Assessment of the uterine and ovarian cycles during the window of implantation

Zsofia Fedorcsak

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Department of Gynecology, Oslo University Hospital
The Faculty of Medicine

UNIVERSITY OF OSLO

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Abstract

Precise determination of point in the uterine cycle is of importance when timing embryo transfer, as the state of the endometrium can affect treatment outcomes. The stage of the endometrial cycle can be approximated through hormonal changes, body temperature, and ultrasound imaging, but studies have found that such indirect measures may deviate from the true state of the endometrium when assessed histologically. This project aimed to further elucidate the concurrence and deviation between the uterine and ovarian cycles by comparing self-reported day in cycle, progesterone levels and histological slides evaluated semi-quantitatively using Noyes criteria.

Our findings suggest that the uterine and ovarian cycles are often asynchronous, both between patients and between different cycles in the same patient. Indeed, 55% of samples evaluated histologically appeared underdeveloped compared to what would be expected from the self-reported day in cycle. The deviation between the uterine and ovarian cycles occurred irrespective of serum progesterone levels, indicating that untimely or abnormal progesterone release was unlikely to cause the delayed maturation. The observed asynchrony between the uterine and ovarian cycles may have implications for female reproductive health, especially for patients trying to conceive.

1.0   Introduction

The female reproductive organs undergo physiological changes in response to the fluctuating hormone levels of the menstrual cycle. The uterus experiences especially drastic morphological changes during this time, in large part caused by the endocrine actions of the ovaries. A deeper understanding of ovarian and uterine physiology is therefore needed to appreciate how these organs interact and influence each other.

1.1 The female reproductive system

The female reproductive system has several components, each with their own important functions. This thesis will focus on the functions of the ovaries and the uterus, but brief introductions to the other organs will be given as well.

1.1.1 Internal reproductive organs

The internal reproductive organs include the vagina, cervix, uterus, fallopian tubes, and ovaries (**Figure 1**).
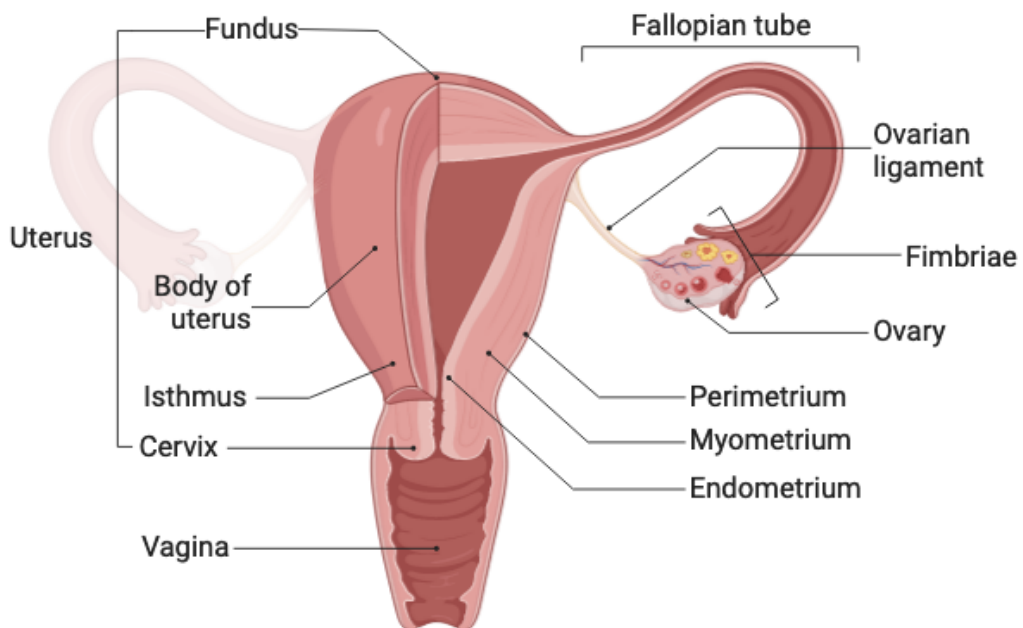

Figure 1. Illustration showing the internal reproductive organs. Created with BioRender.com.

The vagina is a muscular tube connecting the external genitalia to the cervix. It has important functions for sexual intercourse, where it can act as a reservoir for sperm prior to deposition in the cervix. Although the sperm must pass through the vaginal canal for fertilization to occur,

the natural vaginal environment is hostile to these cells due to its low pH (between 3.8 and 4.5). This acidic environment is largely due to colonization by Lactobacillus spp., which secrete acidic products. This low pH, although hostile to sperm, contributes substantially to protection from bacterial infections. There is therefore a balancing act happening in the vaginal canal, where the need for protection from infection and the need for sperm entry are at odds [1]. Additional roles of the vagina include acting as an excretory canal for menses, and expulsion of the fetus during childbirth [2].

The cervix connects the vagina and the main body of uterus. In contrast to the low pH of the vagina, the cervical pH is largely neutral, at around 7. The environment is therefore more hospitable for any deposited sperm [1]. The cervical secretions also play an essential role in their interactions with sperm. The qualities of the cervical mucus changes throughout the menstrual cycle, regulated by the secretion of estrogen and progesterone from the ovaries. Around the time of ovulation, rising levels of estrogen will induce secretion of mucus that is clear, thin and watery, making it easier for sperm to pass through. During the later phases of the menstrual cycle, progesterone levels will rise and become dominant. This induces production of a mucus that is thicker and stickier, making the passage of sperm more challenging[3]. In addition to its function as a "gatekeeper" for sperm, the cervix also limits the ascent of bacterial infections, though through different mechanisms than the vaginal canal [1].

The fallopian tubes connect the uterus and ovaries. They have important functions in reproduction, and pathology in this area is a common cause of infertility. Their main purpose is acting as the site of fertilization and the subsequent transport of the ovum into the uterus for further development [4]. This is facilitated through the actions of the tubal epithelial cells, some having secretory functions and others being ciliated. The secretions create a favorable environment for the ovum, while the beating action of the cilia transport the content of the tubes down towards the uterus. As is the case for the cervix, the fallopian tubes also undergo physiological changes in response to the fluctuating hormone levels of the menstrual cycle [5]. When estrogen-levels are high, the beating action of the cilia and the rate of mucosal secretion increases. This creates a favorable environment for fertilization and transport of the ovum. The opposite can be seen when progesterone-levels rise, with marked cellular deciliation and atrophy of secretory cells [5].

1.1.2 The uterus

The uterus is a hollow organ connected to the vagina through the cervix distally, and to the fallopian tubes proximally (**Figure 1**). The non-pregnant uterus is relatively small, being approximately 8 cm long, and 5 cm wide [6]. Most of its mass is made up of muscle tissue called the myometrium, which has a total weight of around 60 grams. The inner lining of the uterine cavity consists of glandular and vascular tissue, known as the endometrium. The structure of the uterus largely reflects its important role in supporting pregnancy.

As previously mentioned, fertilization usually occurs in the fallopian tubes, where the ovum is subsequently transported downward towards the uterus. The embryo itself will develop in the uterus following implantation in the endometrial wall. The glandular tissue of the endometrium secretes mucus rich in both nutrients and growth factors, important for implantation and initial embryonic development [7]. The myometrium will expand significantly during pregnancy to accommodate the growing fetus. During labor, the muscle tissue will contract in an organized, rhythmic fashion to help expel the neonate [8].

The different areas of the uterus can be divided into sections. The superior part, the area above the uterine connection to the fallopian tubes, is the fundus. The area below the fallopian tubes is the uterine body. The body and the cervix are connected by a small "neck" called the isthmus. The cervix makes up the most inferior part of the uterus [9].

The uterine wall itself is divided into different layers: the perimetrium, myometrium and endometrium. The perimetrium denotes the outermost layer, the myometrium is the muscle tissue found in the middle of the uterine wall, and the endometrium is the glandular tissue lining the uterine cavity.

1.1.2.1 Perimetrium

The outermost layer of the uterus, or the perimetrium, is composed of a serous layer of mesothelial cells continuous with the peritoneum. The perimetrium envelopes the uterus and its supporting ligaments, allowing for frictionless connection to adjacent intraperitoneal organs.

1.1.2.2 Myometrium

The myometrium denotes the bundles of smooth muscle found directly beneath the perimetrium. The muscle fibers run in different directions across the uterine wall, comprised of an outer-, middle-, and inner layer. The outer layer consists of longitudinal and transverse muscle fibers. The longitudinal fibers run along the antero-posterior midline of the uterus, while the transverse fibers envelop the entire organ. Some of the transverse fibers will continue beyond the walls of the uterus, and form a number of the ligaments securing it to surrounding structures [10]. The middle layer, in addition to muscle fibers, has many venous sinuses and is therefore also called the *stratum vasculosum.* The muscle fibers in this layer run in different directions with an irregular arrangement [11]. The inner layer, similarly to the outer layer, contains both longitudinal and transverse muscle fibers. These fibers remain within the uterine wall and do not contribute to the formation of any supporting ligaments [10].

The characteristics of the myometrium display periodic changes. The tissue will expand greatly during gestation to accommodate the growing fetus. The muscle tissue will also experience physiological changes in response to hormonal stimulation. The myometrium is usually at its thinnest during menstruation and will gradually thicken, reaching its peak during the proliferative phase [12].

Leiomyomas are common tumors arising from the myometrium. Although oncologically benign, these tumors can significantly impair the quality of life of those affected. Complications include infertility, heavy menstrual bleeding, constipation, and chronic pelvic pain. A majority of hysterectomies are performed to manage the symptoms of myomas [13].

1.1.2.3 Endometrium

The endometrium is the innermost layer of the uterine wall. It is the site of embryonic implantation and initial development, and will shed every month in the absence of pregnancy. It consists of a richly vascularized glandular tissue, which undergoes significant changes throughout the menstrual cycle.

