Chapter 1

Novel 3-D Imaging Techniques in Early Pregnancy

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Introduction

Recent advancements in ultrasound technology have significantly shifted the focus of antenatal ultrasound screening toward the critical first trimester of pregnancy. However, this period remains somewhat enigmatic, primarily due to the small size of the developing embryo and the constraints imposed by current scanning technologies. This scenario underscores an acute need for more refined imaging of early embryonic anatomy to enhance our comprehension of this crucial developmental phase.

Innovative imaging techniques are not just pivotal in obtaining superior images for research purposes, but they also play a crucial role in medical education and clinical practice. Enhanced images of embryonic and fetal development are invaluable resources for training professionals like sonographers and fetal surgeons. Moreover, they serve as vital educational tools for parents, especially those expecting a child with a fetal anomaly, helping them to understand and prepare for the challenges ahead.

The creation of resources such as the 3D Embryo Atlas has been a significant step forward in the field of human embryology [1]. This, coupled with ongoing research into fetal development, has brought to light the vast extent of what we have yet to learn about our developmental processes. With the increasing reliance on ultrasound screening in the first trimester, there is a growing imperative to comprehensively map fetal anatomical development throughout the entire gestation period [2].

Imaging Early Pregnancy in High Detail

3-D Ultrasound for In Vivo Imaging

Ultrasound stands out for its ability to provide detailed structural information from the first trimester, coupled with its affordability, widespread availability and real-time examination capabilities. While traditional 2-D ultrasound is commonly used, there is a growing shift toward 3-D ultrasound for anomaly evaluation, volumetric measurements, and surface anatomy visualization, offering the advantage of revisiting stored 3-D volumes. Recent advancements in 3-D-ultrasound technology, including innovative visualization software, have greatly enhanced the clarity and lifelikeness of images, aiding clinicians, students and parents [3]. Additionally, these advancements include see-through 3-D rendering applications that enhance the contrast of internal structures, further improving the utility and accuracy of ultrasound in early pregnancy [2, 3].

Micro-CT Imaging for Ex Vivo Imaging

Microfocus computed tomography (micro-CT), an evolving tool in the biomedical field, offers high-resolution imaging without material disruption, making it useful in various nonmedical fields like precision engineering and geosciences [4]. Similar to conventional CT in its use of X-ray attenuation, micro-CT typically involves a fixed radiation source and a rotating sample platform. The application of iodine-based staining, known as diceCT (diffusible iodine-based contrast-enhanced CT), enhances soft-tissue imaging and is increasingly used for studying ex vivo human embryonic and fetal anatomy, having been widely employed in animal research [5]. The versatility of diceCT is a key advantage, effective across various developmental stages, from 6 to 24 weeks of gestation [6, 7, 8]. This method provides detailed anatomical insights and has been adopted by multiple research groups as a virtual autopsy tool, particularly beneficial for parents who decline invasive autopsy following fetal loss [9]. It has been evaluated in prospective studies comparing it to conventional autopsy for gestations

up to 20 weeks [10]. Additionally, research demonstrates the feasibility of studying human fetal anatomy as early as eight weeks' gestation, facilitating virtual autopsies in the early first trimester [8]. By obtaining precise phenotypic data, especially from miscarriage specimens, this approach supports reverse phenotyping, enabling the selection of variants of interest in genome-wide analyses [8]. This offers the potential for genetic counseling to bereaved parents, adding a layer of support during a difficult time. Additionally, its minimally invasive nature allows for its use on rare or invaluable samples, including those with unique abnormalities or museum specimens [2].

Scientific Research on Early Pregnancy

In 2017, the Dutch Fetal Biobank was initiated by researchers at the Amsterdam University Medical Center of the University of Amsterdam, the Netherlands [6]. This significant venture, which has since extended its reach to other hospitals across the Netherlands, focuses on collecting embryonic and fetal specimens for medical scientific research. High-resolution imaging techniques are a key part of this research. Acknowledging the sensitive nature of this field, the Dutch Fetal Biobank places a strong emphasis on ethical practices and compliance with all relevant regulations and laws. This includes ensuring that parents are fully informed about the donation process and that all tissue use is conducted respectfully and responsibly [6].

The biobank opens its doors to donations from parents facing a range of circumstances, including elective pregnancy termination, ectopic pregnancy or the birth of an immature fetus. In the affiliated hospitals, gynecologists or midwives discuss the option of donation with the parents, providing detailed information and guiding them through the decision-making process. Importantly, the biobank maintains strict anonymity for the donors, meaning no personal information from the parents is linked to the research tissues [6]. More information can be found at www.3Dhumandevelopment.com.

