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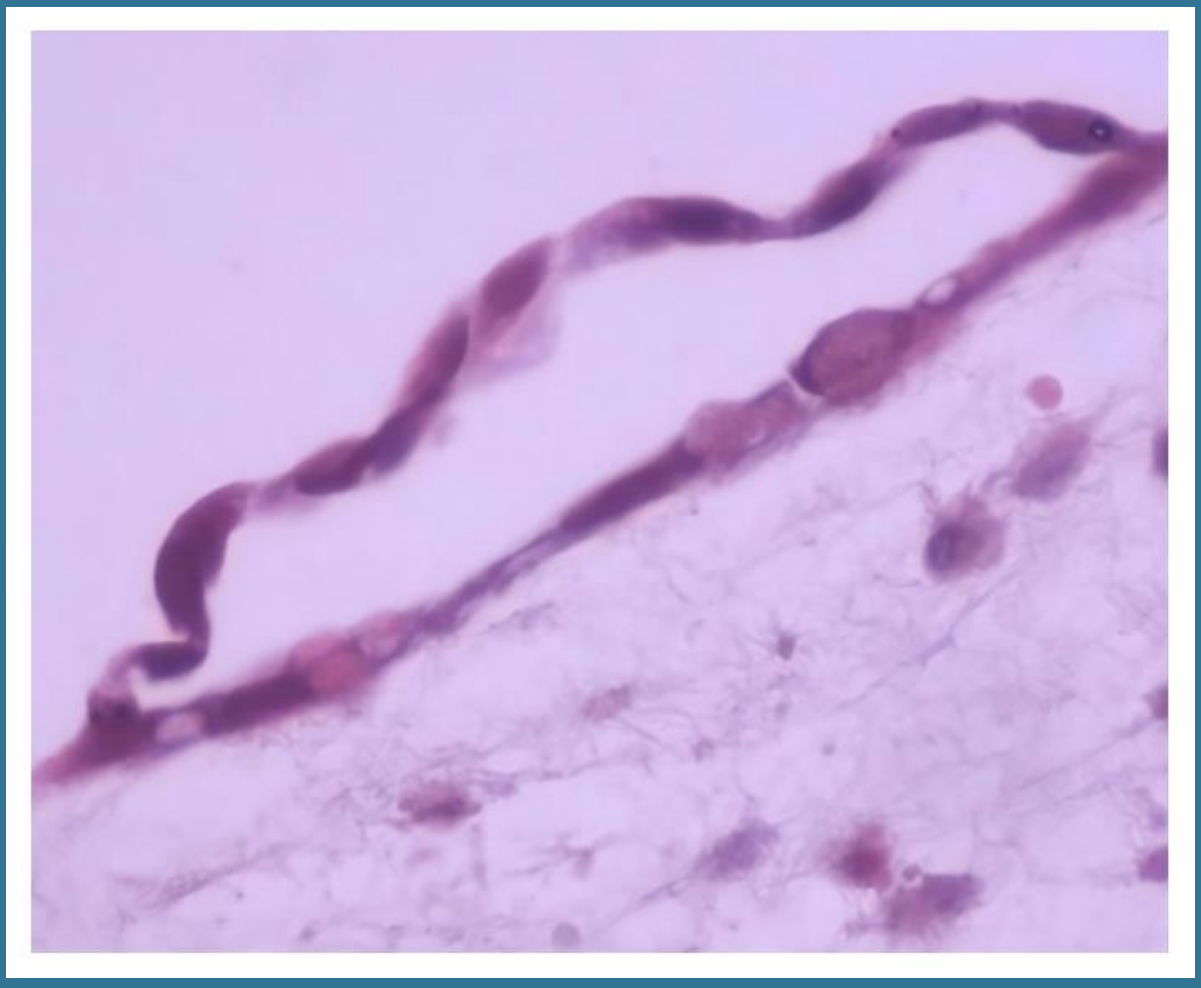

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The Journal of Reproduction

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The Journal of Reproduction possess the ISSN registry granted by the UNESCO (2022). Moreover, complies the requirements of the Berlin Declaration on Open Access to Knowledge in the Sciences and Humanities that promote and provide cultural and scientific information worldwide (2021). With social responsibility our Journal defunds medical education, fomenting the professional excellence and improving the care of patients. Academy does matter, be welcomed to our project!