1.1.2.4 Overview of endometrial histology

The endometrial tissue is primarily composed of epithelial, endothelial, and stromal cells. A variety of immune cells are also present throughout the menstrual cycle. The endometrium can be divided into two sections: the *stratum basale* (basal layer) and the *stratum functionale* (functional layer). The basal layer remains largely unchanged during the menstrual cycle and is not shed. The functional layer can be further divided into two layers, the *stratum spongiosum* and *stratum compactum.* The spongiosum is immediately adjacent to the basal layer, and has a "spongy" appearance. The compactum is denser, and makes up the superficial layer of the *functionale.* The entire functional layer is shed during menstruation, and will regenerate from the remaining basal layer in the next cycle [14].

There are two main types of endometrial epithelial cells: the luminal and the glandular. The luminal cells coat the innermost surface of the endometrium. They are pseudostratified columnar epithelial cells that delve into the deeper tissue layers to form simple tubular glands made up of glandular epithelium. The glandular epithelium of the functional layer undergoes more profound changes throughout the menstrual cycle, but subtle changes can also be seen in the luminal cells [15]. The glands of the endometrium secrete various substances, collectively referred to as *histotroph.* The histotroph consists of cytokines, hormones, ions, enzymes, nutrients such as glucose and lipids, and growth factors, which have been implicated in endometrial stromal maturation, the facilitation of implantation and early embryonic development [16].

The uterine vascular supply is of great functional importance. Two types of arteries, straight and spiral, supply the basal and functional layers respectively. The straight arteries are mostly unresponsive to hormonal fluctuations, but the spiral arteries change significantly in appearance in response to the fluctuating levels of steroid hormones seen throughout the cycle [14, 17]. The transformation of spiral arteries is necessary for successful pregnancy [18].

The stromal cells make up the bulk of the endometrial tissue. As the other endometrial cell types, they undergo changes in response to fluctuating steroid hormones. This includes considerable tissue remodeling in the form of both proliferation and breakdown. The stromal cells have also been implicated in local immune modulation in the endometrium [19].

1.1.3 The ovaries

The ovaries are the female gonads, and play a fundamental role in fertility and the regulation of the menstrual cycle. A natural pregnancy cannot occur without proper ovarian function, as this is the site of oogenesis. The regulation of the menstrual cycle happens through the secretion of ovarian steroid hormones, mainly estrogens, androgens and progestins. Regulation of hormone release from the ovaries is mediated through the hypothalamic-pituitary-ovarian axis (see section *1.2 The menstrual cycle*).

1.1.3.1 Follicular development

During female embryonic development the precursor cells to the oocytes, the oogonia, migrate to the fetal ovary. Here, the oogonia will undergo mitosis, creating around 7 million germ cells total. This rapid division takes place between the second and seventh months of gestation and is followed by a major decline in cell numbers caused by widespread apoptosis. The surviving cells undergo their first meiotic division and will arrest in prophase I as primary oocytes. A layer of supporting granulosa cells form around the primary oocyte, making the primordial follicles. In the later stages of folliculogenesis, and outer layer of mesenchymal thecal cells will form around the follicle. The widespread follicular degeneration (atresia) seen in the fetal ovary will continue after birth, resulting in only about 400 follicles completely maturing during the woman's lifetime [20]. A schematic overview of ovarian follicular development can be seen in **Figure 2**.
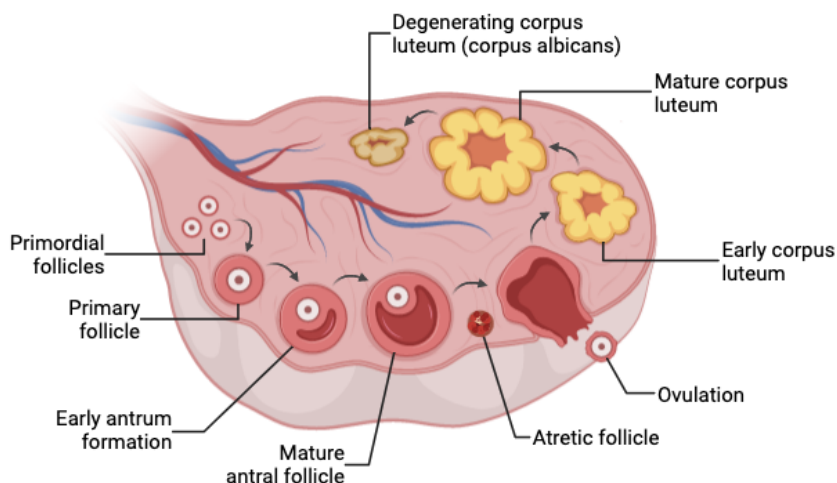
Figure 2. Initial follicular development and subsequent maturation during the ovarian cycle. The primordial follicle, containing an oocyte, develops into a preantral follicle. Upon cyclical hormonal stimulation, the follicle will develop into a mature antral follicle, which during ovulation will release its oocyte into the fallopian tube. Ovulation marks the end of the ovarian follicular phase. The ovulated follicle will then develop into a corpus luteum, marking the beginning of the luteal phase. The corpus luteum will eventually degenerate into a corpus albicans. Created with BioRender.com.

The primordial follicles will develop into preantral follicles, at which stage most will undergo atresia. Follicular development is initiated continuously, but the controlling mechanisms are poorly understood [21]. Initial follicular development lasts around two months, where the follicles will go from primordial to primary to preantral, and is characterized by the growth of both the granulosa and thecal cell layers. During this time the oocyte will also grow in size, acquiring the nutrients, mRNAs, organelles and enzymes necessary for further development [20]. Surrounding the oocyte, the granulosa cells produce a cell-free zone rich in glycoproteins, termed the *zona pellucida*. Importantly, the progression of the follicle from primordial to preantral is largely independent of gonadotropin regulation.

During the next stage of follicular development, preantral follicles mature into antral follicles. In the late preantral stage, the follicular cells acquire hormone receptors. The granulosa cells become responsive to follicle stimulating hormone (FSH) and estrogen, while the thecal cells respond to luteinizing hormone (LH). Thus, subsequent development of the follicle becomes dependent on the serum levels of these hormones. If the hormonal stimulation is insufficient, the follicle may become atretic and degrade. However, if progression through the preantral phase coincides with rising serum levels of pituitary hormones, the follicle may progress to the antral stage. The antral phase sees rapid proliferation and growth of both granulosa and thecal cells, creating an even thicker layer around the oocyte. This phase is characterized by the formation of a fluid-filled space (the antrum) in the granulosa layer of the follicle. The follicular fluid of the antrum is produced by the granulosa cells, and contains hyaluronan and proteoglycans [22].

During the antral stage, the follicle becomes a potent endocrine organ, secreting large amounts of steroid hormones. The ovarian follicular phase is marked by a dominance of estrogen, with estrogen biosynthesis taking place in the granulosa cells. Androgens are needed for estrogen synthesis, but the granulosa cells cannot access the necessary cholesterol substrate for androgen production. De novo androgen synthesis therefore happens in the thecal cells, with the subsequent conversion of androgens into estrogens happening in the granulosa cells. Androgen synthesis is regulated through LH, while the conversion into estrogen is FSH-dependent [23]. The granulosa cells are also sensitive to the estrogens themselves, which have a proliferative effect on the cells. A positive feedback loop therefore develops, with estrogen-levels increasing as more cells proliferate to convert androgens into estrogens. This culminates in sustained high estrogen levels at day 10-12 of the menstrual

cycle. Above a certain threshold, estrogen exerts positive feedback effects on the LH-secretion of the anterior pituitary, inducing the LH surge during the pre-ovulatory phase. In the early follicular phase, when estrogen levels are low, the steroid hormone exerts a negative feedback effect on FSH and LH release [24].

The oocyte is released from the follicle into the fallopian tube during the ovulatory phase. The precise mechanism inducing ovulation is incompletely understood, but rising serum levels of progesterone and the LH-surge during the pre-ovulatory phase are likely important.
The high estrogen levels seen during the late follicular phase lead to a subsequent surge in LH. Ovulation is dependent on the LH surge, with oocyte release consistently occurring 42-46 hours after LH levels peak. The granulosa cells of the follicle acquire sensitivity to LH through increased expression of LH-receptors, mediated by FSH in the presence of estrogen. The LH-sensitized granulosa cells produce progesterone in response to the mid-cycle LH surge. The combined production of progesterone by luteinized granulosa cells and thecal cells contribute to the subsequent mid-cycle FSH surge [25].

Rising LH levels also affect the oocyte and follicle itself. The LH surge triggers the oocyte to complete its first and begin its second meiotic division. It will remain arrested in metaphase II of its second meiotic division until it is fertilized. During this time there is also a profound weakening of the follicular wall, owing to increased activity of proteolytic enzymes in response to rising LH and progesterone levels. This allows the oocyte to pass through the ruptured wall, thus completing ovulation.

Ovulation is followed by the luteal phase, which is characterized by the formation of the corpus luteum composed of the remaining thecal and luteinized granulosa cells. It secretes large amounts of progesterone into the circulation, which helps prime the endometrial lining for implantation and pregnancy until the placenta can assume the maintenance of progesterone levels. There is concomitant estrogen secretion, which reaches a peak during the mid-luteal phase. Maintenance of the corpus luteum is dependent on the continued secretion of LH, but progesterone has a negative feedback effect on LH release at the level of the hypothalamus and the anterior pituitary. Thus, LH secretion decreases with increasing progesterone levels. Over time, and in the absence of the pregnancy-specific hormone human chorionic gonadotropin (hCG), the corpus luteum will degenerate into a lump of scar tissue (corpus albicans). This happens through a process called luteolysis, which marks the end of

the ovarian cycle. It is unknown what exactly triggers luteolysis, but some believe the estrogen surge at mid-cycle is of importance [26]. During the late luteal phase progesterone secretion will first decline and then cease, triggering the shedding of the endometrial lining, which marks the end of the uterine cycle.