Imaging Ectopic Pregnancies

Over the past seven years, the Dutch Fetal Biobank has amassed a rare collection of ectopic pregnancies [11], meaning excised fallopian tubes containing complete pregnancies, yolk sac and placental tissue. These specimens undergo micro-CT imaging, producing high-resolution images of human embryos covered by their fetal membranes (Figure 1.1). The first ectopic pregnancy imaged with contrast-enhanced micro-CT offered an ultrahigh resolution of 3 μm, revealing a 3-mm-long embryo with distinguishable features like the otic pit, brain vesicles and heart tube [12]. When compared to the 3D Embryo Atlas [1], it matched a 28-day-old Carnegie stage 12 embryo, equivalent to 6 pregnancy weeks but uniquely showcased in its natural setting with a yolk sac, fetal membranes and in a vascularized fallopian tube. The iodine staining particularly highlighted both maternal and embryonic vessels, including the pharyngeal arch arteries and dorsal aorta. This pioneering and award-winning study highlighted the possibility of using micro-CT

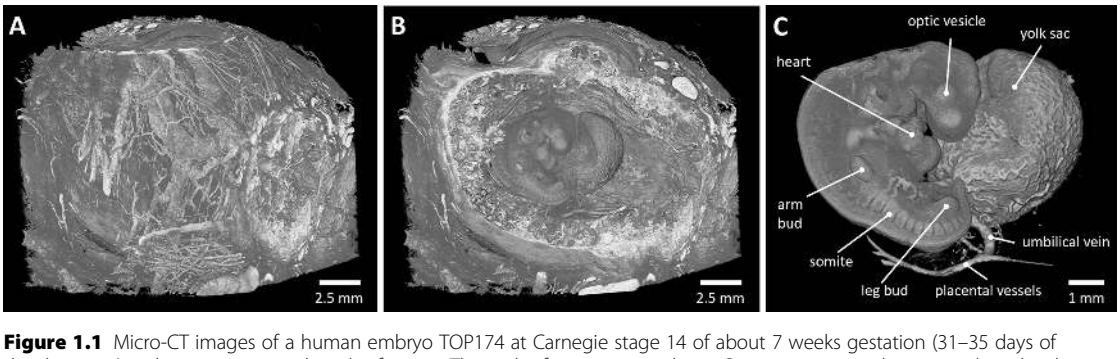

Figure 1.1 Micro-CT images of a human embryo TOP174 at Carnegie stage 14 of about 7 weeks gestation (31–35 days of development) with a crown–rump length of 6 mm. This is the first time ever that a Carnegie stage 14 human embryo has been captured within the intimacy of the fetal membranes. A: Volume rendering an overview of the fallopian tube containing the pregnancy. Note the extensive vascularization. B: The volume rendering has been cropped, which shows the embryo and yolk sac. C: Details of the embryo and yolk sac, showing the various embryonic structures. (Source: B. S. de Bakker and S. C. Visser, Dutch Fetal Biobank, Amsterdam University Medical Center)
For the color version, please refer to the plate section. In some formats, this figure will only appear in black and white.

imaging to study early embryogenesis within the extraembryonic structures [12].

The Future in Imaging Early Pregnancies

The Use of AI in Ultrasound

Artificial intelligence (AI), particularly deep learning (DL), has made significant strides in the last decade, showing great promise in medical fields that rely heavily on imaging. Artificial intelligence's application in fetal imaging has been the subject of recent reviews and various studies, focusing on uses like fetal diagnosis and estimating gestational age. Most of these studies emphasize implementing DL in 2-D ultrasound, such as automatic detection of 2-D planes, probe-motion tracking and enhancing biometric measurements such as head circumference [2].

Artificial intelligence is expected to further enhance embryonic and fetal imaging, particularly in complex classifications and diagnosing congenital defects. We anticipate that AI, trained on models like those from the 3D Embryo Atlas, could assist sonographers in analyzing 2-D and 3-D fetal images. This would involve comparing real-time scans with existing models to identify deviations in fetal anatomy, potentially indicating birth defects. The integration of AI in prenatal imaging promises to improve the detection of congenital abnormalities, leading to better prenatal diagnosis and enhanced postnatal care [2].

