hypocaloric diet intervention on P4 ($P_{INTERVENTION}$=0.013), but not E2 concentrations ($P_{INTERVENTION}$=0.179).

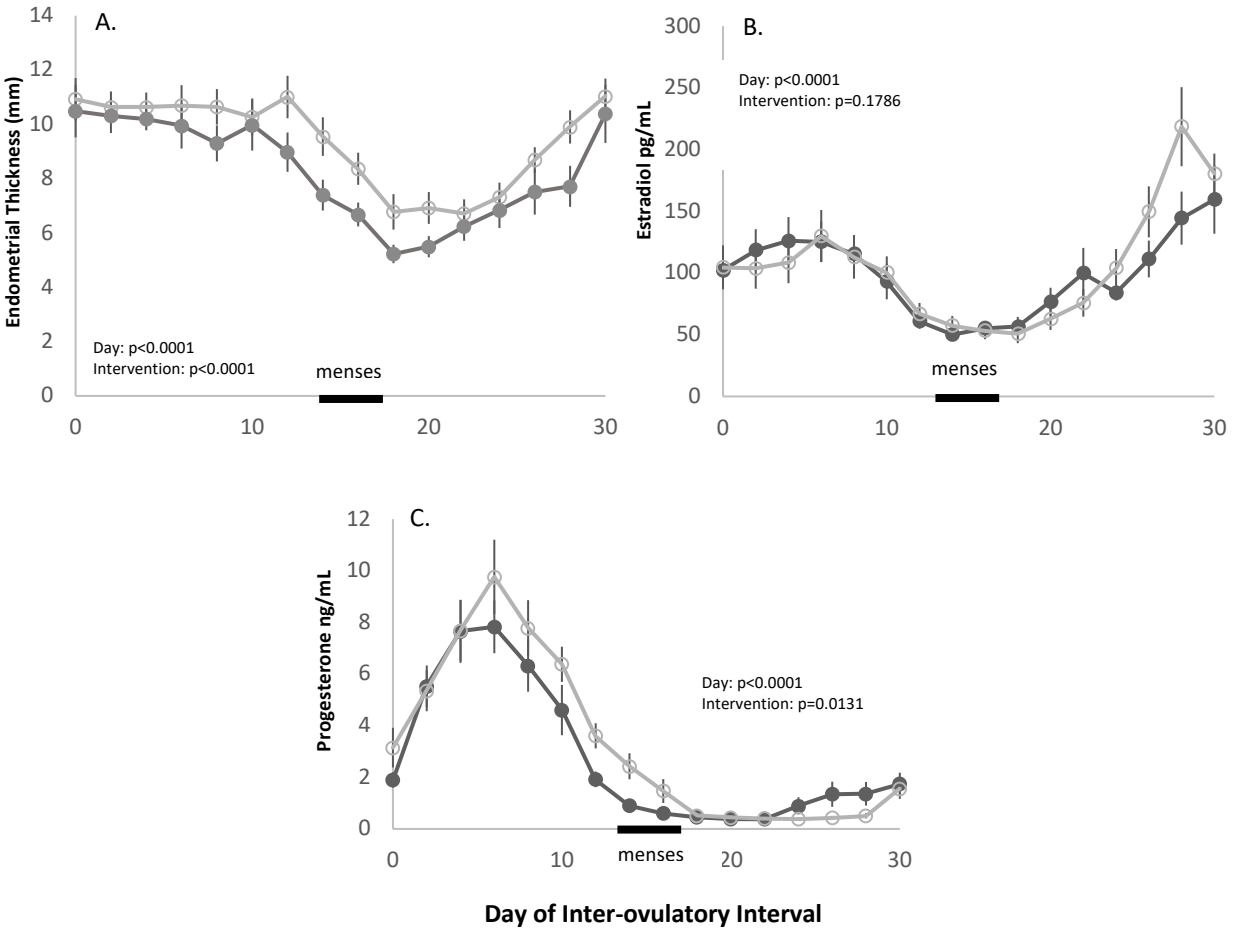

Figure 3.3. ***Longitudinal profiles of endometrial thickness (A) and estradiol (B) and progesterone (C) concentrations over an inter-ovulatory interval (IOI) in Baseline (●) and Month 7 (○).*** Mixed models showed a day effect for endometrial thickness and estradiol and progesterone concentrations, and an intervention effect for endometrial thickness and progesterone levels.

Differences in non-normalized endometrial data between participants before and after the intervention during the proliferative (follicular) and secretory (luteal) phases are presented in Table 4. The endometrium was significantly thicker across Month 7 IOI compared to Baseline IOI (Month 7 vs. Baseline: 9.4 ± 1.3 vs. 8.2 ± 1.2 mm; P=0.0005) with the total percent change in ET

across the IOI being significantly less in Month 7 (59.8 ± 1.3 vs. 71 ± 1.2%; P=0.014). During the secretory-menstrual transition, no differences in the changes of P4 concentrations were noted before and after the intervention (90.7 ± 8.4 vs. 88.6 ± 7.6%; P=0.593). The proliferative phase was significantly shorter by an average of 2.4 days in Month 7 compared to Baseline (P=0.024). In the proliferative phase, in Month 7 IOI minimum ET was increased (P=0.006), mean proliferative phase ET was increased (P=0.0003), and there was a significantly decreased percent endometrial change (P=0.021). In contrast, there were no differences in the day of ET minimum, and measurement or day of maximum ET during the proliferative phase. During the secretory phase there were no differences in the secretory phase length or ET between Month 7 and Baseline IOIs.

Table 3.4. Endometrial characteristics across the proliferative and secretory phases.

	Baseline IOI (n=11)	Month 7 IOI (n=11)
Proliferative Phase		
Proliferative phase length (day)	17.3 ± 3.3	14.9 ± 3.7*
ET Mean (mm)	7.0 ± 1.4	8.4 ± 1.3**
% ET change	65.5 ± 10.1	55.3 ± 12.7*
ET Min (mm)	3.8 ± 1.1	5.5 ± 1.9**
Day of ET Min	6.7 ± 2.4	5.9 ± 3.7
ET Max (mm)	11.2 ± 3.3	12.2 ± 1.8
Day of ET Max	10.7 ± 8.3	11.3 ± 6.1
Secretory Phase		
Secretory phase length (day)	11.7 ± 1.6	12.4 ± 3.7
ET Mean (mm)	10.0 ± 1.5	10.9 ± 1.7
% ET change	45.5 ± 10.4	35.7 ± 17.9
ET Min (mm)	7.0 ± 1.6	8.2 ± 2.4
Day of ET Min	5.6 ± 4.1	5.9 ± 4.9
ET Max (mm)	13.1 ± 2.3	12.9 ± 1.8
Day of ET Max	4.4 ± 2.4	5.5 ± 4.3

Data are presented as mean ± standard deviation. Within rows, * denote significant differences between groups, *P<0.05, **P<0.01, ***, P<0.001 **** P<0.0001.

