Age-Related Changes in Major Ovarian Follicle Wave Dynamics: Morphologic and Endocrinologic Characteristics

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ABSTRACT

Context: Reproductive aging results from depletion of the ovarian reserve and is marked by profound changes in hormone production and menstrual cyclicity. Age-related changes in antral follicle count and hormone production are well-documented. However, follicle wave dynamics and associated changes in hormone production have not been evaluated as women age.

Overall Objective: To test the overall hypothesis that major follicle wave dynamics change as women age, and underlie changes in hormone production as women approach menopause.

Materials and Methods: A prospective, observational study was conducted in 58 women of Reproductive Age (RA; 18-35 years; n=27), Advanced Reproductive Age-1 (ARA1; 36-44 years; n=10) and Advanced Reproductive Age-2 (ARA2; 45-55 years; n=21). The numbers and diameters of all follicles $\geq$2mm were recorded ultrasonographically every 2-3 days for 1 interovulatory interval. In a subset of women (RA, n=10; ARA2, n=17) blood samples were collected at each visit and serum was assayed for FSH, LH, estradiol, inhibin A and B, AMH and progesterone concentrations. Changes in AFC, follicle wave dynamics, and hormone production were compared between groups (SPSS v19.0, α=0.05).

Results: The prevalence of Follicular and Luteal Phase Major Waves (FPMWs, LPMWs) was not different between RA, ARA1, and ARA2 groups [FPMW: 27/27(100%), 10/10(100%), 20/21(95%); LPMW: 10/17(37%), 3/10(30) %, 10/21(48%); P>0.050). All FPMWs were ovulatory. One LPMW ovulated at the time of menses in the ARA2 group; all other LPMWs were anovulatory. Dominant follicles in LPMWs emerged earlier (day -6, -2, -2; P=0.049), grew longer (11, 3, 6 days; P=0.005) and developed to a larger diameter (24, 11, 11 mm; P=0.032) in the ARA2 versus ARA1 and RA groups. In both RA and ARA2 groups, peak luteal phase

estradiol concentrations were greater in women with (139.1, 177.8 pg/mL) versus without (78.0, 95.0 ng/mL) LPMWs (P=0.016, 0.074). In the RA group, 5/10 women developed LPMWs in association with higher mean (90.5 versus 53.3 pg/mL, P=0.028) and peak (139.1 versus 78.0 pg/mL, P=0.016) luteal phase estradiol concentrations versus women in the RA group without LPMWs, respectively. In the ARA2 group, LPMWs developed in association with atypical (>200ng/mL) elevations in estradiol concentrations in 4/8 women (50%). In all women (RA and ARA2 combined), the max diameter of the LPMW dominant follicle positively correlated with luteal phase estradiol (r=0.71, P<0.01) and negatively correlated with max luteal phase LH (r=-.52, P<0.05). In the ARA2 group, max LPMW dominant follicle diameter was negatively correlated with mean luteal phase progesterone (r=-.78, P<0.05). Progesterone was lower in women with (9.65 ng/mL) versus without (13.0 ng/mL, P=0.027) LPMWs in the ARA2, but not RA, group. Women in the RA group with LPMWs had a higher mean AFC ≥6mm and inhibin B versus those without LPMWs. Polyovulatory FPMWs (n=3) were detected only in the ARA2 group and were associated with higher maximum follicular phase estradiol production and elevated luteal phase progesterone when polyovulation occurred at ovulation #1 of the IOI (n=2). **Conclusions:** The prevalence of FPMWs and LPMWs did not differ as women age. However, in women of advanced reproductive age, dominant follicles in LPMWs emerged earlier, grew longer and to a larger diameter. Approximately 50% of cases of LPMWs that developed in women of advanced reproductive age were associated with atypically high luteal phase estradiol and decreased progesterone. Women of reproductive age with a higher AFC ≥6mm and inhibin B were more likely to develop LPMWs.

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DEDICATION

In memory of my dear grandma, Katherina Schmidt-Archibald
and
To my mom, Helena Vanden Brink

Thank you for your lessons on unconditional love, resilience, and for believing in me when I did not.

TABLE OF CONTENTS

PERMISSION TO USE i

ABSTRACT ii

ACKNOWLEDGEMENTS iv

DEDICATION v

TABLE OF CONTENTS vi

LIST OF TABLES viii

LIST OF FIGURES ix

LIST OF ABBREVIATIONS xi

1. GENERAL INTRODUCTION 1

2. LITERATURE REVIEW 3
   - 2.1 Human Ovarian Anatomy 3
   - 2.2 The Hypothalamic-Pituitary-Ovarian Axis 4
   - 2.3 Folliculogenesis 6
     - 2.3.1 Pre-antral Follicle Development 7
     - 2.3.2 Early Antral Follicle Growth 8
     - 2.3.3 Late Antral Follicle Growth 8
     - 2.3.4 Dominant Follicle Selection 10
     - 2.3.5 Preovulatory Follicle Growth 15
     - 2.3.6 Ovulation 15
     - 2.3.7 Anovulation 17
     - 2.3.8 Luteal Dynamics 17
   - 2.4 Reproductive Aging 20
     - 2.4.1 The Menopausal Transition 20
     - 2.4.2 The Ovarian Reserve 21
     - 2.4.3 Hormonal Changes 21
       - 2.4.3.1 Ovulatory Cycles 22
       - 2.4.3.2 Anovulatory Cycles 24
     - 2.4.4 Antral Folliculogenesis 25
     - 2.4.5 Clinical Consequences of Reproductive Aging 26
   - 2.5 Ultrasonographic Imaging of Ovarian Function 28
     - 2.5.1 Ultrasound Physics 28
     - 2.5.2 Ovarian Image Characteristics 30
   - 2.6 Immunoassays 31
     - 2.6.1 Radioimmunoassays 31
     - 2.6.2 Enzyme-Linked Immunosorbent Assays 32
     - 2.6.3 Validation 34

3. RATIONALE 35

4. GENERAL OBJECTIVES AND HYPOTHESES 36

5. Age-Related Changes in Major Ovarian Follicular Wave Dynamics: Morphologic Characteristics 38
5.1 Abstract 38
5.2 Introduction 39
5.3 Materials and Methods 43
5.3.1 Study Participants 45
5.3.2 Study Procedures 46
5.3.3 Definitions 48
5.3.4 Statistical Analyses 49
5.4 Results 50
5.4.1 Participant Demographics 50
5.4.2 Follicle Wave Dynamics 51
5.4.2.1 Luteal Phase Major Waves (LPMWs) 54
5.4.2.2 Follicular Phase Major Waves (FPMWs) 55
5.4.3 Antral Follicle Count (AFC) 56
5.5 Discussion 58
5.5.1 Cycle Length 59
5.5.2 Luteal Phase Major Waves (LPMWs) 60
5.5.3 Follicular Phase Major Waves (FPMWs) 61
5.5.4 Antral Follicle Count (AFC) 62
5.5.5 Limitations and Future Directions 63
5.5.6 Summary 65

6. Age-Related Changes in Major Ovarian Follicular Wave Dynamics: Endocrinologic Characteristics 66
6.1 Abstract 66
6.2 Introduction 67
6.3 Materials and Methods 71
6.3.1 Study Participants 71
6.3.2 Study Procedures 72
6.3.3 Definitions 75
6.3.4 Statistical Analyses 75
6.4 Results 76
6.4.1 Participant Demographics 76
6.4.2 Luteal Phase Major Waves (LPMWs) 77
6.4.3 Follicular Phase Major Waves (FPMWs) 83
6.4.4 Hormone Production and Diameter of the LPMW Dominant Follicle 85
6.4.5 Major Waves and Markers of Ovarian Reserve 85
6.5 Discussion 85
6.5.1 Luteal Phase Major Waves (LPMWs) 84
6.5.2 Follicular Phase Major Waves (FPMWs 88
6.5.3 Limitations and Future Directions 89
6.5.4 Clinical Applications 90
6.5.5 Summary 91

7. GENERAL DISCUSSION 93
7.1 Luteal Phase Major Waves 94
7.2 Follicular Phase Major Waves 99
7.3 Major Follicular Wave Dynamics and Menstrual Cyclicity 100
7.4 The Influence of Ovarian Reserve on the Development of LPMWs 101
7.5 Future Directions and Clinical Applications 101

8. GENERAL CONCLUSIONS 105

9. REFERENCES 106

LIST OF TABLES

Table 5.1 Participant Demographics and Cycle Characteristics Compared among Age Groups.....48

Table 5.2 Prevalence of Major Follicle Waves During the IOI.....49

Table 5.3 Characteristics of Luteal Phase Major Waves.....53

Table 5.4 Characteristics of Follicular Phase Major Waves.....54

Table 6.1 Participant Demographics and Cycle Characteristics Compared among Age Groups.....75

Table 6.2 Endocrine Characteristics of Luteal Phase Major Waves.....80

Table 6.3 Endocrine Characteristics of Follicular Phase Major Waves.....82

Table 6.4 Relationship between LPMW Dominant Follicle Diameter and Hormone Concentrations during the Luteal Phase.....84

LIST OF FIGURES

Figure 2.1 Antral folliculogenesis and the corresponding changes in reproductive hormones during 2 waves of follicle development. Two waves of follicle development occur in approximately 68% of reproductive-aged women. One full menstrual cycle (i.e., the follicular phase and luteal phase) is shown. (Redrawn from Baerwald, et al., 2003)(33).....14

Figure 5.1 Examples of follicular profiles from participants in the RA (a,b), ARA1 (c,d) and ARA2 (e,f) groups. Data are centralized to day of first ovulation (day 0=1st ovulation, ov = ovulation). The largest 3 ndividually-identified follicles in each wave are shown (as determined using the ID Method). The solid bar along the x-axis represents the days when the CL was visualized. The solid vertical line represents the first day of menses.....50

Figure 5.2 An example of a follicle growth profile from a woman in the ARA2 group. The IOI was 17 days long. An ovulatory LPMW was detected. No FPMW was observed. Data are centralized to day of first ovulation (day 0=1st ovulation, ov = ovulation). The solid bar along the x-axis represents the days when the CL was visualized. The solid vertical line represents the first day of menses.....51

Figure 5.3 An example of a follicle growth profile from a woman in the ARA2 group. A lag phase (i.e., period of time in which no follicles $\geq$6mm developed) was detected from ovulation and lasted 38 days. This participant had a 48 day IOI. Data are centralized to day of the first ovulation (ov = ovulation). The solid bar along the x-axis represents the days when the CL was visualized. The solid vertical line represents the first day of menses..... 51

Figure 5.4 Mean AFC in RA, ARA1, ARA2 groups over the entire IOI.....55

Figure 5.5 Mean AFC over the entire IOI in women among the RA, ARA1, and ARA2 groups with versus without LPMWs.....56

Figure 6.1 A-B Growth profiles of the largest 3 follicles (determined using the ID Method) and corresponding hormone profiles during the IOI for individual participants in the RA group. The first day of menses is indicated by a dashed vertical line for each example (top row). Data are centralized to first day of ovulation (0=day of 1st ovulation, x-axes for all graphs, ov= ovulation (top row)). Dominant follicles of the LPMW are indicated by open circles, and dominant follicles of the FPMW are indicated by closed circles on the follicle growth profiles (top row). The days that the CL was detected ultrasonographically are

indicated by a solid bar on the x-axis. In Example A, follicle and endocrine profiles for a single woman without a LPMW are shown. In Example B, profiles for a single woman with a LPMW are shown. The IOI was 30 days in Example A and 25 days in Example B.....78

Figure 6.2 A-D Examples of growth profiles of the largest 3 follicles (determined using the ID Method) and corresponding hormone profiles during the IOI for 4 participants in the ARA2 group. The first day of menses is indicated by a dashed vertical line for each example (top row). Ov=ovulation (top row). 0=day of 1st ovulation (x-axes for all graphs). Dominant follicles of the LPMW are indicated by open circles, and dominant follicles of the FPMW areindicated by closed circles on the follicle growth profiles (top row). The days that the CL was detected ultrasonographically are indicated by a solid bar on the x-axis. Follicle and endocrine profiles for a single woman without a LPMW are shown in Example A. Profilesfor a single woman with 2 dominant follicles developing within an anovulatory LPMW are shown in Example B. A long IOI (40 days) was observed in Example B. Example C includes follicle and endocrine profiles for a second woman in whom 2 dominant follicles also developed within the anovulatory LPMW. In Example C, a short IOI (20 days) was observed. In Example D, the LPMW resulted in ovulation and a 17 day IOI.....79

Figure 6.3 A-C: Differences in mean AFC $\geq$6mm (A), inhibin B (B), and AMH (C) over the IOI in women with versus without LPMWs. Comparisons within each age group are shown. Differences between women with versus without LPMWs are indicated (*).....85

Figure 7.1 A proposed model of the morphologic and endocrinologic changes which occur during reproductive aging. Dashed lines around follicles indicate a pattern of follicle development occurs in some women. A: A 3rd anovulatory major wave occurs in some reproductive aged women (not shown). Modified from Broekmans et al., (167), Soules et al., (176), and Baerwald et al (unpublished). 98

LIST OF ABBREVIATIONS

3ß-HSD	3-ß-Hydroxysteroid Dehydrogenase
AFC	Antral Follicle Count
ALARA	As Low As Reasonably Achievable
AMH	Anti-Mullerian Hormone
ARA1	Advanced Reproductive Age-1
ARA2	Advanced Reproductive Age-2
B-mode	Brightness-Mode
BMI	Body Mass Index
BMP-6	Bone Morphogenetic Protein-6
BMP-15	Bone Morphogenetic Protein-15
cAMP	Cyclic Adenosine Monophosphate
CF	Co-dominant Follicle
CL	Corpus Luteum
DF	Dominant Follicle
E1G	Estrone-3-Glucuronide
E_2	Estradiol
ELISA	Enzyme-Linked Immunosorbent Assays
ERα	Estrogen Receptor α
ERß	Estrogen Receptor ß
FPMW(s)	Follicular Phase Major Wave(s)
FSH	Follicle Stimulating Hormone
GDF-9	Growth Differentiation Factor-9
GnRH	Gonadotropin Releasing Hormone
HAF	Hemorrhagic Anovulatory Follicle
hCG	Human Chorionic Gonadotropin
HPO	Hypothalamic-Pituitary-Ovarian Axis
Hz	Hertz
ID	Identity
IGF-I	Insulin Growth Factor-I
IGF-II	Insulin Growth Factor-II
IGFBP	Insulin Growth Factor Binding Protein
IOI	Interovulatory Interval
IVF	In Vitro Fertilization
kg	Kilogram
LH	Luteinizing Hormone
LP	Luteal Phase
LPDF	Luteal Phase Dominant Follicle
LPMW(s)	Luteal Phase Major Wave(s)
LUF(s)	Luteinised Unruptured Follicle(s)
m^2	Meters squared
Max	Maximum
MI	Mechanical Index
MHz	Megahertz

mIU	Milli-International Unit
mL	Milliliter
mm	Millimeter
mRNA	Messenger Ribonucleic Acid
MT	Menopausal Transition
ng	Nanogram
NonID	Non-Identified
ov	Ovulation
pg	Picogram
$PGF_{2\alpha}$	Prostaglandin-$F_{2\alpha}$
RA	Reproductive Age
RIA	Radioimmunoassay
rpm	Rotations Per Minute
SPSS	Statistical Package for the Social Sciences
STRAW	Stages of Reproductive Aging Workshop
TI	Thermal Index
TSH	Thyroid Stimulating Hormone
VEGF	Vascular Endothelial Growth Factor

Chapter 1

GENERAL INTRODUCTION

The Menopausal Transition (MT) is a period of marked physiologic changes leading up to menopause. Women enter the MT in their mid-forties, with the final menstrual period occurring at the mean age of 51 (1, 2). More Canadian women belong to the 45-54 year age group (15.7%) than any other 10 year age group and this trend is increasing (3). Therefore, a large proportion of Canadian women are entering the MT. Reproductive aging in women occurs in association with the depletion of the ovarian oocyte population. Each oocyte is housed within an ovarian follicle. A decline in the number of oocytes is reflected by a reduction in follicles available for cyclic growth and leads to a progressive decline in Anti-Mullerian Hormone (AMH) and inhibin B and subsequent rise in Follicle Stimulating Hormone (FSH) (4, 5). Gradual changes in hormone production and follicle number are well documented during the MT. More recently marked variability in estrogen secretion has been reported in follicular and/or luteal phase (1, 6, 7). The origins of the erratic changes in estrogen production are not known. Unpredictable fluctuations in estrogen production during the MT are speculated to be the underlying cause of unwanted symptom such as hot flashes and night sweats (8). Unwanted symptoms occur in approximately 85% of women during the MT and can have a significant and negative impact on a woman's quality of life (2, 9). It is plausible that fluctuations in hormone production during the MT originate from changes in follicular dynamics (10). However, follicular dynamics during the transition to menopause over the luteal and follicular phases have not been well studied.

We characterized and compared ovarian follicular dynamics and corresponding hormone secretion in women of reproductive age versus women of advanced reproductive age (i.e., presumed to reflect those undergoing the MT). Serial changes in major follicle wave dynamics over one interovulatory interval (IOI) were evaluated in a group of women from 18-55 years of age (n=57). Changes in hormone production during the IOI were quantified in a subset of the women (n=27). Our findings have been presented in 2 manuscripts. Morphologic (i.e., ultrasonographic) characteristics of major follicle wave dynamics in women of reproductive and advanced reproductive age are described in the first manuscript (Chapter 5). Corresponding endocrine features of major ovarian follicle wave dynamics are described in the second manuscript (Chapter 6).

We anticipate that a greater understanding of the physiological origins of profound fluctuations in menstrual cyclicity and hormonal production will enable the development of effective strategies to prevent and/or treat unwanted symptoms experienced during the MT.

Chapter 2

LITERATURE REVIEW

2.1 Human Ovarian Anatomy

The ovary is the master gland of the female reproductive system. The two main functions of the ovary are to nurture the development of healthy gametes capable of fertilization and to produce steroid hormones to support the development of a healthy baby (11, 12). The adult ovary is approximately 4 x 2 x 1 cm and oval in shape in the adult woman (13-15). A woman's ovaries are situated bilateral to the uterus inferior to the left and right fallopian tubes and suspended within the pelvis by the suspensory ligament, utero-ovarian ovarian ligament, and broad ligament (13, 16, 17).

The outer connective tissue of the ovary is called the cortex and is covered by the surface epithelium and the tunica albuginea (12, 15). The ovarian medulla is the inner region which contains connective tissue, stromal cells, nerves, vasculature, and lymphatics (11). Growing and regressing ovarian follicles at various stages of development are located in the ovarian cortex (11). Follicles are spherical structures which house the oocytes. Each oocyte is enclosed by one follicle. The temporary hormone producing glands formed from follicles after ovulation (i.e., follicle rupture and oocyte release) are called corpora lutea (corpus luteum, singular) (12). Regressing, non-functional corpora lutea are called corpora albicantia (corpus albicans, singular) (12). Both growing and regressing luteal glands can be visualized in an ovary at any stage of the menstrual cycle.

Blood, lymph, and nerves enter the ovary through the hilus (12). The ovarian artery branches off from the abdominal aorta, is contained in the suspensory ligament, and enters the ovary via the mesovarium of the broad ligament (13, 16). The ovarian veins exit the ovary as the

pampiniform plexus, travel through the mesovarium and suspensory ligament to merge with the inferior vena cava on the right side and renal vein on the left side (13). The ovaries receive sympathetic and parasympathetic innervation (16). Sympathetic innervation originates from the thoracic spinal region, segments 10 and 11, whereas parasympathetic innervation originates from the hypogastric plexuses (16).

2.2 The Hypothalamic-Pituitary-Ovarian Axis

The hypothalamic-pituitary-ovarian axis (HPO) co-ordinates female reproductive function (15). Hormones and growth factors produced from the ovaries and/or anterior pituitary provide positive and/or negative feedback at the level of the pituitary and hypothalamus to regulate hormone production during the menstrual cycle.

Gonadotropin Releasing Hormone (GnRH) is a decapeptide hormone produced in the hypothalamus (18). GnRH is released from neurons in the median eminence into the pituitary portal network (19). GnRH travels to gonadotrope cells in the anterior pituitary where it binds to G-protein coupled receptors on the plasma membrane (19). GnRH is released in a pulsatile manner and stimulates the production and secretion of the gonadotropins Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) (20). Slow GnRH pulse frequencies stimulate FSH synthesis, whereas fast GnRH pulse frequencies stimulate LH synthesis (21, 22). FSH and LH are heterodimeric glycoproteins comprised of a common α subunit and unique ß subunits and are synthesized and secreted by the gonadotropes in the anterior pituitary (23, 24). LH binds to the transmembrane LH receptors located on follicular thecal cells, mural granulosa cells in the later stages of follicle development, and on luteinized thecal and granulosa cells of the corpus luteum (CL) (11, 25-28). LH binds to theca cells at all stages of follicle development to stimulate androgen production (11). LH binds to granulosa cells in large antral follicles (i.e., $\geq$10 mm in

diameter) to stimulate granulosa cell proliferation, growth, and estradiol production (11, 27, 28). Luteinized cells of the CL stimulate progesterone production under LH stimulation (26, 29). LH receptor binding initiates an intracellular cAMP-dependent signalling pathway (11). FSH binds to FSH receptors located on the cell surface of granulosa cells and simulates aromatase activity via the G-protein coupled cAMP-dependent signalling cascade (11, 30-32). FSH is suppressed by inhibin B, follistatin, estradiol, and progesterone (described below) (21). FSH induces the emergence of antral follicle waves during the menstrual cycle (11, 33). FSH increases granulosa cell proliferation and follicle growth (11, 30-32). Insulin Growth Factors (IGF) augment the effects of FSH and LH in the granulosa and thecal cells(34-37). Estradiol is an 18-carbon steroid hormone synthesized from cholesterol. Beginning in antral follicles 6-8 mm aromatase converts the androgens produced in thecal cells to estrogens, mainly estradiol (11, 30-32, 38). Estradiol is produced by the granulosa cells of antral follicles. Estradiol synthesis requires both FSH and LH acting on the granulosa and thecal cells within the follicle, respectively (reviewed in (27, 39)). Estradiol crosses the plasma membrane of its target cells and binds to one of two nuclear receptors (ERα or ERß) (40). Estradiol at low concentrations provides negative feedback to the pituitary which suppressing gonadotropin secretion thereby preventing continued follicle growth in smaller FSH-dependent follicles (27, 41-43). On the contrary, estradiol at high (i.e., preovulatory) concentrations is likely to exert positive feedback. However, the role estrogen in providing positive feedback at the hypothalamus is still uncertain (43).

Progesterone is a 21-carbon steroid hormone synthesized in the luteinized granulosa and thecal cells of the preovulatory follicle and CL (44). Progesterone production is mainly under LH control. Progesterone provides negative feedback to the pituitary, suppressing gonadotropin secretion in women during the luteal phase (20, 45).

Inhibins are peptide hormones and members of the transforming growth factor-ß family. Inhibins are heterodimers which share a common alpha subunit linked to a ß -A (inhibin A) or ß-B (inhibin B) subunit through a disulfide link (46, 47). Inhibin B is produced by antral follicles ranging from 2-12 mm in diameter (48). Inhibin A is produced by dominant follicles (i.e. follicles $\geq$10mm) and the human CL (49). Inhibin A and B suppress FSH secretion (46, 50, 51). Inhibin B production by the granulosa cells is dependent on FSH stimulation which then acts directly on the anterior pituitary to suppress FSH (46, 52).

AMH is a dimeric glycoprotein peptide growth factor and a member of the transforming growth factor-β family. AMH is produced by the granulosa cells in early growing follicles up until a diameter of 8-10 mm (48, 53, 54). Preliminary studies have postulated a role for AMH in antral follicle growth and selection by mediating FSH responsiveness in granulosa cells (55, 56).

2.3 Folliculogenesis

Folliculogenesis is defined as the process of follicle development within the ovaries (38). Folliculogenesis is initiated around the 24th week of embryonic development (57). A resting follicle requires approximately 3 months to achieve preantral status (58). A preantral follicle grows and matures into a preovulatory follicle over another 85 days (58). Thus, the entire process of folliculogenesis occurs over approximately 175 days (11, 58). Descriptions of each of the stages of folliculogenesis are described in more detail below. Preantral and early antral follicle dynamics are not the focus of this thesis and will therefore not be discussed in detail.

2.3.1 Pre-antral Follicle development

Prior to puberty, follicles develop to the early antral stage of development (39). Resting follicles, referred to as primordial follicles, are 35-38 µm in diameter and characterized by a single layer of flattened granulosa cells (59). Resting follicles enter a growth phase through a

process called initiation (59). Initiation of the primordial follicle growth is not well understood; however, there are a number of identified hormones and growth factors involved (38, 60-64). Early growing follicles are divided into primary and secondary follicles. Primary follicles are approximately 46μm in diameter and are characterized by a single layer of cuboidal granulosa cells. At the primary follicle stage, the zona pellucida develops around the oocyte (38). A secondary follicle is approximately 80-100μm in diameter and contains multiple layers of granulosa as well as theca cells. Secondary follicles are the first to receive vascular supply (38, 65). Secondary follicle granulosa cells begin to express FSH, estradiol, and androgen receptors and the differentiated theca interna cells express LH receptors. Gougeon (58, 59, 66) has used histological methods to describe classes of follicle growth. These classes are still accepted today (67). Early growing follicles (i.e., secondary) which have entered Class 1 of development are considered preantral follicles. Preantral follicles in Class 1 are 0.1 – 0.2 mm in diameter and several layers of cuboidal granulosa cells surround the oocyte (59). Class 1 preantral follicles are capable of the LH and FSH induced androgen and estrogen synthesis (59). Preantral follicles are gonadotropin sensitive but are able to grow independently of FSH stimulation (68). Preantral follicle development is mediated by autocrine and paracrine growth factors from the oocytes such as GDF-9 and BMP-15 and AMH from the granulosa cells (15, 62).