Synchrotron Imaging

Synchrotron radiation-based X-ray phase-contrast tomography (sCT) is emerging as a promising technique for ex vivo embryonic and fetal imaging. This method is based on the principle that both amplitude and phase of an X-ray beam are altered as they pass through an object [2].

The key advantage of sCT lies in its sensitivity to X-ray phase shifts, which provides clear visualization of soft tissues at a micrometer resolution, without the need for contrast agents or staining, positions it as an invaluable tool in biomedical research. This technique offers detailed insights into embryonic and fetal structures, potentially revolutionizing our understanding of early human development [2].

Conclusion

Since Leonardo da Vinci's pioneering depiction of a fetus in utero, imaging technology has significantly advanced, providing deeper insights into embryonic development [2]. Despite these advancements, the early first trimester remains challenging due to the embryo's small size. To enhance our understanding, combining ultrasound with ex vivo methods like micro-CT is crucial for detailed fetal structure analysis. This, along with large-scale data sharing, sets the stage for integrating AI in ultrasound, potentially enabling the automatic detection of abnormalities in 3-D ultrasound and thus improving the identification of congenital anomalies in these critical early stages of development [2].

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Chapter 2

What Is Endometrial Receptivity?

Hilary Friedlander and Steven L. Young

Endometrial receptivity to human embryo implantation is simple in concept: it can be defined as the ability of the maternal endometrium to support appropriate attachment, invasion and maintenance of the early human embryo. Our understanding of the complex processes involved in early embryo implantation is severely limited because of differences between humans and experimental animals and ethical and technological barriers prohibiting direct observation of human implantation in vivo and even ex vivo. Thus, most of our knowledge is gleaned from animal studies (despite differences), observational studies in couples attempting conception and those undergoing assisted reproduction and, a few examples of human implantation sites unexpectedly found at hysterectomy. The knowledge is augmented by experimental studies in women undergoing assisted reproduction.

The process of human embryo implantation involves direct physical interaction between the trophoblast cells and the maternal endometrial epithelium, stroma, bone marrow-derived immune cells, maternal vascular endothelium and maternal blood circulation. These interactions are complex and require a myriad of maternal adaptations that are directed by both maternal and embryonic factors. Thus, implantation is not a brief or single event but rather a dynamic process of maternal and embryonic interactions that lay an important foundation for a successful pregnancy. It is becoming increasingly clear that abnormal implantation can lead to pregnancy disorders, underlining the importance of understanding the process more completely [1]. Implantation events demonstrated in areas outside of the uterus suggest that the uterus controls the timing of implantation and can prevent implantation from occurring at non-optimal times [2, 3, 4].

The temporal window in each menstrual cycle, during which the endometrium is receptive to embryo implantation is finite, though the precise length in a given individual and between person and between cycle variations remains to be fully determined. Although embryos can implant outside the uterus, apparently irrespective of timing, data discussed below demonstrate that the endometrium limits implantation to a specific temporal window.

Data from almost 70 years ago, using hysterectomy specimens that unexpectedly contained an early pregnancy, showed that embryos did not attach until about day 20 of an idealized 28-day cycle [5]. Most data collected in the ensuing seven decades suggest that normal embryo implantation occurs between cycle days 20 and 24 of an idealized 28-day menstrual cycle, equivalent to post-ovulatory days 6–10. The strongest evidence for a discrete window of implantation in medically unassisted pregnancies was presented by Wilcox et al., as shown in Figure 2.1 [6]. In this study, daily urine samples from 221 women trying to conceive were collected after ceasing all forms of contraception. The urine collected from 126 women who conceived and had a live birth was then analyzed for levels of urinary estriol, pregnanediol glucuronide and human chorionic gonadotropin (hCG). The initial rise in hCG occurred 6–12 days after ovulation, with the rise in 84% of patients occurring on day 8, 9 or 10, and essentially none occurring earlier [6]. Since urinary hCG becomes detectable only after trophoblast invasion, the tight temporal clustering suggests a small window during which implantation is successful. However, those embryos that implanted after day 10 resulted in a very high chance of pregnancy loss prior to six weeks. Those women experiencing pregnancy loss after six weeks showed no differences in implantation timing from those experiencing a live birth. The mean time of implantation was 10.5 days postovulation for the early pregnancy loss group, compared to 9.1 days in the live birth group and 9.2 days in the later pregnancy loss