1.2 The menstrual cycle

The menstrual cycle occurs in most women of reproductive age. On average it's about 28 days long, but the cycle length may vary both within the same person and among individuals. The variable length is mainly attributed to the follicular phase, as the luteal phase is assumed to be more consistent in duration [27]. Menstruation marks the beginning of the cycle, and typically lasts for five days. During this time, the functional layer of the endometrium is shed along with around 60 ml of blood [28]. Both the length of menstruation and the amount of blood loss can vary significantly.

The menstrual cycle can be viewed as a result of two concurrent, interlinked, and cyclic processes: the ovarian and endometrial cycles. The first phase of the menstrual cycle is termed either the follicular or proliferatory phase, depending on the organ system in question. The first phase of the ovarian cycle is the follicular phase, while the corresponding phase in the uterine cycle is the proliferatory phase. The follicular and proliferative phases prepare the reproductive system for pregnancy, by stimulating the maturation of ovarian follicles and rebuilding the endometrial lining. The last phase of the menstrual cycle is the luteal or secretory phase, where the reproductive system accommodates for possible pregnancy through progesterone release and further endometrial maturation. The entirety of the menstrual cycle is tightly controlled by the hormones of the hypothalamic-pituitary-ovarian (HPO) axis (**Figure 3A**).
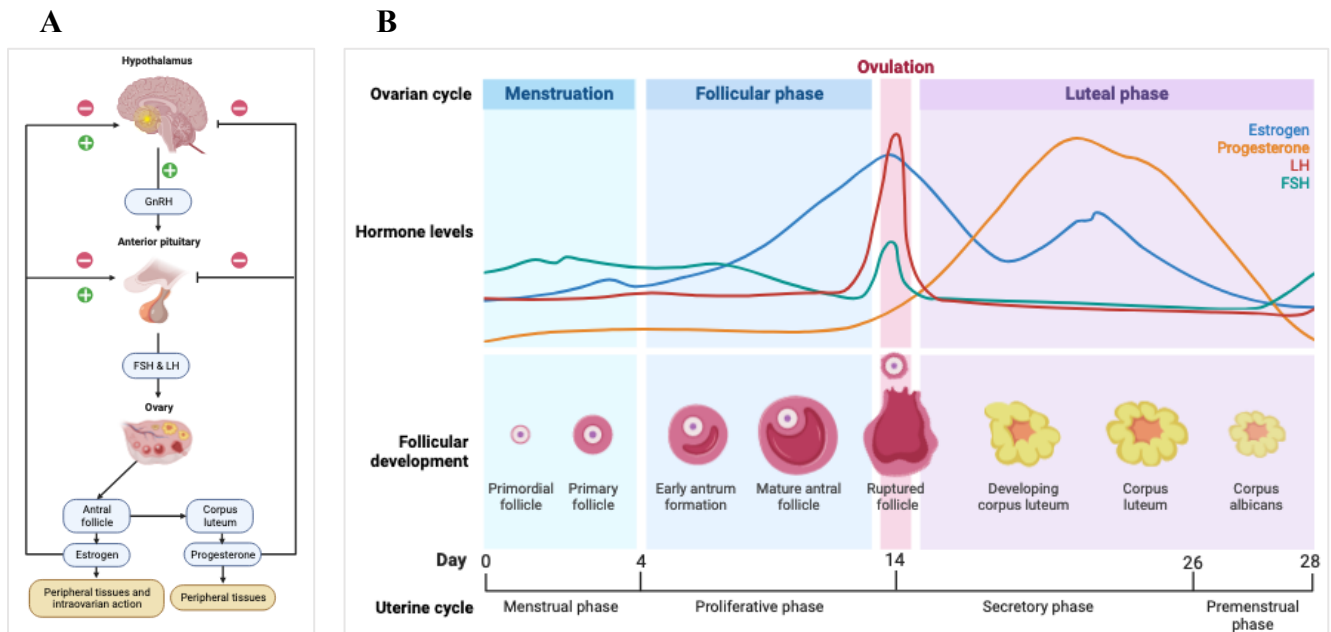
Figure 3. Hormonal control of the menstrual cycle. **A**, illustration of the hypothalamic-pituitary-ovarian axis, including the numerous positive- and negative feedback loops. Adapted from *Human Physiology* (2018)*, by Pocock et al. Created with BioRender.com. **B**, Overview of serum hormone levels during the ovarian cycle. Its relation to the uterine cycle is shown at the bottom. Adapted from "Ovarian hormones throughout the menstrual cycle" by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates.

1.2.1 Hormonal control

The menstrual cycle is regulated through the action of several hormones, including gonadotropin releasing hormone (GnRH), follicle stimulating hormone (FSH), luteinizing hormone (LH), estrogen, and progesterone. The fluctuation of these hormones throughout the menstrual cycle, and their relation to the ovarian and endometrial cycles, can be seen in **Figure 3B**. However, the regulation of the menstrual cycle is complex, and involves a multitude of other hormones, cytokines, and growth factors as well.

GnRH is released by hypothalamic neurons in a pulsatile fashion, and travels to the anterior pituitary through the blood. The frequency of the pulses varies throughout the menstrual cycle, being between 1-2 hours during the follicular phase and around 4 hours during the luteal phase [29]. The pulsatile stimulation of the anterior pituitary results in concomitant pulsatile release of FSH and LH.

LH-secretion is stimulated by high GnRH frequencies. The frequency of LH release will increase slightly in the pre-ovulatory phase, and subsequently decrease after ovulation. In addition to frequency-changes, the amount of secreted LH also varies. LH-levels are initially

low during the early follicular phase, but start to increase during the mid-follicular phase in response to rising estrogen levels, culminating in the LH-surge prior to ovulation [25]. Following luteinization of the granulosa cells after ovulation, estrogen levels decrease while progesterone levels increase. This releases the anterior pituitary from the positive feedback effects of estrogen, decreasing LH secretion throughout the luteal phase.

FSH-secretion is stimulated at low GnRH frequencies. Peak FSH-levels are reached at mid-cycle, with a surge similar to the one seen for LH – although FSH reaches a significantly lower serum concentration [30].

Secretion of steroid hormones from the ovaries is induced by circulating levels of FSH and LH. Estrogens, which dominate the first half of the menstrual cycle, have important proliferatory effects throughout the reproductive tract. During the follicular phase, estrogen is secreted by the follicular granulosa cells in response to circulating FSH. Lower levels of estrogen are released during the luteal phase by the corpus luteum. Serum levels of estrogens peak twice during the cycle: once in the pre-ovulatory phase and once in the mid-luteal phase. Several estrogens are produced, including estrone (E1), 17β-estradiol (E2), and estriol (E3). E2 is the predominant estrogen in pre-menopausal non-pregnant women, and is the primary driver of the estrogen-dependent changes seen during the menstrual cycle.

Progesterone is especially important for successful embryo implantation in the endometrium, and subsequent maintenance of pregnancy. Progesterone dominates the second half of the menstrual cycle, with hormone levels reaching a peak in the mid-luteal phase. The feedback effects exerted by progesterone have the opposite effect on the hypothalamus and anterior pituitary compared to estrogens. At high serum progesterone levels, release of GnRH and the gonadotropins is inhibited, while low progesterone levels seemingly increase secretion [31].

1.2.2 Uterine response to hormonal fluctuation

The cells of the endometrium are sensitive to both estrogen and progesterone, triggering morphological changes throughout the menstrual cycle. The ratio of estrogen/progesterone, the concentration of hormone-sensitive receptors, and location in the uterus are all important for specific endometrial morphological changes to occur [32]. A rough overview of endometrial development in response to steroid hormones is shown in **Figure 4**.
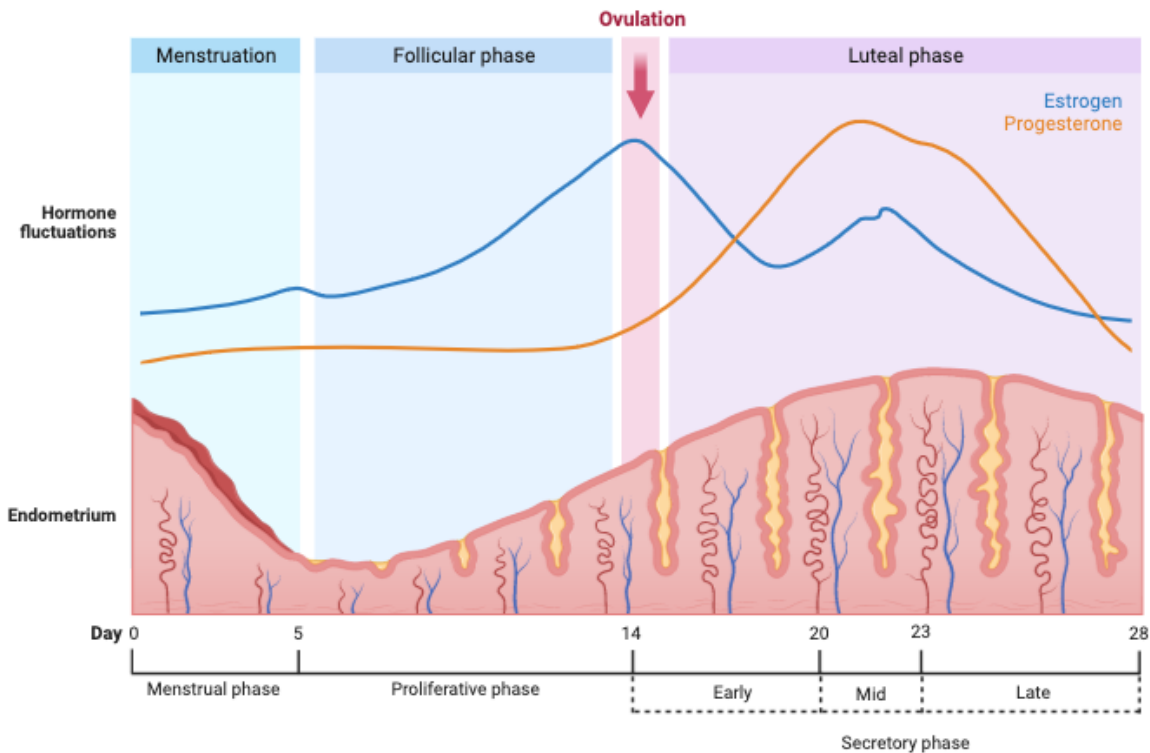

Figure 4. Overview of the fluctuation of steroid hormones and endometrial tissue throughout the menstrual cycle. The follicular/proliferative phase is characterized by estrogen dominance, causing the functional layer to regenerate following tissue loss at menstruation. The progesterone dominance of the luteal/secretory phase prepares the endometrial lining for possible implantation and maintenance of pregnancy. Created with BioRender.com.