Cover photo

Photograph of a mouse blastocyst adhering to and initiating stromal invasion of a 3D human endometrial culture system (day 2 of co-culture). The 3D system was bioengineered using a mixture of fibrin–agarose as a scaffold, with the epithelial cells (Ishikawa) on top, and the stromal cells (HESCs) residing within the matrix, as described by Wang et al (2 ref). Two-cell mouse embryos were obtained from B6D2F1 males × B6C3F1 females and grown to the blastocyst stage. Expanding blastocysts were transferred to the top of the 3D system and evaluated for adherence and invasion of the endometrial model on a time-dependency fashion. (HE x 400).

Bibliographical references of the cover photo

Sex steroids regulate epithelial-stromal cell cross talk and trophoblast attachment invasion in a three-dimensional human endometrial culture system. Wang H, Bocca S, Anderson S, Yu L, Rhavi BS, Horcajadas J, Oehninger S. Tissue Eng Part C Methods. 2013 Sep;19(9):676-87. doi: 10.1089/ten.TEC.2012.0616. Epub 2013 Feb 19.PMID: 23320930.

A novel model of human implantation: 3D endometrium-like culture system to study attachment of human trophoblast (Jar) cell spheroids. Wang H, Pilla F, Anderson S, Martínez-Escribano S, Herrer I, Moreno-Moya JM, Musti S, Bocca S, Oehninger S, Horcajadas JA. Mol Hum Reprod. 2012 Jan;18(1):33-43. doi: 10.1093/molehr/gar064. Epub 2011 Oct 11.PMID: 21989169.

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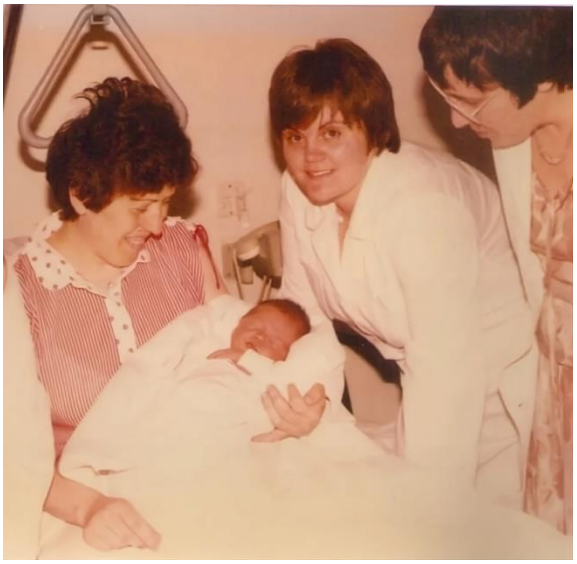
In Memoriam

Ms. Tatjana Kniewald



Clinical embryologists, mourns the loss of their well-known, honored colleague Ms. Tatjana Kniewald who passed away in Nasice, Croatia, on May 4th, 2024, just before her 79th birthday.

Ms. Tatjana was a scientist par-excellence, a clinical embryologist who was responsible for the birth of many IVF babies - directly through her work as well as through the many embryologists she trained, an innovator and an entrepreneur; all at a time when iVF was itself a budding field.

It is almost impossible to try and describe all the achievements of Tatjana in a few sentences, but I will indeed make an attempt Tatjana who was really a brilliant scientist, embryologist – or as she used to present herself: reproductive biologist. As an IVF scientist, Tatjana was " the mother" of the first IVF baby – born in Germany in 1979 as well as to many thousands of babies born afterwards.

Tatjana Kniewald was born in Osijek, Croatia. She studied biology in Zagreb, and later in Vienna and Erlangen. From 1979 Tatjana started her work at the women's clinic in the University Hospital Erlangen as a reproductive biologist in the group of Prof. Trot now. Being a leading scientist, her intensive work resulted in the first IVF pregnancy in Germany (1981) followed by the birth of the first German IVF baby, Oliver, on April 16, 1982.

On 1981, during the period of the famous first IVF pregnancy in Germany, Tatjana, visited the group of Alan Trounson in Melbourne, Australia, sharing with them her knowledge and experience of her journey via the magical pathways of the IVF-ART field.