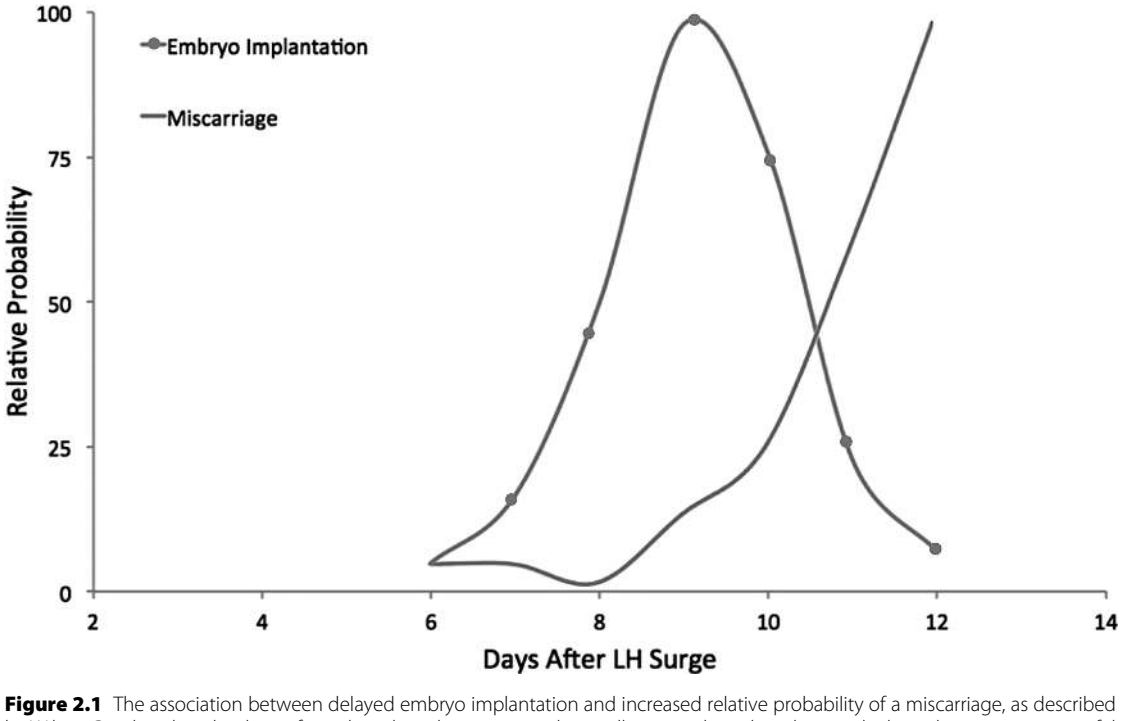

Figure 2.1 The association between delayed embryo implantation and increased relative probability of a miscarriage, as described by Wilcox, Baird et al. and redrawn from their data, demonstrates the small temporal window during which implantation is successful in unassisted cycles. (Source: Wilcox et al. 1999)

group. Multiple mechanisms may account for the association between later hCG detection and early pregnancy loss, including failure to adequately "rescue" corpus luteum progesterone (and other hormone) production, an abnormal embryo that produces hCG in lower amounts or in a delayed manner (as seen with ectopic pregnancies) or an abnormal endometrial–embryo interaction that slowed trophoblast invasion [6]. Of note, previous studies clearly document reduced corpus luteum function with later hCG rise [7].

The use of donor or frozen oocytes or embryos, in conjunction with an artificial cycle without the presence of a corpus luteum, allows further dissection of the relevant mechanisms governing endometrial receptivity and defining the temporal window for endometrial transfer. In the earliest of these studies, Navot et al. reported the percentage of pregnancies across a 6-day period (cycle days 15–20 in a 28-day cycle) extending the window of implantation and of embryos of constant developmental stage (within 24 hours) onto endometria of differing maturational stages. Day-16 transfers had the highest viable pregnancies; however, no specific day appeared to provide a more optimal environment for a 2 to 12-cell stage embryo, concluding that implantation of the embryo, rather than fertilization, is the pivotal process separating fertile from nonfertile cycles [8]. Prapas et al. reported an observational study of pregnancy rates of donor oocyte embryo transfers performed at the four–eight cell cleavage stage phase after different lengths of progesterone exposure in an otherwise identical uterine environment [9]. The investigators observed a two-day transfer window over which more than 80% of the pregnancies were achieved, corresponding to cycle days 18 and 19, which were preceded by 4 or 5 days of progesterone administration, respectively. Since there was no corpus luteum and all the embryos were at a similar stage of development, these data further support endometrial timing as a critical issue, with the length of progesterone exposure determining that timing.