Abbreviations: ET, endometrial thickness.

Discussion

Provided in this study is a longitudinal evaluation of endometrial development in eumenorrheic women across the adiposity spectrum, and further an evaluation of the impact of a hypocaloric dietary intervention on ET. To this point, prospective analyses of the impact of obesity on ET have been limited to cross-sectional studies of post-menopausal women (Barboza et al. 2014; Serdar Serin et al. 2003; Wei et al. 2021) and those with presumptive infertility (Zeng et al. 2013). We provide evidence that despite natural ovulatory cycles, the ET in women with obesity is persistently increased across the entire IOI with an overall lower percent change in ET during the secretory-proliferative phase transition. This increased ET across the IOI occurred alongside decreased P4 production in obesity. Further, increased P4 production following a hypocaloric dietary intervention did not offset a decreased percent change in ET at the secretory-proliferative phase transition. Rather, ET across the IOI further increased in this cohort of participants after a hypocaloric dietary intervention. Nevertheless, the length of the proliferative phase was reduced on average by 2.4 days.Together, these new findings suggest that despite regular ovulatory menstrual cycles, women with obesity display altered endometrial dynamics that may underlie a risk of endometrial hyperplasia in this population which cannot be completely offset with weight loss in the short-term.

Endometrial thickness was persistently increased across the IOI in women with obesity compared to those without obesity. To our knowledge, this is the first documentation of a comparison of endometrial dynamics in eumenorrheic women across the adiposity spectrum. While a relationship between obesity and ET has been shown in anovulatory women (Hsu et al. 2011; Liao et al. 2013; Souter et al. 2011), herein we report a similar direct impact of obesity on ET in

those with a self-reported history of regular menstrual cycles. This relationship between obesity and ET was most pronounced in the proliferative phase when rising E2 concentrations from the growing dominant follicle induced its usual proliferation effects on the endometrial lining (Monis and Tetrokalashvili 2019). Estrogen concentrations are known to be higher in women with obesity due to the peripheral conversion of androgens to estrogens by adipose tissue, as well as decreased sex hormone binding globulin concentrations leading to more free, unbound estrogens (Mair, Gaw, and MacLean 2020). Unlike others who reported increased E2 in women with obesity (De Pergola et al. 2006; Yeung et al. 2013), we did not note higher E2 concentrations in the women with obesity and eumenorrhea. However, we suspect that other estrogen molecules, such as estrone and estriol, may have been elevated in the women with obesity in our study population. Several estrogen molecules have been shown to bind to uterine estrogen receptors and induce proliferation of the endometrium (Yu et al. 2022) and are collectively posited to contribute to the thicker endometrium described in amenorrheic women with obesity (Hsu et al. 2011; Liao et al. 2013; Souter et al. 2011). Whether less biologically active estrogens contributed to the thicker ET noted across the IOI in our study is an area for future research

A decreased percent change in ET at the secretory-proliferative phase transition was noted with women with obesity. This smaller change in ET was accompanied by a greater minimum ET following menses. Menses results from vascular changes and necrosis of the stratum functionalis following regression of the corpus luteum and declines in P4 concentrations (Critchley et al. 2020). Women with obesity in our study had decreased P4 concentrations across the IOI that others have posited may be due to an increased blood volume, a decreased LH pulse amplitude, or selective partitioning of ovarian hormones into adipose tissue in obesity (Jain et al. 2007a). Our study of follicle dynamics in obesity (Chapter 1) would also suggest a potential for impaired corpus luteum function resulting from alterations in granulosa cells of the dominant follicle leading to

impaired large luteal cell P4 production. We noted a decreased percent change in P4 concentrations during the secretory to proliferative phase transition (i.e., the P4 withdrawal), which may imply an insufficient trigger for the complete shedding of the endometrial lining. Our findings suggest either an incomplete shedding of the functionalis or a persistent thickening of the basalis over time in obesity. Our methods did not allow for a differentiation between the layers of the endometrium. Therefore, we cannot comment on which layer(s) are contributing to the overall thickening of the endometrium but nonetheless, there appears to be thickening over time despite monthly shedding in eumenorrheic women with obesity.

We hypothesized that after a hypocaloric dietary intervention, participants would show increased P4 concentrations and in turn, improvements in endometrial dynamics defined by a thinner endometrial lining post-menses. However, an increased ET across the IOI and a decreased percent change in ET compared to Baseline IOI was noted after the intervention – despite improvements in P4 concentrations. Estradiol is primarily responsible for the proliferation of the endometrial lining leading to an increase in ET (Groothuis et al. 2007). We found that estradiol concentrations remained unchanged following the intervention, suggesting that any impact of estrogens on endometrium would be driven by peripheral versus ovarian sources. As referenced above, obesity is considered a hyperestrogenic state with adipose tissue directly contributing to increased peripheral estrogens (Mair et al. 2020). While the participants experienced a 10.5% decrease in total weight, and a 5.2% change in total percent fat, this change in adiposity may not have been sufficient to alter estrogen exposure of the endometrium to offset any persistent thickening in the context of obesity. While all but one participant lost a clinically meaningful amount of weight during the intervention, the majority retained their obesity status (55%) and the remaining transitioned to an overweight category (45%) consistent with maintenance of excessive adiposity across all participants in Month 7. Although P4 concentrations increased after the

intervention, there was no difference in the percent change of P4 concentrations during the secretory to proliferative phase transition. The magnitude of the P4 withdrawal is known to impact the degree of menses, as different doses of P4 have been shown to affect withdrawal bleeds in anovulatory women (Puscheck 2015). Given that the magnitude of the P4 withdrawal signal was similar pre- and post-intervention, this may have contributed to a persistence in the incomplete shedding of the endometrial lining after the intervention. Collectively, our data provide evidence that even after a 6-month hypocaloric dietary intervention, changes in P4 production induced by weight loss may not be sufficient to restore endometrial dynamics in eumenorrheic women.

This study had several strengths. It included a well-characterized cohort of women recruited from the general Ithaca population. As noted in both Chapters 1 and 2, we employed a more accurate measure of adiposity by using DEXA which we felt would provide a more valid assessment of the relationship between obesity and endometrial dynamics. Further, a subset of the participants with obesity underwent a 6-month hypocaloric dietary intervention which enabled interrogation any functional consequences of weight loss on the ET differences we noted in the observational component of this study. As described in Chapter 2, these women were determined to be at a stage of weight-loss readiness and a substantial amount of weight reduction was noted. The intervention was sufficient to induce weight loss and metabolic improvements in our study population consistent with good compliance to the intervention. However, this study was not without limitations. Again, the population was primarily Caucasian and reflected the general population of the Ithaca, NY area. This limits the generalizability of our findings to other racial and ethnic groups. Our study was also limited to measures of ET. Therefore, we cannot comment on changes in endometrial vascularization or endometrial receptivity with obesity and weight loss which have implications for fertility (Lessey and Young 2019). Further, our weight loss intervention was short-term and this study did not include a long-term follow-up. Therefore, any sustained

changes in endometrial development and/or endocrine dynamics following this short-term weight loss intervention are unknown.