On 1986, she left Erlangen and, together with her husband, Alfred Kniewald who himself is a talented biologist of human geneticist they established the first private IVF center in Germany in Würzburg, together with 2 clinicians.

Tatjana was also an innovator. In cooperation with a cryology engineer, she designed a machine for embryo freezing (slow freezing!) which was meant to bypass the seeding damage.

This was also the time when I visited their center to learn embryo freezing. Tatjana was an extraordinary and dedicated teacher. I learned a lot from her and not only freezing. Moreover, I spent with the Kniewalds, in their own home feeling as a part of the family, this was a "deal package" for most of their colleagues.

On 1987, the Kniewald couple opened their own IVF center in Prien, where they successfully worked and lead the laboratory for 17 years. In parallel, they managed to lead the IVF lab in in Bad Dürrnberg, Austria (1990-2000).

Tatjana was a very hard worker. She gained international recognition due to her numerous publications. However she was always dedicated to the process of training other embryologists, motivating and

inspiring them; Tatjana and her husband Alfred, were also involved in the establishment of IVF laboratories in 25 IVF centers, in various countries, including for instance, Spain and the Balkan region.

Tatjana Kniewald was a very special person, enthusiastic, open and very warm. She was always ready to help in troubleshooting and to answer everyone's questions about IVF. However, on top of all she was so human and a loyal friend; I remember once when she was an invited speaker, and instead of participating in a dinner which was planned for her she came to visit us in our modest apartment, climbing the stairs of 4 floors to share with us the joy of our new-born son.

This was Tatjana Kniewald. an extraordinary person, a visionary with a lot of joy in life and tireless drive.

As embryologists, we will always remember Ms. Tatjana Kniewald an extraordinary scientist and visionary. We express our condolences to her husband Alfred – the love of her youth, her son Adrian and his wife, Elisabeth and grandson Dominik.

Dr. Yona Barak, BSc, MSc, PhD.

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Editorial

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Dear Readers,

The endometrium is the only tissue capable of regenerating every month to be functional, or rather, useful, very few times, sometimes none, during the course of life. Its uniqueness has made it a subject of study for decades as it aligns several endocrine organs and coordinates with the development of the embryo to reach its stellar moment in the process of embryonic implantation. As studies using new technologies have been conducted, knowledge about the functioning of the layer that lines the human uterus has increased. Still, implantation of an embryo remians one of the greatest incognitas in the field of human reproduction.

From imaging studies to gene expression analyses or knowledge about the microbiome, data have been provided to understand the physiology and also the changes that occur in the different pathologies that affect the endometrium. Finally, reproductive medicine has assigned its role in the reproductive process, not diminishing its importance in the development of the earliest stages of pregnancy. Understanding its functioning has led to better methods of preparing the luteal phase and understanding and addressing the two main medical problems related to infertility: implantation failure and recurrent miscarriage.

Although there are multifactorial components, not only derived from the endometrial function itself, knowing the characteristics of a healthy endometrium allows the development of protocols to maintain or improve its functionality and also to establish red lines that mark the clinical limits of its normality, such as its thickness or its microbiological balance. Despite these progresses, the difficulties to study human implantation are huge. Thus the need of developing methods and technologies such as the one shown in our cover of this volume are required.

The endometrium is a dynamic, variable, and extraordinarily sensitive tissue to changes. As a complex tissue, its study, the anamnesis of its pathologies, and its implication in reproductive problems are also complex. Establishing guidelines for its treatment for a successful pregnancy is also complex. Nowadays, there are no clear guidelines for the treatment of a dysfunctional endometrium, with empirical protocols existing for, for example, the treatment of a thin endometrium that range from the use of sildenafil to treatment with growth factor-rich plasma, Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) therapy, or stem cell transplantation.

One can find in the literature publications both for and against with favorable or null results, leaving the reproductive specialist with the doubt of their true efficiency. William Osler said that "medicine is the science of uncertainty and the art of probability." That phrase takes on even more significance in the

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treatment of a non-functional or refractory endometrium. But that is precisely what makes Medicine an art and not an exact science. In the development of Personalized Medicine, it is necessary to apply knowledge about the endometrium in the most scientific way possible, clearly understanding what we must do, leaving aside subjective criteria. In our world of assisted reproduction, innovation is a constant reality, a continuous challenge. It is our perpetual curiosity that leads us to innovate and seek solutions, that leads us to think we can do more than we do.