The critical role of progesterone in uterine receptivity and pregnancy maintenance has been repeatedly confirmed since the discovery and purification of progesterone almost 100 years ago [10]. Produced almost exclusively by the

corpus luteum and placenta, progesterone's endometrial action is essential for transforming the endometrium from proliferative to secretory morphology, including driving the profound changes in endometrial stromal cell morphology and function, termed decidualization. These endometrial changes dictate a limited temporal period of receptivity to embryo implantation. Not long after progesterone's discovery, pioneering studies by Georgeanna Seegar Jones, John Rock and Arthur Hertig suggested that insufficient progesterone could lead to failed implantation [11, 12, 13]. The idea that progesterone deficiency could cause the endometrium to become poorly receptive or non-receptive due to an inadequate progesterone effect became known as luteal phase deficiency (LPD) [11, 12]. Foundational work, a decade later, by Noyes et al. established histologic criteria for endometrial dating that in the luteal phase reflected progesterone effects, as shown in Figure 2.2 [13]. Thus, it was proposed that histological examination of an endometrial sample during the mid to late luteal phase could be used to diagnose LPD, which was supported by hundreds of subsequent publications. However, increasing skepticism in the early 1990s, based partially on concerns about the precision of histological assessment [14], led to two landmark studies that demonstrated the ineffectiveness and imprecision of using histologic assessment of progesterone effect to distinguish infertile from fertile women, as shown in Figures 2.3 and 2.4 [15, 16]. Other proposed approaches to assessing progesterone sufficiency included assessing single or repeated serum progesterone concentrations. However, the demonstration of serum pulses of progesterone, entrained to luteinizing hormone pulses, that result in large swings in serum progesterone concentration over short time intervals make such an approach inherently infeasible [17].

While endometrial receptivity is largely orchestrated by progesterone on an estrogen-primed endometrium, it is not simply a result of direct progesterone effects. Paracrine signals, generated by estrogen and progesterone action, are essential for acquisition of endometrial receptivity, normal endometrial functionality and functional interactions with the embryo [18]. In fact, estrogen and progesterone are the only two hormones necessary to generate a receptive endometrium [19]. The sequential actions of estrogen and progesterone drive endometrial receptivity, which is attained by most women in the mid-luteal phase. Any asynchrony between this balance may result in the pathophysiologic sequelae demonstrated in clinical problems such as endometriosis, infertility, pregnancy loss, endometrial cancer and abnormal uterine bleeding [1]. Studies at the cellular and molecular level have demonstrated a number of other factors that are critical to endometrial receptivity. A downregulation of endometrial epithelial estrogen receptor-α (ESR1 gene product) at the time of embryo implantation has been found so far in all placental mammals studied and thus is likely a crucial molecular event for receptivity [20, 21 22, 23]. In human endometrium, overexpression of estrogen receptor-α in the endometrium during the window of implantation has been associated with unexplained infertility [23].

A large number of molecular changes have been proposed as single or collective markers of receptivity in the human endometrium. S100P, a protein originally detected in the human placenta, is considerably upregulated during the window of implantation [24]. When cultured endometrial cells were exposed to estrogen and progesterone, a 100-fold increase in expression in S100P was demonstrated in response to progesterone in both primary endometrial epithelial and stromal cells; there was no significant effect in response to estrogen [24]. This indicates S100P participates in the endometrial changes of the window of implantation and suggests its use as a unique biomarker for endometrial receptivity. Endometrial integrins, specifically $\alpha 4\beta 1$ and $\alpha v\beta 3$, participate in the cascade of molecular events contributing to endometrial receptivity and effectively define the window of implantation, considered as markers for the time of maximal uterine receptivity [25, 26].