2.3.2 Early Antral Follicle Growth

Early antral follicle growth comprises Classes 2 – 4. During this phase, follicles grow from a diameter of 0.2 to 2 mm in diameter. An antrum (i.e., fluid-filled cavity) begins to form from the amalgamation of fluid collections within the follicle (38, 59). The cumulus oophorus complex is comprised of the oocyte surrounded by a layer of specialized granulosa cells called ‘cumulus cells’. The cumulus oophorus can be observed once a follicle has entered Class 2. The

progression from a Class 2 to 4 follicle results in an increase in granulosa cell number approximately 3000 to 750000 (58).

2.3.3 Late Antral Follicle Growth

With the onset of puberty, the hypothalamic-pituitary-ovarian axis becomes activated and gonadotropin-dependent continued follicle development occurs. Antral follicles 2-5 mm in diameter are have been detected throughout the cycle in women of reproductive age (30, 69-71) and are dependent on gonadotropins for continued growth (58, 69, 72). Growing follicles 2-5 mm undergo atresia in the absence of gonadotropin stimulation (30, 58). Atresia occurs via apoptosis, a form of programmed cell death (73). More than 99% of all growing follicles will ultimately undergo atresia while approximately 1% ovulate (57). An increase in FSH of 10-30% above the threshold is required to stimulate antral follicle beyond the gonadotropin-independent stage (72, 74). The magnitude of the absolute rise in FSH is variable between women (2.5 fold in variation) (75). If FSH falls below the minimum concentration of FSH (i.e., the FSH threshold) required for continued development of a follicle it is unable to maintain its growth and undergoes atresia (72). Granulosa cells of 2-5 mm follicles are highly mitotic under FSH stimulation (58, 76). Therefore, a rise in FSH above the follicular threshold leads to the growth of a cohort of follicles referred to as a follicular wave.

Waves of follicle development (also referred to as ‘cyclical recruitment of the follicular cohort’) have been described histologically (77) and ultrasonographically in women 2-3 times during an interovulatory interval (IOI) (33, 70, 78, 79). An IOI is defined as the interval between one ovulation and the subsequent ovulation. The first phase of the IOI is the luteal phase and the second phase of the IOI is the follicular phase. The luteal and follicular phases are separated by menses. Therefore, an IOI is a menstrual cycle that has been shifted by approximately 2 weeks.

A wave of antral follicle development is defined as the synchronous growth of a cohort of antral follicles that occurs at regular intervals during the menstrual or estrous cycle. (33, 70, 78, 79). In women, waves of follicle development have been detected in antral follicles ≥5 mm (70). Waves of follicle development in women are comparable to follicle wave patterns described in domestic farm animals (80, 81). Major waves are those in which a dominant follicle is selected for preferential growth (described below). Minor waves are those in which a dominant follicle is not selected (33). Women with 3 waves of follicle development had a mean IOI length of 29 days, with waves emerging on days 1, 12, and 18 (day 0 = ovulation) (33). The mean IOI length in women with 2 waves was 27 days; wave 1 emerged on day 1 and the ovulatory wave emerged on day 15 (33). Repeatability of wave patterns has not been studied in women. However, repeatability of wave patterns has been shown to exist in 70% in cows (82).

Each wave of follicle development is preceded by a nadir and subsequent rise in serum FSH (33, 83, 84). Granulosa cells of antral follicles in the recruited cohort (i.e., 2-12mm in diameter) produce and secrete inhibin B (48, 50). Inhibin B acts directly on the anterior pituitary to inhibit FSH secretion (50, 75). In this way, the recruited cohort regulates its own growth (discussed below). In addition to inhibin B, AMH has been shown to play a role in follicle wave emergence/ recruitment (85, 86). AMH is detected in primary follicles until approximately 10mm (48). AMH has been shown in vitro to inhibit the responsiveness of human and rodent granulosa cells to FSH by reducing FSH receptor mRNA and aromatase expression and in a dose dependent manner (55, 56, 87). Therefore, AMH is involved in preventing follicles from being recruited into a wave and regulating their aromatase activity (55, 56, 87). Continued research is required to understand the role of AMH in human follicle wave emergence.

The physiologic status of luteal phase wave follicles is not fully elucidated. Results from early studies suggest that the proportion of healthy follicles ≥1 mm is lower in the luteal versus follicular phase (66, 76, 88). The low proportion of functional follicles in the luteal phase was believed to be due to suppression of FSH by the CL since a higher proportion of healthy follicles was observed in the late luteal phase in association with the regressing CL and rising FSH (76). Continued research is required to confirm these preliminary findings.

2.3.4 Dominant Follicle Selection

Major waves of follicle development are those in which a dominant follicle is selected for preferential growth. Selection of a dominant follicle is defined as the preferential growth of one follicle from a wave, while the remaining antral (termed 'subordinate') follicles regress (33, 89, 90). In women of reproductive age, the final wave of each IOI (i.e., in the follicular phase) is an ovulatory major wave. All waves of the IOI that precede the ovulatory wave (i.e., in the follicular or preceding luteal phase) are major or minor anovulatory waves. Anovulatory major waves preceding the ovulatory wave have been detected in 22% of women (33).

The physiologic mechanisms underlying selection have been investigated in both humans and domestic farm animals. The number of follicles selected for preferential growth is dependent upon the amount of time (i.e., window) that FSH remains above the threshold of follicle growth (72, 91). The duration FSH remains above the threshold for follicle growth determines the number of dominant follicles selected, which is referred to as the FSH Threshold/Window theory (72, 74). Selection of the dominant follicle is associated with a fall in FSH.

In ovarian stimulation therapy, exogenous gonadotropins (e.g., recombinant FSH) are administered beyond the normal physiologic fall in FSH. The extended FSH window results in the ability of multiple follicles to achieve dominance and become selected (72). FSH, in

association with IGF, stimulates aromatase activity in granulosa cells of the dominant follicle (31, 53, 92). FSH is unable to stimulate aromatase activity prior to this stage, attributed in part to the inhibitory effects of epidermal growth factor and AMH (56, 93).

Estradiol synthesis by the physiologically selected follicle is preceded by LH-stimulated androgen production in theca cells. Aromatization of androgens to estradiol within growing follicle increases local and systemic estradiol concentrations (31, 53, 94, 95). Estradiol produced by the dominant follicle also stimulates proliferation and development of the endometrial lining in preparation for ovulation and subsequent conception each cycle (39). Increased aromatase activity in the dominant follicle induces LH receptors on the granulosa cells (28, 96). Thus, the selected follicle switches from FSH to LH dependent growth (97-99). Estradiol and inhibin B production from the dominant follicle provide negative feedback to the anterior pituitary which suppresses FSH (100, 101). Thus, selection occurs in association with a decrease in FSH (102, 103). Follicle development shifts from inhibin B/activin to inhibin A/follistatin dependence at the time of selection (49, 50, 104). Subordinate follicles under the dominant follicle are unable to continue growth in an environment of declining FSH (and have not developed LH receptors) and therefore succumb to atresia (101, 102). The term ‘deviation’ has been defined as the point at which the growth trajectories of the dominant and subordinate follicles diverge (90).

Two different theories exist regarding the role of FSH in the selection process. Studies in domestic farm animals have shown that the future ovulatory follicle had an early size advantage over all other follicles of the wave (81, 90). The first theory proposed that the largest follicle at the time of wave emergence may have the greatest number of granulosa cells and is therefore most capable of responding to FSH and aromatase; thus, it becomes selected. According to a

second theory, the follicle with the lowest FSH threshold is thought to be the first to initiate growth, induce LH receptors, and be selected for preferential growth (67, 72, 74, 94).

Future dominant follicles have higher intra-follicular estradiol and IGF-II concentrations at the time of selection (30, 92, 105). Low Insulin Growth Factor Binding Proteins (IGFBP) results in increased IGF-II bioavailability which further stimulates aromatase activity. An inverse relationship between follicular estradiol and AMH production has been documented at the time of selection (48, 54, 85, 87, 106). AMH concentrations decline in antral follicles becoming undetectable in follicles >10mm (i.e., at selection) and has been recently shown to inhibit FSH-induced aromatase expression in human granulosa cells in vitro (48, 54, 56). However, the precise mechanisms and significance of this relationship are not understood.

Selection of a dominant follicle in a major wave occurs during the mid-follicular phase of each menstrual cycle in all women and during the luteal phase in 22% of women (33). The reasons why some, but not all, women develop major follicle waves in the luteal phase are not understood. The role of the CL in influencing the development of luteal phase waves has been investigated (107). No differences in luteal phase length, progesterone, or luteal estradiol production women with versus without luteal phase major waves were detected (33, 107). In contrast, early luteolysis in cows is thought to be responsible for the development of 2 versus 3 follicular major waves during the IOI (108). Cows with 3 waves have a longer inter-wave interval versus cows with 2 major waves (108). Luteal regression occurred later in cows with 3 waves luteal regression occurred after the second wave had regressed and 3 days after the third and final (ovulatory) major wave emerged (108). Consequently, in cows with 3 waves, the ovulatory follicle grew for a shorter period of time and ovulated at a smaller diameter verses cows with 2 waves (108). In women, the day of selection was not different in luteal versus

follicular phase major waves and occurred 3 days after wave emergence (i.e., at 4-5mm) (33). However, all waves that developed in the luteal phase were anovulatory presumably due to the absence of an LH surge (10, 20). In the presence of progesterone, estradiol slows GnRH and therefore LH pulse frequency thereby preventing ovulation (10, 20, 98). Although no dominant follicles in the luteal phase ovulated, it is plausible that they have the capacity to ovulate. Luteal phase antral follicles which have acquired the ability to synthesize estradiol in vitro have been detected (31). From a clinical perspective, the initiation of ovarian stimulation in the early luteal phase in patients undergoing In Vitro Fertilization (IVF) has resulted in the retrieval of oocytes and corresponding embryos (109). Therefore, healthy follicles exist in the luteal phase and can be stimulated for continued growth in the presence of a CL. From additional studies, it has been shown that ultrasonographic image attributes of dominant follicles from ovulatory waves are different compared to dominant follicles from anovulatory waves. (110) The clinical significance of the differences in image attributes, however, requires further investigation. Two and three waves of antral folliculogenesis during the human menstrual cycle are shown in Figure 2.2.

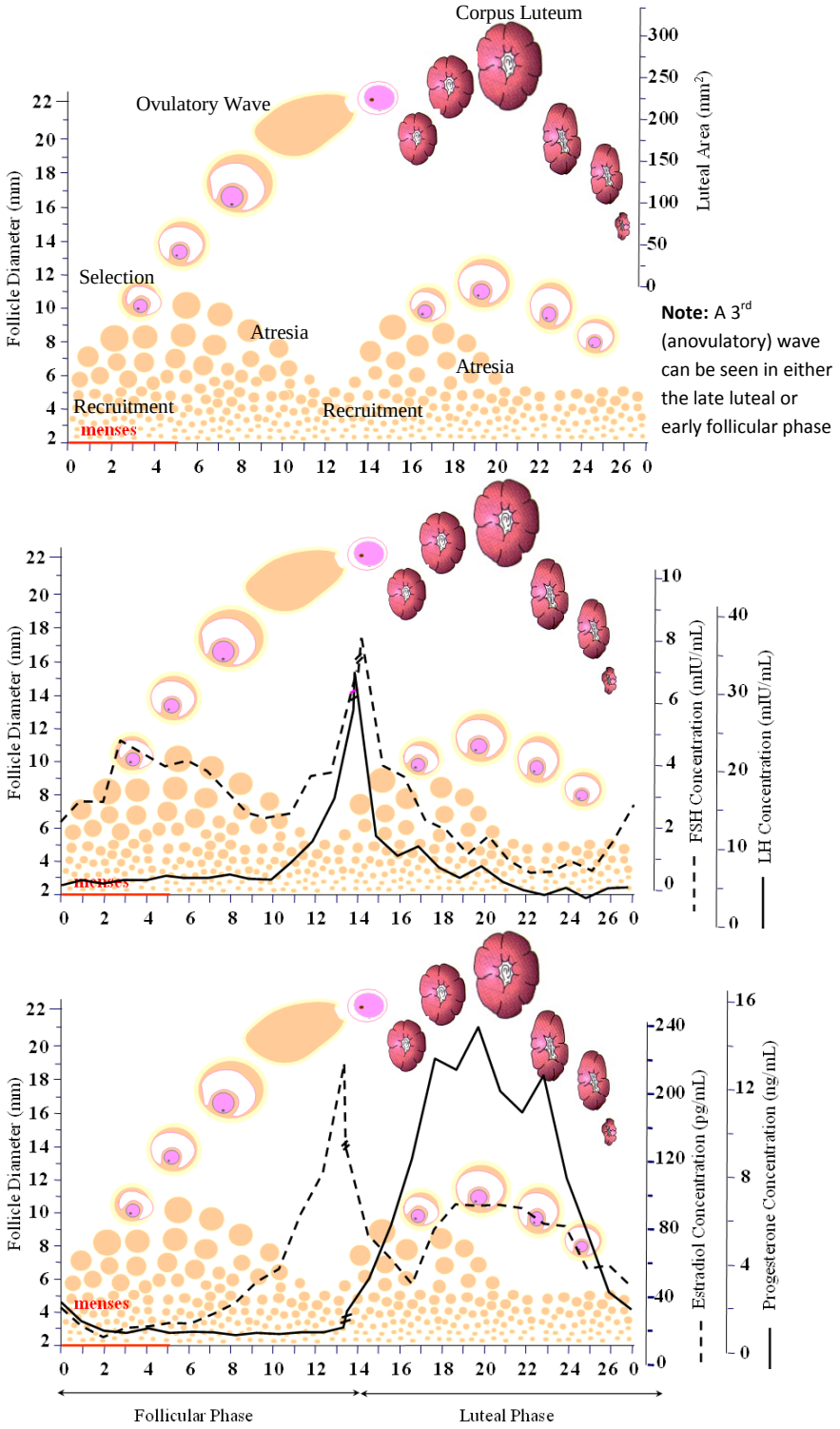

Figure 2.1 Antral folliculogenesis and the corresponding changes in reproductive hormones during 2 waves of follicle development. Two waves of follicle development occur in approximately 68% of reproductive-aged women. One full menstrual cycle (i.e. the follicular phase and luteal phase) is shown. (Redrawn from Baerwald, et al., 2003) (33)

2.3.5 Preovulatory Follicle Growth

The dominant follicle grows at an accelerated rate after selection and reaches a preovulatory diameter of 16-29 mm in the late follicular phase of the cycle (33, 58, 111, 112). Granulosa and thecal cell proliferation continues to occur in association with increased antral volume and (31, 58, 59). Post-selection follicle development is LH dependent and is associated with increasing estradiol production (97-99). Insufficient LH production prevents the dominant follicle from continuing to grow whereas premature luteinisation of the ovulatory follicle and disrupted oocyte maturation may occur if LH is too high (113). After selection of the dominant follicle, estradiol causes an increase in LH pulse frequency which further drives aromatase activity (20). Estradiol production is positively correlated with follicle diameter whereas the androgens are negatively correlated with follicle diameter, (31, 32, 53, 75). The pre-ovulatory follicle produces >90% of estradiol in the late follicular phase (114). In addition to LH, continued growth of the preovulatory follicle depends on inhibin A from the follicle and other growth factors such as GDF-9, BMP-15, and BMP-6 from the oocyte (31, 104, 115). Inhibin A is produced by the dominant follicle and has been shown to increase LH-induced androgen production in the thecal cells (51). The persistently elevated serum estradiol concentration triggers an LH surge from the anterior pituitary (88, 116). The LH surge induces a re-initiation of oocyte meiosis as well as final maturation and ovulation of the dominant follicle.

2.3.6 Ovulation

Ovulation is comprised of a series of morphologic, endocrinologic, and biochemical changes that culminate in the release of the oocyte. The LH surge inhibits granulosa cell proliferation (98) and granulosa cells develop lipid droplets in the cytoplasm-evidence of increased steroidogenic activity (117). Granulosa cells enlarge, become disorganized within the

follicle wall, and begin to produce progesterone leading up to ovulation (66, 88). A sharp decline in estradiol and increase in progesterone production occurs at the time of the LH surge and work collectively to induce enzymes and proteases which degrade the follicle wall (117). The surface epithelium of the follicle sloughs off from the degrading tunica albuginea following the surge (117). The cumulus oocyte complex undergoes expansion and is released from the follicle wall into the follicular fluid (117). Granulosa cells dissociate from each other and the follicle wall and are also sloughed off into the follicular fluid (117). The LH surge also initiates an inflammatory signalling cascade (117). The LH surge stimulates Prostaglandin E_2 and $F_{2\alpha}$ synthesis in the granulosa cells which play an important role in ovulation by increasing blood flow to the preovulatory follicle, reducing blood flow to the apex of follicle rupture, and contributing to COC expansion (117-120). Increased white blood cells from the inflammatory (i.e., increased local histamines) response induce vasodilation of the capillaries surrounding the ovulatory follicle (26). Increased blood flow from the surrounding capillaries increases hydrostatic pressure in the capillaries surrounding the preovulatory follicle (118). Proteolytic enzymes degrade the follicle wall such that the hydrostatic pressure in the follicles overcomes the weakened tunica albuginea thus causing follicle rupture (118). Real time ultrasonographic observations have shown that expulsion of 70% of follicular fluid occurs in 0.9 ± 0.3 minutes and the remaining evaluation occurs during an additional 6 minutes (121). The COC complex is released into the peritoneal cavity where it is taken up by the fimbriae into the fallopian tube.

2.3.7 Anovulation

Anovulation occurs in approximately 10% of natural cycles, with an increasing prevalence as women approach menopause (1, 6, 122, 123). Three types of anovulatory follicles have been documented ultrasonographically: 1) simple anovulatory follicles; 2) haemorrhagic

anovulatory follicles (HAFs); and, 3) luteinised unruptured follicles (LUFs) (118, 124). Simple anovulatory follicles can grow beyond a preovulatory follicle diameter and form a cyst. Simple anovulatory follicles do not luteinize or haemorrhage and are characterized by thin walls and an anechoic follicular antrum (124). A HAF is a dominant follicle which undergoes continued growth and internal haemorrhaging after failing to ovulate (118, 125-127). HAFs are not associated with endocrine or ultrasonographic evidence of ovulation (124). The LUF syndrome has been defined as the lack of rupture of a preovulatory follicle, with luteinisation of the thecal and granulosa cells (118, 128, 129). Haemorrhage may or may not occur (130). Fibrous strands in the antrum are typical of hemorrhagic follicles (HAFs) beginning to clot. In contrast to a HAF, thickened walls are indicative of luteinisation of the LUF (118). LUFs have been better characterized in women and have been considered a cause of infertility (123, 131). LUFs are associated with a normal or blunted LH surge, and a delayed increase in serum progesterone (132, 133). A LUF is believed to develop as a consequence of one of the following events: 1) the follicle's inability to respond to the LH surge (133); 2) an insufficient LH surge (118, 132, 134, 135); 3) poorly timed human chorionic gonadotropin (hCG) dosing in ovulation induction (132); 4) insufficient induction of LH receptors (136); 5) NSAID use which prevents the inflammatory process involved in ovulation (137); and/or, 6) stress (138).

2.3.8 Luteal Dynamics

The Corpus Luteum (CL) is a temporary endocrine gland that develops from the preovulatory follicle following ovulation. Haemorrhage into the cavity of the CL occurs in approximately 50% of ovulations (118). The CL with internal haemorrhage forming a cystic cavity is called a corpus haemorrhagicum. The presence of a central cystic cavity does not affect luteal function (139).

Luteal development, referred to as ‘luteinisation’, is a time of significant growth, hypertrophy, and differentiation (29). During luteinisation, granulosa cells of the former follicle are transformed into granulosa lutein cells (also referred to as ‘large cells’). Similarly, the theca cells of the follicle develop into theca lutein cells (‘small cells’) (140). In humans, the two cell types remain segregated in the CL (29). Luteinized granulosa and thecal cells gain the ability to convert pregnenolone to progesterone through activation of the 3ß-HSD enzyme (44). The granulosa-lutein cells expand up to 10 times their pre-luteal size and are the primary source of progesterone from the CL (29, 140). Luteinized thecal and granulosa cells also retain their abilities to synthesize androgens and estradiol, respectively (44). Angiogenesis represents a significant portion of luteal growth forming a dense network of capillaries in the CL (29). Steroid hormone transport from the thecal and granulosa lutein cells occurs from the dense vascular network in the CL (29, 44). The primary function of the CL is to synthesize and secrete progesterone which acts on the endometrial lining of the uterus to provide an environment conducive for implantation and development of an embryo. Cholesterol is delivered to the CL by high and low density lipoproteins for steroid hormone production (44). Estradiol suppresses LH secretion in the presence of progesterone. Inhibin A also is a product of the CL; however, the role of inhibin A is not well understood (29, 50).

Luteinisation is regulated by autocrine, paracrine, and endocrine factors. The oocyte plays an important role in preventing premature luteinisation (44). Prostaglandin E_2 is produced by the large cells of the primate CL and is important in the development and maintenance of the CL (141). Other regulatory factors of CL formation include LH, VEGF, IGF, BMP and cyclins (142-144). Progesterone production, luteal diameter, and vascular development peak in the CL 6-7 days after ovulation (107). Luteolysis is initiated approximately 7 days after ovulation, in

association with a decline in production of estradiol and progesterone. Human chorionic gonadotropin (hCG) is produced by the conceptus and is an endocrine signal that implantation has occurred (144). Progesterone production by the CL continues when hCG is produced. In the absence of conception and hCG production the CL begins to regress during a process referred to as 'luteolysis. Prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$) is the main signal for luteolysis. In women, luteolysis is an ovary-mediated event (145). $PGF_{2\alpha}$ is produced by the CL reduces blood flow to the CL and disrupts the production of progesterone thereby inducing functional luteolysis (141, 144, 146). Endothelin-1 is a vasoconstrictor and is involved in inhibiting steroidogenesis and inducing luteal regression in concert with $PGF_{2\alpha}$ (144, 147). Structural luteolysis, or involution of the CL, occurs via apoptosis (146). $PGF_{2\alpha}$ is the main signal identified to trigger apoptotic signalling cascades (146).

The fall in progesterone and estradiol and increase in $PGF_{2\alpha}$ cause vasoconstriction and atrophy of the endometrial blood supply (148). Reduced progesterone signals the release of lysosomal, proteolytic, and fibrinolytic enzymes which break down the stratum functionalis of the endometrium (148). Collectively, the lack of continued blood supply and degradation of the lining results in the shedding of the stratum functionalis which is referred to as menstruation (148). The fall in progesterone and estradiol results in an increase in FSH production from the anterior pituitary, signalling emergence of the next follicular wave (20, 98).

2.4 Reproductive Aging

2.4.1 The Menopausal Transition

The menopausal transition (MT) represents the 4-7 years in a woman's life leading up to reproductive senescence (2, 122). The MT begins at the age of 45-47 and culminates in menopause at an average age of 51 years (77, 149, 150). The first detection of changes in menstrual cyclicity is the first noticeable sign of entry into the MT (122). Typically, a shortening of the menstrual cycle is the first detected in the MT and is due to a shortened follicular phase (122, 151-153). As the MT progresses, increased variability in menstrual cyclicity occurs with eventual lengthening of the menstrual cycle due to an increasing incidence of delayed ovulation and anovulatory cycles (6, 122, 150, 154-158). The current understanding of changes in ovarian physiology as women approach menopause is based primarily on endocrine investigations with limited research on follicular development. Further research to characterize age-related changes in folliculogenesis will provide valuable insight into changes in hormone production and menstrual cyclicity that occur with advancing reproductive age.

2.4.2 The Ovarian Reserve

Reproductive aging occurs as a result of the depletion of the ovarian reserve (77, 149, 159, 160). The maximum complement of ovarian follicles (i.e., approximately 7 million) is attained at approximately 20 weeks gestation (11). By the 24^{th} week of gestation, germ cell production ends and the oogonia within each primordial follicle are arrested at the prophase stage of meiosis (11, 57). At birth, each ovary contains between 250,000 and 500,000 resting (non-growing) follicles (161, 162). The total number of oocytes is reflected by the number of ovarian follicles and is referred to as the ovarian reserve. The ovarian reserve declines continuously until menopause, when there are approximately 1000 non-growing follicles per ovary (160, 161).

Depletion of the ovarian reserve occurs as a result of both initiation of follicular growth and follicle atresia (11).