Incomplete shedding and persistent thickening of the endometrium puts individuals at risk for abnormal uterine bleeding and endometrial hyperplasia (Jones and Sung 2020). This study provides evidence that regardless of cycle status, obesity is a risk factor for increased ET. Further, we provide evidence that a 6-month hypocaloric dietary intervention increases P4 concentrations, but not to a level sufficient to improve endometrial development. Together, our findings challenge the notion that regular menstrual cycles in obesity reflect an unperturbed reproductive axis and endometrium. Whether eumenorrheic women with obesity benefit from P4 supplementation to regulate the endometrial response, and prevent increased endometrial thickening over time, requires further investigation.

SUMMARY AND FUTURE DIRECTIONS

To this point, reproductive dysfunction in eumenorrheic women with obesity has been largely limited to endocrine assessments. The notion that regular menstrual cyclicity is indicative of normal reproductive function in those with obesity should be reconsidered. The work presented in this dissertation utilized serial transvaginal ovarian and uterine ultrasonography and hormonal assessments to reveal the degree to which antral follicle and endometrial development are impaired in obesity. The major findings and implications of the research are as follows:

We showed the feasibility of using serial transvaginal ultrasonography to effectively capture antral follicle growth and endometrial development in women with excess adiposity ***(Chapters 1, 2, and 3)***. Given that increased adiposity may impact ultrasound image quality, we showed that it is possible to consistently visualize the ovaries and reliably count and track unique follicles in this clinical population. Further, we were also able to consistently resolve and take serial measurements of the endometrium with high retention rates across all studies. Together, our approaches provide a basis for future evaluations of antral follicle and endometrial dynamics in women with obesity to more completely understand the impact of excess adiposity and metabolic disturbances on reproductive health.

We showed that altered reproductive function is a constant feature in eumenorrheic women with obesity as evidenced by the ovary ***(Chapters 1 and 2)*** and the uterus ***(Chapter 3)***. In conjunction with a known decrease in progesterone, we affirmed a global downregulation of follicle development, increased prevalence of luteal phase defects and a thickened endometrial lining in those with obesity. Specifically, we showed an increased proportion of recruitable follicles and a decreased proportion of selectable follicles in women with obesity compared to the non-obese group. The women with obesity further displayed alterations in major key events of antral

folliculogenesis. Namely, a decreased number of recruitment events and a smaller ovulatory follicle diameter at selection. While eumenorrheic women with obesity displayed evidence of the cyclic recruitment and experience all key events of folliculogenesis, follicle development was deemed suppressed as evidenced by the proportionally fewer number of dominant follicles that merged in this population. This new knowledge provides the first model of antral follicle development in obesity and regular ovulatory cycles ***(Chapter 1)***. These data are critical in being more representative of our current demographic in which there is a growing number of reproductive age women with overweight and obesity. Our data on follicle and endocrine dynamics in obesity may aid in improving contraception and infertility treatments, as both are known to be suboptimal in women with obesity. Further, our data point to a higher rate of luteal phase defects in those with obesity and regular cycles and suggest a benefit for screening of luteal dysfunction in those with obesity and desire for conception. Future research should focus on the metabolic mechanisms in obesity that may underlie these alterations in follicle development, as well as the impact of improper luteinization.

We showed that weight loss induced by a hypocaloric dietary intervention improved some of the alterations in antral follicle dynamics that were detected in eumenorrheic women with obesity ***(Chapter 2)***. Our assessment showed favorable changes in ovulatory follicle size at selection, a decreased total antral and recruitable follicle pool, an increased number of dominant follicles, and increased progesterone production over the cycle ***(Chapter 2)***. As such, we provide direct evidence of enhanced follicle development with weight loss which supports lifestyle intervention as a first-line treatment to improve reproductive health outcomes even in eumenorrheic individuals with obesity. Our data point to improvements in glucoregulatory status as potential contributors to enhance folliculogenesis with weight loss. Future research should focus on the actual mechanisms by which changes in metabolic status could alter follicle development and luteinization with weight loss. We noted improvements in some but not all

aspects of follicle and endocrine dynamics which supports the need to better understand the role for degree of weight loss, type of lifestyle intervention and any short versus long-term benefits of weight loss on ovarian function.

We extended on our new knowledge of ovarian follicle development in eumenorrheic women with obesity to understand any potential impact on functional changes in the endometrium. We showed that eumenorrheic women with obesity had a significantly thicker endometrium across the cycle, with a smaller percent change in thickness at the secretory-proliferative phase transition ***(Chapter 3)***. Our data suggest that regular menses in women with obesity may not result in complete shedding of the endometrial lining and that a consistent build-up of this lining is possible over time. We extended on our findings of improved follicle development with weight loss to investigate any direct impact on the endometrium ***(Chapter 3)***. Despite the improvements in follicle dynamics, increased progesterone production and clinically significant decreases in adipose tissue, we showed that the endometrial lining progressively thickened in the presence of obesity consistent with the conclusion that these phytological changes with weight loss were not sufficient induce complete shedding of the endometrial lining. Evidence of an incomplete shedding of the endometrium despite regular menses suggests an increased risk for hyperplasia in this population that persists even after a short-term hypocaloric dietary intervention. Future research should investigate the cellular characteristics of the endometrium in eumenorrheic women with obesity to better delineate any actual risks for endometrial hyperplasia. It may be necessary to develop criteria for hyperplasia (risk) specific to this population as current recommendations are best suited for amenorrheic and/or post-menopausal women. Further, the degree to which progesterone supplementation could promote endometrial health in this population warrants consideration.

Collectively, this dissertation provides new insight into the mechanisms of reproductive dysfunction in eumenorrheic women with obesity. Given the clinical nature of our work, these

findings can be immediately translated into practice to improve the management of women with obesity and regular menstrual cycles who may experience sub-fertility or abnormal uterine bleeding. This new knowledge has implications for understanding mechanism by which obesity could exacerbate reproductive disorders, such as PCOS. Further, our data provide an important basis for promoting the use of lifestyle intervention as first-line treatment of reproductive dysfunction in obesity. Ultimately, our current obesity epidemic calls for prioritization of research focused on the broad-spectrum effects of obesity on women's reproductive health. Our data are conclusive in determining that even in the context of regular menstrual cyclicity, risks for adverse reproductive health outcomes are present.