1.2.2.1 Estrogens

Estrogens interact with the cells of the endometrium through estrogen receptors (ERs). These receptors are found in both glandular and stromal cells, but are more prevalent in the latter [33]. The level of ER expression is highest during the proliferative phase and declines during the secretory phase. The ER receptors can be found intracellularly, as the isoforms ERα and ERβ, and on cell membranes in the form of the G-protein coupled estrogen receptor GPER. The estrogenic hormone estradiol (E2) is the main substrate of ERs in non-pregnant women [34].

The intracellular receptors ERα and ERβ are hormone-induced transcription factors found in the cytoplasm and nucleus of estrogen-responsive cells. Estrogens therefore have to pass the cell membrane in order to bind to these receptors. Since estrogen is a steroid hormone, and thus lipophilic, it can pass freely through the cell membrane in its unbound form. Upon ligand binding, the ERs will bind to specific loci on the genome termed estrogen response elements (EREs) [35], which in turn affect transcription of specific genes in the cell.

The G-protein coupled estrogen receptor GPER does not affect gene transcription in the same way ERα and ERβ does, but has important downstream effects nonetheless. GPER is mainly localized on intracellular membranes, such as the endoplasmic reticulum, Golgi, and the nucleus, where it exerts effects on intracellular pathways. It can also affect transcription indirectly, by increasing or decreasing the activity of the transcription factor NF-KB [36].

Although the exact action of estrogen in the endometrial cells is incompletely understood [37], the effects on the endometrial tissue are well established. Estrogen stimulates cell proliferation and hypertrophy throughout the endometrium. This results in a significant thickening of the endometrial lining, which doubles in diameter by the late proliferative phase [14]. The spiral arteries in the functional layer also respond to the rising estrogen levels by proliferating, growing in size to accommodate the expanding stroma. Stimulation of the ERs also prepare the tissue for the secretory phase by increasing the expression of progesterone receptors (PRs), thus making the tissue progesterone sensitive.

1.2.2.2 Progesterone

Progesterone receptors (PRs), which exist in the isoforms PR-A and PR-B, become abundant in the endometrial tissue in response to stimulation by estrogen in the proliferative phase [38]. This allows for extensive tissue remodeling in response to the progesterone dominance of the secretory phase. The remodeling, although progesterone dependent, involves both endocrine and paracrine pathways [34].

PRs share many similarities with ERs, as they are abundant in both stromal and glandular endometrial cells and are hormone induced transcription factors. When bound to progesterone, PRs modulate transcriptional activity either directly by binding to DNA, or through interaction with other transcription modulators [34].

Progesterone antagonizes the general tissue proliferation seen during estrogen dominance and inhibits further growth of glandular epithelial cells. Stromal cells, however, will continue to proliferate upon stimulation of the PRs, allowing for predecidualization to occur [38]. Progesterone also promotes capillary growth in the endometrium, greatly increasing its vascular supply [39]. These changes bring about a favorable endometrial environment for the implantation and growth of an embryo.

1.3 Pregnancy

Pregnancy begins when an ovulated oocyte is fertilized by a sperm in the fallopian tube. During its transit to the uterus, the fertilized ovum will complete its second meiotic division and develop from a single-celled zygote to a 50-150 cell blastocyst ready for implantation. The blastocyst contains the inner cell mass which will develop into the fetus, and the trophectoderm which will become part of the placenta. Upon successful implantation, both sets of cells will develop over the course of 40 weeks, becoming and supporting a fully formed fetus. Initiation and development of a successful pregnancy is dependent on the embryo, circulating hormones, and the state of the endometrium.

1.3.1 Factors needed for successful implantation and pregnancy

Progesterone is an especially important hormone during induction of pregnancy, as evidenced by the higher rates of infertility among women with low luteal-phase progesterone levels [40]. Indeed, progesterone triggers important changes in the uterus through effects on both the myometrium and endometrium. Quiescence of the myometrium is necessary throughout pregnancy, as to not expel the embryo prematurely. The myometrium responds to rising levels of progesterone by blocking muscle contractility through transcriptional modifications [41]. This effect is maintained over time, provided serum progesterone levels remain high.

Endometrial receptivity to the embryo is at its highest during the *window of implantation* (WOI). Endometrial changes associated with the WOI include development of glands, secretion of histotroph, increased vascular supply and predecidualization of the stromal cells, which together create a favorable environment for the endometrium-embryo crosstalk conducive to implantation. There is some disagreement as to the exact timing of the WOI,

with suggestions raging from day 19-21 [42] to day 20-24 [43] of the menstrual cycle. Nevertheless, the WOI is thought to occur during the mid-secretory phase (**Figure 5**).
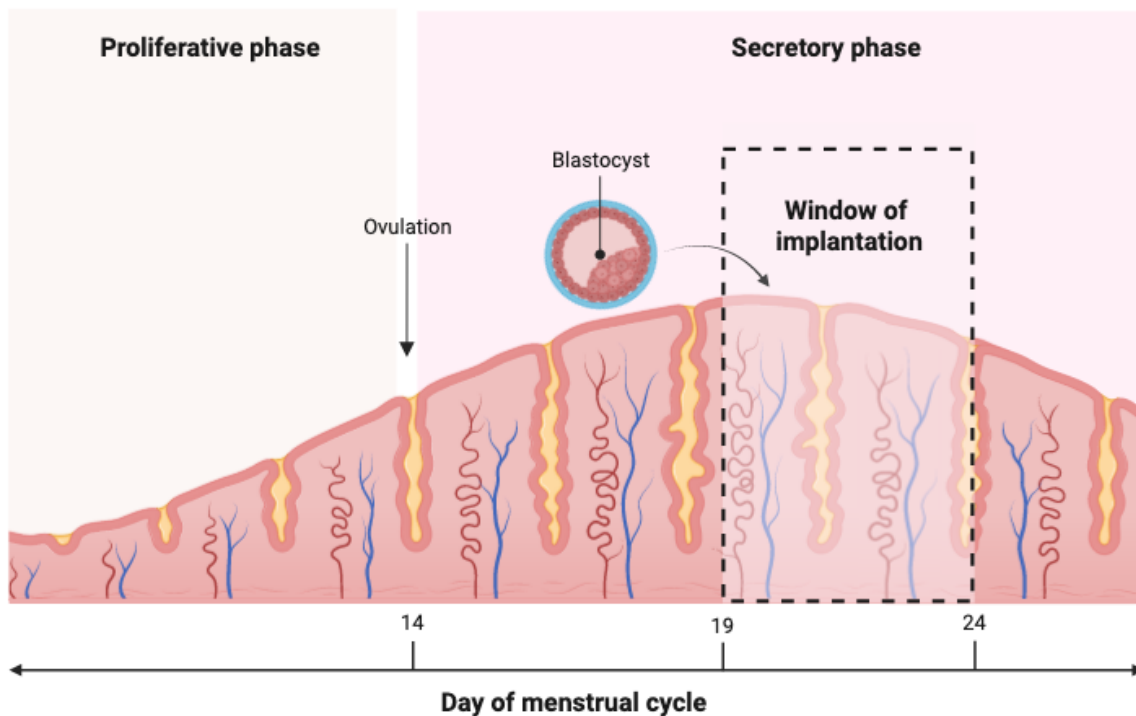

Figure 5. Schematic representation of the window of implantation (WOI). The WOI is the timeframe of maximal endometrial receptivity. There is still contention as to the actual interval of the WOI, with suggestions ranging from day 19-21 to 20-24. Created with BioRender.com.

1.4 Histological changes of the endometrium during the menstrual cycle

Cyclic changes in morphology can be seen in most of the organs of the female reproductive system. In the uterus, the functional layer of the endometrium changes significantly in response to the shifting levels of estrogen and progesterone. During the menstrual cycle, the endometrium will go through three phases: menstruation, proliferatory phase, and the secretory phase (**Figure 4**).

1.4.1 Menstrual phase endometrium

The onset of menstruation is initiated by decreasing progesterone levels following the degeneration of the corpus luteum, signaling absence of pregnancy. Cessation of hormonal support causes the spiral arterioles to constrict, causing local ischemia in the functional layer [14]. This is reflected in ischemic changes in the tissue, such as microembolization of smaller

vessels and general tissue degradation. Necrosis, compaction of tissue, widespread neutrophilic infiltration and increased parenchymal hemorrhage are all hallmarks of the endometrium in the menstrual phase [44]. An example of neutrophilic infiltration is shown in **Figure 6G**.

1.4.2 Proliferatory phase endometrium

The proliferative phase coincides with the ovarian follicular phase, with endometrial tissue remodeling occurring in response to rising estrogen levels. The end of the proliferative phase is marked by ovulation, usually at day 14 of the cycle. It is important to note that 14 days is the average length of the follicular phase, but this can vary greatly both within and between individuals [45]. The morphological changes observed during this phase are not sufficiently distinct to allow classification with daily precision. However, the changes may be classified in early-, mid-, and late phases [46].

The early proliferatory phase (approx. day 4-7) is characterized by short, straight glands with numerous mitoses in the glandular epithelium [44]. The stromal cells divide, thus creating a compact supporting tissue. The surface epithelium is especially thin during this time [47].

The mid proliferative phase (approx. day 8-10) is marked by significantly more stromal edema than the early phase. The glands also grow in size, becoming long and curved. Mitoses are frequent in both the glandular epithelium and the stroma [44].