Recently, investigators have shown that the depletion of the ovarian reserve occurs as a gradual acceleration in the rate follicle depletion (163, 164). This contrasts with the previously accepted theory in which an abrupt increase in the rate of acceleration of follicle loss occurred at 37-38 years of age (4, 161, 164). In addition to a reduction in the ovarian reserve, the risk of chromosomal abnormalities in oocytes increases as women age (165, 166). Thus, both the quality and quantity of the ovarian reserve declines with reproductive aging (167).

2.4.3 Hormonal Changes

The earliest detectable endocrine change during the MT is a decline in AMH which occurs in association with a shortened menstrual cycle (5). AMH is produced by granulosa cells, and is expressed from initiation of follicle growth until antral follicles achieve a diameter of approximately 8-10 mm (48, 54). AMH is correlated with the ovarian reserve because it is produced by all growing follicles <10mm (4). The next endocrinologic change characterized in women of advanced reproductive age is a fall in inhibin B and rise in FSH (5). The loss of inhibin B from the antral follicle wave results in a rise in FSH secretion from the anterior pituitary (48, 50, 75, 168-174). The monotropic rise in FSH due to the decline in inhibin B supports the theory that the decline in ovarian follicle number is the origin of reproductive aging in women (reviewed in (175)). An alternative notion to the ovarian origin theory is that an age-related slowing of the GnRH pulse generator in the hypothalamus results in a rise in basal FSH. The latter theory of the origins reproductive aging has been predominantly supported in animal models (reviewed in (176, 177)). Evidence of hypothalamic/pituitary aging in women has been demonstrated in the later stages of reproductive life (158, 178-180). The rise in FSH is detected

in some, but not all, studies before the onset of menstrual cycle irregularity (169, 181-183). To date, some evidence exists to support a hypothalamic origin of reproductive aging, however the largest body of scientific research conducted in women supports the theory of an ovarian origin of reproductive aging.

2.4.3.1 Ovulatory Cycles

Ovulatory cycles occur in decreasing frequency throughout the transition to menopause (5, 6, 184). As the transition progresses, increased variability and lengthening of the menstrual cycle is documented in association with falling inhibin B, AMH, and rising FSH (5, 150, 154, 156-158, 185-187). An overall gradual but marked decline in follicular phase estrogen is documented as women approach menopause---the fall in estrogen is one of the last endocrine changes to occur prior to menopause (188). Luteal progesterone production is generally thought to decline in women undergoing the MT with ovulatory cycles of (5, 6, 155, 182, 189). Although LH concentrations rise gradually as reproductive senescence approaches, intra-cycle variability in LH concentrations has also been documented (discussed below) (190, 191).

The earliest changes associated with reproductive aging in most women are a modest shortening of cycle length in association with a decline in AMH, inhibin B, and rise in FSH (5, 192). The shortened menstrual cycles result from a shortening of the follicular phase (153, 192, 193) which, in turn, has been attributed to either earlier (153, 193, 194) or accelerated follicle development (175, 192). Shifted ovulatory follicle dynamics were attributed to the monotropic rise in FSH and consequently an earlier rise in estrogen (153, 172, 192, 193, 195). Elevated mid-cycle estrogen during the MT has been shown to be associated with the development and ovulation of multiple dominant follicles (182, 194, 196).

Atypically high estrogen (>20ng/mL of the urinary estradiol metabolite EIG) in the early follicular phase has been associated with suppressed FSH and short (19 days) inter-menstrual intervals (155). A more detailed characterization of hormone production during the MT has revealed atypically high estrogen in the late luteal and early follicular phases of the cycle (10). The luteal phase pattern of secretion, termed a Luteal-Out-Of-Phase event (LOOP), was marked by a secondary acute rise in estrogen (i.e., >900 pmol/L), which began in the mid-luteal phase and lasted into the early follicular phase (10). Ovulation occurred in the early follicular phase of half (3/6) of the women with a LOOP event by recording an increase in serum progesterone (10). The origins of the acute increases in luteal phase estrogen are not known. However, it is speculated that major waves of antral follicle development may be responsible for the atypical estrogen fluctuations (10).

In women of advanced reproductive age with regular ovulatory/menstrual cycles, progesterone was normal (191, 195) or elevated in association with lower luteal LH (153). Increased progesterone in the luteal phase of women undergoing the MT may be explained by an increased incidence of polyovulation; however, notion this has not been directly investigated. An increase in cycle length (the interval between subsequent menses) during the MT is associated with anovulation and lag phases (6, 10, 197). Lag phases (10) are also referred to as delayed ovulations (155) or inactive phases (198, 199) and are defined as a period of time with no evidence of ovarian activity during an ovulatory cycle (6). Lag phases are comprised of elevated FSH and LH and low estrogen throughout the follicular phase (1, 10, 181, 198-200). The end of a lag phase is demarcated by a rise in estrogen (155, 198). Ovulatory cycles with lag phases preceding ovulation have also been associated with subsequently low progesterone and high estrogen in the luteal phase (10, 155, 200). Low luteal phase progesterone has been attributed to

either insufficient luteinisation (155) or luteolytic effect of estrogen (10). Ultrasonographic characterizations of follicle and luteal growth during ovulatory cycles with lag phases have not yet been conducted in women. Therefore, it is not known whether lag phases occur due to a lack of recruitable follicles or the inability of the antral cohort to respond to the elevated FSH.

2.4.3.2 Anovulatory Cycles

The prevalence of anovulation is more closely associated with the stage of reproductive aging than with chronologic age. Anovulation occurs more frequently in the late versus early stages of the MT (5, 197, 201). Anovulatory cycles consist of either short (i.e., <21 days) or long (i.e., >35 days) inter-menstrual intervals (10, 155, 202, 203) that occur in the presence of elevated gonadotropins (1, 5, 10, 155, 180, 201). The hormonal characteristics of three types of anovulatory cycles have been described (158, 180). The first type of anovulatory cycle is characterized by a normal rise in follicular phase estrogen followed by an LH surge but no evidence of luteal function (10, 158, 180). This type of anovulatory cycle reflects the insensitivity of the preovulatory follicle to the LH surge (158). The second type of anovulatory event occurs when there is normal rise in follicular phase estrogen without evidence of an LH surge or luteal function (10, 155, 158, 180). This type of anovulatory cycle is presumed to reflect the inability of elevated concentrations of dominant follicle estrogen to stimulate an LH surge (158) or pituitary desensitization to estrogen (178-180). The third anovulatory cycle type is characterized by low estrogen concentrations and elevated FSH and LH (5, 158, 180, 201). Cycle length and estrogen production have been shown to be more variable in anovulatory versus ovulatory cycles (199). To date, most studies have characterized anovulation using endocrine assays or by recording the length of the inter-menstrual interval and the occurrence of irregular

bleeding patterns. Follicle growth dynamics during anovulatory cycles in women have not been reported.

2.4.4 Antral Folliculogenesis

There is a well documented decline in AFC in association with reproductive aging (204-208). Early emergence (153, 193, 194), and both accelerated (195) and normal (153) preovulatory follicle growth early in the MT have been reported from limited ultrasonographic data. Both normal (193, 195) and smaller preovulatory follicle diameters (153, 194) have also been reported. The likelihood of multiple dominant follicle growth increases as women age (194). The development of multiple dominant follicles in the follicular phase is believed to result in the age-related increase in dizygotic twinning, however an increased prevalence of polyovulatory cycles has not been characterized ultrasonographically (196, 209). Age-related changes in follicular dynamics have also been observed using animals models. Similar to studies conducted in women, early emergence, slowed growth and smaller peak diameter of the preovulatory follicle have been observed in middle aged-mares (210). As reproductive aging in mares continues, cycle lengthening occurs due to delayed emergence of the ovulatory wave (210, 211). In cows, no change in follicle wave dynamics was observed with age despite a decline in oocyte competence, AFC, and increased FSH (212, 213).

2.4.5 Clinical Consequences of Reproductive Aging

Reproductive aging occurs in association with the depletion in the number of follicles in a woman's ovaries. The ovarian reserve is associated with a woman's fertility potential otherwise known as the capacity or potential to conceive. The ovarian reserve is used as a clinical marker of her proximity to menopause and loss of the ability to conceive, regardless of age (160, 162, 214). Direct measurement of the ovarian reserve is not practical in the clinical setting where

measurements need to be made non-invasively. Therefore, indirect markers of ovarian reserve have been developed. The Antral Follicle Count (AFC) has been defined as the number of follicles 2-5mm (11, 162, 215), 2-9 (216), or 2-10 mm (204, 207, 217) detected ultrasonographically. The AFC correlates positively with the size of the ovarian reserve (4, 208). Both low (218) and high (219, 220) inter-observer variability has been reported when recording the AFC. Thus, efforts to standardize the method to determine AFC have been made (221). High inter-cycle (222), intra-cycle (33) and inter-woman (215) variability in AFC has further been reported which emphasizes the need for standardized criteria for interpreting clinical AFC measurements. AMH has been shown to decline with age and thereby strongly correlates with the ovarian reserve and AFC (4, 223, 224). It has been generally thought that AMH showed little intra- and inter-cycle variability and was therefore a more reliable tool than AFC for predicting reproductive status (225, 226). However, more recently investigators have demonstrated considerable intra-cycle variability in AMH in women of reproductive age (20-32 years old) (227) and late reproductive age (45-55 years old) (226). The origins of the underlying changes in AMH are not understood. To date, AFC and AMH are thought to be the best predictors of ovarian reserve (62, 208, 224, 228).

Inhibin B is also produced by the antral follicles available for cyclic emergence into a wave (48, 70). Inhibin B declines in women approaching and entering into the MT (172). Inhibin B has been shown to independently predict early follicular phase FSH as a function of age in women 40-50 years old and to a lesser extent 20-50 (when assessed between days 3 to 5) (172). However, the fall in inhibin B has only been consistently detected in women undergoing the MT therefore inhibin B is not a reliable predicator of entry into the MT (72, 172, 224).

FSH was the first hormone to be measured clinically as a marker of reproductive aging (181, 189). FSH increases as a result of the decrease in inhibin B with the depleting ovarian reserve and concomitant AFC (188, 195, 229). The rise in FSH has been detected by some investigators before the onset of menstrual irregularity (182, 183). However, other investigators have reported a poor correlation between FSH and age as FSH began to increase only in women >40 or in the late MT (i.e. periods of amenorrhea of 3-11 months) (72, 172, 224). Inter-cycle, intra-cycle, and inter woman variability in FSH secretion exists (72, 197, 229, 230). Between-woman variance in early follicular phase FSH has been shown to comprise most (65-97%) of the total variance by age (229). Age, ethnicity, BMI, as well as the numbers and growth dynamics of follciles and corresponding estrogen concentrations have been shown to affect serum FSH concentrations in women as they transition to menopause (33, 231, 232). Therefore, FSH alone is not recommended as a reliable marker for ovarian reserve (230-232).

The Stages of Reproductive Aging Workshops (STRAW) were held in 2001 and again in 2011 to develop a staging system to provide a standardized set of criteria for the clinical and scientific evaluation of reproductive aging in women (122, 183). The STRAW criteria are based on changes in menstrual cyclicity, hormone production and AFC that occur in women throughout the reproductive lifespan independent of age (122).

The origins of the changing hormone production during progression through the STRAW stages are not known. It is possible that the previously-reported fluctuations in steroid hormone production during the MT may be due to aberrant follicle wave dynamics. It has been further proposed that atypical and acute changes in hormone production during the MT may be associated with the development of unwanted symptoms such as vasomotor symptoms (i.e., hot flashes, vaginal dryness, night sweats) and associated symptoms of fatigue and alterations in

mood. Research in this area is important for developing evidence-based, safe, and effective treatments to alleviate symptoms reported in women undergoing the MT.

2.5. Ultrasonographic Imaging of Ovarian Function

2.5.1 Ultrasound Physics

Ultrasound waves are longitudinal mechanical sound waves. The frequency of ultrasound waves (>20,000 kHz) is greater than the frequency of audible sound (20-20,000 kHz) (233). Sound waves are generated when an electric charge is applied to a crystal with natural or synthetic piezoelectric properties (233). Ultrasound waves transmitted from the crystals propagate through a medium (233). The frequency (the number of cycles per second, measured in hertz (Hz)) at which sound waves travel is dependent on the properties of the piezoelectric crystal (233).

The energy (amplitude and intensity) of a sound wave becomes lost (i.e., attenuated) as sound waves propagate through a medium (233). A high frequency transducer provides better resolution of structures in close proximity to the transducer; however, a high frequency attenuates very quickly and limits spatial resolution of deeper structures (14). Lower frequency transducers permit visualization of deeper structures---the longer wavelength that occurs as a consequence of using lower frequency transducers limits the spatial (i.e., axial) resolution of a structure (233).

Ultrasonography enables the real-time non-invasive visualization of stationary and moving structures (234). Ultrasound has been used transvaginally and transabdominally for clinical and research purposes to visualize structure and function (14, 70). Transvaginal ultrasonography is the most commonly-used clinical and research tool for imaging the female reproductive system. Transvaginal transducers allow the piezoelectric crystals to be placed in

close proximity to the ovaries and uterus. Transducers are shaped and designed to optimize the field of view of interest (14). Selection of a higher frequency curvilinear array transducer (i.e., 6-9MHz) allows for a wide field of view which is necessary to visualize the reproductive tract with good spatial resolution (14).

Transducers emit timed pulses of ultrasound waves (233). The degree of difference between the acoustic impedances of the first medium and the reflector medium determines how much of the sound wave is transmitted, refracted, or reflected (233). Scattering of the sound wave occurs uniformly in all directions when the wavelength is larger than the reflector (233). The returning echoes are weak since only a small portion of the transmitted wave is returned back to the transducer (233). Sound waves reflect at the angle equal to the angle of incidence when the sound wavelength is smaller than the reflector (233). Thus, the strength of the returning echo is dependent on the angle of transmission. When there is a difference in the acoustic impedance between 2 media transmission of some of the wave occurs and bends (233). The bending of a sound wave as it crosses from 1 medium to another is called refraction (233). In B-mode, or brightness-mode ultrasound imaging, the reflected echo is converted to an electronic signal in a digital scan converter. The electronic signal is stored as binary digits which represent the amplitude of the returning signal (235). Most B-mode systems are able to detect 256 different amplitudes and assign the signal a grey-scale value (235). The grey-scale value of the signal is based on the returning amplitude of the sound wave whereas the location of the signal is based on the time travelled (236). Multiple pulses of ultrasonographic sound waves are sent out along the transducer crystal array to obtain a field of view on the display screen (236).

There are no known adverse effects reported using diagnostic ultrasonography at recommended frequencies and intervals (237). Standards are in place to ensure that diagnostic

and research ultrasonography is as safe as possible. Thermal and mechanical indices are used by sonographers indicators of patient safety during an ultrasonographic examination (237). Thermal effects are largely due to absorption of the ultrasound energy causing attenuation of the wave intensity (237). Mechanical energy is converted to heat which may raise the local temperature of the tissues (237). The mechanical index (MI) reflects the likelihood cavitation will occur. Cavitation is the mechanical stress caused by the presence of small bubbles in the medium (237). The thermal index (TI) is the ratio between the power provided to the medium (tissue) and the power required to raise the temperature of the medium (tissue) 1º C (237). The MI and TI are displayed on the output screen so they can be monitored by the ultrasonographer. MI and TI values below 1.0 are considered acceptable for clinical and research purposes in humans (237). The ALARA ('as-low-as-reasonably-acceptable') principle is recommended for conducting ultrasonographic evaluations (237). The ALARA principle requires that ultrasonography be conducted only if necessary and using the lowest power for the required image.

2.5.2 Ovary Image Characteristics

Echotexture refers to the pattern of echogenicity of a tissue. The stromal tissue of the ovary has a low-intensity, 'grainy' echotexture (14). Antral follicles appear as anechoic round structures in the ovarian stroma (13, 14). Ultrasonographic waves propagate through fluid with minimal attenuation or reflection. Therefore, a fluid filled follicle is visualized as an anechoic structure (233). Often, acoustic enhancement is seen just distal to the antral follicle because there is less attenuation as the sound wave propagates through follicular fluid (233). Ultrasonography can be used reliably to detect follicles $\geq$ 4 mm in diameter..

Four types of ultrasonographic descriptions of corpora luteal have been described (139). Corpora lutea are described as having an outer wall of luteinized tissue and an inner central area

when visualized ultrasonographically (238, 239). The outer wall is echogenic, has an irregular shape, and can be distinguished from the ovarian stroma due to its echotexture (238). The outer wall can either be thin (<3mm) or thick (>3mm). The inner central area can be hypo-echoic or hyper-echoic(238). Echotextural features of the CL have been shown to reflect CL functionality (i.e., progesterone production) in women (238) and domestic farm animals (240). A thin-walled CL with a hypo-echoic central region is associated with lower luteal progesterone (238) .

2.6 Immunoassays

The most common method of measuring hormone concentrations in serum is immunoassays. Immunoassays involve using antibodies (or antigens) of known concentrations to bind to the unknown concentration of hormone in the serum. Assays operate on the principle of Law of Mass Action which states that at equilibrium the ratios between the concentrations of bound and unbound hormone (ligand) will be constant. Therefore, given a known quantity of binder (i.e., the antibody), the ratio of bound to free ligand at equilibrium will be quantitatively related to the total amount of ligand.

2.6.1 Radioimmunoassays

Radioimmunoassays (RIAs) use an antibody as the binder and measure antigens or antigenic compounds such as hormones in serum samples (241). In an RIA, a radioactive label is bound to a purified antigen (what is to be measured) to function as the tracer. The basis of the RIA is competitive binding between the tracer antigen and the unknown test antigen or ligand onto antibodies (241). Often the antibody is bound to the sides of a plastic or glass tube (241). When a known concentration of tracer is added to a tube with an unknown amount of ligand in a test sample, the tracer and ligand bind competitively to the antibody (241). Competitive binding will eventually reach equilibrium between the bound and free phases of the tracer and ligand

(241). Standard curves are generated for each RIA and the percent bound tracer is compared to the standard curve to infer the amount of ligand in the sample (241). If a large percentage of the labelled antigen binds to the antibody only a small amount of antigen was present in the sample being assayed; the percent bound tracer and amount of ligand present in the sample are inversely related (241).

2.6.3 Enzyme-Linked Immunosorbent Assays

Enzyme-Linked Immunosorbent Assays (ELISAs) are a subgroup of enzyme immunoassays in which an enzyme is coupled to an antibody, antigen, or hapten such that the immunological and enzymatic activities are retained (241). In an ELISA, the tracer is an enzyme bound to an antibody which contrasts RIA, where there is an identical molecule to the ligand which has a radioactive isotope substituted in or attached to the ligand. An ELISA involves the sequential addition of reagents separated by wash steps to remove any unbound reagents (241, 242). Immunoassays utilize the binding properties of an antigen to its antibodies which can either be antigen specific (monoclonal) or less specific binding to different antigens (polyclonal) (241). The ligand is measured through detection of color after a chromophore reacts with the enzyme (241). Three different forms of competitive ELISAs exist- direct, indirect, and sandwich ELISAs. However, all forms have certain common features: 1) the unknown compound (ligand) is attached to a solid surface; 2) a detection antibody linked to an enzyme is added and washing removes any unbound antibody; and, 3) the amount of bound antibody is quantified by measuring the colour of the bound antigen-antibody-enzyme (242). To quantify the amount of ligand (antigen) the colour change can be read using a spectrophotometer. A spectrophotometer transmits light at a specific wavelength through the fluid in the wells and measures the amount of

absorption of the light by the color of the enzyme which correlates with the amount of unknown ligand (241).

A direct ELISA is the simplest ELISA (242, 243). The ligand (unknown compound or antigen) to be measured is diluted in a buffer and then added to the plate or well (242, 243). The ligand passively adsorbs to the plate surface during the incubation period (242, 243). Uncoated (unbound) antigen is washed away. The conjugated antibody-enzyme tracer is added which binds directly to the antigen adsorbed to the plate or well (242, 243). After incubation, free conjugate is washed away and a substrate (i.e., chromophore) is added (242, 243). The enzyme in the conjugate catalyzes the reaction in the chromophore/substrate solution, yielding color (242, 243). The reaction is stopped and is quantified using a spectrophotometer. Direct ELISAs are most commonly used for immunohistological staining (243).

An indirect ELISA involves addition of the sample with unknown ligand (antigen) to the plate or well. The well is then incubated allowing the antigen can adsorb to the solid phase (242). Unlabelled antibodies are added in a buffer solution which specifically bind to the antigens during an incubation period (242). After another washing step to remove any free antibody, the conjugate (what will be detected / measured) diluted in a buffer solution is added. The mixture incubates to allow the conjugate to specifically bind to the antibodies which are bound to the antigens adsorbed to the solid phase (242). After a wash step, the substrate/chromophore is added, binds to the conjugate, is stopped and the colourmetric reading is completed (242).This test is used to detect the presence of antibodies in sera and is most suitable for diagnostic purposes to screen large numbers of samples (242). A disadvantage to this method is that there can be increased risk of non-specific binding in different sera with the second antibody (244).

A sandwich ELISA begins with the adsorption of antibodies to the solid phase referred to as capture antibodies (242, 243). After a wash, the antigens are diluted in blocking buffer and added to the antibodies in the wells on the plate (242, 243). The antigens attach to the antibodies bound to the solid phase (242, 243). Following another wash, the enzyme-antibody conjugate within the blocking buffer is added and incubated (242, 243). Excess conjugate is washed away and chromophore is added to react with the enzyme (242, 243). The reaction is thereafter stopped and read with a spectrophotometer (242, 243). The disadvantage to this method is that the ligand of interest (or antigen) must have two binding sites, or epitopes, because it must bind to both a capture and tracing antibody (242).

2.6.3 Validation

Determining how well an immunoassay performs is critical and must be identified when reporting results. Sensitivity, range, intra- and inter- assay precision, linearity (assaying samples under different dilutions), recovery (assaying at increasing known ligand solutions), and specificity should be determined and reported for assays used in labs (245-247) . All ELISA results are compared with the RIA results. Correlations between the two results are made with a correlation statistic to determine if there are differences between the values (246). Sensitivity, specificity, and precision are characteristics of all immunoassays that contribute to accuracy (246).

Chapter 3

RATIONALE

The number of women entering the MT is increasing (3). The physiologic changes women undergo as they progress through the MT are not fully understood. The current understanding of the MT is limited to changes in AFC, menstrual cyclicity and hormone production. Profound variability in menstrual cyclicity and hormone production has been observed; however, the physiologic origins of these changes are not understood. Follicles are the functional units of the ovary. Follicles produce hormones which act to regulate cyclic changes in reproductive function throughout life. Therefore, it is plausible that the origins of the profound variability in hormone production can be elucidated by characterizing the changes in follicle dynamics. A greater understanding of the physiologic origins of the changes in hormone production during the MT will enable the development of more effective preventative and therapeutic strategies for minimizing hormonal fluctuations. Acute elevations in estradiol secretion during the MT have been proposed to be associated with unwanted symptoms (in particular vasomotor symptoms) and may also place women at greater risk for estrogen-dependent reproductive cancers. Alleviating unwanted symptoms and health risks associated with atypical hormone production during the MT will ultimately improve the quality of life of women as they age.

Chapter 4

GENERAL OBJECTIVES AND HYPOTHESES

The overall objective of this research was to characterize the age-related changes in major follicular wave dynamics and corresponding changes in reproductive hormone secretion throughout the human menstrual cycle. The first objective was to compare the prevalence and growth dynamics of major follicular waves in women as they age (Chapter 5). The second objective was to characterize and compare hormone production associated with major follicular wave dynamics in women of reproductive versus advanced reproductive age (Chapter 6).

The general hypotheses tested were: 1) changes in major follicular wave dynamics would occur with age; and, 2) changes in reproductive hormone production would occur with age in association with major follicle wave dynamics. The null hypothesis was that follicle wave dynamics and associated hormone production were the same across age groups.

The following specific hypotheses were tested:

A. Study 1 (Chapter 5): Age-Related Changes in Major Ovarian Follicular Wave Dynamics: Morphologic Characteristics.

1. *Follicular Phase Major Waves:* The dominant follicle of the *Follicular Phase Major Wave (FPMW)* would emerge, become selected, and ovulate earlier in women of advanced reproductive versus reproductive age; and,
2. *Luteal Phase Major Waves:* In women of advanced reproductive age, the prevalence of (LPMWs) would not differ, the growth dynamics of LPMWs would differ, and anovulatory or ovulatory LPMWs would develop.

B. Study 2 (Chapter 6): Age-Related Changes in Major Ovarian Follicular Wave Dynamics: Endocrinologic Characteristics,

1. *Follicular Phase Major Waves:* Elevated serum estradiol and inhibin A would be detected in advanced reproductive versus reproductive-aged women in association with a greater incidence of codominance and/or polyovulation;

2. *Luteal Phase Major Waves:* Atypically high estradiol and inhibin A secretion as well as decreased progesterone production would be associated with the development of LPMWs in women of advanced reproductive age; and,

3. *Major Waves and Markers of Ovarian Reserve:* Greater luteal phase inhibin B and AMH concentrations would be detected in women with versus without LPMWs, irrespective of age.