APPENDIX A

IMPACT OF OBESITY ON ANTI-MULLERIAN HORMONE (AMH) LEVELS IN WOMEN OF REPRODUCTIVE AGE

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Abstract

Obesity negatively impacts reproductive health, including ovarian function. Obesity has been posited to alter Anti-Müllerian hormone (AMH) production. Understanding biological factors that could impact AMH levels is necessary given the increasing use of AMH for predicting reproductive health outcomes in response to controlled ovarian stimulation, diagnosing ovulatory disorders, onset of menopause, and natural conception. In this narrative review we evaluate the impact of obesity on AMH levels in healthy, regularly cycling reproductive-age women (18-48years). Thirteen studies (n=1214 women; [811, Non-obese {body mass index; BMI <30kg/m^2}; 403, Obese {BMI>30kg/m^2}]) were included, of which five reported decreased AMH levels with obesity, whereas eight showed comparable AMH levels between groups. Studies reporting lower AMH levels were limited by small sample sizes, cross-sectional designs, and emphasis on higher obesity classes which may not represent most obesity cases worldwide. Including women with higher obesity classes (Class 3 versus Class 1) may have been a factor in studies reporting lower AMH levels. Ultimately, the notion of a negative impact of obesity on AMH in otherwise healthy women with regular menstrual cycles should be deemed uncertain at this time. This conclusion is prudent considering the biological basis for any impact of obesity on AMH production is unknown.

Introduction

Obesity remains a persistent and growing public health concern, with current rates nearing 40% of reproductive-aged women in the United States (Hales et al. 2020). Obesity impacts a broad array of health risks in women across the lifespan (Aronne et al. 2009), including adverse reproductive health outcomes such as menstrual cycle irregularity, abnormal uterine bleeding, endometrial hyperplasia, infertility, and pregnancy complications (Kazemi et al. 2020; Klenov and Jungheim 2014; Kyrou et al. 2000; Practice Committee of the American Society for Reproductive Medicine 2015; Reproductive 2015). Further, women with obesity are 20% more likely to experience later onset of menopause, which in part, may underlie the increased risk of breast, ovarian, and uterine cancer seen in this population (J. Zhu et al. 2018). While the impact of obesity on reproductive health is known to be multi-factorial, many of the adverse reproductive outcomes may be linked to endocrine disruptions that reflect an impaired ovarian function (Silvestris et al. 2018). Specifically, infertility observed in women with obesity is commonly associated with ovulatory disturbances and irregular menstrual cyclicity (Dağ and Dilbaz 2015). However, even women with obesity and regular menstrual cycles exhibit a longer time to spontaneous pregnancy (Gesink Law, Maclehose, and Longnecker 2007; Moy et al. 2015a; Rich-Edwards et al. 2002; Van Der Steeg et al. 2008) and lower success rates of controlled ovarian hyperstimulation compared to their normal-weight counterparts (Fedorcsák et al. 2004). This potential for subfertility aligns with previous reports of an altered reproductive hormone profile in women with obesity and regular cycles including, decreased follicle stimulating hormone (FSH) levels (De Pergola et al. 2006), decreased luteinizing hormone (LH) pulse amplitude (Jain et al. 2007b), increased estradiol levels (De Pergola et al. 2006) and decreased luteal phase progesterone production (Jain et al. 2007b). Despite strides toward characterizing the nature of reproductive disturbances in obesity, several questions remain to be answered on how and why obesity may drive disordered ovarian function.

To that end, an altered ovarian follicular environment has been confirmed in women with obesity

and involves disruptions in multiple systems, including steroidogenic action, metabolism, and inflammation, all of which can impact folliculogenesis and ovulatory potential (Robker et al. 2009). The degree to which obesity impacts ovarian reserve is more controversial as available data has largely focused on sub- or infertile populations wherein studies have not shown consistent associations between serum markers of ovarian reserve and body mass index (BMI) (Kazemi et al. 2020; Moslehi et al. 2018). Anti-Mullerian hormone (AMH), a glycoprotein primarily produced by the granulosa cells of primary and early-stage antral follicles, is a marker whose association with obesity is controversial (Almeida et al. 2018; Dewailly et al. 2014; Jeppesen et al. 2013) – albeit a single meta-analysis suggests a negative association of AMH with BMI [19]. A growing interest in the use of AMH to predict reproductive health outcomes related to response to controlled ovarian stimulation (Zheng, Liu, and Chen 2015), diagnosis of ovulatory disorders (Abbara et al. 2019), the onset of menopause (Kruszynska and Slowinska-Srzednicka 2017), and even natural conception (Steiner et al. 2011) necessitate an understanding of biological factors, such as obesity, that could impact the predictive power of AMH for such reproductive outcomes.

The mechanisms through which obesity may adversely affect AMH production are unknown. One possibility relates to the altered metabolic regulation of ovarian granulosa cells. Obesity is commonly associated with systemic insulin resistance and compensatory hyperinsulinemia. Excessive insulin levels have been shown to alter granulosa cell receptivity, and subsequently, AMH production (Nardo et al. 2009). Likewise, the increased leptin production associated with obesity could directly suppress AMH production. This observation is derived from the inhibitory effects of leptin administration on AMH and AMH receptor gene expression in cultured granulosa cells from patients undergoing controlled ovarian hyperstimulation (Merhi et al. 2013). More indirect in nature is the notion that lower AMH levels in women with obesity may result from a hemodilution effect of increasing body size (Palomaki et al. 2020). Another possibility includes an impact of obesity on AMH catabolism and excretion. Obesity is known to alter the excretion of

other reproductive hormones such as FSH, estradiol, and progesterone (Spandorfer et al. 2004). However, the exact mechanisms of AMH excretion are unknown (Griesinger et al. 2012). Last, obesity may have an increased apoptotic effect at the ovarian follicle level (Nteeba, Ganesan, and Keating 2014). While this posited mechanism may explain reduced ovarian follicle pool and AMH levels, it seems less likely based on existing data of a later time to ovarian senescence in women with obesity.

Our current demographic necessitates elucidating and a consideration of the impact of obesity on AMH given its growing use as a marker of reproductive potential in healthy women of reproductive age. Most of the available data on AMH levels has been focused on women with infertility and/or polycystic ovary syndrome (PCOS) (Safdarian et al. 2018; Saxena, Ramani, and Singh 2018). Of the data available in otherwise healthy women, AMH levels have been more commonly reported in women of lean BMI or women of advanced reproductive age (Care 2019; Meczekalski et al. 2016). Some women with obesity have regular cycles, yet their reproductive hormone profile suggests some level of ovarian dysfunction that could manifest as disordered AMH production compared to their lean counterparts (Moy et al. 2015b). Differences in AMH production across the adiposity spectrum could lead to inaccurate conclusions about the ability of AMH to adequately inform reproductive health outcomes in women. To address the current knowledge gap, we conducted a review to provide an up-to-date account of AMH levels in lean and obese women with regular menstrual cycles with the goal of establishing the degree to which obesity impacts AMH production in healthy, potentially fertile women.