Due to the rapid glandular growth outpacing the stromal development during the late proliferative phase (approx. day 11-14), there is a marked increase in the tortuosity of glands [44]. The glandular expansion is reflected in rising mitotic activity, and the epithelium will start to become increasingly pseudostratified. The supporting tissue also sees continued expansion, with more mitoses and a reduction in stromal edema.

Examples of proliferatory phase endometrium are shown in **Figure 6A** and **B**. Numerous mitoses can be seen in both sections. An overview of the development of endometrial tissue characteristics throughout the menstrual- and proliferative phases is shown in **Figure 7A**.
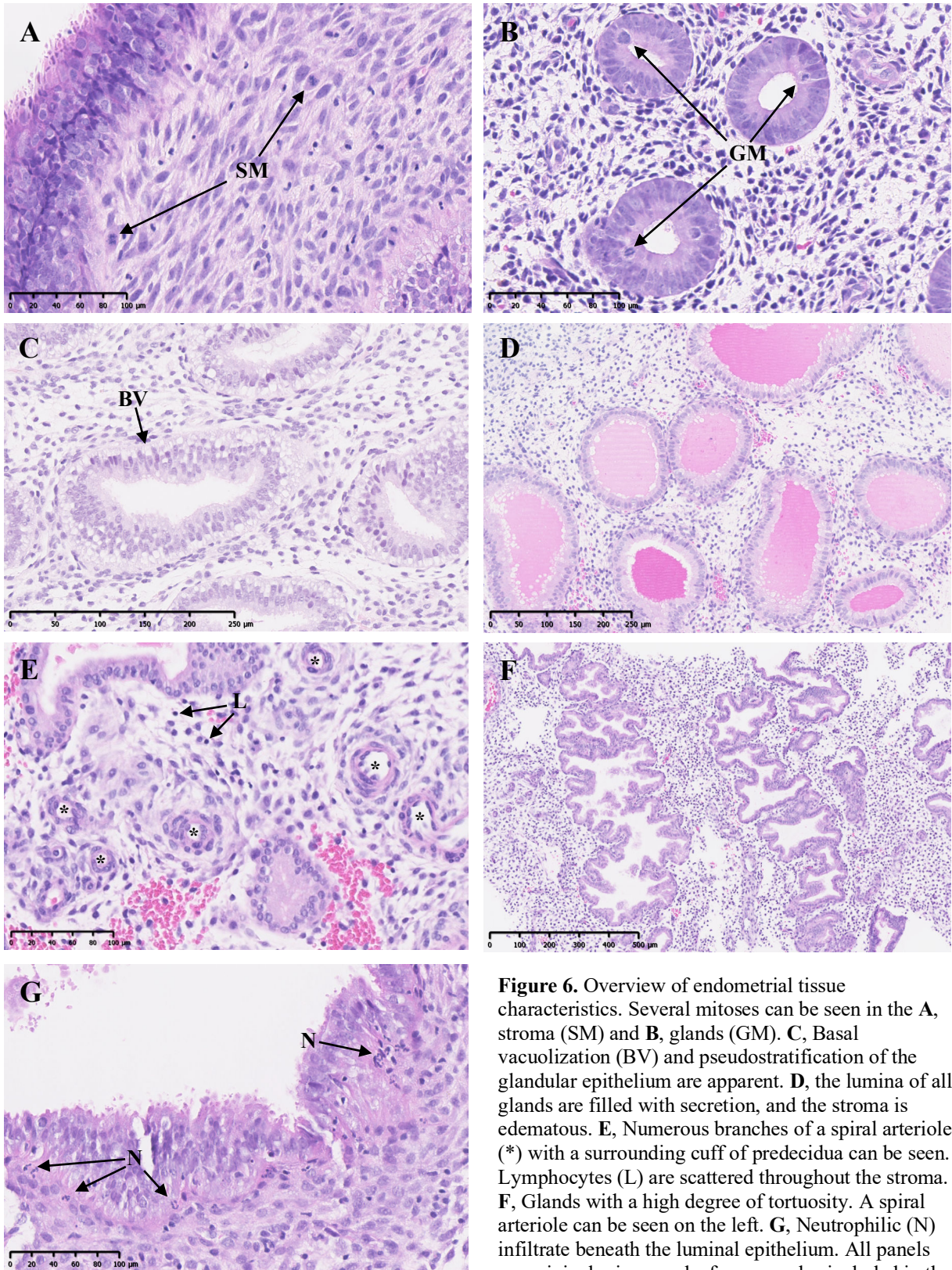
Figure 6. Overview of endometrial tissue characteristics. Several mitoses can be seen in the **A**, stroma (SM) and **B**, glands (GM). **C**, Basal vacuolization (BV) and pseudostratification of the glandular epithelium are apparent. **D**, the lumina of all glands are filled with secretion, and the stroma is edematous. **E**, Numerous branches of a spiral arteriole (*) with a surrounding cuff of predecidua can be seen. Lymphocytes (L) are scattered throughout the stroma. **F**, Glands with a high degree of tortuosity. A spiral arteriole can be seen on the left. **G**, Neutrophilic (N) infiltrate beneath the luminal epithelium. All panels are original micrographs from samples included in the present study.

1.4.3 Secretory phase endometrium and Noyes criteria

Noyes et al. published a seminal article in 1950, where they presented a histological method of dating secretory phase endometrial biopsies on a "day-to-day" basis [46]. They evaluated 8 different endometrial characteristics, including 4 glandular and 4 stromal features. Glandular characteristics included gland mitoses, pseudostratification, basal vacuolation and the presence of luminal secretion. Stromal changes comprised edema, tortuosity of glands, presence of predecidual reaction, and degree of leukocytic infiltration. The early secretory phase (day 16-19) was primarily dated based on glandular changes, while the mid (day 20-22) and late (day 23-28) secretory phases were assessed by stromal development. An overview of endometrial tissue characteristics throughout the secretory phase is shown in **Figure 7B**.

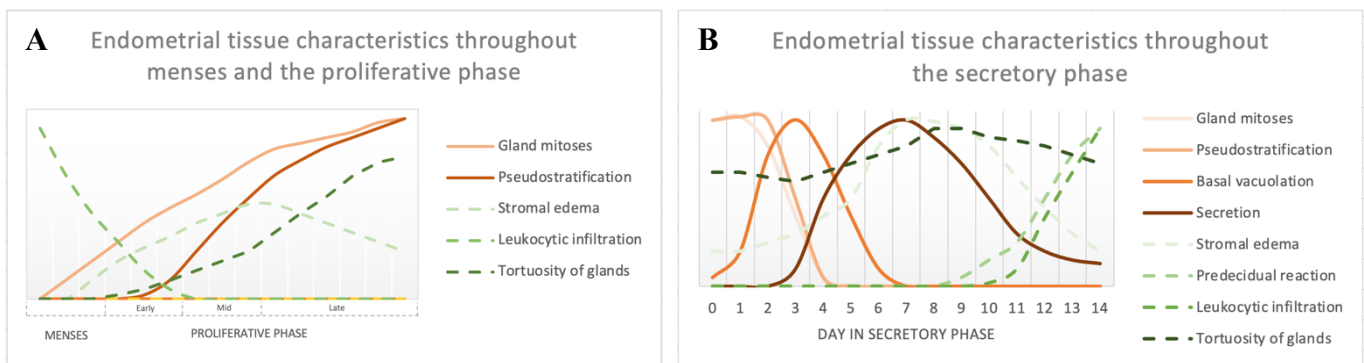**Figure 7.** Prevalence of endometrial tissue characteristics throughout the menstrual cycle. **A**, tissue characteristics through menstruation and the proliferative phase. Prevalence of basal vacuolation and secretion is not included, as these do not develop until the secretory phase. **B**, tissue characteristics through the secretory phase. Adapted from Noyes et al. (1950).

Dating of the secretory phase begins on day 16, where basal vacuolation starts to become prevalent. To classify a gland as having undergone basal vacuolization, vacuoles should be present in at least 50% of the epithelial cells [46]. As the vacuoles push the nuclei upwards in an irregular pattern, the glandular epithelium may appear as pseudostratified. This is gradually corrected by day 18, where the basal vacuoles steadily translocate to an apical position, once again making the epithelium appear as a single layer [44]. The vacuolar contents are slowly discharged into the glandular lumen in the form of secretion. Thus, by day 19, few vacuoles remain in the epithelial cells, with glandular secretions becoming increasingly prominent [44]. In the early stages of the secretory phase some mitotic activity can still be observed in the glands. This continued proliferation increases glandular tortuosity and size towards the later stages of the early secretory phase. The stroma, which in the early stages is relatively compact, becomes more edematous as the tissue approaches the mid secretory phase [25].

Some of these characteristics are shown in **Figure 6C**, which displays typical early secretory phase endometrium.

Luminal secretion reaches its peak on day 20, marking the beginning of the mid-secretory phase [46]. Few glands exhibit basal vacuolization, pseudostratification or mitotic activity. The stromal edema will gradually increase during this time, reaching maximum levels at day 22 [25]. Endometrial appearance typical of the mid secretory phase is shown in **Figure 6D**. Glandular tortuosity will continue to increase throughout the rest of the secretory phase.

On day 23, spiral arterioles become apparent in the stroma. This is caused by the enlargement of periarteriolar cells, which later become the prominent cuffs of predecidua characteristic of the late secretory phase. Predecidual cells are precursors to decidual cells, which develop following embryo implantation in the endometrium. Decidual cells facilitate blastocyst implantation, expansion of the trophoblast, and maternal-fetal interaction throughout pregnancy [48]. The success of their precursors is therefore necessary for induction and maintenance of pregnancy. Thick cuffs of predecidua appear around the arterioles at day 24, and areas of predecidual cells begin to form under the luminal epithelial cells around day 25. By day 27, the areas of periarteriolar and sub-epithelial predecidua coalesce, creating large sheets of compact tissue. The expansion of predecidualization is accompanied by increasing lymphocytic infiltration in the stroma. An example of a spiral arteriole with surrounding predecidua and lymphocytes is shown in **Figure 6E**.