From the classic study of the endometrium through pathological anatomy to observe the ancient Noyes criteria, whose first studies date back to 1950, to the development of artificial intelligence for the diagnosis of endometrial receptivity through imaging, the physiopathology of the endometrium has been investigated at the biological level, dissecting at the molecular level the processes that take place in the endometrium throughout the menstrual cycle, especially focusing on the window of implantation that leads to the possibility of an embryonic implantation and a healthy pregnancy. Invasive molecular tools aimed at diagnosing and evaluating endometrial functionality have been developed, highlighting the clinical need to observe the endometrium objectively, trying to improve existing techniques.

The fact that the different tests developed that analyze different genes with different technologies and different algorithms end up obtaining similar results makes us think that, indeed, endometrial gene expression is an objective molecular criterion both for defining endometrial receptivity and for finding factors related to the physiopathology of endometrial tissue. We could say, without fear of being wrong, that in the field of endometrial tests, "all roads lead to Rome" and that the spirit of their conception is to provide the physician with a useful tool. However, it is obvious that although all roads lead to Rome, not all roads are the same, and something even more important than the road is the "way of facing it."

Every invention or development has its function and usefulness, from scissors to a molecular study, and if used incorrectly or in the wrong place or time, undesirable results are obtained. In recent years, knowledge of the endometrial microbiological ecosystem has resulted in a new unknown facet for clinical study.

The implication of the microbiome in uterine functionality and especially in its alteration has brought to light another variable to consider in certain cases of infertility. The endometrium never ceases to surprise us, and thinking that for decades it was established that the scenario of the beginning of pregnancy was a sterile place. And it is not so. This part of the study of the endometrium may have no end due to its complexity and also to its connections with the intestinal microbiome and gynecological alterations, which in some cases seem to have a dysbiotic origin.

Molecular tools are objective and precise, which allows setting limits, margins, thresholds, and critical levels, but we must not forget the biological variability between individuals and the phenotype associated with lifestyle. Similarly, the latest non-invasive developments for image analysis give the reproductive specialist more data to improve the efficiency in treating the infertile couple. In this monograph, some pathologies related to this unique tissue are also addressed. Pathologies that, likewise, present a varied spectrum of manifestations that affect endometrial functionality at different levels.

This special edition on the endometrium of The Journal of Reproduction only shows some aspects of its complexity but serves to establish the direction in which the efforts of clinicians and researchers are headed in knowing, diagnosing, and above all interpreting the signals that the endometrium shows to treat the patient in a personalized and efficient way for a successful embryonic implantation and a safe pregnancy.

Warm regards,

Ricardo Héctor Asch Schuff

José Horcajadas, PhD

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The endometrium: basic aspects of a complex tissue

Sergio Oehninger

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ABSTRACT

This monograph reviews the orderly sequence of events that characterize the human endometrial cycle, the known key molecules and mechanisms involved during the proliferative and secretory phases, as well as the validation of the determinant processes of decidualization and the establishment of the window of implantation. A new look into old dogmas is delivered, and new challenges are posed based on contemporary and objective scientific and clinical evidence. This highly detailed narrative constitutes an in-depth academic work that presents original research, analysis, and arguments aimed to solve current dilemmas or to open the path for further analysis and debate. The understanding and development of new and robust experimental paradigms of genomics, proteomics, and transcriptomics has led to the development of novel high-throughput methodologies aimed directly at interrogating biomolecules, their cellular locations, and interactions, at previously unattainable levels. We remain enthusiastic that the information provided herein will become more and more significant not only as basic physiologic advances but importantly, critical from a clinical point of view, for improved infertility management, and perhaps with an impact on women's health.

KEYWORDS: Endometrium, implantation, blastocyst, transcriptomics.

MANUSCRIPT

Introduction

The endometrium is composed of two layers. The functional layer adjacent to the uterine cavity, contains the luminal epithelium, glands, and stroma, and responds to the sequential effects of circulating estradiol (E2) and progesterone (P4) to proliferate and then become secretory during each menstrual cycle, and differentiates to sustain blastocyst implantation. The basal layer contains stem cells which regenerate the functional layer upon menstruation. The functional endometrium consists of a single layer of columnar epithelium and a layer of connective tissue (stroma) containing a rich blood supply provided by the spiral arteries, that varies in thickness according to cyclic sex hormonal influences.