Methods

This work represents a narrative review. The methods have been summarized herein.

Review question

The PEO (Population [P], Exposure [E], Outcome [O]) criteria of our review were defined before the literature search. To that end, our study question was, in lean and obese regularly cycling women (P), are AMH levels (O) lower in reproductive age women with obesity and regular menstrual cycles compared to their lean (non-obese) counterparts (E)?

Primary outcome

Our primary outcome was serum AMH levels.

Data Sources and Search Strategy

A search of published literature was conducted in electronic databases of MEDLINE (PubMed), Institute for Scientific Information (ISI) Web of Science, and Scopus through July 27, 2020, using a search strategy based on the PEO framework, as described above. In short, studies included for review were limited to original research articles in which (1) the study was conducted in healthy reproductive-aged (18-48 years) regularly cycling women, (2) the exposure was obesity, and (3) AMH levels were reported as an outcome for lean and obese groups. Only articles published in English were included. Studies must have used BMI as a categorical term, with obesity defined as a BMI>30 kg/m^2 and lean defined as some value <30 kg/m^2. Where AMH levels were reported separately for overweight women (BMI >25 and <30 kg/m^2), data were pooled with lean women where possible. Every record retrieved by this search strategy underwent a title and abstract screening to confirm that it aligned with the inclusion criteria. Articles that were relevant and appropriate were downloaded for full-text review, and data on the general characteristics of the study, patient population, study design, obesity definitions, AMH levels, and inclusion and exclusion criteria were extracted.

Inclusion and Exclusion Criteria

Briefly, observational (cross-sectional, case-control, cohort) studies or cross-sectional analysis of baseline measures from randomized controlled trials on women with regular menstrual cycles included wherein the influence of obesity (non-obese and obese subtypes) as an exposure variable was evaluated on our study outcomes of interest. Non-peer-reviewed studies; studies without the design of interest; studies wherein our outcomes of interest were not compared between lean and obese women with regular cycles; studies that were not conducted on healthy women; studies in children (<17 years); pregnant women; or menopausal-aged women (>48 years); and, where study data were irretrievable after contacting their corresponding authors were excluded.

Data Extraction

The following data were extracted using a standardized protocol (1) first author's name; (2) study publication year; (3) participants' characteristics, including total sample size and the sample size of participants in the lean and obese groups; (4) study design and setting and type of data analysis/collection (prospective/retrospective); (5) participants' age; (6) participants' body mass index (BMI); and (7) AMH levels.

Results

Literature Screening

One thousand nine hundred fifty-six studies were identified using the search strategy through electronic databases. Duplicates found using multiple databases, keywords, and sources were removed (n=985). The titles and/or abstracts of the remaining records (n=971) were screened, of which 615 studies were deemed irrelevant. The full texts of the remaining 356 studies were assessed for eligibility. Of these, 329 were further excluded due to the full text not available (n=7), study design not appropriate (n=105), no comparison between obese and non-obese women

(n=150), study not performed on reproductive-aged women (n=51), AMH not reported as an outcome (n=13), and duplicate reports of the same study data (n=3). Twenty-seven studies remained, and 14 studies were further excluded due to an inconsistent definition of obesity. Ultimately, 13 studies were included in the review. A description of each study and its relevant characteristics and findings are summarized in Table 1.

Study Characteristics

Of 13 studies identified in this review, 8 involving a total of 193 obese and 261 lean women with regular menstrual cycles documented no significant differences in AMH levels between groups. Percent differences in AMH levels between groups ranged -70.4% to 62.5% (Mean: -5.5%; Median: 2.5%). BMI of the lean participants ranged from 21.6 to 25.6 kg/m^2, and BMI of the obese participants ranged from 31.7 to 34.3 kg/m^2, consistent with the inclusion of women with strictly Class 1 (30 to <35 kg/m^2) obesity. Studies were conducted across a broad array of countries and included diverse ethnic populations from North America (Roth et al. 2014; Shaw 2011), South America (Woloszynek et al. 2015), Asia (Kurek Eken et al. 2019; Sahin Ersoy et al. 2017; Shahin et al. 2019), and Africa (Al-Eisa et al. 2017; Halawaty et al. 2010). Participants ranged in age from 23.8 to 46.2 years, with the mean age across studies being approximately 29 years. Studies were largely cross-sectional in nature and involved an assessment of serum AMH levels at a single time point during the menstrual cycle. The timing of the AMH assessment was not standardized to a particular stage of the cycle for all studies. However, 6 (Halawaty et al. 2010; Olszanecka-Glinianowicz et al. 2015; Peigné et al. 2020; Shahin et al. 2019; Steiner et al. 2017; Su et al. 2008; Woloszynek et al. 2015) of the 13 studies did measure AMH during the earliest part of the follicular phase (days 2–7). According to the most recent position statement by the American Society for Reproductive Medicine (ASRM), intracycle variation in AMH is considered minimal and standardizing timing of assessments is not a requirement at this time (Pfeifer et al. 2015).

Five out of thirteen studies involving 210 obese and 550 lean women with regular menstrual cycles documented either significantly lower AMH levels in the obese compared to lean groups and/or a negative association between AMH and BMI. Percent differences in AMH levels between groups ranged -9.7% to -76.7% (Mean: -27.4%; Median: -21.8%). BMI of the lean participants ranged from 20.7 to 22.4 kg/m^2 and that of the obese participants ranged from 33.0 to 46.0 kg/m^2, consistent with inclusion of women across Class 1 (30 to <35 kg/m^2), Class 2 (35 to <40 kg/m^2) and Class 3 (40 kg/m^2 or higher) obesity. Studies were also conducted across a broad array of countries and included diverse ethnic populations from North America (Steiner et al. 2017; Su et al. 2008) and Europe (Chiofalo et al. 2017; Olszanecka-Glinianowicz et al. 2015; Peigné et al. 2020). Participants ranged in age from 23 to 46 years, with the mean age across studies being approximately 30 years. Studies were largely cross-sectional in nature and involved an assessment of serum AMH levels at a single time point during the menstrual cycle. Collectively, this group of studies included a similar number of obese women, but more than double the number of lean women, compared to the studies that reported no difference in AMH across BMI groups. A broader range of obesity was represented, but studies were more limited in their geographic representation.

Table 1. Characteristics of studies reporting AMH levels in lean and obese reproductive-aged women with regular menstrual cycles.