Chapter 5

Age-Related Changes in Major Ovarian Follicular Wave Dynamics: Morphologic Characteristics

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5.1 Abstract

Context: The menopausal transition results from depletion of the ovarian reserve and is marked by profound changes in hormone production and menstrual cyclicity. Follicle wave dynamics have not been studied in women during the menopausal transition.

Objective: To test the hypothesis that major follicular wave dynamics change during the transition to menopause.

Materials and Methods: A prospective, observational study was conducted in 58 women of Reproductive Age (RA; 18-35 years; n=27), Advanced Reproductive Age-1 (ARA1; 36-44 years; n=10) and Advanced Reproductive Age-2 (ARA2; 45-55 years; n=21). The numbers and diameters of all follicles $\geq$2mm were reported ultrasonographically every 2-3 days for 1 interovulatory interval. Changes in AFC and follicle development were compared between groups (SPSS v19.0, α=0.05).

Results: The prevalence of Follicular and Luteal Phase Major Waves (FPMWs, LPMWs) was not different between RA, ARA1, and ARA2 groups [FPMW: 27/27(100%), 10/10(100%), 20/21(95%); LPMW: 10/17(37%), 3/10(30) %, 10/21(48%); $P>0.050$). All FPMWs were ovulatory. One LPMW ovulated following menses in the ARA2 group; all other LPMWs were anovulatory. Dominant follicles in LPMWs emerged earlier (day -6, -2, -2; P=0.049), grew longer (11, 3, 6 days; $P=0.005$) and developed to a larger diameter (24, 11, 11 mm; $P=0.032$) in the ARA2 versus ARA1 and RA groups.

Conclusions: The prevalence of FPMWs and LPMWs does not differ as women age. However, in women of advanced reproductive age, dominant follicles in LPMWs emerge earlier, grow for a longer period of time and to a larger diameter compared to younger women.

Keywords: Aging - Ovarian follicle - Menstrual cycle - Ovary - Antral follicle count

5.2 Introduction

The transition to menopause is a time of profound changes in reproductive physiology and menstrual cyclicity (150, 231). The Menopausal Transition (MT) begins, on average, in a woman's mid-forties and lasts approximately 4-7 years (150). Menstrual irregularity is currently the hallmark feature signalling the onset of the MT (122, 150, 157). As a woman enters the MT, a shortening of the menstrual cycle occurs (150-153, 157, 182). With progression through the MT, menstrual cycle variability increases and cycles eventually lengthen until the final menstrual period (122, 150, 156-158, 186, 248). Documentations of cycle variability associated with reproductive aging in women are consistent with those reported using animal models of reproductive aging (211, 249, 250). Changes in menstrual cyclicity during the MT result from the age-related decline in ovarian function. Throughout a woman's lifetime, there is a continuous depletion in the number and quality of oocytes, referred to as the ovarian reserve (77, 149, 159,

162, 163, 175). The reduction in the ovarian reserve and resultant changes in reproductive hormone production culminate in menopause at approximately 51 years of age when the number of follicles comprising the ovarian reserve falls below a critical level of approximately 1000 (160, 161).

It is not possible to quantify the numbers of primordial follicles (and oocytes within) using currently-available imaging techniques. However, the ultrassonographic determination of Antral Follicle Count (i.e., AFC) has been shown to accurately reflect the ovarian reserve (122, 204, 207). The AFC has been defined as the number of follicles 2-5 (58), 2-9 (216), or 2-10 mm (207, 217, 221, 251), visualized using transvaginal ultrasonography (122). The depletion of the ovarian reserve and concomitant decline in AFC with age leads to a decrease in the amount of circulating Antimullerian Hormone (AMH) and inhibin B (168, 170, 172, 252). AMH is produced by granulosa cells of pre-antral and early antral follicles (48, 253), and thereby declines with age (208). AMH is positively correlated to AFC, and these 2 endpoints have been shown to be the best predictors of ovarian reserve and reproductive status (85, 224, 225, 254). Inhibin B is thought to be produced by the antral follicles of each follicular wave during the menstrual cycle (48). Inhibin B secretion inhibits FSH production which mediates follicular regression and selection of a dominant follicle (in cases of major follicular waves) (172). Thus, a loss of inhibin-mediated negative feedback on gonadotropin production as a woman ages results in a rise in systemic FSH.

Atypically high estradiol secretion during the luteal phase has been documented in addition to gradual changes in inhibin B, AMH and FSH during the MT (10, 155, 255). The luteal phase pattern of estradiol secretion, termed a Luteal-Out-Of-Phase (LOOP) event, has been characterized as an atypically high rise in estradiol, which begins in the mid-luteal phase and

persists into the early follicular phase (10). Ovulation, defined as a positive LH surge and subsequent progesterone rise, occurred in the early follicular phase of half (3/6) of the women with LOOP events (10). Documentation of atypically high estradiol secretion and endocrinologic evidence of ovulation have important clinical implications for women as increased estrogen exposure is associated with reproductive cancers (201) and unwanted symptoms experienced during the MT (256). The origin of atypical luteal phase estradiol secretion, however, is not known.

Several studies have been conducted to characterize the decline in AFC and altered endocrine environment during the MT. However, little is known about changes in ovarian follicle dynamics that occur with reproductive aging. It has been shown that antral follicles in the follicular phase of regularly cycling women in their early to mid-forties emerge earlier (153, 193, 194), and grow at either an accelerated (172, 192), slowed (194), or normal (153) rate when observing the follicular phase of regularly cycling women of reproductive age. It has also been reported that women $\geq$45 years were twice as likely to have multiple dominant follicles $\geq$10mm throughout their cycle; however, the origin and fate of the multiple dominant follicles was not ascertained (194). Ovulation at a smaller preovulatory follicle diameter and an increased incidence of polyovulation has also been associated with reproductive aging (153, 196). Anovulatory cycles and cycles with lag phases (delayed emergence of the ovulatory follicle) have been demonstrated endocrinologically to become more frequent across the MT; however limited ultrasonographic data are available to confirm these findings (1, 10, 155, 194). Few studies have been conducted to characterize the serial growth of individual follicles during the entire menstrual cycle, in particular during the luteal phase. Furthermore, luteal development during the MT has not been characterized.

Two-three waves of antral follicles develop during each menstrual cycle in women of reproductive age (70). Major waves have been defined as those in which selection and preferential growth of at least 1 dominant follicle occurred (33). Minor waves are those in which selection was not manifest. Currently, it is not known whether wave patterns of antral follicle development over the menstrual cycle change during the transition to menopause. It is plausible that major follicle waves developing in the luteal phase may be responsible for the atypically high estradiol secretion during the MT (10, 155, 255). A greater understanding of the origins of atypical hormone production during the MT has important clinical implications for preventing the associated health risks women face during the MT. Continued research to elucidate the physiologic changes occurring during the MT will enable the development of safe and effective strategies for treating unwanted symptoms and thereby optimize the quality of life of women as they age. The growing proportion of Canadian women over the age of 45 years makes research in this area is particularly relevant (257).

The objective of the present study was to characterize major follicular wave dynamics as women age. The following hypotheses were tested: i) the dominant follicle of the follicular phase major wave would emerge, become selected, and ovulate earlier in women of advanced reproductive versus reproductive age; ii) the prevalence of luteal phase major waves would not differ with age; iii) the growth dynamics of Luteal Phase Major Waves (LPMWs) would differ with age; and iv) anovulatory and ovulatory LPMWs would develop in women of advanced reproductive age. Our hypotheses were based on previous research conducted by our group and others, as well as clinical observations in women of advanced reproductive age undergoing natural cycle monitoring or ovarian stimulation for the treatment of infertility.

5.3 Materials and Methods

A prospective, observational study was conducted from 2006-2011. The study protocol was approved by the Biomedical Research Ethics Board at the University of Saskatchewan and the Strategic Priorities and Planning Committee of the Saskatoon Health Region. All study procedures were conducted in accordance with the Tri-Council Policy Statement on the Ethical Conduct for Research Involving Humans. Informed consent was obtained by all participants before study procedures were initiated.

5.3.1 Study Participants

Women were recruited using electronic and paper advertisements posted throughout the Saskatoon Health Region, University of Saskatchewan, and Women's Midlife Health Centre of Saskatchewan. Inclusion criteria included: 18-55 years of age; a normal complete blood count; and normal serum TSH, prolactin, and β-hCG findings. To be eligible, women aged 18-44 had a history of regular menstrual cycles (i.e. 21-45 days long). However, women aged 45-55 were eligible if they had a history of no more than 12 months amenorrhea. Exclusion criteria included: use of any type of hormone therapy within 3 months of study participation, current smokers, documented ovarian failure and/or currently-diagnosed infertility of unexplained or female origin, medical conditions or use of medications known or suspected to interfere with reproductive function, presence of only one ovary, ovaries which we were unable to visualize ultrasonographically, pregnancy, lactation, and/or participation in an investigational drug trial within 30 days of study participation.

Seventy-one women were enrolled in the study. Only ovulatory cycles were included in our analyses. Data from 13/ 71 women enrolled in the study were excluded for the following

reasons: pregnant during the study (n=1), unable to complete study procedures for personal reasons (n=7), anovulatory cycles documented during the study (n=3), and failure to meet inclusion criteria following pre-study screening (n=2). Data from the remaining 58 participants were evaluated. Women were divided into the following age categories: reproductive age (RA; aged 18-35), advanced reproductive age 1 (ARA1; aged 36-44), and advanced reproductive age 2 (ARA2; aged 45-55). The age of the RA group was chosen to represent a reference group of ovulatory women in the peak reproductive stage of life. The ARA 1 group represented women during the late reproductive stage of life (36-44 years of age).The ARA2 age group was chosen to represent ovulatory women undergoing the transition to menopause.

5.3.2 Study Procedures

Transvaginal ultrasonography was conducted to evaluate ovarian function in each participant (6-9MHz curvilinear array transducer, Ultrasonix RP, Burnaby, BC, Canada). Ultrasound exams were performed by 1 of 2 investigators (ARB, HKV). Scans were initiated in the early-mid follicular phase (i.e. day 8-15) and continued every Monday, Wednesday, and Friday for one complete Interovulatory Interval (IOI). An IOI was defined as the time period from one ovulation to the subsequent ovulation. When a preovulatory follicle was detected at 14-16mm, ultrasound exams were performed daily in order to determine the day of ovulation. Ovulation was defined as the disappearance of a dominant follicle seen on the previous day and subsequent visualization of the corpus luteum (CL) (107, 258).

During each examination, video clips of each ovary in both the sagittal and transverse planes were recorded. All video clips were reviewed retrospectively by a single investigator (HKV) using customized imaging software (Santesoft DICOM Editor Version 3.1.0). All follicles $\geq$4 mm and the number of follicles $\geq$2 mm were tabulated at each visit for each

participant. Measurements were taken separately for the right and left ovaries. Follicle diameter measurements were made when the longest and widest follicle dimensions were observed. The diameter of each follicle <10 mm was calculated as the average of the length and width. When a follicle achieved a mean diameter of 10 mm in one plane, the same follicle was identified in the video clip recorded in the other plane, and the length and width measurements were made again. Thus, the diameter of follicles ≥10 mm was calculated as the overall mean of the average diameter measurements in the sagittal and transverse planes.

Serial changes in follicle diameter during the IOI were tabulated using both the ID and NonID methods, as previously described (33, 70, 259, 260). Briefly, the ID method involved sketching all follicles ≥4 mm in each ovary (70, 259-261).The day-to-day identities of all individual follicles which grew to ≥7 mm during the IOI were retrospectively identified and growth profiles were graphed for each participant (Windows Office Excel, 2003). In contrast, the NonID method involved sorting the diameters of all follicles ≥4 mm in decreasing order at each visit. Each day of the IOI comprised a single row of a word processing spreadsheet (Windows Office Excel, 2003) and each column represented a single follicle that developed during the IOI. It was assumed that the largest, second largest etc. follicles on the first day of the IOI became the largest, second largest etc. follicles on all subsequent days. No sketches of the ovaries were made for the NonID method, and the day-to-day identities of individual follicles were not determined. The growth profiles of all individual follicles, as determined using the NonID method, were then graphed.

The numbers of follicles (i.e. AFC) across the entire IOI were reported for each participant for the following size categories: 2-5 mm, 2-9 mm, 2-10 mm, ≥4 mm, ≥5 mm, and ≥6 mm. Major follicular waves were characterized by quantifying changes in both the number

and growth dynamics of individual follicles. Minor waves were not characterized in the present study.

Diary cards were completed by participants to record adverse events and medications taken during the study.

5.3.3 Definitions

A **Dominant Follicle (DF)** was defined as a follicle which grew to a diameter of ≥10 mm and exceeded the next largest follicle by at least 2mm. A **major follicle wave** was defined as an increase followed by a decrease in the total number of follicles ≥6mm occurring in association with the emergence of at least one dominant follicle. An increase in follicle number was defined as at least 2 increasing data points (1-3 days between data points) between a nadir and a peak. In 13% of cases an increase in AFC was not evident in follicles ≥ 6 mm. In these cases the increase in follicle number was characterized using follicles ≥ 4 or 5 mm. A decrease was defined as 2 decreasing data points (1-3 days between data points) following the preceding rise in AFC of the wave. In 14% of all major waves the decrease in AFC was not evident in follicles ≥ 6mm, therefore the decrease was characterized using AFC 2-9, 2-10, ≥4 or ≥5 mm.

The **day of wave emergence** was defined as the day that the DF was identified retrospectively at 4 or 5mm. A **codominant follicle (CF)** emerged from the same wave as the DF, attained a diameter of ≥10 mm, and exceed the next largest follicle by ≥2 mm. **Subordinate follicles** (SF) were defined as all other follicles that emerged in association with the AFC rise from which the dominant follicle emerged. The first subordinate (SF1) was considered the second largest (non-dominant) follicle in the wave, SF2 the third largest, etc. The **day of selection of the DF** was defined as the day immediately preceding a difference in growth

trajectories between the DF and SF1 (i.e., the DF continued to grow while the SF1 initiated static or regressing phase).

The **Follicular Phase (FP)** was defined as the period of time from the first day of menses until the last day the preovulatory follicle was observed. The **Follicular Phase Dominant Follicle (FPDF)** emerged from the **Follicular Phase Major Wave (FPMW).** The **Luteal Phase (LP)** was defined as the period of time from the day of ovulation until the day before the first day of menses. A **Luteal Phase Dominant Follicle (LPDF)** emerged from the **Luteal Phase Major Wave (LPMW)**.

A **lag phase** was defined as a period of no follicular development >6 mm for greater than 20 days from menses or ovulation.

5.3.4 Statistical Analyses

The primary endpoint was follicle diameter. Secondary endpoints included follicle number, growth intervals, cycle lengths, and single point measures of physiologic interest. Follicle diameter and number data were centralized to the first day of ovulation for statistical and illustrative purposes. Normality was assessed for all endpoints using Shapiro-Wilks tests. Non-parametric tests were used for all analyses because our data were not normally distributed. Continuous variables at predetermined times (i.e., on specific days of the IOI; over the entire IOI, follicular phase, or luteal phase) were compared between groups using Mann-Whitney U tests and Kruskal-Wallis tests. When significance was detected using Kruskal-Wallis tests, post hoc comparisons were made using Mann-Whitney U tests with a Bonferonni adjustment. Fisher's Exact tests were used to compare categorical endpoints across age groups. Continuous data are reported as median (interquartile range). Categorical data are reported as percentage of

age group (proportion of group). Alpha was set at 0.05. All statistical analyses were conducted using SPSS (IBM, Version 19.0).

5.4 Results

5.4.1 Participant demographics

Participant demographics and cycle features are compared in Table 1. Age, gravidity, and parity increased across age groups (P <0.001). BMI was lower in the ARA1 versus RA and ARA2 groups (P =0.033). The prevalence of past smokers was not different across age groups *(P>0.05).* Mean IOI, follicular phase and luteal phase lengths did not differ among age groups (P>0.05).

Table 5.1 Participant Demographics and Cycle Characteristics Compared between Age Groups

	RA n=27	**ARA1** n=10	**ARA2** n=21	P-value
Demographics				
Age (years)	27.0[a] (18.0-35.0)	40.0[b] (36.0-43.0)	48.0[c] (45.0-50.0)	**<0.001**
BMI (kg/m2)	25.6 [a] (21.0-35.0)	22.4[b] (21.0-30.0)	25.9[a] (20.0-33.0)	**0.033**
Gravidity (#)	0[a] (0-3)	2[b] (0-5)	3[b] (0-5)	**<0.001**
Parity (#)	0[a] (0-3)	2[b] (0-4)	2[b] (0-4)	**<0.001**
Ever-smoker (n)	1	0	2	>0.050
Cycle Characteristics				
IOI (days)	27.0 (25.0-30.0)	26.5 (23.0-32.0)	26.0 (17.0-48.0)	0.545
Follicular Phase Length (days)	14.0 (11.0-25.0)	14.0 (10.0-18.0)	13.0 (4.0-33.0)	0.465
Luteal Phase Length (days)	14.0 (5.0-16.0)	13.0 (10.0-15.0)	13.0 (7.0-17.0)	0.602

[a,b,c] Within rows, values with different superscripts are different. Day 0 = day of first ovulation.

5.4.2 Follicular Wave Dynamics

Examples of follicular wave profiles that developed in individual participants within each age group are shown in Figure 5.1. One (36/58, 62%) or two (22/58, 38%) major follicular waves were observed during the IOI in all women evaluated. Cycles in which LPMWs developed and those in which LPMWs were not detected are compared within and between age groups. The prevalence of major waves in the follicular and luteal phases is shown in Table 5.2. In the RA group, 27/27 women developed an ovulatory FPMW, while only 10/17 women developed an anovulatory LPMW. Similarly, in the ARA1 group, 10/10 women developed a FPMW (ovulatory) and 3/10 women developed a LPMW (anovulatory). In the ARA2 group, 20/21 women developed a FPMW (ovulatory) and 10/21 women developed a LPMW (anovulatory). The prevalence of FPMWs and LPMWs did not differ among age groups (P >0.05; Table 5.2, supplemental). In 22/23 cases (irrespective of age group), LPMWs were anovulatory. In the remaining case (1/23), one woman in the ARA2 group developed a LPMW which resulted in ovulation at the time of menses; ovulation of the LPMW was confirmed by ultrasonographic visualization of the CL. A subsequent FPMW was not observed in this participant (Figure 2).

Lag phases were documented in 2/21 (10%) women in the ARA2 group. An example of a lag phase in shown in Figure 5.3.

Table 5.2 Prevalence of major follicle waves during the IOI

	RA	**ARA1**	**ARA2**	P-value
Follicular Phase Major Waves	100% (27/27)	100% (10/10)	95% (20/21)	>0.05
Luteal Phase Major Waves	37% (10/17)	30% (3/10)	48% (10/21)	>0.05

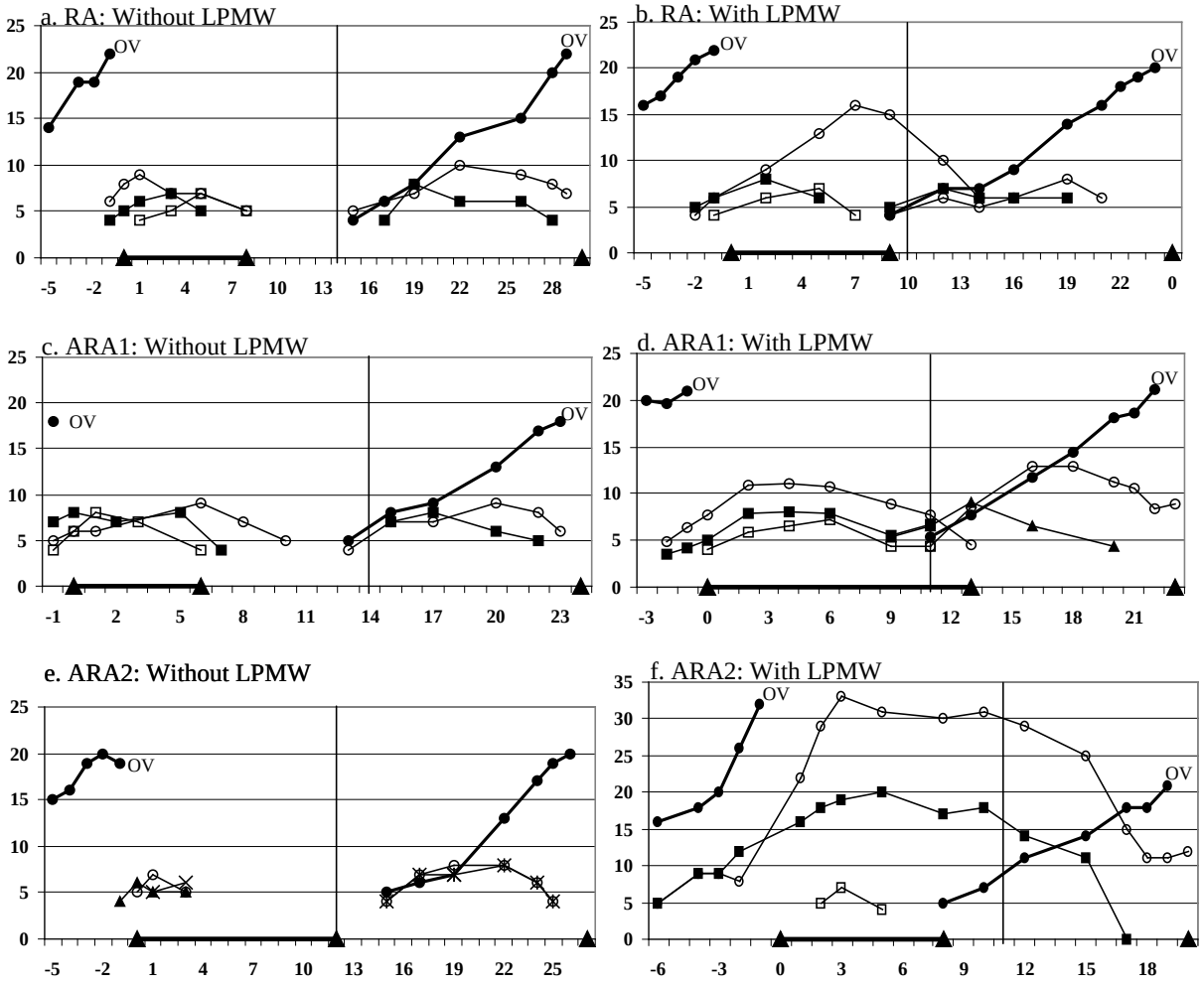

Figure 5.1 a-f Examples of follicular profiles in individual participants among each of the age groups. Examples from the RA (a,b), ARA1 (c,d) and ARA2 (e,f) groups are shown. Data are centralized to day of first ovulation (i.e., day 0=1st ovulation). OV=ovulation. The largest 3 individually-identified follicles in each wave are shown (as determined using the ID Method). The solid bar along the x-axis represents the days when the CL was visualized. The solid vertical line represents the first day of menses

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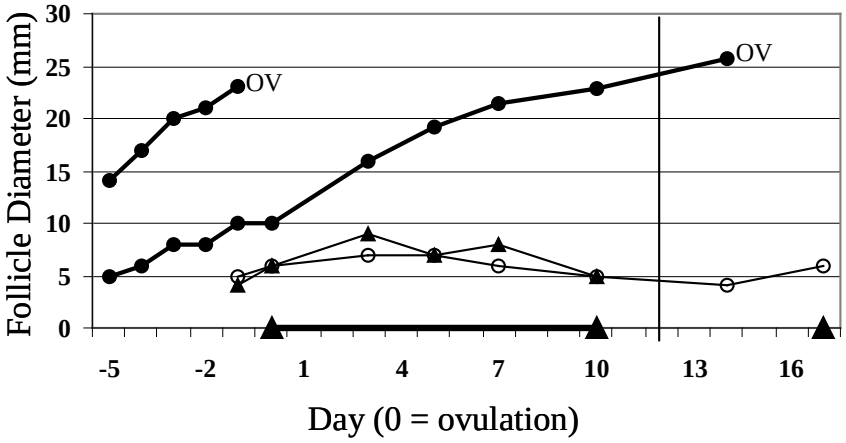

Figure 5.2: An example of a follicle growth profile from a woman in the ARA2 group. The IOI was 17 days long. No FPMW was observed during the IOI. Data are centralized to day of first ovulation (day 0). OV = ovulation. The solid bar along the x-axis represents the days when the CL was visualized. The solid vertical line represents the first day of menses.