As the tissue approaches the end of the secretory phase, glands become "saw-toothed" and ragged in appearance. Subtle degenerative changes of the glands and stroma become more apparent by day 27 and 28, during the pre-menstrual stage [44]. Typical appearance of pre-menstrual endometrial tissue is shown in **Figure 6F**.

1.5 Aims of the study

This study was part of the PhD project of Thea Falkenberg Mikkelsen, which aimed to examine the effect of submucosal leiomyomas and myomectomy on markers of endometrial receptivity. Accurate endometrial dating was needed, as the prevalence of the examined markers changes throughout the menstrual cycle.

Earlier studies have uncovered variation between the ovarian and uterine cycles, but little is known about the extent of asynchrony [49, 50]. The work presented in this study aimed to quantify the temporal gap between the ovarian and uterine cycles. This was achieved by comparison of semi-quantitatively assessed endometrial samples, representing the endometrial cycle, and self-reported day in cycle and progesterone levels, which represent the ovarian cycle.

2.0 Materials and methods

This was a prospective observational study that recruited patients between 2020 and 2022 at the Department of Gynecology, Oslo University Hospital.

2.1 Ethical approval

The study was approved by the Regional Committees for Medical and Health Research (REK, No. 66064) as part of the PhD work of Thea Mikkelsen.

2.2 Study population

67 female volunteers participated in this study. The participants were between 18 and 40 years of age, with varying gynecological histories. 42 of the patients had leiomyomas and were scheduled for excision by either hysteroscopy or laparoscopy. Endometrial samples from these participants were collected both before and after (3-6 months) surgery. An additional 25 healthy controls were also included, and provided one endometrial sample each. All participants had regular menstrual cycles (28 ± 7 days), at least one live born child, and no history of infertility. Exclusion criteria included recent pregnancy (in the last 3 months), ongoing lactation, and use of hormonal treatment. Written consent was obtained from all participants prior to the collection of data and biological material.

2.3 Data collection

All participants were surveyed using a standardized questionnaire. Menstrual bleeding pattern, cycle length, and current day in cycle (self-reported) were recorded. Many participants were able to provide detailed information through the use of cycle-tracking apps.

2.4 Sample collection and preparation

All participants provided blood samples on the day of endometrial sample collection. Serum levels of progesterone were then analyzed by the central laboratory of Oslo University Hospital using ELISA.

All participants underwent a clinical gynecological exam and transvaginal ultrasound to affirm the absence of unknown pathology. Samples of the endometrial lining were obtained using suction catheters (Pipelle®, Laboratoire C.C.D, France), between day 19-23 of the menstrual cycle (self-reported). The examiner aimed to collect samples from the fundus of the

uterus. The biopsies were subsequently preserved in formalin, embedded in paraffin, sectioned, and stained using hematoxylin and eosin (HE).

PhD candidate Thea Falkenberg Mikkelsen recruited patients, obtained consent, and arranged for collection and initial processing of all endometrial tissue samples. My role in the project was to comprehensively analyze endometrial histology, and precisely date the samples.

2.5 Histological examination

Stained glass-mounted sections were digitalized using NanoZoomer XR scanner (Hamamatsu Photonics, Japan). I assessed all the histological samples in a semi-quantitative manner using NDP.view2 software [51], while being blinded to the findings of the standardized questionnaire.

I first screened the slides for suitability for endometrial staging. Samples with extensive fixation artifacts, scarce volume or failed staining were excluded. I conducted subsequent analysis of suitable slides by using a semi-quantitative approach based on Noyes criteria [46], utilizing eight distinct tissue characteristics to date the endometrial samples. Glands were assessed for pseudostratification, basal vacuolation, the presence of luminal secretions, and number of mitoses of the glandular epithelium. The stromal changes were assessed by degree of edema, glandular tortuosity, predecidual reaction, and leukocytic infiltration. The distribution of glandular and stromal features for each sample is presented in *6.0 Appendix*. For each slide, approximately 100 randomly chosen glands were outlined and examined for any of the aforementioned glandular characteristics. For ease of analysis, I marked each gland with color coded pins corresponding to the glandular characteristics present (**Figure 8**). When scoring the stromal changes, the entirety of the tissue sample was assessed.

The interpretation of ambiguous or uncertain glandular and stromal features were resolved through discussion with a pathologist.

Glandular characteristics were quantitatively assessed, while stromal features were graded on a three-point scale. The scoring system rated changes on a scale of – (not present), + (present), and ++ (prevalent).

Frequency of each glandular characteristic and score of stromal features were recorded for every slide, and subsequently used to determine the endometrial cycle date. Samples determined to be in the proliferative phase were classified in early-, mid-, and late phases.

Secretory phase samples were classified in the same three phase-system, and further dated within a two-day interval of the secretory phase. The first of the two timepoints was used for calculating summary statistics.
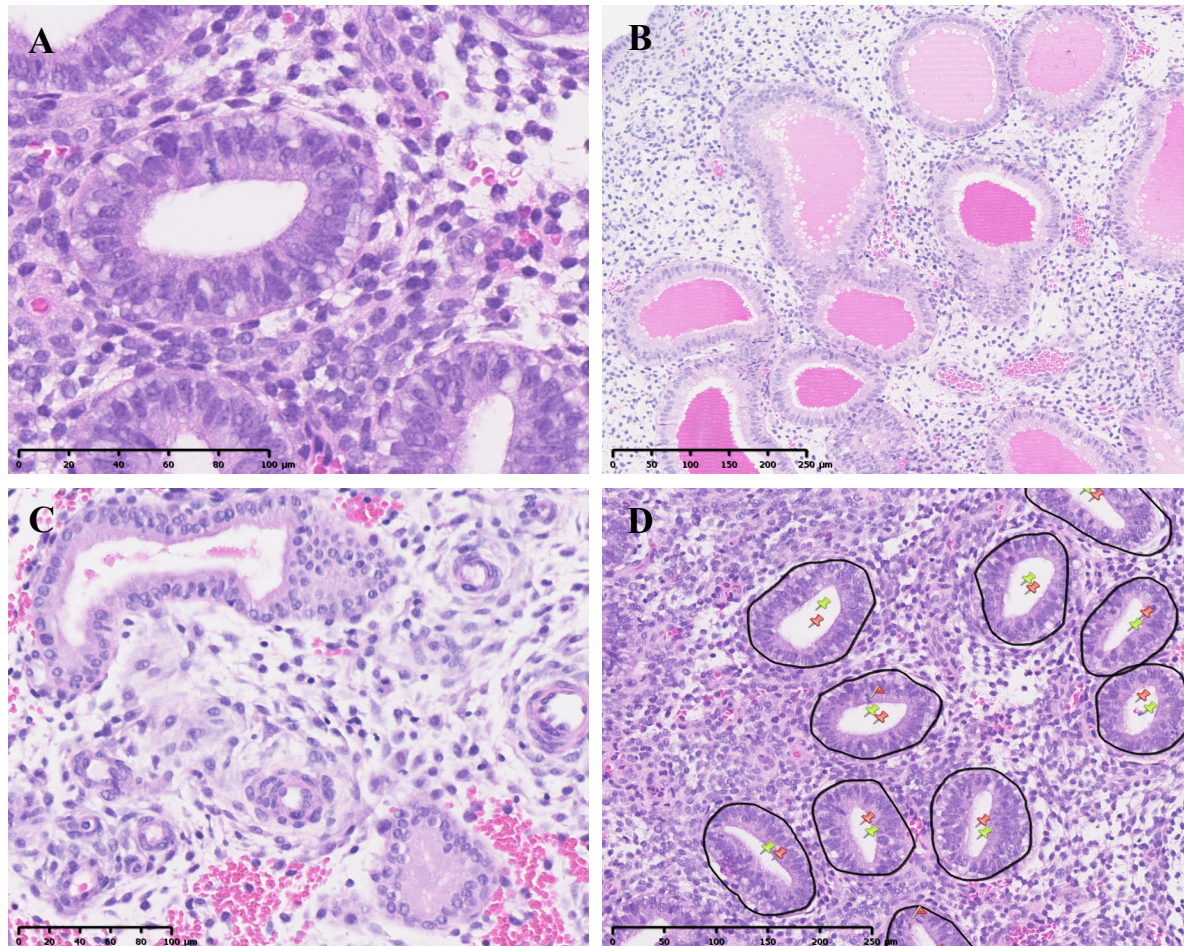

Figure 8. Overview of histological assessment. **A**, example of a section classified as early secretory phase. Note the glandular mitosis, prominent basal vacuoles and beginning pseudostratification. The stroma is compact. **B**, example of a section classified as mid secretory phase. Luminal secretion is prevalent, and the stroma is loose and edematous. **C**, example of a section classified as late secretory phase. A spiral arteriole runs diagonally across the section, with a surrounding cuff of predecidua. Hemorrhage and lymphocytic infiltrate can be seen in the stroma. **D**, method for collection of quantitative data. Glands were outlined and marked with color coded pins corresponding to specific glandular characteristics. Here, red pins represented the presence of basal vacuoles, green the presence of pseudostratification, while red flags marked gland mitoses. The section was later classified as early secretory. All panels are original micrographs from samples included in the present study.

2.6 Data analysis

Summary statistics (mean and standard deviation) were calculated using Microsoft® Excel (2022). Spline/LOWESS curves were fit to serum progesterone levels in **Figure 12** using GraphPad Prism 10.

3.0 Results

To assess the correlation and deviation of the ovarian and uterine cycles, histologically dated endometrial biopsies were compared to self-reported day in menstrual cycle in fertile participants with regular 28-day (range 23-35) cycles.