Lead Author, Publication, Year (Country)	Participants' Characteristics * (n, age [year], BMI [kg/m^2])	Group Definitions based on BMI (kg/m^2)	Study Design	Assay Type, Method	Cycle Day or Stage	AMH Levels			Correlation (*p*-Value) Adjustment for Confounders	Exclusion Criteria
						Obese	Lean	*p*-Value across BMI groups*		
Al-Eisa 2017 (Egypt) (Al-Eisa et al. 2017)	Lean group (n, 30; age, 28.7; BMI, 22.8) Obese group (n, 30; age, 27.6; BMI, 31.7)	Lean: 20-29 Obese: 30-35	Cross-sectional analysis of a non-randomized trial	Beckman Coulter ELISA	Day 2-3	4.60 (3.11-6.09)	2.83 (0.03-5.63)	>0.05	NR	Infertility, concomitant diseases, ovarian issues, or use of drugs that affect hormone levels
Chiofalo 2017 (Italy) (Chiofalo et al. 2017)	Lean group (n, 19; age, 30; BMI, 22) Obese group (n, 26; age, 33; BMI, 46)	Lean: <25 Obese:>30	Cohort	Gen II Beckman Coulter ELISA	Random	2.14 (0.81-3.47)	2.37 (0.17-4.57)	<0.0001	NR	Use of estro-progestin, metformin or inositol, hyperprolactinemia, and endocrine disorders
Eken 2019 (Turkey) (Kurek Eken et al. 2019)	Lean group (n, 38; age, 26.66; BMI, NR) Obese group (n, 31; age, 26.03; BMI, NR)	Lean: 18.5-24.9 Obese: >30	Cross-sectional	Ansh Labs AMH ELISA	Early follicular phase	2.56 (1.78-3.34)	2.30 (1.58-3.02)	>0.05	NR	Androgen-producing tumors, 21-hydroxylase deficiency, adrenal hyperplasia, hyperprolactinemia, thyroid disease, Cushing's, and use of insulin sensitizers and/or medications that interfere with reproduction

Ersoy 2017 (Turkey) (Sahin Ersoy et al. 2017)	Lean group (n, 36; age, 26.4; BMI, 21.6) Obese group (n, 26; age, 26.7; BMI, 32.8)	Lean: 18.5-24.9 Obese: >30	Cross-sectional	Ansh Labs AMH ELISA	Day 2-4	3.10 (2.10-4.10)	3.10 (2.10-4.10)	NR	NR	Smoking, alcohol abuse, diabetes, Cushing's, adrenal hyperplasia, androgen-secreting tumors, thyroid dysfunction, and hormonal drug use
Halawaty 2010 (Egypt) (Halawaty et al. 2010)	Lean group (n, 50; age, 46.1; BMI, 25.6) Obese group (n, 50; age, 46.2; BMI, 32.9)	Lean: <30 Obese: 30-35	Cross-sectional	DSL AMH ELISA	Day 2-5	2.55 (1.74-3.36)	3.39 (3.15-3.63)	0.56	NR	Use of hormones, smoking, pregnancy, lactation, hysterectomy, previous ovarian surgery, PCOS, endometriosis, and other medical conditions that could affect ovarian function
Olszanecka-Glinianowicz, 2015 (Poland) (Olszanecka-Glinianowicz et al. 2015)	Non-PCOS group (n, 36/67 obese; age, NR; BMI, NR)	Lean: 18.5-24.9 Obese: >30	Observational	Immunotech E LISA	Day 3-5	3.90 (1.60-6.20)	5.10 (2.70-7.50)	<0.05	-0.075 (P<0.05) Age	Hyperandrogenism, PCOS and infertility
Peigne 2020 (France) (Peigné et al. 2020)	Lean group (n, 21; age, 32.0; BMI, 20.7) Obese group (n, 16; age, 31.5, BMI, 33.7)	Lean: <25 Obese:>30	Case-control	DXI sandwich che mi-luminescent immunoassay	Early follicular phase	0.87 API: 34.6%	0.92 API: 39.02%	P>0.05 P<0.001	NR API -0.557 (P<0.01)	Irregular cycles, hyperandrogenism, abnormal ovaries, use of medications that affect metabolism or ovarian function within 3 months

Roth 2014 (United States) (Roth et al. 2014)	Lean group (n, 10; age, 27.3; BMI, 22.3) Obese group (n, 10; age; 32.5, BMI; 34.3)	Lean: 18.5-25 Obese: >30	Cross-sectional	Gen II Beckman Coulter ELISA	Mid-cycle	0.02 (0.01-0.06)	0.05 (0.02-0.10)	0.10	NR	Chronic diseases, use of exogenous sex steroids or medications known to affect reproductive hormones, regular exercise exceeding m ore than four hours weekly, or attempting pregnancy
Shahin 2020 (Jordan) (Shahin et al. 2019)	Lean group (NR) Obese group (NR)	Lean: 18.5-25 Obese: >30	Case-control	Roche Cobas ECLIA	Day 2-4	3.11 (0.92-5.3)	2.91 (-0.16-5.98)	0.70	NR	Congenital adrenal hyperplasia, Cushing's, malabsorptive or eating disorders, menopause, history of bariatric surgery
Shaw 2011 (United States) (Shaw and Edelman 2013)	Lean group (n, 31; age, 23.8; BMI, 22.2) Obese group (n, 36; age, 27.3; BMI, 33.4)	Lean: <25 Obese: >30	Case-control	Beckman Coulter ELISA	Random	0.64	0.61	0.76	NR	NR
Steiner 2017 (United States) (Steiner et al. 2017)	Lean group (n, 461; age, NR; BMI, NR) Obese group (n,114; age, NR; BMI, NR)	Lean: 18.5-24.9 Obese: >30	Cohort	Gen II Beckman Coulter ELISA	Day 2-4	2.20 (0.90-4.00)	2.85 (1.50-5.50)	0.06	NR	Known fertility problems (sterilization, PCOS, tubal blockage), endometriosis, previous or current use of fertility treatments, partner with a history of infertility, lactation, recent use of

										injectable hormonal contraception
Su 2008 (United States) (Su et al. 2008)	Lean group (n, 18; age, 45; BMI, 22.4) Obese group (n, 18; age, 45.1; BMI, 37.6)	Lean: <25 Obese:>30	Cross-sectional	DSL AMH ELISA	Day 1-4	0.07 (0.03-0.15)	0.30 (0.14-0.63)	0.01	P=0.02	Hormonal therapy, contraception, PCOS
Woloszynek 2015 (Brazil) (Woloszynek et al. 2015)	Lean group (n, 66; age, NR; BMI, NR) Obese group (n,10; age, NR; BMI, NR)	Lean: <25 Obese: >30	Cross-sectional	Gen II Beckman Coulter ELISA	Day 2-7	1.90 (0.40-10.90)	2.90 (0.30-11.20)	0.29	NR	Chronic diseases, menstrual irregularity, PCOS, infertility, hysterectomy, oophorectomy, serum LH and FSH concentrations out of the reference ranges

Abbreviations: PCOS, polycystic ovary syndrome; BMI, body mass index; ELISA, enzyme-linked immunosorbent assay; NR, not reported; OCP, oral contraceptive pill; LH, luteinizing hormone; FSH, follicle-stimulating hormone. ECLIA; electrochemiluminescence immunoassay; API, AMH prohormone index

AMH levels expressed as ng/mL. Mean (±SD) or Median (25-75th) are presented as provided by the manuscript.