More recently, research has demonstrated that expression of transcription factor FOXO1 (Forkhead box O1) in the endometrial epithelial cells in combination with the loss of progesterone receptor during the window of implantation are interrelated and critical to endometrial receptivity and subsequent embryo implantation [27]. It is thought that the yin-and-yang relationship of FOXO1 and progesterone receptors is necessary for the establishment of pregnancy, and it underscores the importance of FOXO1 as a marker and regulator of endometrial receptivity. However,

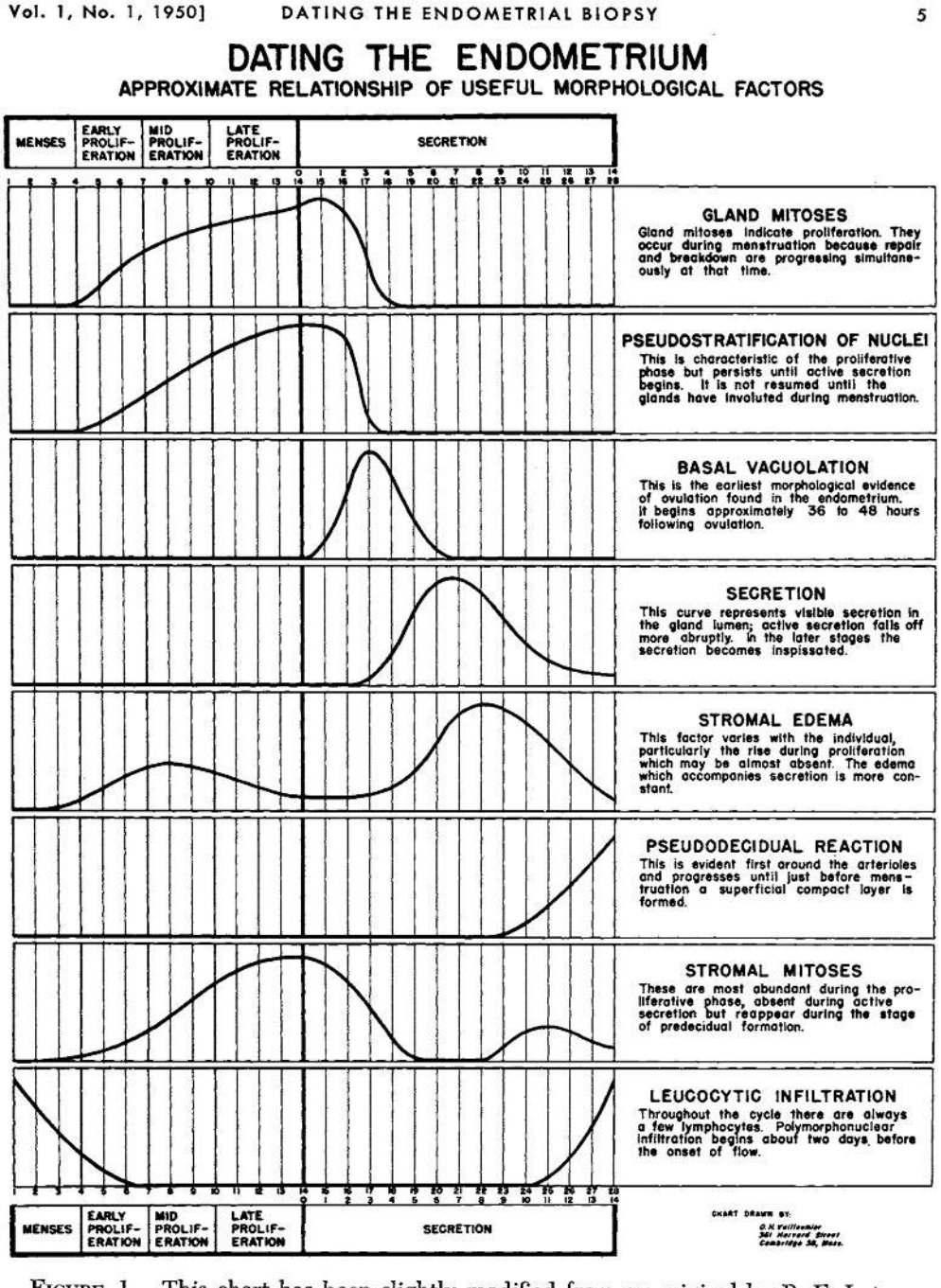

FIGURE 1. This chart has been slightly modified from an original by P. F. Latour. The curves represent approximately estimated quantitative changes in each of eight factors we consider most helpful in dating endometrium.