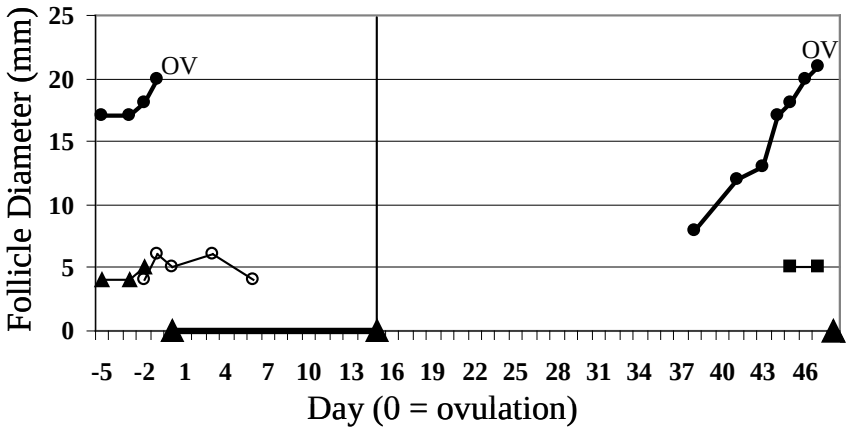

Figure 5.3 An example of a follicle growth profile from a woman in the ARA2 group. A lag phase (i.e., period of time in which no follicles ≥6mm developed) was detected from ovulation and lasted 38 days. This participant had a 48 day IOI. Data are centralized to day of the first ovulation. OV = ovulation. The solid bar along the x-axis represents the days when the CL was visualized. The solid vertical line represents the first day of menses.

5.4.2.1 Luteal Phase Major Waves (LPMWs)

Characteristics of LPMWs are shown in Table 4. Luteal phase major waves emerged earlier (relative to the day of the first ovulation) in ARA2 versus RA group (day -6.0, -2.0, respectively; P=0.049). Similarly, the dominant follicle of the LPMW was larger in the ARA2 versus RA group (10.0 versus 6.5 mm, respectively; P=0.022; Table 4) on the day of the first ovulation. The ovulatory follicle from the previous follicular phase (i.e., preceding ov #1) on the day of LPMW emergence tended to be smaller in the ARA2 versus RA and ARA1 groups (P=0.066).

The day of selection (2.0, 3.0, 0.0; P=0.180) and diameter of the DF (9.0, 10.5, 9.7 mm; P=0.631) and SF1 (8.0, 7.5, 7.0 mm; P=0.248) on the day of selection were not different in RA versus ARA1 versus ARA2 groups (Table 5.3). The DF of the LPMW grew for a longer period of time (11.0, 6.0 days; P=0.005) and to a larger diameter (24.0, 11.0 mm; P=0.032) in the ARA2 versus RA group. In contrast, the SF2 of the LPMW developed to a smaller diameter in the ARA2 versus RA group (6.0, 7.5 mm; P=0.013)

Table 5.3 Characteristics of Luteal Phase Major Waves

	RA n=10	**ARA1** n=3	**ARA2** n=10	P-value
Day of emergence (day)				
DF	-2.0[a] (-3.0--0.8)	-2.0[a,b] (-2.0--2.0)	-6.0[b] (-7.0--3.3)	0.049
SF1	-1.0 (-2.0-0.3)	2.0 (2.0-2.0)	-1.5 (-6.0--0.3)	0.255
SF2	-1.0 (-3.0--0.8)		-1.0 (-4.0--0.8)	0.728
Diameter of the ovulatory follicle on LPMW day of emergence (mm)	19.5 (16.5-22.8)	20.0 (20.0-20.0)	12.5 (9.5-16.0)	0.066
Diameter of the LPDF on day of ovulation #1 (day 0)	6.5[a] (5.0-8.3)	8.0[a,b] (5.0-9.0)	10.0[b] (10.0-12.0)	0.008
Day of Selection	2.0 (0.5-4.5)	3.0 (2.0-4.0)	0.0 (0.0-2.0)	0.180
Diameter on day of Selection (mm)				
DF	9.0 (6.5-11.0)	10.5 (10.0-11.0)	9.7 (8.0-10.0)	0.631
SF1	8.0 (7.5-9.5)	7.5 (7.0-8.0)	7.0 (5.0-9.0)	0.442
SF2	7.0 (6.0-7.8)	6.0 (6.0-6.0)	5.0 (4.0-6.5)	0.129
Maximum Diameter (mm)				
DF	11.0[a] (10.0-12.0)	11.0[a,b] (11.0-12.0)	24.0[b] (13.0-27.0)	0.032
SF1	8.0 (7.8-10.0)	7.5 (7.0-8.0)	7.0 (5.3-9.0)	0.248
SF2	7.5[a] (7.0-9.0)	7.0[a,b] (7.0-7.0)	6.0[b](4.0-6.0)	0.013
Growth interval of DF (days)	5.5[a] (4.8-8.3)	3.0[a,b](2.0-4.0)	11.0[b](8.0-15.0)	0.005

[a,b,c] Within rows, values with different superscripts are different. Day 0 = day of first ovulation.

5.4.2.2 Follicular Phase Major Waves (FPMWs)

Characteristics of FPMWs are shown in Table 3. The day of emergence of the DF, SF1 and SF2, and the day of selection were not different among the 3 age groups. Similarly, the diameter of the DF on the day of selection and the maximum pre-ovulatory diameter of the DF were not different between groups. The SF1 was smaller at the time of selection. Both SF1 and SF2 reached a smaller maximum diameter in the ARA2 versus RA and ARA1 groups. No

obvious differences in the growth interval of the DF in FPMWs were detected between age groups.

The incidence of CFs within the FPMW was not different between age groups (P>0.050). In contrast, the incidence of ovulatory co-dominant follicles in the FPMW (i.e., polyovulation) tended to be higher in ARA2 (3/21, 14%) versus RA (0/27, 0%) group (P=0.070).

Table 5.4 Characteristics of Follicular Phase Major Waves

	RA n=27	**ARA1** n=10	**ARA2** n=21	P-value
Day of emergence (day)				
DF	13.0 (11.0-15.0)	15.0 (10.0-19.0)	13.5 (12.0-16.3)	0.708
SF1	14.5 (11.8-16.0)	14.0 (9.0-17.0)	15.0 (13.5-19.0)	0.216
SF2	14.0 (11.0-16.0)	15.0 (11.0-20.0)	15.0 (14.8-19.3)	0.134
Day of Selection	19.0 (16.8-21.8)	18.0 (17.0-20.3)	19.0 (16.8-21.3)	0.927
Diameter on day of Selection (mm)				
DF	10.1 (8.0-11.5)	9.2 (7.8-10.3)	10.1 (6.5-14.0)	0.723
SF1	9.0[a] (8.0-10.3)	9.0[a] (8.8-9.0)	7.0[b] (5.8-8.0)	<0.001
SF2	8.0 (6.8-9.0)	7.0 (5.3-8.0)	6.0 (4.8-8.3)	0.157
Maximum Diameter (mm)				
DF	21.0 (19.0-21.0)	19.0 (16.0-21.3)	20.0 (17.3-21.8)	0.400
SF1	9.0[a] (8.0-10.5)	9.0[a] (9.0-9.0)	7.0[b] (5.0-8.0)	<0.001
SF2	9.0[a] (8.0-10.0)	8.0[a] (7.0-8.3)	7.0[b] (5.0-8.0)	0.001
Growth interval of DF (days)	12.5 (12.0-15.0)	11.0 (10.0-14.0)	12.0 (11.3-13.0)	0.050

[a,b,c] Within rows, values with different superscripts are different. Day 0 = day of first ovulation.

5.4.3 AFC

Changes in the distribution of AFC (2-9 mm and ≥6 mm averaged over the entire IOI) with age and in women with versus without LPMWs are shown in Figures 4 and 5, respectively. A decrease in mean AFC 2-9 mm occurred with age (P<0.001; Figure 5.4). Similar decreases in mean AFC ≥4, ≥5, and ≥6 mm over the entire IOI were observed with age (P <0.001). Women in the RA group with LPMWs had a higher mean AFC ≥5 mm and ≥6 mm (18.5, 9.2) compared to

those without LPMWs (13.5, 5.5); (P=0.015, 0.025; Figure 5.5). A similar trend was detected for AFC 2-9 mm (P=0.079). No differences were detected in the mean AFC 2-9, ≥4, ≥5, and ≥6 mm among women with versus without LPMWs in the ARA1 and ARA2 groups (P>0.050).

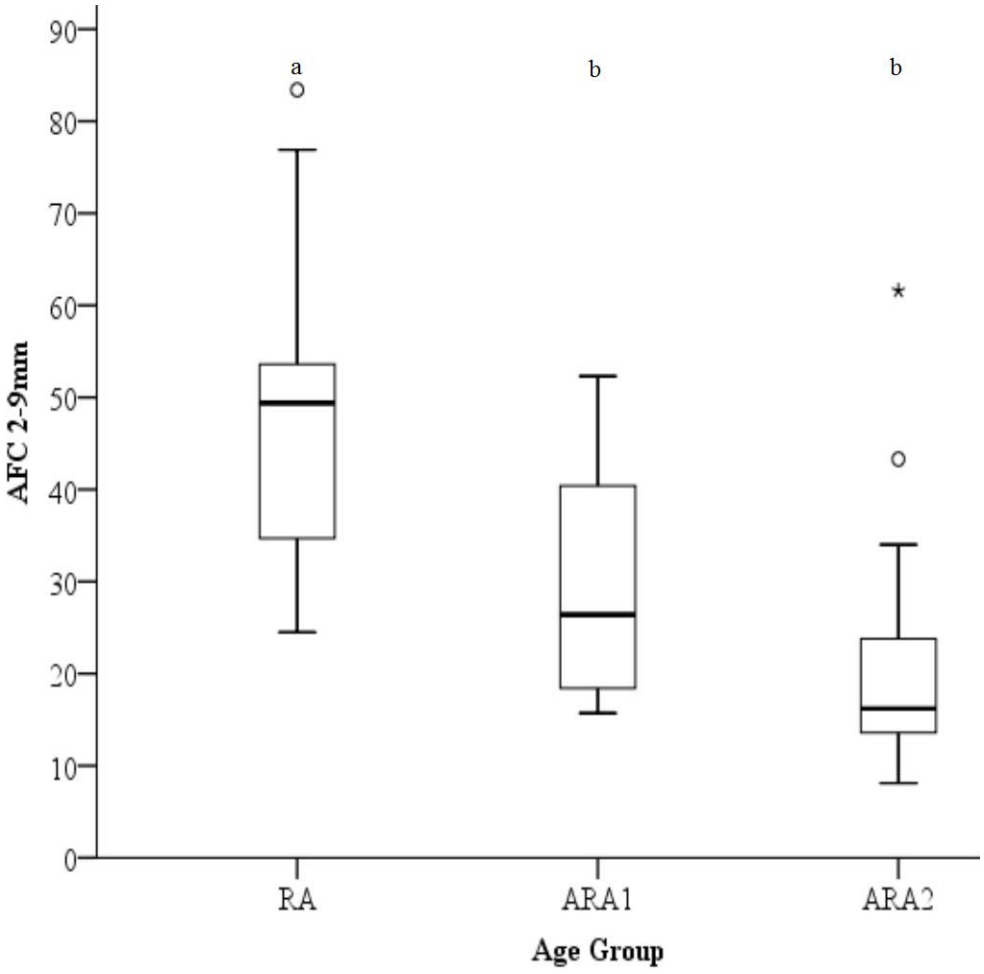

Figure 5.4 Mean AFC in RA, ARA1, ARA2 groups over the entire IOI.

º,* Indicate outliers. Across groups, values with no common superscripts within each graph are different (P<0.001).

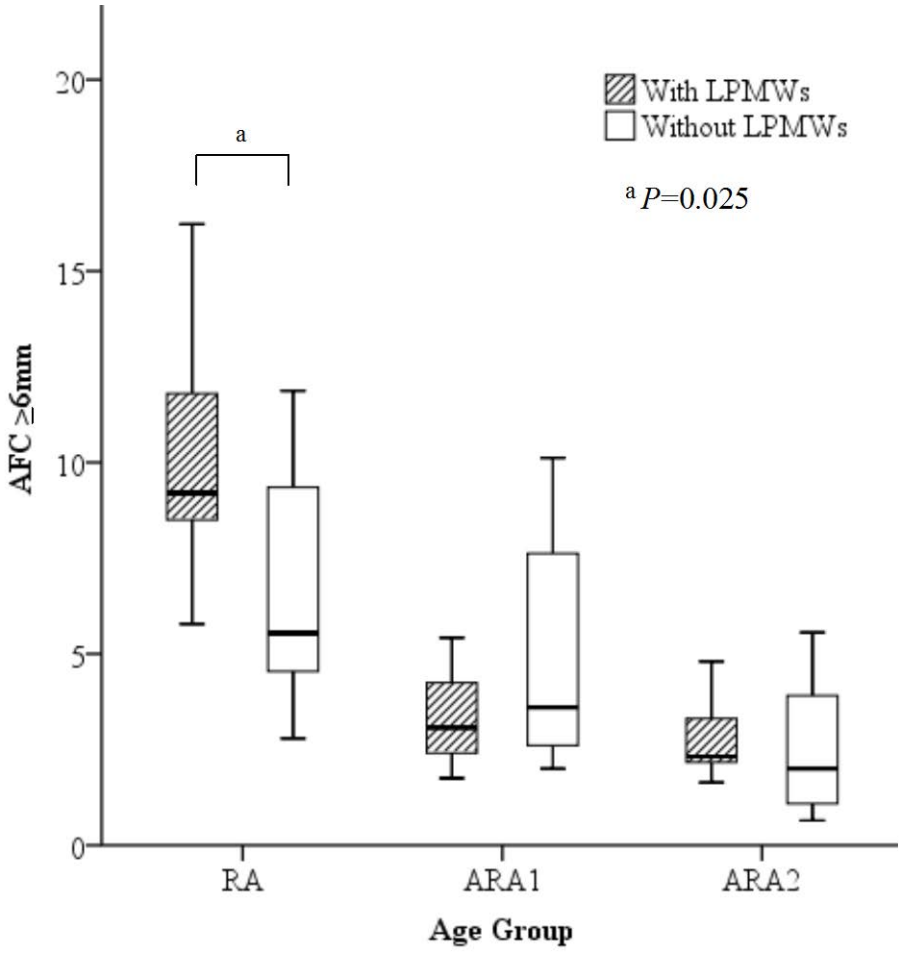

Figure 5.5 Mean AFC over the entire IOI in women among the RA, ARA1, and ARA2 groups with versus without LPMWs.

º Indicates outliers.

5.5 Discussion

Our results supported the hypothesis that major waves of follicle development occur in women approaching and/or during the MT. The incidence of both luteal phase and follicular phase major waves was not different in women of reproductive age compared to those undergoing the transition to menopause. The dynamics of luteal phase major waves did change in women during the transition to menopause. Luteal phase major waves emerged earlier, grew

longer, and to a larger diameter in women in the ARA2 versus RA and ARA1 groups. In the RA and ARA1 groups, all LPMWs were anovulatory. In contrast, in the ARA2 group, LPMWs were either ovulatory or anovulatory. Polyovulations and smaller subordinate follicles in the FPMW were detected in the ARA2, but not RA and ARA1 groups.

5.5.1 Cycle Length

Our finding that the IOI and FP lengths were not different across age groups, contrasts with changes in menstrual cyclicity that have been previously associated with the MT in women (122), domestic farm animals (211), and non-human primates (249, 250). However, we did observe within-group variability in IOI and FP length with age. An abnormally long follicular phase in women of advanced reproductive age in our study was the consequence of lag phases, consistent with previous endocrine studies (10, 155). An abnormally short IOI was detected in one participant in our study. The shortened IOI resulted from the development of an ovulatory LPMW after which a follicular phase wave did not emerge. Documentation of an ovulatory LPMW in women with shortened cycles supports previous reports of ovulatory Luteal out of Phase (LOOP) endocrine events (10). Our finding that LP length did not change across age groups is supported by previous studies which report no change in luteal phase length across age groups in women (10, 168, 189, 192, 262) and domestic farm animals (210, 211). Decreased luteal function (i.e., production of Progesterone (5, 6) and inhibin A (173) is one of the final changes detected in women undergoing the transition to menopause occurring in association with an increased incidence of anovulation (6, 202) and increased cycle length (5, 6, 155). We attribute the lack of changes in the length of the IOI, FP, and LP in our study to recruiting by age group versus STRAW stage and therefore including women at different stages of the MT.

5.5.2 Luteal Phase Major Waves

The prevalence of LPMWs in our study ranged from 30-50% across age groups, similar to previous reports of follicular waves in women (70, 263). Interestingly, the frequency of LPMWs did not differ with age. However, the growth dynamics of the LPMWs were markedly different in the older women. In reproductive age women, a major or minor wave of follicle development has been shown to emerge at the time of ovulation in association with the preovulatory rise in FSH (263). In the present study, women in the RA and ARA1 groups exhibited emergence of a LPMW at the time of ovulation, the dominant follicle grew to a median diameter of 11.0mm and then regressed in the early-mid luteal phase. However, in the ARA2 group, LPMWs emerged earlier (i.e., in the mid-late follicular phase of the preceding IOI) and grew to large, often cystic diameters (range: 10.0-37.0 mm). It appeared that the LPMW in the ARA2 group may have emerged from a minor follicular phase wave preceding the first ovulation of the IOI. The largest follicle(s) of the LPMW had already achieved dominance at the time of the pre-ovulatory gonadotropin surge. Therefore, we postulate that the largest 1 or 2 follicles recruited into the LPMW responded to the preovulatory LH surge, which resulted in their continued growth during the luteal phase.

Although the LPMW growth dynamics in ARA1 women (aged 36-44) were not different compared to the RA and ARA2 groups, visual inspection of the data suggests the differences between ARA1 and ARA2 were not detectable due to a very small sample size in the ARAR1 group (n=3). In ARA1 women, the LPMW day of emergence, the growth interval and maximum diameter of the DF very similar to the RA group.

Follicle waves in the luteal phase of the menstrual cycle in reproductive age women have not been shown to ovulate (70, 263). However, in the present study, we documented ovulation of a dominant follicle from a LPMW during the MT. This novel finding supports the notion that functional (i.e., FSH responsive, estrogenic) dominant follicles can grow in the luteal phase (76). Furthermore, growth and ovulation of LPMW dominant follicles supports previous reports of successful oocyte retrieval from follicles in the luteal phase in women undergoing ovarian stimulation for the treatment of infertility (109, 264). Further, documenting ovulation of LPMWs during menses has clinical implications for understanding contraceptive needs of women as they age.

5.5.3 Follicular Phase Major Waves

Follicular phase major waves occurred in all but one woman in our study. In general, the growth dynamics of FPMWs were conserved across age groups. The day of emergence, day of selection, and diameter of the dominant follicle at selection within FPMWs did not change across age groups. No obvious differences in growth interval were detected between age groups. Our findings are not consistent with reports of earlier emergence of the ovulatory follicle (153, 193, 194), earlier ovulation (193) or slowed preovulatory growth (194) in older women. We attribute differences in findings between our study and others in part to inconsistencies in methods used to evaluate follicle dynamics. In the present study, serial changes in individually identified follicles were quantified over an entire IOI. In contrast, previous studies involved endocrine endpoints as an indirect measure of follicle development and/or limited assessment of dominant follicle growth. Furthermore, differences in data categorization (i.e., age versus cyclicity) may have lead to differential findings between studies. Lag phases have been observed endocrinologically during the late MT (10, 155). However, follicular growth during lag phases had not been

previously evaluated. We observed a prolonged phase of no follicle growth > 6mm prior to the development of the FPMW in 2 women in the ARA2 group. Antral follicles 2-5 mm represent the cohort of follicles available for recruitment (also referred to as 'wave emergence') (58). The absence of follicle growth >6 mm during the lag phase demonstrates the inability to develop major follicle waves.

The diameter of SF1 was smaller on the day of selection and the maximum diameter of SF1 and SF2 were smaller in the ARA2 versus ARA1 and RA. These findings are consistent with previous research in mares (211). In 5/20 cases in the ARA2 group, emergence and selection of the DF at 4-5 mm occurred on the same day because SF1 had already reached its peak diameter before regressing on the day of wave emergence. We believe smaller subordinate follicle peak diameters reflect the reduced number of recruitable follicles available at the time of wave emergence, attributed to the decreased ovarian reserve.

Documentation of codominance and increased incidence of polyovulation in the FPMW supports earlier reports of increased twinning rates with age in women (196) and domestic farm animals (211). The increased incidence on polyovulation as women approach menopause is thought to occur due to the elevated FSH (or 'widened FSH window') during wave emergence of the FPMW during the MT, which allows more than one follicle to escape atresia and become dominant (74, 196, 265).

5.4 AFC

A progressive decrease in AFC occurred in all age groups in the present study, as has been previously well-documented (162, 164, 175, 204). In the RA group, women with LPMWs had a greater AFC $\geq$6 mm compared to those without LPMWs. It appears that a greater ovarian

reserve may increase the likelihood of LPMWs in young women, but not those in the MT. Previous investigators have also proposed that a threshold for ovarian reserve exists, below which dysregulation and atypical hormone fluctuations causes aberrant follicle growth dynamics (10, 266).

5.5.5 Limitations and Future Directions

The inability to collect ultrasonographic data every day was one of the biggest challenges in our study, as it may have led to error in determining the timing of emergence of follicles within a wave. However, a scanning frequency of more than 1-3 days for a period of 6 weeks would not be considered practical or ethical by research volunteers and/or investigators. Collection of serial ultrasonographic data from 71 women in our study was conducted over a 5 year period. Even with a large sample size, there were relatively small sample sizes on a given day of the cycle within each of the 3 different age groups. Evaluation of changes in the number and growth dynamics of follicles <4 mm involves much more error compared to follicles ≥4 mm, especially when clear images of the ovaries cannot be obtained. Future research should incorporate 3D ultrasonographic and/or high-resolution technologies for improved imaging of ovarian follicular dynamics in women.

Recently, criteria have been developed to characterize the physiologic stages of reproductive aging, originating from the Stages of Reproductive Aging Workshops (STRAW) (122, 267). The objectives of the staging systems are to provide standardized criteria for the clinical and scientific evaluation of reproductive aging in women. The STRAW criteria are based on changes in menstrual cyclicity, hormone production and antral follicle count (i.e., AFC) that occur in women throughout the reproductive lifespan (122). According to STRAW, the first

indication of reproductive aging is a change in menstrual cyclicity (122). We grouped our participants into STRAW categories of reproductive aging; however, the resulting limited sample size did not provide statistical power to test our hypotheses. Studies of this nature require significant financial resources and time commitments from both participants and investigators.

The findings from the present investigation have included the morphologic (i.e., growth) aspects of follicular dynamics as women age. No functional (i.e., endocrinologic) data have been presented. We felt that inclusion of endocrine data was beyond the scope of the present manuscript. We have completed corresponding endocrine evaluations for a subset of women that participated in our study. The endocrine data will be published as a separate manuscript (Chapter 6). Atypical and acute elevations in luteal phase estradiol (termed 'LOOP' events) have been reported in approximately 40% of women during the MT (10). The origins of the atypical luteal phase elevations, however, are not known. It is speculated that the development of luteal phase major waves may underlie the atypical elevations in luteal phase estradiol as women age. Insight into the origins of atypical estrogen production during the MT has important clinical implications for understanding the causes of vasomotor symptoms and estrogen-mediated risks of reproductive malignancies (201). Future research in this area will enable the development of safe and effective therapies for treating unwanted symptoms that will ultimately improve the quality of life of women as they age.

5.5.6 Summary

Major follicular waves developed during the follicular and/or luteal phases of ovulatory menstrual cycles during the transition to menopause. The prevalence of major waves in the follicular and luteal phases was not different in reproductive age women compared to those of advanced reproductive age. Luteal phase major waves emerged earlier, grew longer, and to a larger diameter as women progressed through the menopause transition. An increased incidence of polyovulation and smaller subordinate follicles were detected in FPMWs of women of advanced reproductive age. Lag phases were detected only in women of the ARA2 group. These novel findings may provide insight into the origins of erratic and unpredictable changes in ovarian hormone production and associated unwanted symptoms (e.g., vasomotor symptoms) and risks of reproductive malignancies that women experience as they approach menopause.

Chapter 6

Age-Related Changes in Major Ovarian Follicular Wave Dynamics: Endocrinologic Characteristics

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6.1 Abstract

Context: Acute and atypical elevations in luteal phase estradiol secretion (i.e. >245pg/mL) have been reported in ovulatory women during the MT. However, the origins of the profound changes in estradiol production are not known.

Objective: To test the hypothesis that profound and acute changes in hormone production during the MT are associated with the development of major follicular waves.

Materials and Methods: A prospective, observational study was conducted in women of Reproductive Age (RA; 18-35 years; n=10) and Advanced Reproductive Age-2 (ARA2; 45-55 years; n=17). The numbers and diameters of all follicles $\geq$2 mm were determined ultrasonographically every 2-3 days for 1 interovulatory interval (IOI). Blood samples were collected at each visit, and serum was assayed for FSH, LH, estradiol, inhibin A and B, AMH

and progesterone concentrations. Follicle wave dynamics and hormone production over the IOI were characterized and compared between age groups (SPSS v19.0, α=0.05).

Results: In the ARA2 group, LPMWs developed in association with atypical elevations in estradiol concentrations in 4/8 women (50%). In the RA group, 5/10 women developed LPMWs in association with high, but not atypical, estradiol. In both RA and ARA2 groups, luteal estradiol was higher in women with (139.1, 177.8 pg/mL) versus without (78.0, 95.0 ng/mL) LPMWs (P=0.016, 0.074). Max diameter of the LPMW dominant follicle positively correlated with luteal phase estradiol (r=0.71, P<0.01) and negatively correlated with max luteal phase LH (r=-.52, P<0.05) when both age groups were combined. Max diameter of the LPMW dominant follicle was negatively correlated with mean luteal phase progesterone in the ARA2 group (r=-.78, P<0.05). In the ARA2, but not RA group, progesterone was lower in women with (9.65 ng/mL) versus without (13.0 ng/mL, P=0.027) LPMWs.