Spearman's correlation is presented where available.

Discussion

In the present work, we aimed to provide an updated review of whether obesity *per se* differentially affects AMH production in healthy, potentially fertile women. Our study comprised a total of 13 studies involving 403 obese and 811 lean women, respectively. Overall, we did not detect a difference in AMH levels in obese versus lean women with regular menstrual cycles. Specifically, of the 13 studies included in this review, only one was prospectively designed to evaluate the potential for differences in AMH levels between lean and obese women with regular menstrual cycles [35]. The remaining studies reported AMH levels for healthy lean and obese women with regular menstrual cycles as part of their (baseline) clinical characteristics for studies aimed at: (1) contrasting reproductive and metabolic features of women with or without PCOS (Al-Eisa et al. 2017; Chiofalo et al. 2017; Kurek Eken et al. 2019; Olszanecka-Glinianowicz et al. 2015; Peigné et al. 2020; Roth et al. 2014; Sahin Ersoy et al. 2017; Shahin et al. 2019), (2) predicting onset of menopause (Jain et al. 2007b) or time to natural conception (Steiner et al. 2011), or (3) assay validation and reference range development (Woloszynek et al. 2015).

These findings contrast those of Moslehi et al. (Moslehi et al. 2018), which is, to our knowledge, the only review exploring an impact of obesity on a broad array of ovarian reserve markers, including AMH. The authors showed lower AMH levels in obese women based on a pooled analysis involving 211 obese and 233 non-obese, fertile women (weighted mean differences, -0.94, 95% CI -1.14, -0.73 ng/mL**)** and a negative relationship between AMH and obesity in a group of "*fertile non-PCOS*" women [19]. Several reasons may have contributed to differences in findings between Moslehi et al. and our observations. We aimed to build upon data from Moslehi et al. study by identifying newer studies, thus increasing our cohorts' sample sizes that had reported on AMH levels in "*lean*" and "*obese*" women with regular menstrual cycles. Our approach to identifying relevant studies differed from that of Moslehi et al. in having used different definitions for lean and obese groups. We defined obesity as strictly being a BMI >30 kg/m^2 and included

overweight women in the lean group when available. Unlike Moslehi et al., we did not include overweight women in the obese group since we and others have not detected significant variations in antral follicle development in overweight women during natural inter-ovulatory intervals when compared to lean women (Heidi Vanden Brink et al. 2013). Further, we did not include women with reasonable potentials for sub-fertility and accounted for more recent studies since their search was limited to those published by 2016. Ultimately, we identified nearly twice as many studies (13 versus seven studies) reporting AMH levels in otherwise healthy reproductive-aged women with regular menstrual cycles, with a majority (54%) being published after 2016. Moslehi et al. noted significant heterogeneity in AMH levels as part of their evidence synthesis in women with PCOS (Moslehi et al. 2018). Others have similarly noted high heterogeneity in AMH levels in reproductive-aged women–albeit their focus has been in sub-fertile populations such as women with PCOS (Dumont et al. 2015). Consistently the recent observations of a working group on the International Evidence-Based Guideline for the Diagnosis and Management of PCOS confirmed high heterogeneity in AMH levels across studies in women with PCOS (Han and goleman, daniel; boyatzis, Richard; Mckee 2019). However, unlike Moslehi, the working group elected not to quantitively pool AMH levels across studies; instead, they provided a qualitative assessment of the collective evidence and concluded that high heterogeneity is a major factor impacting the development of consensual AMH thresholds to diagnose PCOS (Han and goleman, daniel; boyatzis, Richard; Mckee 2019) which is consistent with our approach in the work presented herein.

Most Studies Showed no Difference in AMH levels with Obesity. Included in this group of eight studies was the lone study whose primary aim was to evaluate differences in AMH levels between obese (n=50) and non-obese (n=50) groups. Mean AMH levels were 32.9% lower in the obese group compared to the lean group, but differences did not reach statistical significance (Halawaty et al. 2010). While this study used stringent criteria to corroborate the healthy

reproductive status of the participants, Halawaty and colleagues used a narrow definition for obesity (30-35 kg/m^2), which primarily included women with Class 1 obesity. Further, the mean and range of the BMI of the non-obese group were 25.6 and 24-29 kg/m^2, respectively, possibly indicating a small number of women with BMI 18.5-24.9 kg/m^2 in the lean group. Ultimately, the spectrum of adiposity in the study by Halawaty et al. may not have been sufficient to capture a significant effect of obesity on AMH production (Halawaty et al. 2010). It must also be noted that this study focused on establishing an impact of obesity on the markers of ovarian reserve, specifically in older reproductive-aged women during the early transition phase of the late premenopausal state. As such, all women demonstrated regular menstrual cycle length (22-35 days) but also variability in cycle length by seven days in either direction for at least two cycles. The mean age of the non-obese and obese groups was 46.1 and 46.2 years and may not wholly reflect AMH production in younger women that are well outside the perimenopausal transition.

In a study by Woloszynek et al., involving 100 younger women with a mean age of 31, median AMH levels were 52.6% lower in obese versus lean groups, but differences across BMI groups (lean, overweight, and obese) were not statistically significant (Woloszynek et al. 2015). The study was designed to validate the use of the Gen II AMH immunoassay for use in reproductive-aged males and females and develop normative reference ranges for these populations. As secondary aims, any potential influence of hormonal contraceptives, smoking, and BMI on AMH levels was evaluated. Woloszynek and colleagues did not detect a significant association between AMH and BMI even after accounting for age in their analysis. This study involved a small number of women with a BMI >30 kg/m^2 (n=10), and AMH values for this group were highly variable (95% CI: 0.4-10.9 ng/mL), in line with its minimal contribution to the pooled analysis. Further, approximately 30% of all females included in the study used oral hormonal contraceptives (OCP), albeit the degree to which the groups of interests were on OCP was not reported. The unbalanced representation of obesity in this cohort alongside any confounding effects of OCP use may have

impacted the ability to detect an impact of obesity on AMH levels.