In the endometrium, simple tubular glands reach from the endometrial surface through to the base of the stroma, and upon differentiation provide an optimum environment for implantation and growth of the embryo. The endometrium is central, echogenic (detectable using ultrasound scanning), has a typical trilaminar appearance reaching an average thickness of 7 to 10mm during maximal estrogenic effect, and cohabitates with a unique microbiota. Upon fertilization, the egg may implant into the uterine wall and provides feedback to the ovaries through human chorionic gonadotropin (hCG) secreted by the trophoblast, that rescues and maintains the corpus luteum span, which will continue its role of secreting P4 and E2 and other hormones until the newly formed placenta takes over the endocrine functions.

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At a cellular-molecular level, implantation is nowadays recognized as a controlled inflammatory response. After ovulation, a dramatic differentiation process of endometrial stromal cells into decidual stromal cells is induced. Decidualized stromal cells are key players which initiate a cascade of events leading to recruitment and local differentiation of the decidual uterine natural killer cells (uNK cells). These cells mediate embryo recognition and immunotolerance, and secrete cytokines required to activate and control trophoblast invasion. Decidual uNK cells also participate in spiral artery remodeling and activate and control trophoblast invasion[1].

Luteal phase dysfunctions, uncontrolled trophoblast invasion of stroma and arteries, and abnormal decidualization and placentation, can derive in severe pathologies ranging from lack of implantation to miscarriage, development of trophoblast tumors, and can be the culprits of later obstetrical complications such as fetal growth retardation and preeclampsia. Despite active research, particularly derived from the experience and knowledge gained in infertility treatment via in vitro fertilization and embryo transfer (IVF-ET), the critical regulatory elements that determine implantation and regulate its different phases remain incompletely resolved.

Here, it is our primary aim to discuss the state-of-the-art of the orderly sequence of events that characterize the human endometrial cycle, the key molecules and mechanisms involved, their cellular origins and temporal-spatial interactions, and the characterization of the determinant processes of decidualization and the window of implantation (WOI).

The human endometrial cycle: proliferative and secretory phases and establishment of embryo receptivity

The intraovarian processes of follicular recruitment, selection, and dominance, that occur during the proliferative phase allow for an increasing and sustained secretion of E2, which is the main endometrial regulator in the first half of the cycle. Importantly, whereas many ovarian follicles begin their developmental course at each cycle, typically only a single follicle sustains its inherent gametogenic potential; all others succumb to atresia finally having forfeited their latency[2,3]. Upon the completion of the follicular phase and ovulation, the dominant follicle undergoes dramatic morphological and functional changes to become the corpus luteum. This robust endocrine organ biosynthesizes and secretes large amounts of P4, the key hormone that prepares the estrogen-primed endometrium for embryo implantation[4]. During the mid secretory phase, the endometrium transforms into a temporally receptive tissue that is suitable for embryo adhesion and attachment for a limited period[5,6].

There is a complex and reciprocal paracrine communication between the pre-implanting blastocyst and the endometrium. The pre-implantation embryo signals its presence to the mother by endocrine modulators, such as hCG, and paracrine growth factors, which act locally on the endometrium to facilitate attachment. The intimate mechanisms that determine initial adhesion between trophectoderm cells and the endometrium are not completely understood. In the sheep, it is likely that embryonic L-selectins and their specific ligands present in the luminal epithelium, as well as trophoblastic integrin receptors and the reciprocal epithelial integrin molecules (also including osteopontin and arginylglycylaspartic acid -RGD) play an important recognition role[8]. The significance of L-selectins, glycodelin, and other complex sialylated N-glycans and other carbohydrate moieties in human embryo recognition and adhesion has been described[9,10]. Following attachment, the embryo penetrates the luminal epithelium, breaches the basal membrane, and invades into the underlying stroma, while in synchrony endometrial stromal cells begin full decidualization[7].