Conclusions: The development of LPMWs during the MT is associated with acute and atypical elevations in estradiol in association with decreased luteal progesterone production.

Keywords: Aging - Ovarian follicle - Menstrual cycle - Ovary - Estradiol

6.2 Introduction

The Menopause Transition (MT) is a time of profound changes in reproductive physiology (150, 231). The MT begins at the age of 45-47, lasts approximately 4-7 years, and leads up to menopause which occurs at an average age of 51 years (77, 149, 150). Changes in menstrual cyclicity are the first clinical signs of entry into the MT (122). A shortening of the menstrual cycle usually occurs in the early MT (122, 151-153). As the MT progresses, increased cycle length variability and eventual lengthening of the menstrual cycle occurs due to an

increasing incidence of delayed ovulation and anovulatory cycles (6, 122, 150, 154-158). The ovarian origins of age-related changes in menstrual cyclicity are not fully understood.

Reproductive aging occurs as a result of the depletion of the ovarian reserve (77, 149, 160, 162). A reduction in the number of primordial follicles comprising the ovarian reserve leads to a decrease in in the antral follicle count (AFC) and a correspondent decrease in serum AMH and inhibin B production. AMH is produced by granulosa cells of pre-antral and early antral follicles up to 8-10mm (48, 54), while inhibin B is produced from all antral follicles of the recruited 'cohort' or 'wave' (i.e., 2-12mm)(48). The loss of negative feedback of inhibin B on FSH with age results in an overall rise in serum FSH (162, 168, 172, 195). The fall in AMH and inhibin B and corresponding rise in FSH are the first endocrine changes that occur during reproductive aging (122).

Inhibin A is produced by the dominant follicle in the mid-follicular phase and the CL in the luteal phase (50, 51). Serum concentrations of inhibin A decrease towards the later stages of the MT in association with an increased incidence of lag phases (i.e., periods of no follicular estrogen production), anovulation, and cycle length variability (168, 172, 173, 256, 268).

A gradual increase in estrogen occurs early in the MT (i.e., up until 2 years prior to menses) followed by a marked decline as women enter the late MT and menopause (7, 228, 269). The fall in estrogen is one of the last hormonal changes to precede menopause, in association with increased cycle length, reduced luteal progesterone and elevated gonadotropins (6, 155, 188, 229). Contrary to the notion of a gradual rise and fall in estrogen, great inter-cycle and intra- / inter-woman variability in follicular and luteal phase estrogen secretion has been documented during the MT (6, 10, 182). Elevated follicular phase estrogen has been associated with the

growth of multiple dominant follicles (193, 194) and dysregulation of FSH production (169). Low follicular phase estrogen has been associated with lag phases and anovulatory cycles (1, 6, 10). Acute and atypically high luteal phase estrogen has been described in association with either normal progesterone, or decreased luteal progesterone and lower luteal LH (1, 6, 10, 151, 182). In a recent study, Luteal-Out-Of-Phase (LOOP) events were detected as a rise in estrogen which began in the mid-luteal phase and persisted into the early follicular phase in association with decreased luteal progesterone (10). The LOOP events (defined as estrogen concentrations outside the normal luteal phase reference range or >900pmol/L) occurred in approximately 40% of women during the MT (10). An LH surge (interpreted to represent ovulation) was detected in the early follicular phase in 3/6 (50%) women with a LOOP event (10). The origins of the atypical luteal phase estradiol are not known. However, it has been speculated that major waves of antral follicle development underlie the atypical estradiol fluctuations (10).

We have previously documented 2-3 waves of antral folliculogenesis during the menstrual cycle in reproductive age women (70, 107). Major waves were defined as those in which selection of a dominant follicle occurred from a recruited cohort of antral follicles $\geq$5mm (33). Minor waves were those in which dominance did not occur (33). Similarly, we have recently documented that more than one major follicle wave may develop during a given cycle in women during the MT (270). The incidence of major waves in the follicular and luteal phases was similar in reproductive versus advanced reproductive aged women. Polyovulation and smaller subordinate follicles were detected in the Follicular Phase Major Waves (FPMWs) in women of advanced reproductive age ($\geq$45 years old). The FPMW day of emergence, selection, and diameters at selection and ovulation of the dominant follicle were similar across age groups. However, in advanced reproductive age women, Luteal Phase Major Waves (LPMWs) emerged

earlier (i.e., prior to ovulation) compared to reproductive age women (i.e., at the time of ovulation). In advanced reproductive aged women the largest follicle of the LPMW had already achieved dominance at the time of ovulation and then continued to grow in the luteal phase. The dominant follicle of the LPMW grew for a longer period of time (11 versus 6 days) and to a larger diameter (24 versus 11 mm) during the MT compared to women of reproductive age. Most (9/10) LPMWs during the MT were anovulatory. However, in one case, a LPMW resulted in ovulation at the time of menses. Reproductive age women with LPMWs had a higher AFC $\geq$6 mm compared to those without LPMWs. However, no effect of AFC on the development of LPMWs was detected during the MT.

The physiologic origins of acute and atypical elevations in luteal phase estrogen production during the MT are not known. It is plausible that these profound alterations in hormone production during the MT may originate from the emergence of major follicle waves. Associations between age-related changes in hormone production and follicle wave dynamics have not been investigated. The objective of the present study was to characterize the endocrine changes that occurred in association with major follicular wave dynamics as women age. Our general hypothesis was that changes in major follicular wave dynamics would underlie changes in hormone production as women approached menopause**.** The following specific hypotheses were tested:

1. Follicular Phase Major Waves Elevated serum estradiol and inhibin A would be detected in advanced reproductive versus reproductive-aged women, in association with a greater incidence of codominance and/or polyovulation;

2. Luteal Phase Major Waves Atypically high estradiol and inhibin A secretion as well as decreased progesterone production would be associated with the development of LPMWs in women of advanced reproductive age; and,

3. Major Waves and Markers of Ovarian Reserve Greater luteal phase inhibin B and AMH concentrations would be detected in women with versus without LPMWs, irrespective of age.

6.3 Materials and Methods

A prospective, observational study was conducted from 2006-2011. The morphologic (i.e., ultrasonographic) aspects of this study have been described in detail elsewhere (270). The study protocol was approved by the Biomedical Research Ethics Board at the University of Saskatchewan and the Strategic Priorities and Planning Committee of the Saskatoon Health Region. Study procedures were conducted in accordance with the Tri-Council Policy Statement on the Ethical Conduct for Research Involving Humans.

6.3.1 Study Participants

Participants were recruited using electronic and paper advertisements posted throughout the Saskatoon Health Region, University of Saskatchewan, and Women's Midlife Health Centre of Saskatchewan. Thirty women (n=30) were enrolled in this study as a subset of a previous study to characterize age-related changes in follicular wave dynamics (270). Data from 3/30 women were excluded for the following reasons: incomplete IOI during the study period (n=1) or the development of anovulatory cycles (n=2). Data from the remaining 27 participants were included in our analyses. Women were divided into the following age categories: 1) Reproductive Age (RA; 18-35 years, n=10); and, 2) Advanced Reproductive Age 2 (ARA2; 45-55 years, n=17). The RA age group was chosen to represent a reference group of ovulatory

women in the peak reproductive stage of life. The ARA2 age group was chosen to represent ovulatory women undergoing the transition to menopause. The Advanced Reproductive Age 1 group represented women during the late reproductive stage of life (36-44 years of age). Women in the ARA1 group were evaluated in a previous study (270) to characterize age-related changes in follicle wave dynamics; however, women in the ARA1 group were not evaluated in the present study. Informed consent was obtained by all participants before study procedures were initiated. Diary cards were completed by participants to record adverse events or medications taken during the study.

Inclusion criteria included a normal serum complete blood count, serum TSH, prolactin, and ßhCG. Women aged 18-35 were eligible if they had a history of regular menstrual cycles (i.e., 21-45 days long). However, women aged 45-55 were eligible if they had no more than 12 months of amenorrhea. Exclusion criteria included the use of any type of hormone therapy within 3 months of study participation, current smokers, documented ovarian failure, infertility of unexplained or female origin, medical conditions known or suspected to interfere with reproductive function, presence of only one ovary, ovaries which we were unable to visualize ultrasonographically, use of any medications known or suspected to interfere with reproductive function, pregnancy or lactation, and/or participation in an investigational drug trial within 30 days of study participation.

6.3.2 Study Procedures

Transvaginal ultrasonography was conducted to evaluate ovarian and uterine function in each participant (6-9MHz curvilinear array transducer, Ultrasonix RP Burnaby, BC, Canada). Ultrasound exams were performed by 1 of 2 investigators (ARB, HKV). Scans were initiated in

the early-mid follicular phase (i.e., day 8-15) and continued every Monday, Wednesday, and Friday for one complete interovulatory interval (IOI). An IOI was defined as the interval from one ovulation to the subsequent ovulation. When a preovulatory follicle was detected at 14-16mm, ultrasound examinations were performed daily in order to determine the day of ovulation. Ovulation was defined as the disappearance of a dominant follicle seen on the previous day and subsequent visualization of the Corpus Luteum (CL), followed by a rise in serum progesterone to at least 5.0 ng/mL (10, 107, 258). Anovulation was defined as the development of a dominant follicle which failed to ovulate determined by ultrasonographic visualization of ovulatory failure and a serum progesterone concentration of <5.0 ng/mL.

During each examination, video clips of each ovary in both the sagittal and transverse planes were recorded. All video clips were reviewed retrospectively by single investigator (HKV) using customized imaging software (Santesoft DICOM Editor Version 3.1.0). The number of follicles ≥2mm and the diameter of all follicles ≥4mm were tabulated at each visit for each participant. Measurements were taken separately for the right and left ovaries. Follicle diameter measurements were made when the longest and widest follicle dimensions were observed. The mean diameter of each follicle < 10 mm was calculated as the average of the length and width. When a follicle achieved a mean diameter of ≥10 mm in one plane, the same follicle was identified in the other plane, and the length and width measurements were made again. Thus, the overall mean diameter of follicles ≥10mm was calculated as the mean of the average diameter measurements in the sagittal and transverse planes. Serial changes in follicle diameter during the IOI were tabulated using the ID method. Briefly, the ID method involved sketching all follicles ≥4mm in each ovary (70, 259, 260). The day-to-day identities of all individual follicles which grew to ≥7mm during the IOI were retrospectively identified and

growth profiles were graphed for each participant in Windows Office Excel (2003). The numbers of follicles (i.e., AFC) across the entire IOI were reported for each participant for the following size categories: 2-5 mm, 2-9 mm, 2-10 mm, ≥4 mm, ≥5 mm, and ≥6 mm.

Blood samples were drawn at each study visit. Approximately 5.0mL of blood was collected into a 10 ml clot activated tube and allowed to sit at room temperature for 30-60 minutes before centrifugation for 20 minutes at 2500 rpm. The serum was drawn off and stored at -50 degrees Celsius.

After study completion, serum samples were transported to the Prairie Diagnostics Services Laboratory at the University of Saskatchewan for analyses. Solid-phase two-site chemiluminescent immunometric assays (Immulite; Siemens Healthcare Diagnostics Ltd.,United Kingdom) were performed to measure concentrations of FSH and LH. Double antibody radioimmunoassays (Count-A-Coat, Siemens Healthcare Diagnostics Inc., Los Angeles, CA, US) were performed to measure concentrations of estradiol. Solid-phase radioimmunoassays (Count-A-Coat, Siemens Medical Solutions Diagnostics) were performed to measure concentrations of progesterone. Enzyme-linked immunosorbent assays (Beckman Coulter Inc., Brea, CA, USA) were performed to measure inhibin A, inhibin B, and AMH. FSH and LH assays were performed in singlicate. Inhibin A, inhibin B, estradiol, progesterone, and AMH assays were performed in duplicate. Inter-assay coefficients of variation were as follows: inhibin A (low = 14.4%, high 7.8%), inhibin B (low = 6.02%, high = 7.34%), AMH (low = 7.81%, high = 10.5%), FSH (low = 5.8%, med = 5.8%, high = 6.7%), LH (low = 5.7%, med = 5.3%, high = 3.4%), progesterone (low = 17.1%, med = 9.5%, high 15.2%), estradiol (low = 23.2%, high = 12.9%). Intra-assay coefficients of variation were as follows: inhibin A (5.5%), inhibin B (5.7%), AMH (5.7%), progesterone (low = 6.3%, med = 3.8%, high = 13.0%), estradiol (low = 15.5%, high = 9.9%).

The levels of detection of the assays were 5.0 pg/mL for inhibin A, 2.6 pg/mL for inhibin B, 0.08ng/mL for AMH, 1.4 pg/mL for estradiol, 0.02 ng/mL for progesterone, 0.1mIU/mL for LH and FSH.

6.3.3 Definitions

Major follicular waves were characterized by evaluating changes in both the number and growth dynamics of individual follicles. A **major follicle wave** was defined as an increase followed by a decrease in the total number of follicles ≥6mm, occurring in association with the emergence of at least one dominant follicle as previously described (33, 270).

The **day of wave emergence** was defined as the day that the dominant follicle was identified retrospectively at 4-5mm. A **Dominant Follicle (DF)** was defined as a follicle which grew to a diameter of ≥10mm and exceeded the next largest follicle by at least 2mm. A **Codominant follicle (CF)** emerged from the same wave as the DF, attained a diameter of ≥10mm, and exceed the next largest follicle by ≥2mm. **Subordinate follicles** were those follicles in a wave that did not achieve dominance.

Atypically high estradiol was defined as either luteal phase estradiol >200 pg/mL and/or a secondary rise in estradiol not associated with luteal phase progesterone. This definition is based on the expected range in serum estradiol concentration during the luteal phase and previous characterization of atypically high estradiol (10, 271).

6.3.4 Statistical Analyses

Standardized graphs for the following endpoints over the IOI were created for each participant: 1) follicle diameter, using the ID method; 2) AFC 2-5 mm, 2-9 mm, 2-10 mm, ≥4 mm, ≥5 mm, and ≥6 mm; and 3) serum concentrations of FSH, LH, estradiol, progesterone,

inhibin A, inhibin B, and AMH. Graphs were centralized to the day of the first ovulation. Normality was evaluated for each study endpoint using Shapiro Wilk tests (SPSS Version 19.0, 2010). Mann-Whitney U-tests were conducted to compare study endpoints between age groups (SPSS Version 19.0, 2010). Endocrine concentrations were compared in women with and without cases of codominance (i.e., ovulatory and/or anovulatory). The incidence of FPMWs and LPMWs were compared between groups using Fisher's Exact tests (SPSS Version 19.0, 2010). Continuous data are reported as median (interquartile range). Categorical data are reported as percentage of age group (proportion of group). Bivariate correlations between endocrine and follicular endpoints were conducted using the Pearson or Spearman coefficient as appropriate (SPSS Version 19.0, 2010).

6.4 Results

6.4.1 Participant Demographics

Participant demographics and cycle phase lengths of the RA group and ARA2 group are presented in Table 6.1. As expected, age, gravidity and parity increased with age. Body mass index and prevalence of smoking were similar among groups. The length of the IOI, follicular phase, and luteal phase did not differ in the RA versus ARA2 groups (Table 6.1; $P>0.05$).

Table 6.1 Participant Demographics and Cycle Characteristics Compared Between Age Groups

	RA n=10	**ARA2** n=17	P-value
Demographics			
Age (years)	27.0 (21.8-34.3)	48.0 (46.0-49.5)	0.000
BMI (kg/m^2)	26.5 (24.8-29.8)	25.6 (22.8-27.4)	0.238
Gravidity (#)	0.0 (0.0-1.3)	3.0 (2.0-4.0)	0.002
Parity (#)	0.0 (0.0-0.3)	2.0 (2.0-3.0)	0.003
Ever-smoker (n)	0.0	2.0	0.516
Cycle Phase Lengths			
IOI (days)	27.5 (25.0-30.0)	27.0 (25.5-29.5)	0.667
Follicular Phase Length (days)	14.5 (12.8-16.0)	13.0 (12.0-15.0)	0.333
Luteal Phase Length (days)	14.5 (12.3-15.0)	13.0 (13.0-15.0)	0.759

Day 0 = day of first ovulation of the IOI.

6.4.2 Luteal Phase Major Waves

The prevalence of LPMWs between age groups was not different (RA: 50%; ARA2: 47%; P=0.600).

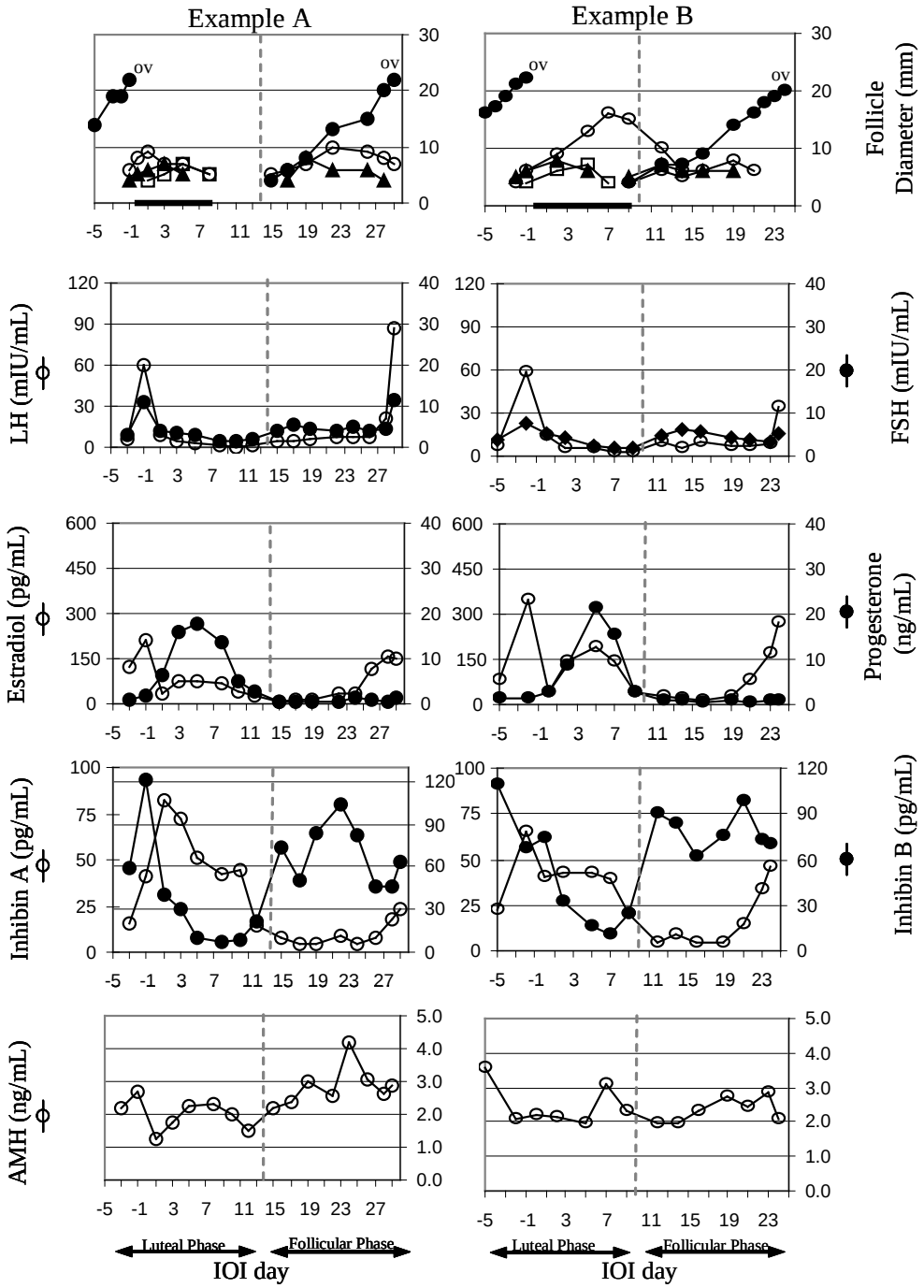
The growth profiles and corresponding hormone profiles of individual participants with and without LPMWs in the RA (Figure 6.1) and ARA2 (Figure 6.2) groups are shown**.** In Figure 6.1 (Example A, RA) and Figure 6.2 (Example A, ARA2), no LPMWs were observed. Luteal phase estradiol remained within normal ranges in association with a normal luteal phase rise and fall in progesterone. In Figure 6.1 (Example B, RA), a LPMW emerged at the time of ovulation in association with the preovulatory rise and fall in FSH and LH. Immediately thereafter, estradiol, inhibin A, and progesterone began to rise. Luteal phase estradiol and progesterone remained within normal ranges. In Figure 6.2 (Examples B-D, ARA2), LPMWs emerged prior to ovulation in the mid follicular phase. The largest follicles of the LPMWs had already become dominant (i.e., $\geq$ 10 mm) at the time of ovulation and continued to grow following the preovulatory LH

and FSH surges. Codominance (i.e., the development of 2 dominant follicles) was observed in Examples B and C. In Figure 6.2 (Example B), inhibin B decreased during the early luteal phase. Two acute rises in estradiol were observed (> 200pg/mL). The first rise began 2 days before ovulation and peaked 12 days after ovulation in association with the growth of 2 LPMW dominant follicles. A smaller, secondary atypical rise in estradiol and inhibin A was observed between days 20-22 (corresponding to the early follicular phase) after one dominant follicle of the LPMW regressed but the other persisted. One dominant follicle of the LPMW regressed in the late luteal phase whereas the second dominant follicle persisted into the subsequent follicular phase. FSH was suppressed in the luteal phase in association with atypically high estradiol production. Progesterone concentrations were normal. In comparison, in Figure 6.2 (Example C), a single acute rise in estradiol began 1 day before ovulation and peaked 2 days after ovulation in association with the growth of 2 LPMW dominant follicles. Both dominant follicles persisted throughout the luteal phase in the presence of declining inhibin B, low progesterone and low inhibin A. In Figure 6.2 (Example D), a LPMW emerged 4 days before ovulation in association with an increase in FSH and subsequent increase in inhibin B. Estradiol rose and progesterone and inhibin A fell in association luteal regression and continued growth of the LPMW dominant follicle. The dominant follicle grew throughout the luteal phase in the presence of a normal rise and fall in progesterone and inhibin A. After luteal regression, the dominant follicle continued to grow and produce estradiol until it ovulated in the early follicular phase. A second rise in progesterone was detected in the early follicular phase.

Endocrine characteristics in the luteal phase of women between age groups are compared in Table 6.2. Luteal phase FSH was greater while inhibin B and AMH concentrations were lower in ARA2 versus RA women ($P<0.05$). Progesterone was higher in ARA2 versus RA women

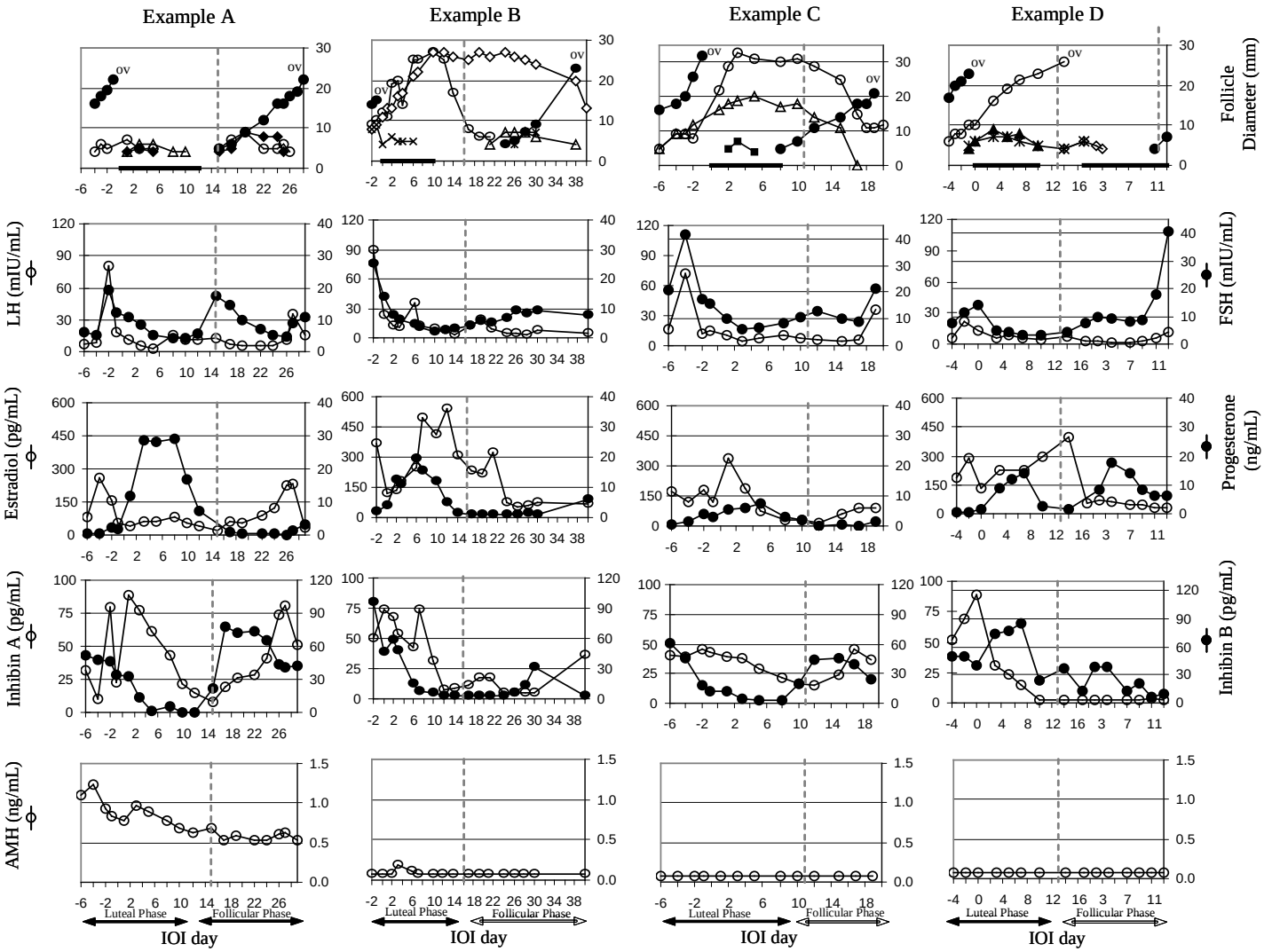
(Table 6.2; P<0.05). There were no differences in LH, estradiol, or inhibin A over the luteal phase between age groups (Table 6.2; P>0.05).