3.1 Histological dating of endometrial biopsies

Noyes criteria were used to estimate the date of the endometrial cycle for all tissue samples. In order to demonstrate concordance of the present assessment with that of Noyes et al., the frequencies of sample glandular characteristics were compared to the expected prevalence as presented in Noyes seminal paper.
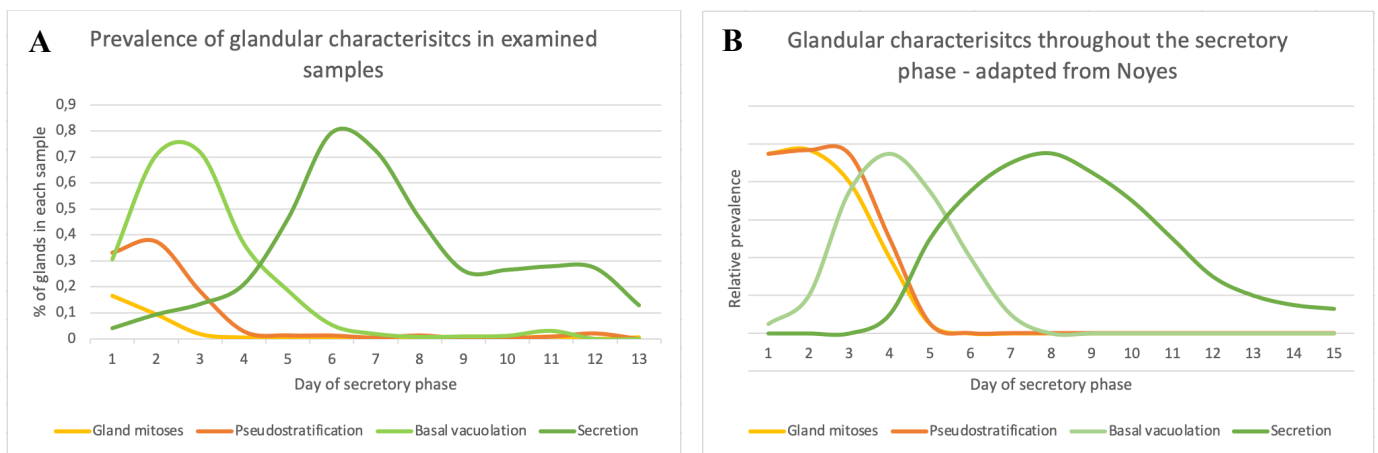

Figure 9. Validation of quantitative histological assessment. **A**, frequency of glands exhibiting specific glandular characteristics in samples dated to different timepoints throughout the secretory phase (n=74). Characteristics include gland mitoses, pseudostratification of glandular epithelium, basal vacuolization, and luminal secretion. **B**, expected prevalence of characteristics throughout the secretory phase. Adapted from Noyes et al (1950). Only glandular features were included, as stromal elements were semi-quantitatively assessed.

As can be seen in **Figure 9**, specific glandular features emerged and peaked in similar order: pseudostratification and mitoses peaked at day 2-3, followed by basal vacuoles at day 3-4, and luminal secretions at day 7-8. Timepoints from study samples peaked 1-2 days earlier than expected. Overall, the correlation between the dating of samples and the expected prevalence of tissue characteristics according to Noyes criteria is strong, affirming proper histological assessment.

3.2 Significant inter-patient variation between histological dating and self-reported day in cycle

Following validation of the histological method, day in cycle of the uterine and ovarian cycles were compared. These comparisons are based on "days of deviation", meaning the difference between the day of the uterine cycle as determined by histological evaluation, and the patients self-reported day in cycle. Thus, 0 days of deviation means the self-reported and histologically determined day in cycle coincided.
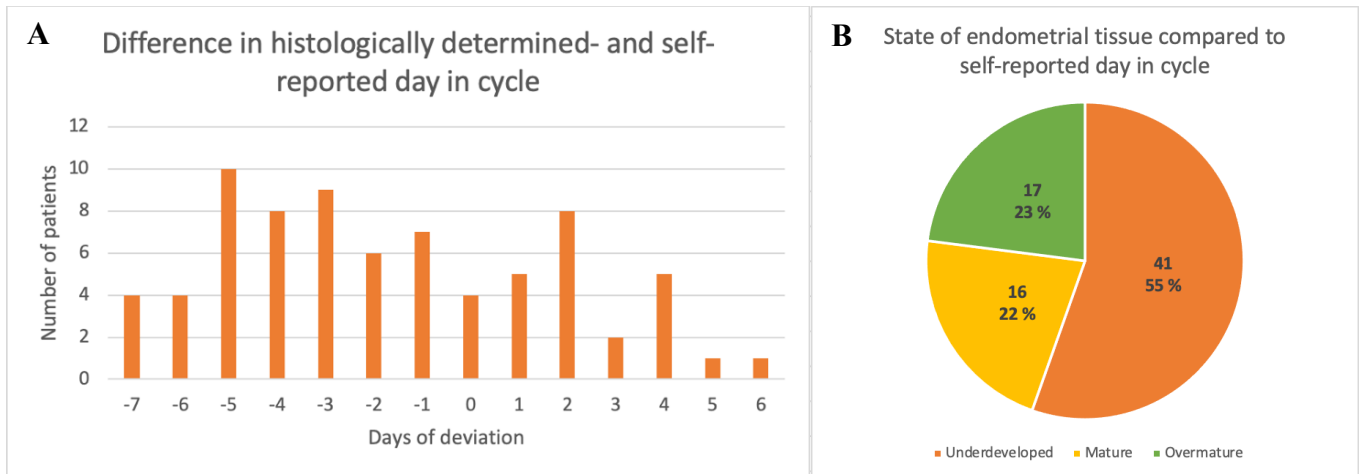

Figure 10. Deviation between estimated cycle day as determined by histological evaluation and self-reporting (n=74 for both panels). **A**, distribution of patients according to number of days of deviation (mean 3.22, SD 1.85). **B**, Prevalence of histologically immature, mature, and overmature endometrial samples when compared to self-reported day in cycle.

We noted frequent asynchrony between the endometrial and ovarian cycles, as shown in **Figure 10A**. Indeed, of the 74 samples examined only 4 had completely matched uterine and ovarian cycles. Most of the unmatched samples tended to be immature; the endometrial tissue was underdeveloped compared to what would be expected at the self-reported day in cycle (**Figure 10B**). This accounted for 55% (n=41) of samples, and an additional 23% (n=17) of samples were overmature. Only 22% (n=16) were mature, and thus in the expected mid-secretory phase. In total, 78% of patients had a significant difference in endometrial state compared to reported day in cycle.

3.3 Significant intra-patient variation between histological dating and self-reported day in cycle

In order to assess whether deviation between the endometrial and ovarian cycles was a stable individual feature or varied between menstrual cycles of the same person, we focused on matched samples from 27 participants who were tested in two different menstrual cycles (**Figure 11**). We found that 3 subjects had the same number of days of deviation between two cycles, while 24 subjects had absolute mean deviation of 3.26 days (SD 2.42), indicating considerable and inconsistent variability between endometrial and ovarian cycles of the same person. Thus, significant deviation in the endometrial and ovarian cycles can be seen both between and within patients.
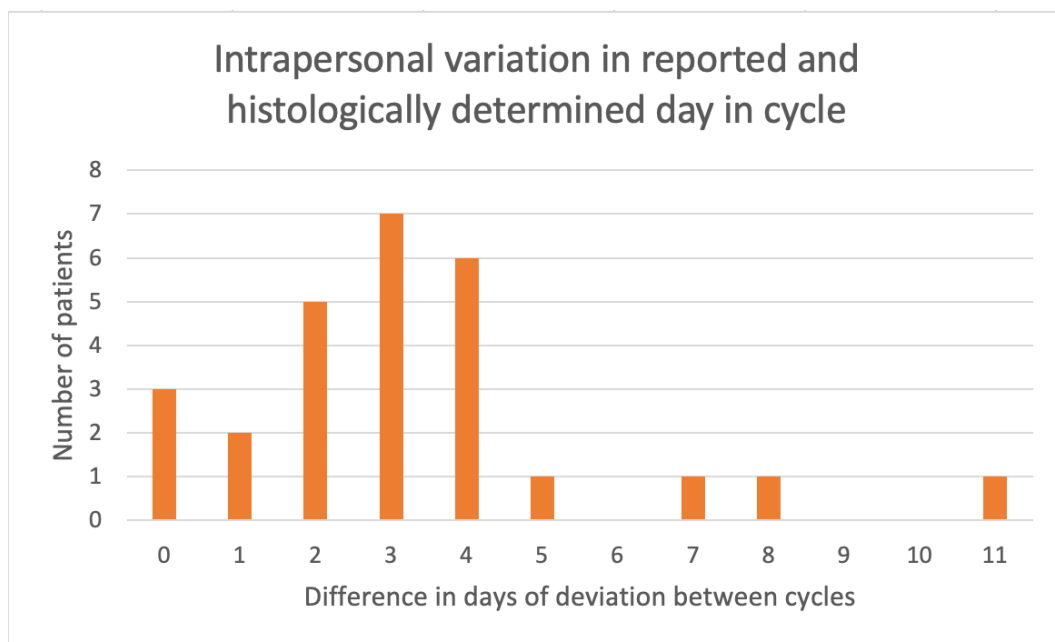
Figure 11. Difference in days of deviation in histologically determined and self-reported day in cycle between two cycles in the same individual (mean 3.26 days, SD 2.43, n=27).

3.4 Serum progesterone levels correlate better with the luteal phase than secretory phase

To examine whether the deviation between the endometrial and ovarian cycles was related to ovarian progesterone release, serum progesterone levels were assessed. As the self-reported day in cycle and serum progesterone levels seem strongly correlated, including the mid-phase maximum (**Figure 12A**), the self-reported time points will also be referred to as "*day in luteal phase*" throughout this section.