Fewer Studies Showed Decreased AMH with Obesity. Except for a single study (Steiner et al. 2017), the remaining four studies included in this group were small, involving ≤50 participants in both lean and obese cohorts combined. While women in the obese and lean groups across all these studies had comparable age distributions, the BMI classes of the groups were variable, especially in those with obesity, and none of the studies included women with overweight. Of these, the studies by Chiofalo et al. (Chiofalo et al. 2017) and Olszanecka-Glinianowicz et al. (Olszanecka-Glinianowicz et al. 2015) showed significantly lower AMH levels in obese versus lean women, with AMH levels being 9.7% (p<0.0001) and 23.5% (p<0.01) lower, respectively. Further, the study by Olszanecka-Glinianowicz and colleagues showed a negative correlation between AMH levels and BMI (r=-0.30, p<0001). Chiofalo and colleagues evaluated AMH levels as part of an intervention study involving bariatric surgery. As such, their obese group consisted of women with Class 3 obesity (mean BMI=46 kg/m^2). In contrast, the study by Olszanecka-Glinianowicz et al. that investigated AMH levels in the context of largely Class 1 obesity. Overall, these results suggest that obesity may have a negative impact on AMH across the obesity spectrum with a dose effect that is not linear.

Further a small study (n=36), Su et al. (2008) examined associations between obesity and serum and ultrasound measures of ovarian reserve in women of late reproductive age (mean age: 45 years) who did not use hormonal therapy and contraceptives and did not have PCOS (Su et al. 2008). AMH levels were a striking 76.7% lower in the obese cohort compared to the lean group (p=0.014). The authors identified BMI as an independent predictor of AMH and concluded that lower AMH levels in obese women of late reproductive age resulted from physiologic processes other than a decreased ovarian reserve. This observation suggests that the impact of obesity on AMH may extend into later reproductive years – albeit more data are needed to corroborate this

hypothesis.

Of the studies with larger sample sizes, Steiner et al. reported a trend (p=0.06) toward differences in AMH levels across BMI groups involving a total of 750 women in underweight, lean, overweight, and obese classes (Steiner et al. 2017). In the case of groups of interest to this review, AMH levels were 29.5% lower in 114 obese women with regular cycles and no history of infertility compared to 461 lean women with similar reproductive health histories. The study was designed to assess any association between the biomarkers of ovarian reserve and time to natural conception in a group of late reproductive age women (30-44 years) in which rigorous approaches were used to exclude known fertility problems, ovaries disorders, and recent hormonal conception use. Ultimately, Steiner and colleagues adjusted their time to pregnancy models for AMH by BMI to reflect obesity as an important covariate.

More recently, in 2020, Peigne et al. reported a new marker of AMH measure, the AMH prohormone index (API) (Peigné et al. 2020). API is an inverse measure of the conversion of proAMH to $AMH_{N,C}$ as the latter form is the only isoform that binds the AMH-receptor complex. API was first introduced as an outcome in 2016 (Pankhurst and Chong 2016) and is thought to be a marker of biologically active AMH. API is calculated by [(proAMH)/(totalAMH)]*100. Sixteen obese control women displayed a significant decreased API of 34.6% compared to the lean group with an API of 39.0% (p<0.001). However, AMH levels between the groups were not statistically significant. Both lean and obese groups of women were those that did not have a history of irregular cycles, hyperandrogenism, abnormal ovaries on ultrasound, use of medication that affected metabolism or ovarian function, or history of significant medical conditions. This study was designed to answer the question, does the relative distribution of AMH isoforms differ from one patient to another depending on their BMI and PCOS status in the serum and the follicular fluid (FF)? The group concluded that API was significantly decreased in the obese controls

compared to the lean controls. However, there was no difference in API across control and PCOS women, so Spearman correlations were performed for all women. A negative association between AMH and BMI was seen (R=-0.557, p<0.001). Further, the group noted that AMH is higher in lean women outside of the follicular fluid compared to their obese counterparts, indicating that the conversion of proAMH into its cleaved isoform likely occurs in extra-ovarian tissues that may be exacerbated in obese women. Ultimately, the authors indicate that using API may help to address new roles of AMH in metabolic disturbances associated with reproductive alterations in obesity.

AMH Assay Variability as the Main Factor Contributing to Inconsistent Reports. Differences in AMH levels between healthy women and women with subfertility have been well documented (Dewailly et al. 2014). This difference is reflected in other markers of ovarian reserve, such as antral follicle counts (Deb et al. 2013). Biological factors, including age, genetic variation within the AMH gene (i.e. *AMH* and *AMH2*)(Gorsic et al. 2019), race and ethnicity (Nelson et al. 2020), and stage of the menstrual cycle (La Marca, Grisendi, and Griesinger 2013) have all been posited to influence AMH production to varying degrees, with some factors garnering more support than others. By contrast, technical factors related to variability in AMH assay performance are an accepted contributor. In our review, seven different assays were used across the 13 included studies. Comparability studies have been performed between some of the assays used but given the large number of AMH assays on the market and the discontinuation of others, not all the assays represented in this review have been evaluated against each other for comparability. Namely, data are available to contrast the performance of the original Beckman Coulter ELISA, Beckman Coulter Gen II ELISA, Roche Elecys Cobas, and the Ansh ELISA. However, data are not available for the DSL ELISA, Immunotech ELISA, or DXI sandwich chemiluminescent immunoassay. Based on the available evidence, AMH levels measured by the different assays can vary drastically (up to 40%) (Melado et al. 2018). This appreciable sample-to-sample variability and the substantial discrepancies in the between-assay conversion factors that have

been proposed suggest assay performance issues. Differential responses to pre-analytical proteolysis, conformational changes of the AMH dimer, or the presence of interfering substances are speculated to play a role (Teede et al. 2018). To that end, Teede and colleagues recently called for an international reference standard for AMH and the more robust independent evaluation of commercial assays in routine use using clinical samples with well-defined sample handling and processing protocols (Teede et al. 2018).

Conclusions

Collectively, our evaluation is consistent with most studies showing comparable AMH levels between obese and lean groups of reproductive-aged women with regular menstrual cycles, wherein women with obesity primarily presented with milder obesity status (Class 1). The findings of this review do not corroborate any negative impact of obesity on AMH. The notion of a negative impact of obesity on AMH should be deemed uncertain at this time, warranting further investigations to address the limitations of current literature. This conclusion is prudent in light of the biological basis for such an impact being largely unknown. Few studies have prospectively evaluated the relationship between obesity and AMH production, and more mechanistic studies are needed to better understand how obesity and/or alterations in metabolic status could regulate AMH. The clinical utility of AMH should be improved by the development of international reference standards for AMH assays. However, immediate issues concerning sample storage and processing could be addressed in the interim to improve assay performance. The inclusion of women across all classes of obesity should also be considered in future research, as current evidence is limited to studies that reported lower levels of AMH in higher obesity classes *per se*, limiting the generalizability of findings. Given the growing prevalence of obesity in reproductive-aged women, improving the predictive power of reproductive health markers remains an important priority. AMH is an integral part of this debate.

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