Figure 2.2 The curves represent quantitative changes in the endometrium according to eight different histologic factors assessed as a means of dating the endometrium. As shown, multiple different factors are required in order to accurately date the endometrium, given the similarities in multiple histologic parameters at various time points. (Source: Noyes et al. 1950)

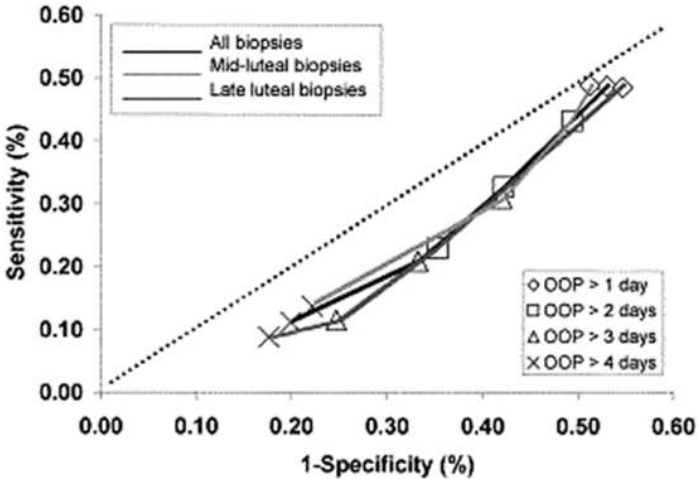

Figure 2.3 Receiver operating characteristic curve depicting the performance of the endometrial biopsy as a diagnostic tool for infertility. The y-axis represents sensitivity, defined as the proportion of infertile women with an out-of-phase biopsy ranging in 1–4 days discrepancy. The x-axis represents specificity, defined as the proportion of fertile women with an inphase biopsy. Each curve runs roughly parallel to the line connecting 0% sensitivity and 100% specificity and 100% sensitivity and 0% specificity, suggesting poor diagnostic utility for isolating fertility from infertility. (Source: Coutifaris et al. 2004) For the color version, please refer to the plate section. In some formats, this figure will only appear in black and white.

none of these markers have been shown to be sufficient for diagnosis or prognosis in the contexts that have been studied.

There are multiple commercially available tests to evaluate endometrial receptivity and defects in endometrial receptivity by way of three general approaches: errors in dating, errors in function and errors in function specifically owing to inflammatory factors [28]. These tests offer personalized treatment for pathologies of the endometrium and are now thought to be utilized by over 4000 reproductive clinics in over 90 countries around the world [29]. While great advancements have been made in the application of personalized medicine to assisted reproductive technologies, limitations still exist, and current research does not support routine endometrial testing for all infertile patients [29].

The endometrial receptivity array (ERA) was reported in 2011 and is purported to identify shifts in the temporal window of receptivity leading to asynchrony between the embryo and the endometrium. The ERA test is based on the measurement of 238 gene transcripts whose collective changes in abundance correlated with cycle phase and receptivity [30]. Although initially it was very promising, recent well-designed clinical trials suggest the lack of usefulness in the patient populations studied [31, 32]. It remains to be seen if the test will still be useful in more narrowly defined groups of patients. The ER Map/ER Grade, a new comprehensive system evaluating endometrial receptivity, is the latest development employing gene expression analysis using quantitative reverse transcription polymerase chain reaction with a new panel of genes specifically involved in endometrial proliferation and the maternal immune response to endometrial implantation [33]. While initial investigation has shown promise, further research with randomized controlled trials is required.

The endometrial function test (EFT) is comprised of both a histologic assessment and an assessment of endometrial development, requiring two endometrial biopsies – one on cycle day (CD) 15 and the other on CD 24. Quantitative immunohistochemical assessment of cycle E (a molecular marker of proliferation) and p27 (a molecular marker of proliferation termination) determined the developmental state of the endometrium [34]. If there is still glandular cycle E in the endometrium on CD 24, then the glands are still proliferating and are considered unreceptive to a blastocyst implantation. Further, a panel of markers can be examined on the two biopsy specimens to discern information on the developmental trajectory, whereby adjustments can be made to the stimulation protocol to correct for these abnormalities. In one study, women who had an abnormal EFT without any form of intervention or correction were found to be 10.5 times less likely to have an ongoing pregnancy compared to women who had a normal EFT or had an intervention after diagnosis of an abnormal EFT [35]. Again, large prospective trials demonstrating prognostic precision in various patient populations and effective treatments remain to be performed.