Figure 12. Serum progesterone levels in relation to day in the menstrual cycle. "*Day in luteal phase*" is equivalent to the self-reported day 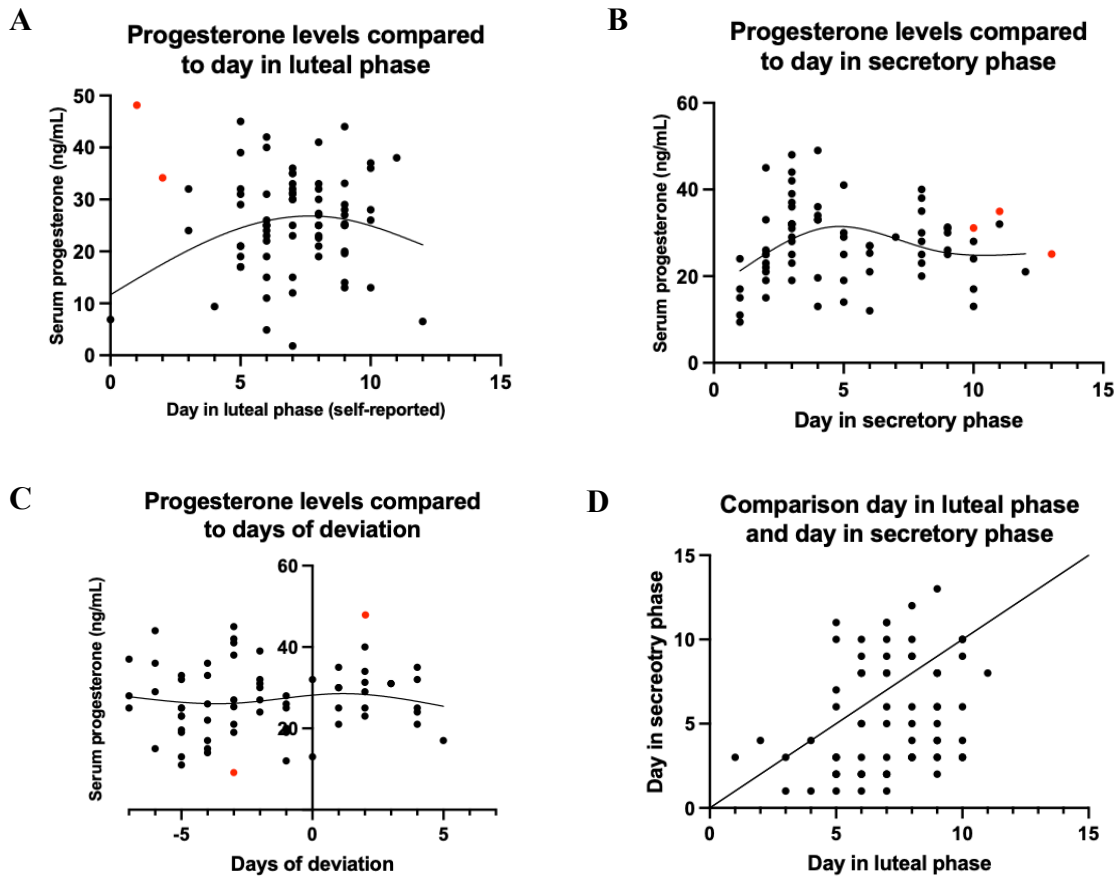in cycle. Red dots represent outliers that were excluded from curve fitting. **A**, serum progesterone levels compared to day in the luteal phase of the ovarian cycle. Peak levels are reached in the middle of the cycle, around day 6-8 (n=74). **B**, serum progesterone levels compared to day in the secretory phase of the endometrial cycle. Peak levels are reached early, around day 3-4 (n=73). **C**, days of deviation represent the difference in day of cycle as determined by histological evaluation (day in secretory phase) and self-reporting (day in luteal phase). Progesterone levels are comparable regardless of the number of days of deviation (n=67). **D**, day of luteal phase compared to day of secretory phase. Samples aligned on the diagonal have expected endometrial maturity. Samples under the diagonal have underdeveloped endometrial tissue at time of sampling, while those above the diagonal are overmature (n=74).

Peak progesterone levels were reached in the mid-luteal phase of the ovarian cycle (self-reported days 6-8, **Figure 12A**), whilst the peak occurred earlier when timed to the secretory phase (histologically determined day 3-4, **Figure 12B**). Hormone secretion appears to be stable across a wide range of days of deviation, implying adequate luteal phase ovarian function. Indeed, histologically immature (days of deviation < -1) and overmature (days of deviation > 1) endometrial samples were present at similar serum progesterone levels (**Figure 12C**).

4.0 Discussion

We found frequent asynchrony between the uterine and ovarian cycles, both between individuals and between cycles in the same individual. Variation in endometrial maturation in the secretory phase occurred irrespective of serum progesterone levels, implying that ovarian luteal phase dysfunction was unlikely to cause the observed asynchrony.

Histological assessment was conducted using Noyes criteria, with quantitative examination of glandular characteristics and a semi-quantitative approach to stromal changes. The method was validated by comparing the temporal development of glandular features to the standard dating by Noyes et al. We found that characteristics of glandular maturation peaked earlier than expected, which was likely due the choice of the first day from the two-day interval provided for dating. Furthermore, the relative prevalence of glandular characteristics seemed to differ between Noyes overview and the study samples. This was likely caused by the adaptation of **Figure 9B** from Noyes original paper, where characteristics were presented on individual graphs [46]. The exact prevalence of each characteristic was not provided, and only relative to itself. Therefore, compared relative prevalence may be misrepresented in the present study. Overall, the evolution of glandular features matched the standard trajectories provided by Noyes et al. well.

However, the use of Noyes criteria may also represent a limitation, as they have been extensively criticized [52]. Some criticism has been directed towards the patient samples used by Noyes et al. Only biopsies from infertile women were examined, which may not be representative of a healthy population [46]. Furthermore, the assumption of a 14-day secretory phase was necessary to implement the day-to-day dating criteria, even though the secretory phase can vary in length [53]. The large interobserver variability in histological evaluation may also impair the efficacy of the criteria [54].

Although other histological criteria have since been proposed [52, 55], Noyes criteria remain the gold standard for endometrial dating. To reduce some of the possible limitations in the use of Noyes criteria, the present study used a semi-quantitative approach to determine the day in cycle. This may reduce the interobserver variability, as evaluation was largely based on relatively objective data collected from individual samples.

Both present and previous data show that endometrial asynchrony is frequent among women with a regular menstrual cycle. A study conducted by Murray et al. (2004) found that delayed endometrial maturation was common among healthy, fertile women, a tendency seen both between individuals and between cycles in the same individual [52]. Alfer et al. (2020, 2021) found similar results, concluding that the endometrial maturation was delayed by 2 days on average [50, 56]. Notably, the average estimated delay varies between studies, and many provide different explanations for the maturation delay. Murray (2004) points to limitations in the use of histological dating criteria, while Alfer (2020, 2021) proposed that the observed delay was simply a normal variant, and thus of negligible pathological importance. Studies conducted by Zorn et al. (1984) and Santoro et al. (2000), who both found asynchrony between the endometrial and ovarian cycles, point to dysfunctional progesterone secretion as a possible explanation for inadequate endometrial maturation [49, 57]. Overall, numerous studies suggest that delayed endometrial maturation is common among healthy, fertile women. However, little is known about the extent of the delay or the underlying cause.

We attempted to relate the observed asynchrony of the uterine and ovarian cycles to serum progesterone levels as a proxy for luteal phase function. Progesterone secretion is usually at its highest during the mid-luteal phase [25] – indeed, we observed peak progesterone levels during the self-reported days 6-8. When timed to the secretory phase, however, peak progesterone levels were reached by days 3-4. There was little correlation between progesterone levels and the degree of deviation, and the asynchrony was therefore unlikely to be caused by a luteal phase defect. Alternatively, the frequently underdeveloped endometrium *could* be explained by an abnormal endometrial response to progesterone. Although possible causes of a deficient endometrial response are outside the scope of this thesis, studies conducted in patients with endometriosis imply abnormal PR-signaling, faulty gene expression, environmental toxins and chronic inflammation as possible causes of progesterone resistance, which is a common feature of endometriosis [58, 59]. The delay in maturation may also represent a normal variant, as postulated by Alfer et al. (2021) [50].

Regardless of the cause of asynchrony, a disorderly uterine cycle has consequences for women's health. A wide range of issues may be encountered in areas such as hormonal treatment, reproduction, and fertility. Our data suggest that, in any given cycle, the probability of being within the window of implantation at the expected days of the mid-luteal phase may be as low as 20%. This is especially problematic for patients undergoing infertility treatment,

as reduced endometrial receptivity is estimated to cause two-thirds of implantation failures [60]. Thus, asynchrony between the uterine and ovarian cycles may significantly decrease the chances of pregnancy.

There are notable limitations to the present study, many related to the partial data analyses. The results were not controlled for age, myoma-status or myomectomy, which can affect the physiology of the endometrial tissue [61, 62]. Since we recruited both healthy fertile women with regular menstrual cycles and participants with myomas, the findings may not be generalized to a broader population, in particular women with irregular menstrual cycles or other gynecological conditions. The sites from which endometrial biopsies were collected may also influence the results, as samples from different locations may show variability in histological dating [63]. Choice of the earliest timepoint from the two-day interval provided for dating may also represent a bias in the analyses. Confirming the endometrial and ovarian cycle status with additional methods, such as gene expression analysis, proteomic mapping, serial hormone assays and assessment of the LH surge may further strengthen our findings [52, 56, 64].

Our data may highlight unmet research and clinical needs, especially as it pertains to infertility. Both the present and earlier studies have found significant asynchrony between the endometrial and ovarian cycles, a tendency seen both between individuals and between cycles in the same individual. Novel methods may therefore be needed to evaluate uterine receptivity to improve outcomes of infertility treatment.

Notably, the observed asynchrony was not associated with a luteal phase defect, which is regarded as an important cause of reduced endometrial receptivity and infertility [65]. Thus, more research is needed to determine the actual cause of the observed asynchrony between the uterine and ovarian cycles.

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6.0 Appendix I

Distribution of specific glandular and stromal features in endometrial samples obtained during the presumed mid-luteal phase from women of reproductive age with regular menstrual cycles. Columns indicate individual tissue samples (n=74), color scale denotes values between 0 (red) and 1 (green).