Endocrine characteristics of women with versus without LPMWs in the 2 study groups are shown in Table 6.2. Within the RA group, luteal phase inhibin B and estradiol were higher in women with versus without LPMWs (Table 6.2; P=0.009 and 0.028). Similarly, in the ARA2 group, max luteal phase estradiol tended to be higher in women with versus without LPMWs (Table 6.3; P=0.074). In both RA and ARA2 groups; the 75% quartile for max luteal phase estradiol was markedly higher in women with LPMWs versus without LPMWs (Table 6.2). In contrast, luteal phase progesterone was lower (Table 6.2; P=0.012, respectively) in ARA2 women with versus without LPMWs.



SI Conversion Factors: FSH and LH: mIU/mL x 1.0 = IU/L; Progesterone: ng/mL x 3.18 = nmol/L; Estradiol: pg/mL x 3.67 = pmol/L; Inhibin A and B: pg/mL x 1.0 = ng/L; AMH: ng/mL x 7.14 = pM

Figure 6.1 A-B Growth profiles of the largest 3 follicles (determined using the ID Method) and corresponding hormone profiles during the IOI for individual participants in the RA group. The first day of menses is indicated by a dashed vertical line for each example (top row). ov= ovulation (top row). 0=day of 1st ovulation (x-axes for all graphs). Dominant follicles of the LPMW are indicated by open circles, and dominant follicles of the FPMW are indicated by closed circles on the follicle growth profiles (top row). The days that the CL was detected ultrasonographically are indicated by a solid bar on the x-axis. In Example A, follicle and endocrine profiles for a single woman without a LPMW are shown. In Example B, profiles for a single woman with a LPMW are shown. The IOI was 30 days in Example A and 25 days in Example B.



SI Conversion Factors: FSH and LH: mIU/mL x 1.0 = IU/L; Progesterone: ng/mL x 3.18 = nmol/L; Estradiol: pg/mL x 3.67 = pmol/L; Inhibin A and B: pg/mL x 1.0 = ng/L; AMH: ng/mL x 7.14 = pM

Figure 6.2 A-D Examples of growth profiles of the largest 3 follicles (determined using the ID Method) and corresponding hormone profiles during the IOI for 4 participants in the ARA2 group. The first day of menses is indicated by a dashed vertical line for each example (top row). Ov=ovulation (top row). 0=day of 1st ovulation (x-axes for all graphs). Dominant follicles of the LPMW are indicated by open circles, and dominant follicles of the FPMW are indicated by closed circles on the follicle growth profiles (top row). The days that the CL was detected ultrasonographically are indicated by a solid bar on the x-axis. Follicle and endocrine profiles for a single woman without a LPMW are shown in Example A. Profiles for a single woman with 2 dominant follicles developing within an anovulatory LPMW are shown in Example B. A long IOI (40 days) was observed in Example B. Example C includes follicle and endocrine profiles for a second woman in whom 2 dominant follicles also developed within the anovulatory LPMW. In Example C, a short IOI (20 days) was observed. In Example D, the LPMW resulted in ovulation and a 17 day IOI.

Table 6.2 Endocrine Characteristics of Luteal Phase Major Waves

	Irrespective of LPMW Development$^{\Psi}$	**LPMW Detected***	**LPMW Not Detected***	P-value*
RA	n=10	n=5	n=5	
Mean FSH (mIU/mL)	4.7 (3.0-5.7)[a]	4.7 (3.1-5.4)	4.8 (2.7-6.0)	0.917
Max FSH	7.4 (5.1-11.4) [a]	7.3 (5.6-11.8)	7.6 (5.6-11.8)	0.602
Mean LH (mIU/mL)	7.1 (5.6-10.5)	8.6 (6.5-16.1)	6.5 (6.5-16.1)	0.175
Max LH	16.4 (8.4-31.2)	18.3 (15.5-43.6)	8.69 (6.33-24.9)	0.117
Mean Inhibin B (pg/mL)	24.66 (17.94-50.05) [a]	48.7 (30.5-55.7)	19.1 (13.4-19.5)	**0.009**
Max Inhibin B	74.45 (42.8-145.2) [a]	125.9 (58.7-205.6)	49.3 (34.7-77.9)	0.117
Mean AMH (ng/mL)	2.35 (1.60-2.97) [a]	2.37 (2.00-4.43)	1.84 (1.11-2.66)	0.251
Max AMH	3.05 (1.87-3.80) [a]	3.60 (2.49-6.19)	2.31 (1.40-3.15)	0.076
Mean E2 (pg/mL)	73.25 (49.3-95.0)	90.46 (73.3-109.9)	53.3 (35.2-67.3)	**0.028**
Max E2	118.0 (72.9-140.0)	139.1 (119.5-166.6)	78.0 (56.1-105.7)	**0.016**
Mean Inhibin A (pg/mL)	38.0 (33.7-44.0)	37.2 (28.1-49.0)	38.9 (36.1-46.4)	0.347
Max Inhibin A	57.9 (49.4-81.0)	53.3 (35.9-93.2)	59.2 (54.1-81.4)	0.465
Mean Prog (ng/mL)	7.97 (6.01-10.05) [a]	9.36 (6.00-10.10)	6.70 (5.75-9.25)	0.602
Max Prog	12.92 (10.07-18.21)	14.98 (8.10-20.3)	11.10 (10.20-16.30	0.602
ARA2	n=17	n=8	n=9	
Mean FSH (mIU/mL)	5.8 (4.5-8.1) [b]	6.6 (5.0-8.4)	5.2 (4.4-7.5)	0.211
Max FSH	10.60 (7.63-16.6) [b]	14.0 (8.7-17.3)	8.2 (7.3-14.4)	0.210
Mean LH (mIU/mL)	6.89 (5.41-8.84)	6.9 (6.1-7.9)	5.8 (4.7-9.4)	0.773
Max LH	13.4 (10.1-16.4)	13.3 (9.5-18.4)	14.8 (10.1-15.4)	0.962
Mean Inhibin B (pg/mL)	8.6 (3.0-20.9) [b]	16.0 (3.4-27.7)	7.1 (2.7-15.6)	0.277
Max Inhibin B	22.9 (4.6-64.5) [b]	39.8 (5.9-82.4)	22.9 (3.1-55.8)	0.573
Mean AMH (ng/mL)	0.10 (0.08-0.34) [b]	0.09 (0.08-0.11)	0.13 (0.08-0.89)	0.173
Max AMH	0.15 (0.08-0.45) [b]	0.12 (0.08-0.21)	0.15 (0.09-1.09)	0.262
Mean E2 (pg/mL)	73.5 (60.47-125.47)	96.9 (64.5-199.6)	71.1 (57.3-99.0)	0.321
Max E2	123.5 (91.4-249.9)	177.8 (105.6-327.0)	95.0 (86.2-144.7)	0.074
Mean Inhibin A (pg/mL)	42.8 (28.5-48.3)	39.4 (26.0-44.7)	45.3 (32.4-57.3)	0.178
Max Inhibin A	62.50 (41.55-87.90)	58.3 (34.3-71.6)	69.6 (51.1-100.0)	0.248
Mean Prog (ng/mL)	11.61 (8.13-13.05) [b]	9.65 (5.87-11.30)	13.0 (10.00-15.90)	**0.027**
Max Prog	19.30 (14.44-25.89)	16.0 (9.87-18.90)	24.5 (18.80-28.30)	**0.012**

SI Conversion Factors: FSH and LH: mIU/mL x 1.0 = IU/L; Progesterone: ng/mL x 3.18 = nmol/L; Estradiol: pg/mL x 3.67 = pmol/L; Inhibin A and B: pg/mL x 1.0 = ng/L; AMH: ng/mL x 7.14 = pM

Data are reported over the luteal phase of the IOI.

* Within rows, P-values represent comparisons between women with and without LPMWs.

Ψ Within column 2, common endpoints between age groups are compared. [a,b]Values with different superscripts are different (P<0.05).

6.4.3 Follicular Phase Major Waves (FPMWs)

The prevalence of FPMWs between age groups was not different (RA: 10/10, 100%; ARA2: 16/17; 94%; P=0.630). All FPMWs were ovulatory and all but 1 woman in the ARA2 group had a FPMW during the IOI. Polyovulatory FPMWs occurred only in the ARA2 group (n=3/17).

The growth profiles and corresponding hormone profiles of individual participants with and without FPMWs in the RA (Figure 6.1) and ARA2 (Figure 6.2) groups are shown**.** In Figure 1 (Example A, RA), a FPMW with a codominant follicle was observed. One dominant follicle regressed, and the other continued to grow preferentially. FSH, inhibin B and AMH rose in association with wave emergence in Figure 6.1 (Example A and B, RA). A secondary rise in inhibin B was observed in the early follicular phase in Figure 6.1 (Example A and B; RA). Inhibin A and estradiol rose in association with the selection and continued growth of the dominant follicle. Progesterone remained low throughout the follicular phase.

Wave emergence was more variable in women belonging to the ARA2 group. The range was from 3 days before to 8 days after the onset of menses. There was no FPMW in Figure 6.2 (Example D) because the LPMW was ovulatory in the early follicular phase. LPMW dominant follicles persisted into the follicular phase in Examples B and C. In Figure 6.2 (Example B), FPMW emergence did not occur until the atypical estradiol secretion associated with the LPMW dominant follicles fell. FSH and inhibin B rose in association with wave emergence in Figure 6.2 (Examples A-C, ARA2). Inhibin A and estradiol rose in association with dominant follicle growth in Figure 6.2 (Examples A-C). However, a break in sampling of one week may have impacted our ability to detect the estradiol rise in Figure 6.2 (Example B).

Endocrine characteristics in the follicular phase of women between age groups are compared in Table 6.3. FSH was higher and inhibin B and AMH were lower in ARA2 versus RA women (Table 6.3; P<0.05). Follicular phase estradiol tended to be higher in the ARA2 versus RA groups, in association with a greater prevalence of polyovulation (Table 6.3, P-value versus adjusted P-value).

Table 6.3 Endocrine Characteristics of Follicular Phase Major Waves

		RA	**ARA2**	P-value	(Adjusted)*
		n=10	n=17		
FSH (mIU/mL)	Mean	7.1 (4.8-8.5)	11.1 (7.4-13.1)	**0.010**	**(0.019)**
	Max	10.2 (8.0-13.2)	19.3 (11.2-21.8)	**0.025**	**(0.033)**
	Day 2-4	7.4 (4.3-7.9)	15.5 (450-19.4)	**0.006**	**(0.016)**
LH (mIU/mL)	Mean	11.9 (7.0-18.6)	12.1 (7.2-13.8)	1.000	(0.953)
	Max	30.1 (18.1-76.1)	36.0 (18.5-53.2)	0.880	(0.861)
	Day 2-4	6.3 (4.2-9.6)	8.7 (6.2-12.0)	0.092	(0.074)
Inhibin B (pg/mL)	Mean	68.5 (61.9-79.9)	50.7 (31.2-74.5)	**0.004**	**(0.001)**
	Max	101.0 (91.3-118.1)	77.2 (46.8-103.2)	**0.027**	**(0.012)**
	Day 2-4	64.8 (49.2-73.8)	39.7 (2.6-67.3)	**0.018**	**(0.030)**
AMH (ng/mL)	Mean	2.53 (1.78-3.11)	0.09 (0.08-0.20)	**(<0.001)**	**(<0.001)**
	Max	3.04 (2.27-4.40)	0.12 (0.08-0.30)	**(<0.001)**	**(<0.001)**
	Day 2-4	2.23 (1.99-2.38)	0.08 (0.08-0.37)	**(<0.001)**	**(<0.001)**
E2 (pg/mL)	Mean	71.4 (60.5-80.5)	81.2 (63.6-132.9)	0.175	(0.292)
	Max	164.8 (144.7-199.6)	225 (147.7-323.9)	0.098	(0.242)
	Day 2-4	16.0 (9.6-26.1)	22.8 (15.23-36.52)	0.075	(0.084)
Inhibin A (pg/mL)	Mean	19.4 (14.8-21.1)	22.3 (15.3-27.4)	0.422	(0.482)
	Max	50.9 (40.0-54.0)	47.3 (35.1-60.2)	0.802	(0.349)
	Day 2-4	5.0 (5.0-7.4)	10.45 (5.0-12.75)	0.331	(0.213)

SI Conversion Factors: FSH and LH: mIU/mL x 1.0 = IU/L; Estradiol: pg/mL x 3.67 = pmol/L; Inhibin A and B: pg/mL x 1.0 = ng/L; AMH: ng/mL x 7.14 = pM
Data are reported over the follicular phase of the IOI. Unadjusted p-value reflects comparisons between groups in all (ovulatory) participants.*Adjusted p-value reflects comparisons after removal of polyovulatory cycles from the dataset.

6.4.4 Hormone Production and Diameter of the LPMW Dominant Follicle

Bivariate correlations between max diameter of the dominant follicle in the LPMW and mean and maximum reproductive hormone concentrations of the luteal phase are reported in Table 6.4. In the RA group, the maximum diameter of the dominant follicle in the LPMW positively correlated with mean luteal phase estradiol and negatively correlated with maximum luteal phase LH (r=0.95, -0.95; P<0.01). In the ARA2 group, the maximum diameter of the dominant follicle in the LPMW correlated negatively with mean luteal phase progesterone (r=-0.78, P<0.05). When age groups were combined, the maximum diameter of the dominant follicle in the LPMW correlated positively with luteal estradiol (mean: r=0.59, p<0.05; max: r=0.71, P<0.01) and negatively with maximum luteal LH (r=-0.52, P<0.05). The maximum diameter of the dominant follicle in the LPMW was not correlated with inhibin A across any of the age groups.

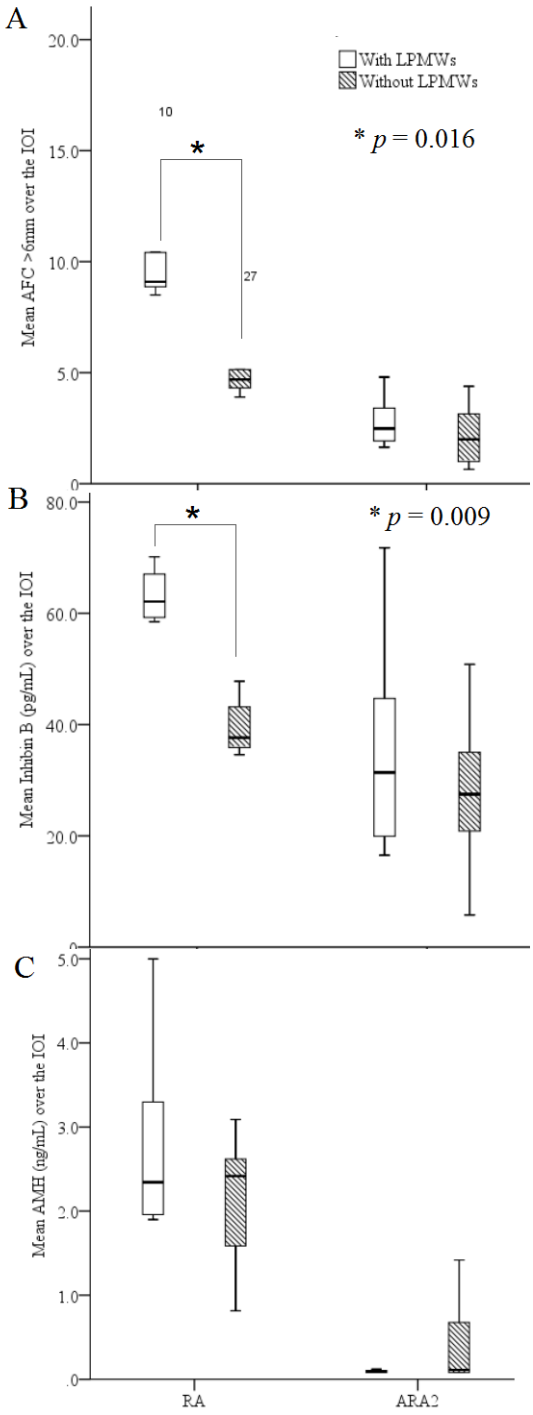
6.4.5 Major Waves and Markers of Ovarian Reserve

In RA women, the mean AFC $\geq$6mm and inhibin B over the IOI were greater in women with LPMWs versus those without (Figure 6.3; p=0.009). In ARA2 women, no differences in AFC, AMH, or inhibin B were detected between women with and without LPMWs (Figure 6.3).

Table 6.4 Relationship between LPMW Dominant Follicle Diameter and Hormone Concentrations during the Luteal Phase

	RA	**ARA2**	**All Women**
	n=5	n=8	n=13
Max LPDF diameter vs.			
Mean LP E2	.95 **	.38 NS	.59 *
Max LP E2	.53 NS	.49 NS	.71**
Mean LP Prog	.53 NS	-.78 *	-.25
Max LP Prog	.53 NS	-.61 T	-.10
Mean LP LH	-.74 T	.16 NS	-.13
Max LP LH	-.95 **	-.18 NS	-.52 *
Mean LP Inhibin A	.00 NS	-.27 NS	.00
Max LP Inhibin A	-.16 NS	-.40 NS	-.10

* P<0.05; ** P<0.01; ***P<0.001; NS, not significant; T, trend ($0.05 \geq P \leq 0.09$). LPDF = luteal phase dominant follicle of the major wave. LP = luteal phase. Tests are one-tailed test.



SI Conversion Factors: Inhibin A and B: pg/mL x 1.0 = ng/L; AMH: ng/mL x 7.14 = pM

Figure 6.3 A-C Differences in mean AFC ≥6mm (A), inhibin B (B), and AMH (C) over the IOI in women with versus without LPMWs. Comparisons within each age group are shown. Differences between women with versus without LPMWs are indicated (*).

6.5 Discussion

In the present study, we characterized endocrinologic changes associated with follicle wave dynamics in women of advanced reproductive age compared to women of reproductive age. Increased estradiol, but not inhibin A, was associated with an increased incidence of polyovulation in the ARA2 versus RA group. Half of the LPMWs were associated with atypically high estradiol and decreased luteal progesterone in the ARA2 group. No difference in inhibin A was detected in women with or without LPMWs which was similar to FPMWs. Greater inhibin B, but not AMH, was detected only in the RA women with versus without LPMWs. Thus, our hypotheses were partially supported.

6.5.1 Luteal Phase Major Waves (LPMWs)

Luteal phase major waves developed in approximately 50% of women evaluated; the prevalence of LPMWs did not change with age. Luteal phase major waves associated with atypically high estradiol secretion were detected prior to noticeable changes in menstrual cyclicity in 2/4 women of advanced reproductive age. The prevalence of LPMWs in reproductive age women in this study was greater than previous reports (33, 103). The greater prevalence of LPMWs in the present study compared to previous reports may have been attributed to a small sample size, differences in participant demographics, lifestyle factors (i.e. diet, exercise) and/or environmental influences (i.e. season, urban versus rural setting) not fully understood.

In contrast to the prevalence of LPMWs across age groups, the growth dynamics of LPMWs did change as women aged. Specifically, the LPMWs emerged earlier (6 versus 2 days before ovulation) in the ARA2 versus RA groups. The largest 1-2 follicles of LPMWs at advanced reproductive age had already achieved dominance at the time of the pre-ovulatory

gonadotropin surges, and then appeared to respond to rising FSH and LH in the early luteal phase. The dominant follicle(s) continued to grow to large, often cystic, diameters (range: 10.0-37.0 mm) and persisted into the late luteal and sometimes early-mid follicular phase. In comparison, LPMWs in the RA group achieved dominance (diameter range:10.0-16.0 mm) and regressed before the emergence of the subsequent FPMW (270). Atypical elevations in estradiol were detected in half of the ARA2 women with LPMWs. In general, we found the larger the dominant follicle, the higher the estradiol and lower the peak LH concentrations. Serum luteal phase estradiol was higher, but not atypically high (i.e., >200 pg/mL), in RA women with versus without LPMWs and in the remaining 4/8 ARA2 women with LPMWs.

There are several reports of an inverse relationship between luteal phase estradiol and progesterone during the MT in women (6, 10, 151, 182) as well as in domestic farm animals (272, 273). However, the physiological mechanisms for this relationship were not understood. In the present study, progesterone concentrations were not different between women with versus without LPMWs in the RA group. However, in the ARA2 group, progesterone was lower in women with versus without LPMWs. It is possible that atypically high estradiol in the luteal phase in the older women may have had a suppressive effect on luteal progesterone production. It is further plausible that the timing of the atypical rise in estradiol influenced its potential effect on luteal function. An early rise in luteal phase estradiol was associated with lower progesterone production; however, a similar observation was not made when estradiol was elevated in the mid-late luteal phase. In contrast to estradiol, luteal phase inhibin A was not different in women with versus without LPMWs in either age group. These data suggest that dominant follicles of LPMWs do not contribute to luteal phase inhibin A production. Alternatively, it is possible that dominant follicle production of inhibin A in the luteal phase may be influenced or masked by

production of inhibin A by the CL. Further research is required to determine if and why dominant follicles from luteal versus follicular phase major waves differentially produce inhibin A.

All but one LPMW was anovulatory. In one woman of advanced reproductive age, an ovulatory LPMW was observed. The ovulatory LPMW emerged 5 days before the first ovulation of the IOI and grew throughout the luteal phase in association with atypically high concentrations of estradiol in the late luteal phase. The LPMW ovulated during menses, followed by a rapid drop in estradiol. A second CL was observed in associated with a second rise in progesterone in the follicular phase. We believe that the high estradiol concentration from the LPMW dominant follicle triggered an LH surge in the early follicular phase, once the inhibitory actions of progesterone were removed. However, due to sampling frequency, we did were unable confirm the presence of a follicular phase LH surge. Ovulation in the early follicular phase has been shown endocrinologically in women undergoing the MT (10).

In the present study, early follicular phase elevations in estradiol were attributed to the development of functional dominant follicles persisting from LPMWs. These findings contradict those of previous studies in which early follicular phase estradiol elevations were postulated to originate from either earlier (153, 193) or accelerated (172, 192) follicular phase follicle development. Moreover, luteal progesterone was greater in the ARA2 versus RA groups, due to a greater frequency of polyovulations from the FPMW. Increased luteal progesterone concentrations have been reported in women of advanced reproductive age previously (6, 153).

In reproductive age women, the occurrence of LPMWs was positively associated with AFC $\geq$6 mm and inhibin B. Women in the RA group with LPMWs had a greater mean AFC

≥6mm and inhibin B over the IOI compared to those without LPMWs. Similarly, individual AFC profiles for follicles ≥6 mm over the IOI closely resembled those of inhibin B. Inhibin B is produced by antral follicles 3-12 mm, and peak at a diameter of approximately 9mm (48). Thus, LPMWs were more likely to develop when a greater number of follicles were recruited into a wave. AMH concentrations were not associated with the development of LPMWs. However, we anticipate that the development of more sensitive AMH assays will allow the conduct of future studies to confirm a relationship between AMH and the development of LPMWs as women age.

6.5.2 Follicular Phase Major Waves (FPMWs)

Follicular phase major waves were detected in all women during the study with the exception of one woman in the ARA2 group. FPMWs consistently emerged at the time of menses onset (i.e., 13-14 days post ovulation) in RA and ARA2 women, and resulted in ovulation. We interpreted these findings to mean that the timing of FPMW emergence in ovulatory cycles appears relatively uniform as women progress from reproductive to advanced reproductive age.