The ReceptivaDx® test evaluates the endometrium for immunohistochemical expression of B-cell CLL/lymphoma 6 (BCL6), a marker of endometriosis

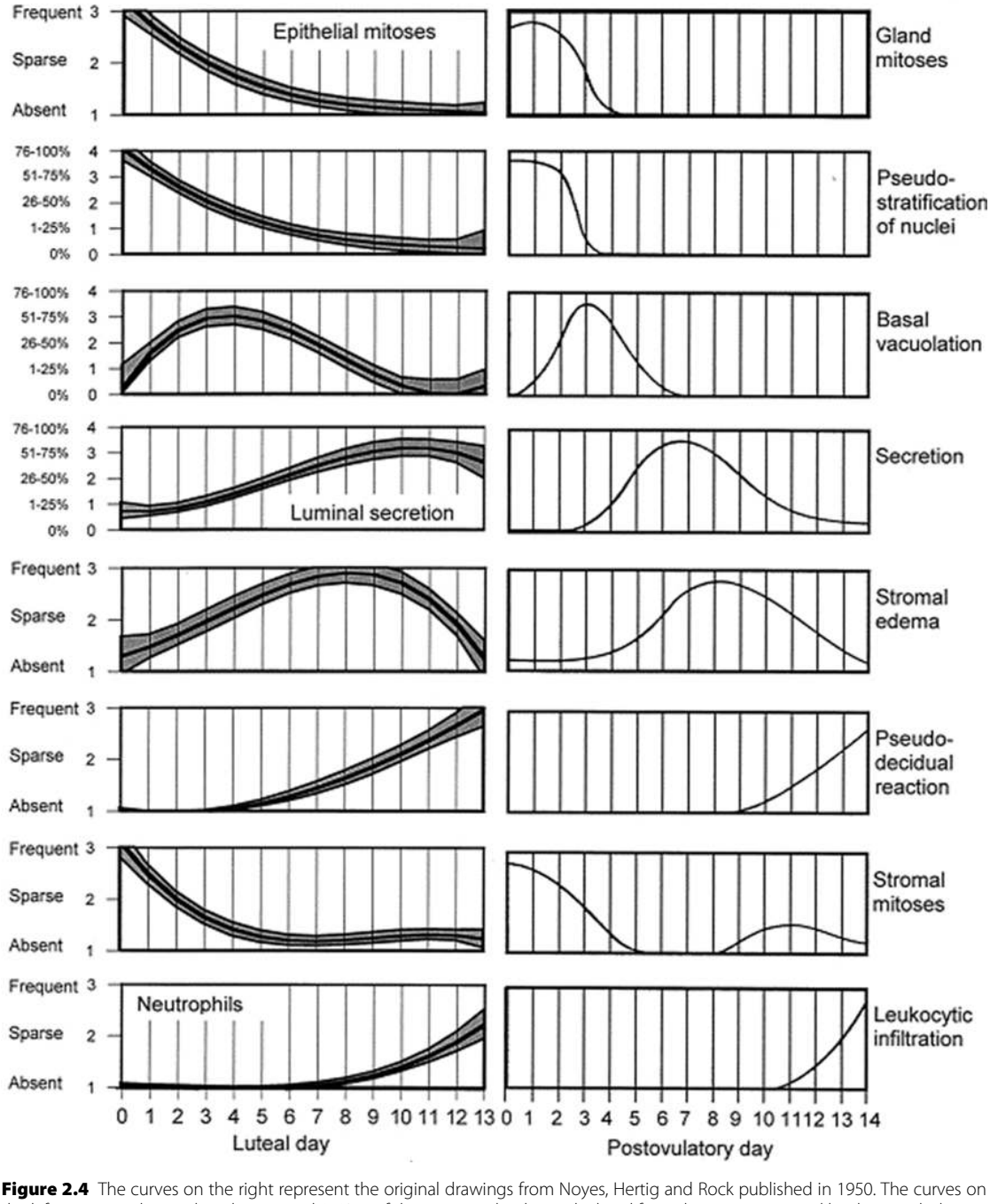

Figure 2.4 The curves on the right represent the original drawings from Noyes, Hertig and Rock published in 1950. The curves on the left represent the predicted mean and 95% confidence intervals when calculated from the scores assigned by three pathologists blinded to luteal day published by Murray et al. in 2004. Polynomial curves were generated for each of the 32 histologic features examined. While the curves on the left generally resemble those on the right, they also demonstrate that the selected histologic features are less temporally distinct than previously proposed. (Source: Murray et al. 2004)

[36]. The endometrial biopsy is obtained 6–10 days postovulation in a natural cycle or 5–10 days after starting progesterone in a programmed cycle. Patients with confirmed increased BCL6 expression on biopsy have been found to have decreased pregnancy and live birth rates compared to women with normal BCL6 expression [37]. Thus, treatment for endometriosis is generally recommended prior to