In the ARA2 group, FPMWs developed under the influence of elevated FSH as well as lower inhibin B and AMH, consistent with previous reports (5, 191, 193). FPMWs during advanced reproductive age were associated with greater estradiol (but not inhibin A) production. Thus, our hypothesis that elevated follicular phase estradiol and inhibin A would be a result of an increased incidence of codominance and/or polyovulation was only partially supported. Greater follicular phase estradiol has been previously postulated to relate to a greater incidence of codominance and polyovulation (182, 274). The results of our research support this notion. We believe that the polyovulation occurred in women of advanced reproductive age due to the loss

of inhibin B-mediated negative feedback to FSH, which then widens the FSH window thereby allowing more than one follicle to become dominant (74, 265).

6.5.3 Limitations and Future Directions

This pilot study was conducted to characterize age-related change in major follicular wave dynamics and associated hormone production. Comparing follicular and endocrine dynamics across age groups was a logical starting point to test our hypotheses. However, harmonized clinical and scientific criteria have recently been developed to characterize the stages of reproductive aging (i.e., Stages of Reproductive Aging Workshops (STRAW) criteria) (122). The STRAW stages of reproductive aging are based on changes in menstrual cyclicity, hormone production and antral follicle count (i.e., AFC) that occur in women throughout the reproductive lifespan (122). When datasets from our study were categorized by STRAW criteria, we found that only 5/17 (30%) women in the ARA2 group were considered to be in the early or late MT. The remaining 12/17 (70%) were determined to be in late reproductive STRAW stages. The low proportion of women in the ARA2 group that had entered the MT was attributed, in part, to the evaluation of ovulatory but not anovulatory cycles. We further attempted to compare follicle development and hormone production across STRAW categories of reproductive aging. However, our relatively small sample sizes provided inadequate power to form meaningful conclusions. Therefore, the next step in our research program is to characterize follicle and corresponding hormone dynamics in women during STRAW stages of reproductive aging. The long-term goal of research in this area is to increase our understanding of the physiologic changes that lead to atypical hormone production and changes in menstrual cyclicity as women approach menopause.

The role of AMH in regulating human antral folliculogenesis is not understood. In women of advanced reproductive age, there were several instances where concentrations of inhibin A, inhibin B, and/or AMH were at or below the sensitivity of the assays. Continued research is required to develop more sensitive AMH and inhibin assays in order to investigate the roles of these hormones in regulating age-related changes in follicle dynamics.

6.5.4 Clinical Applications

Documentation of functional (i.e., estrogenic) luteal phase dominant follicles has clinical applications for optimizing fertility therapies in cancer patients. Results of preliminary studies have shown that luteal phase oocyte retrieval in patients requiring urgent treatment prior to chemotherapy resulted in development of healthy, viable embryos (109). Collectively, findings from the previously mentioned study (49) and the present study suggest that it may be possible to initiate ovarian stimulation in all fertility patients at both the early follicular and early luteal phases of the cycle.

The combination of serial ultrasonographic and endocrinologic data provides insight into the origins of atypical hormone production in women of advanced reproductive age. These findings are clinically relevant because aberrant fluctuations in estradiol during the MT have been speculated to be associated with the development of vasomotor symptoms, which can have a significant and negative impact on a women's quality of life. Furthermore, exposure to unopposed estradiol has been shown to place women at increased risk for endometrial cancer (201, 275). Information about the follicular origins of variability in hormone production during the MT is essential for understanding the pathophysiology of unpredictable and debilitating symptoms experienced by women as they age. Increased knowledge about ovarian aging also has

implications for providing insight into the origins of age-related risks for cardiovascular disease, cognitive dysfunction, and declining musculoskeletal health (8, 188, 275, 276).

6.5.5 Summary

FPMWs developed in 94-100% of women, while LPMWs occurred in 30-50% of women; the prevalence of FPMWs and LPMWs did not change with age. The timing of emergence of FPMWs appeared uniform as women progressed from reproductive to advanced reproductive age, despite reduced AMH and inhibin B and increased FSH. In contrast, the growth and endocrine dynamics of LPMWs markedly changed with age. LPMWs emerged earlier, grew longer, and to a larger diameter in association with atypically high estradiol and decreased serum progesterone in women of advanced reproductive age. It appears that the fall in inhibin B, AMH, and resultant rise in FSH that occurs with age enables the development of large, estrogenic, luteal phase dominant follicles. The relationship between acute elevations in luteal phase estradiol secretion and suppression of progesterone production requires further investigation. A greater understanding about the relationships between follicle wave dynamics and hormone production and unwanted symptoms (i.e., vasomotor symptoms) during the MT will provide valuable insight into the development of safe and effective therapies for alleviating vasomotor symptoms.

Chapter 7

GENERAL DISCUSSION

In the studies presented in this thesis, age-related changes in human antral folliculogenesis and reproductive hormone concentrations were characterized. Major follicle wave dynamics were compared across a single IOI in women of reproductive age (RA: 18-35 years, n=27) and advanced reproductive age (ARA1: 36-44 years, n=10; ARA2: 45-55 years, n=21). Hormone production associated with major follicle wave dynamics was evaluated in a subset of women in the RA group (n=10) and ARA2 group (n=17). The RA group was chosen to represent a reference group of ovulatory women in the peak reproductive stage of life. The ARA1 group represented ovulatory women during the late reproductive stage of life. The ARA2 age group was chosen to represent ovulatory women undergoing the transition to menopause.

We are the first to characterize concurrent and serial changes in ultrasonographic (i.e., morphologic) and endocrine characteristics of ovarian function as women age. Previous studies conducted to characterize reproductive aging and the transition to menopause have been based on urinary or serum endocrine data and/or changes in menstrual cyclicity with follicle data restricted mainly to the selected preovulatory follicle. A decline in AFC, AMH and inhibin B were detected with age in association with a rise in FSH, as previously reported. Major waves of follicle development were detected in both the follicular and luteal phases of the IOI. Ovulatory FPMWs occurred in 95-100% of women and LPMWs occurred in 30-50% of women. The prevalence of LPMWs and FPMWs did not change with age. However, the growth dynamics of follicular and luteal phase major waves did change with age. Therefore, our general hypothesis

that changes in major follicle wave dynamics and hormone production would occur with age was supported.

Our data supported the theory that depletion of the ovarian reserve is responsible for altering the activity of the hypothalamic-pituitary-axis which then initiates the physiologic changes that occur as women enter the transition to menopause (175). This theory of an ovarian origin of reproductive aging contrasts the notion that a deterioration in the 'biological clock' in the suprachiasmatic nucleus of the hypothalamus results in altered GnRH release and gonadotropins secretion that occurs with entrance in the MT (180, 277, 278). However, we did not specifically evaluate hypothalamic and/or pituitary sensitivity to reproductive hormones.

7.1 Luteal Phase Major Waves

Although the prevalence of LPMWs did not change, the growth dynamics and hormone production were markedly different between age groups. The dominant follicle(s) of the LPMWs emerged earlier, grew longer, and to a larger diameter in women of advanced reproductive age ($\geq$45 years old). We were able to determine the exact day of emergence of LPMWs in 7/10 women with LPMWs in the ARA2 group. Of the 7 LPMWs, 5 emerged in the mid-follicular phase (day -6 to -4; day 0 = ovulation) of the preceding cycle. The remaining 2 LPMWs emerged around the time of ovulation (day -1 to 2; day 0 = ovulation). The timing of emergence of follicular, but not luteal phase, major waves has been previously investigated. No evidence of mid- to late- follicular phase emergence of the luteal phase major wave has been demonstrated in animal models. Therefore, the mid-late follicular phase emergence of the LPMW is a unique finding in women of advanced reproductive age in our study. It is plausible that the dominant follicles from LPMWs in older women emerged from a follicular versus a luteal phase wave.

Evidence of minor waves emerging in the mid-late follicular phase (i.e., at the time of selection) has been obtained in mares (279) and women (6). We believe that the decreased inhibin B from the reduced follicular pool and resultant rise in FSH led to the emergence of major waves in the mid-late follicular phase (despite the suppressive effects of estradiol from the dominant follicle). The lack of sufficient FSH suppression during the mid-late follicular phase may reflect a combination of reduced hypothalamic responsiveness to estradiol and/or decreased inhibin B production from antral follicles of the wave. From limited studies, it has been shown that reduced hypothalamic/pituitary responsiveness to estradiol occurs in the later stages of reproductive aging (180). Therefore, it is plausible that estradiol from the dominant follicle of the FPMW may be unable to exert its suppressive effects of FSH production due to reduced hypothalamic/pituitary responsiveness with reproductive aging. It is further plausible that AMH (which is declining with age) may enhance the sensitivity of follicles to FSH. AMH has been shown previously to suppress granulosa cell FSH-induced aromatase activity (56, 87). However, it is unclear if intrafollicular concentrations of AMH in similar sized follicles decline in women of advanced versus reproductive. Continued research is needed to elucidate whether AMH has an autocrine or paracrine role within the ovary with reproductive aging.

The sensitivity of the AMH assay became a limiting factor in comparing AMH in women with versus without LPMWs. The largest follicle of the LPMW had already achieved dominance at the time of the preovulatory gonadotropin surge (i.e., ovulation #1 of the IOI). Therefore, we believe that the LPMW dominant follicle likely had acquired the cellular machinery (i.e., aromatase enzyme activity and LH receptors) which allowed it to respond to the FSH and LH surge and continue to grow to a preovulatory or cystic diameter in the subsequent luteal phase. In all women of reproductive age, the LPMWs were growing in association with developing CL,

therefore we believe did not have the machinery to respond to the LH surge like the dominant follicles of the LPMWs in women of advanced reproductive age. In reproductive-age women, LPMWs were associated with high (but not atypically high) estradiol. However, in women of advanced reproductive age, LPMWs were associated with atypically high estradiol. We were not able to directly measure the follicular versus luteal contributions of estradiol or inhibin A in the present study. However, we observed that the larger the LPMW dominant follicles grew, the more estradiol was produced in the luteal phase. Half (4/8) of the LPMWs in the ARA2 group developed in association with atypically high estradiol secretion. We interpret these data to confirm previous speculations (7) that atypical elevations in luteal phase estradiol during the MT originate from age-related changes in major follicle wave dynamics. Thus, our hypotheses were further supported.

Luteal phase progesterone concentrations were higher overall in the ARA2 versus RA groups. These findings were due to a greater incidence of polyovulation, and multiple luteal glands, in the ARA2 group. Polyovulations did not occur in the RA group. However, when data were further stratified, we found that women of advanced reproductive age with LPMWs had lower luteal progesterone concentrations compared to those that did not have LPMWs even after cycles with multiple CLs were removed. We interpret these findings to suggest that follicular estradiol may have a suppressive effect on luteal function. Luteolytic effects of estradiol have been demonstrated in domestic farm animals (8, 9). Similarly, inverse relationships between luteal phase estradiol and progesterone have been reported previously during the MT (6, 10). After closer inspection of individual profiles, we observed that earlier acute luteal phase increases in estradiol appeared to be associated with lower progesterone whereas mid- to late-luteal phase increases estradiol were not. Thus, we believe that the timing of the acute increase in

estradiol may influence its potentially suppressive effect on luteal development similar to reports in domestic farm animals (272, 273, 280). In both the RA and ARA2 groups, LPMW dominant follicle peak diameter was inversely related to peak luteal phase LH concentrations. In the presence of progesterone, estradiol has a suppressive effect on LH secretion. Thus, we believe that the acute increase in luteal phase estradiol further suppressed LH production. This may also explain why all but one of the LPMWs with atypically high estradiol production failed to ovulate. In one woman of advanced reproductive age, a LPMW was able to grow (in the presence of a CL) and ovulate in the early follicular phase following luteal regression. An LH surge was not confirmed due to the frequency of blood sampling. However, a second rise in progesterone was detected following ultrasonographic documentation of follicle rupture. The physiologic mechanisms allowing some but not all LPMWs to ovulate are not known. We speculate that the acute increase in estradiol production from the luteal phase dominant follicle was able to stimulate an LH surge which then resulted in ovulation. Not all LPMWs resulted in atypically high estradiol production. However, atypically high luteal estradiol production was only observed in association with LPMWs. Documentation of ovulation at the time of menses in women has clinical implications for understanding contraceptive needs of women as they approach menopause.

In summary, LPMWs in women of advanced reproductive age may have one of the following 3 fates:

Type I Emergence around the time of ovulation, normal estradiol and progesterone production, resulting in anovulation (both participants had a 26 day IOI (n=2));

Type II Mid/late follicular phase emergence, normal estradiol and progesterone production, resulting in anovulation (the IOIs ranged from 26-30 days (n=2)); or,

Type III Mid/late follicular phase emergence associated with atypically high estradiol, lower progesterone, resulting in either ovulation or anovulation (the IOIs ranged from 17-41 days (n=4)).

A proposed model of the endocrine changes from the depletion of the ovarian reserve associated with reproductive aging and the consequences on luteal phase major wave emergence are depicted in Figure 7.1.

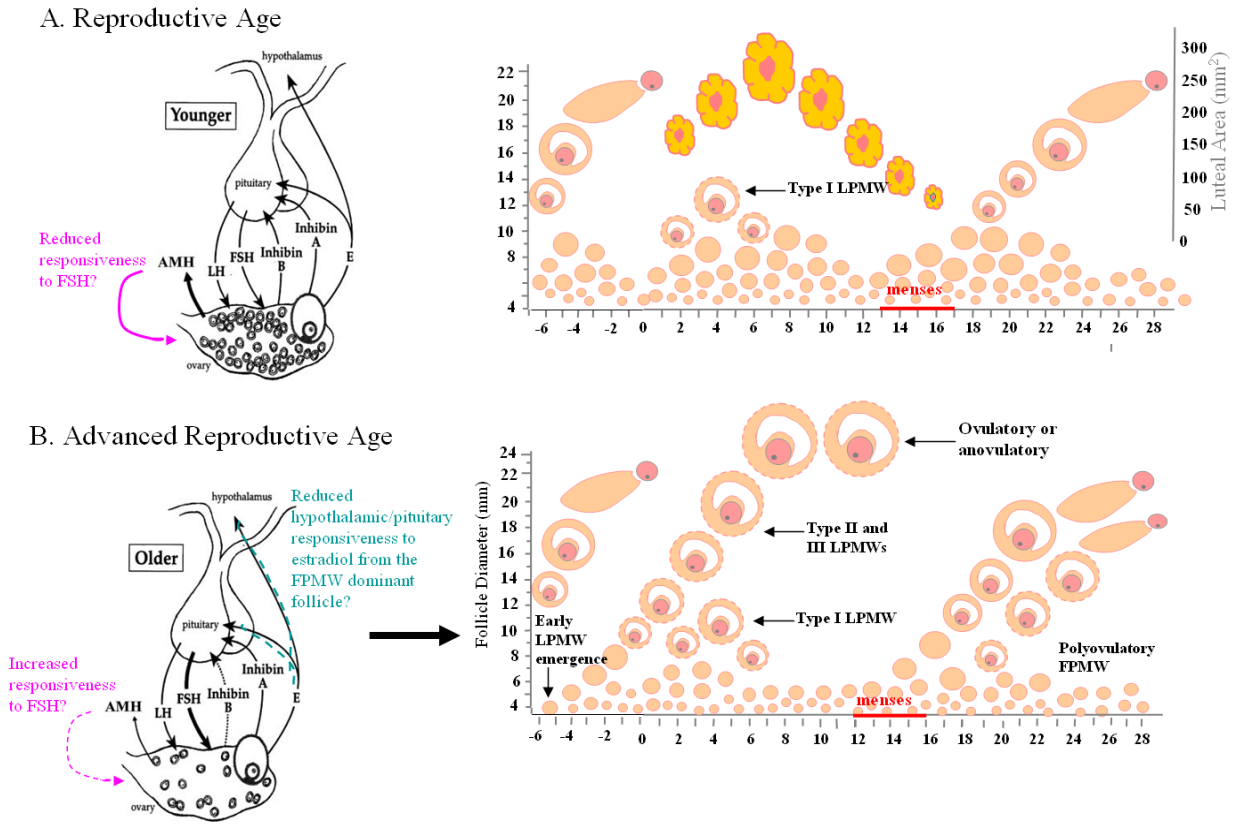


Figure 7.1: A proposed model of the morphologic and endocrinologic changes which occur during reproductive aging. Dashed lines around follicles indicate a pattern of follicle development occurs in some women. A: A 3rd anovulatory major wave occurs in some

reproductive aged women (not shown). Modified from Broekmans et al., 2009 (167), Soules et al., 1998 (176), and Baerwald et al (33).

7.2 Follicular Phase Major Waves

In contrast to LPMWs, fewer differences in the growth dynamics of FPMWs were detected. The day of wave emergence, day of selection, diameter at selection, and preovulatory diameter in the FPMW were similar among age groups. No obvious differences in the growth interval of the FPMW dominant follicle were detected between groups. The lack of differences in timing of emergence, day of selection, and preovulatory diameter of the FPMW across age groups contrasts findings from previous studies (153, 193, 194, 210, 211). We believe that underlying minor patterns of follicle development occur across the menstrual cycle/IOI. It is plausible that the endocrine environment resulting from follicle and/or luteal development in the luteal phase determines the timing of FPMW emergence and whether a minor wave will develop into a major wave. In previous studies, indirect assessments of follicular development based on hormone changes and/or ultrasonographic data limited to the dominant preovulatory follicle were made. In contrast, we assessed serial changes in the growth dynamics of individually-identified follicles over an entire IOI. Therefore, differences in methodology may have contributed to the variation among reported findings. Categorization of data by age in the studies discussed versus the degree of menstrual cycle (ir)regulatory and stage of reproductive aging in other studies may have further contributed conflicting results. We identified two possible sources of elevated estradiol in the follicular phase. Polyovulatory cycles only occurred in the ARA2 group, consistent with previous studies (192, 194, 196). Polyovulatory FPMWs in the ARA2 group were associated higher maximum (i.e., preovulatory) follicular phase estradiol, supporting previous speculation (182). Early elevated follicular phase estradiol has been previously attributed to earlier (153, 193) or accelerated (172, 192) dominant follicle growth. However, we

were able to document that early follicular phase estradiol (i.e., day 2-4) was also associated with development of estrogen-producing LPMWs persisting into the early follicular phase, as opposed to earlier emergence of the FPMWs reported from other investigators(153, 193, 194). We found that subordinate follicles grew to smaller diameters with reproductive aging, similar to domestic farm animals (211). It is plausible that a smaller number of follicles within a wave may limit the diameter to which subordinate follicles can develop.

7.3 Major Follicular Wave Dynamics and Menstrual Cyclicity

Unique patterns of follicle development were characterized during ovulatory cycles in the ARA2 group, consistent with previous research (1, 5, 6). An abnormally short IOI was detected in one participant which resulted from the development of an ovulatory LPMW. In contrast, abnormally long IOIs occurred due to the presence of lag phases. Previous investigators have characterized lag phases as times of temporary ovarian non-responsiveness to pituitary stimulation by FSH and resulting lack of estradiol production (155, 198). It was hypothesized that an increased rate of atresia, slowed early antral follicle growth, or a lack of available follicles accounted for the limited endocrine activity in a lag phase (155, 198, 199). We observed a lack of follicle development beyond 6 mm during lag phases in association with elevated FSH and LH. Major and minor waves of follicle growth have been characterized in follicles $\geq$5mm; however, major waves were those in which growth proceeded beyond 6 mm and a dominant follicle was selected at approximately 10 mm (70). We concluded that major waves of follicle development did not occur during lag phases. Minor waves may have emerged during the lag phase; however, minor waves were not characterized in this study.

7.4 The Influence of Ovarian Reserve on the Development of LPMWs

The factors which determine whether or not a LPMW develops remain poorly elucidated. We observed that reproductive age women with a higher AFC $\geq$ 6 mm and greater inhibin B were more likely to develop LPMWs. Thus, a greater number of follicles available for cyclic emergence into a wave increased the likelihood of developing a LPMW in reproductive age women. To our surprise, no differences in AFC 2-9 mm and serum AMH were detected in women of reproductive age. However, it is possible that the inability to detect a difference in AFC 2-9 mm and AMH in women with versus without LPMW was related to limitations in the sensitivity of AMH assays and the inability to reliably detect serial changes in antral follicles < 4mm. The relationship between LPMWs, AFC, and ovarian reserve is not fully understood.

7.5 Future Directions and Clinical Applications

Age-related changes in major follicle wave dynamics were evaluated in the present study. However, minor waves were not characterized. The age-related decline in the number of antral follicles, frequency of data collection (i.e., 1-3 days), and sensitivity of ultrasonography in the present study limited our ability to reliably characterize minor waves. We observed the emergence of minor waves every 4-6 days throughout the IOI in reproductive age women which appeared to underlie the development of major waves. We believe that the follicles recruited into the LPMWs in women of advanced reproductive age may originate from an underlying minor wave that emerged in the mid follicular phase of the preceding cycle. Emergence of minor waves at the time of selection has been observed previously by our group (unpublished data) and also in mares (279). The development of novel techniques such as ultrasound biomicroscopy for in vivo

use and improved ultrasound technology may further enable research to characterize minor waves of antral folliculogenesis in women.

Categorization of our data by age was a logical starting point to characterize changes in follicular dynamics as women approach menopause. We recruited women in age groups which have been shown to represent the peak reproductive (RA), late reproductive (ARA1), and the menopausal transition (ARA2) stages of life (2, 150).Women with either regular or irregular cycles were recruited into the ARA2 group. However, only ovulatory cycles were evaluated. Ovulatory and anovulatory cycles are a feature of the MT however the prevalence of anovulatory cycles increases as the MT progresses. We believe that exclusion of anovulatory cycles in our study prevented us from including women in the later stages of the MT. We observed that only 30% were found to be in the MT when we categorized data by STRAW criteria for menstrual cyclicity (122). It was not possible to analyze our data according to STRAW categories as the resultant small sample size (27 women distributed over 5 categories of reproductive life) did not provide enough statistical power to test our hypotheses. Therefore, future studies should be conducted to characterize follicle wave dynamics in both ovulatory and anovulatory cycles across the different STRAW stage of reproductive aging.

The serum concentrations of inhibin A, B, and AMH in women of the ARA2 group were frequently at, or below, the sensitivity of the assays. As a result, we were not able to characterize serial changes in AMH concentrations in all of the older women. The development of more sensitive AMH assays is critical for elucidating the role of AMH in regulating antral follicle wave dynamics.

Hormone therapy (HT) is used by women during the MT to treat unwanted symptoms (i.e., vasomotor symptoms, night sweats, vaginal dryness) and to prevent complications related to the low estrogen (i.e., osteoporosis) (281). LPMWs are a source of atypically high estradiol as women age. Future research should be conducted to study relationships between atypically high estradiol production associated with the development of LPMWs, unwanted symptoms, and endometrial hyperplasia. A greater understanding of the pathophysiology of unwanted symptoms and age-related increased risks for endometrial hyperplasia will enable the development of novel therapies for preventing and/or treating these debilitating adverse events. The discovery of inhibin B and its direct role in regulating follicle growth dynamics via FSH offers an exciting avenue to explore a potential new line of therapy for suppressing follicle growth. A detectable decline in inhibin B occurs in the fourth decade of a women's life (170, 172, 173). Impaired folliculogenesis and aberrant hormone production begins following the decline in inhibin B. We propose that incorporating inhibin B into a hormone therapy regimen may be an effective way to suppress aberrant follicle growth without incurring the same health risks associated with long-term estrogen supplementation (282).

In summary, major waves of follicle development occur in ovulatory cycles throughout the reproductive lifespan. The depletion of the ovarian reserve as women age results in a decline in inhibin B, rise in FSH, a consequent alteration in follicle wave dynamics and resulting atypical production of estradiol and progesterone during the menstrual cycle. The growth dynamics of FPMWs remains fairly constant as women age with exception of an increased incidence of polyovulation and smaller subordinate follicles. However, the growth dynamics of LPMWs markedly changed with age. In women of advanced reproductive age, LPMWs emerged earlier,

grew longer and to a larger diameter, in association with atypical elevations in luteal phase estradiol and lower luteal phase progesterone compared to women without LPMWs.

Chapter 8

GENERAL CONCLUSIONS

The results of the studies presented in this thesis have led to the conclusion that follicle wave dynamics and associated hormone production change as women age. The specific conclusions are listed below.

1. Waves of follicle development occur in both the follicular and luteal phases of the IOI. The prevalence of major follicles waves does not change as a woman ages. Approximately 95-100% of women develop FPMWs and 30-50% of women develop LPMWs.
2. An increased incidence of polyovulation and smaller subordinate follicles in FPMWs occurs with age.
3. The growth dynamics of LPMW and associated hormone production change with age:
    a. LPMWs may be anovulatory or ovulatory in women of advanced reproductive age;
    b. LPMWs emerge earlier, grow longer and to a larger diameter in women of advanced reproductive age compared to those of reproductive age; and,
    c. In 50% of women of advanced reproductive age, atypically high luteal phase estradiol and low (but not atypical) progesterone occur in association with the development of LPMWs.
4. In women of reproductive age, an increased AFC $\geq$6mm and inhibin B (which indicates a greater number of follicles available for recruitment into a wave) are more likely to develop LPMWs.

Chapter 9

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