The sequential actions of E2 and P4 after ovulation regulate the formation of a differentiated endometrial stromal tissue, known as the “decidua,” which supports embryo growth and maintains early pregnancy[5,6]. Decidualization occurs during the ovulary cycles, independently of the presence of an embryo in the uterine cavity (as opposed to other animal species). Decidualization consists of the differentiation of elongated, fibroblast-like mesenchymal cells in the endometrial stroma to rounded, epithelioid-like cells. This morphological change is initiated during the mid-secretory phase of the menstrual cycle because of elevated P4 levels and begins with stromal cells surrounding the spiral arteries in the upper two-thirds of the endometrium. Human decidualization begins approximately 6 days after ovulation, at the onset of the putative WOI[11]. The process is accompanied by secretory transformation of the uterine glands, an influx of the specialized decidualized uNK cells, and vascular remodeling to support the maternal blood supply to the growing conceptus[12].

Progesterone is an essential regulator of decidualization and a prerequisite for successful blastocyst implantation. But decidualization is also controlled by complex interactions of transcription factors, cytokines, and signaling pathways. A critical network for the decidualization of endometrial stromal cells is comprised of P4 and its downstream molecules, including the transcription factors FOXO1, HOXA10, C/EBPβ and HAND2, and the protein BMP[5]. During

decidualization, differentiating stromal cells carry a molecular signature of mesenchymal–epithelial transition as they are reprogrammed to become decidualizes stromal cells with widespread changes in gene expression[13]. These decidualized cells contribute to the micro-environment in the human endometrium and have direct and indirect influences on endometrial remodeling, local immune response regulation, antioxidant responses, and angiogenesis[5].

Estradiol-dependent epithelial cells proliferation is a mandatory pre-requisite for adequate decidualization. In these cells, E2 upregulates E2 receptors (ER) as well as P4 receptors (PR). In stromal cells, and after the LH surge, E2 acting via ERα stimulates P4 resulting in proliferation and differentiation of stromal cells. Cyclic AMP (cAMP) of yet unknown but probably stromal origin potentiates the P4 effects. Stromal cells are then included to produce, among others, prolactin and IGFBP-1, that are typically used as biomarkers of decidualization in vitro. Possible epithelial cell signals also participate in stromal decidualization. Importantly, E2 induces angiogenesis by stimulating secretion of VEGF by various cell types (decidualized stroma cells, uNK, and epithelial cells)[14,15].

Most functions are P4-dependent and occur via genomic progesterone signaling pathways mediated by the nuclear PR, although certain signaling occurs via non genomic PR and other pathways that involve adhesion molecules leading to cell-cell interactions (integrins and others), vasoactive factors (prostaglandins and others), cytokines (such as leukemia inhibitory factor -LIF- and interleukins), homeobox genes, and many other transcriptional factors [13]. Further research is needed to determine if these pathways function independently, in parallel, or converge to a common signaling pathway to establish the network of crosstalk between the embryo and endometrium that is necessary for implantation[16].

Prior to implantation, the blastocyst must hatch out of its acellular glycoprotein coat, the zona pellucida. Blastocyst hatching is believed to be regulated by both dynamic cellular components such as actin-based trophectodermal projections, and a variety of autocrine and paracrine molecules originating from the blastocyst and probably also of endometrial origin. Embryonic signals occur via EGF receptor (EGFR) and Cox-2, in coordination with the stimulation of zona pellucida lysins (Heparin sulphate, uPA, Plasmin, MMP-9, and implantation serine proteinase 1 [ISP1]). Pro-inflammatory (IL-6, LIF, GM-CSF) and anti-inflammatory (IL-11, CSF-1) cytokines modulate hatching rates and regulate proteases (MMPs, tPAs, cathepsins and ISP1). There is evidence of endometrial origin for hatching; putative secreted endometrial factors may include Heparin sulphate and EGF, among others[17]. Recent IVF data from time-lapse video cinematography demonstrate blastocyst hatching in association with contraction and zona pellucida rupture as captured in vitro[18].

Extensive paracrine relationships exist among the various cell types of the endometrium[14,15,16]. The epithelial cells possess ERα, ERβ, and PR, and secrete many functionally significant proteins such as glycodelin A and LIF among others. The stromal cells have ERα, ERβ, and PR in the secretory phase., and secrete a variety of regulatory molecules, including Il-15. Estradiol up regulates ER and PR during the proliferative phase, but down regulates ER during the secretory phase. Differentiated uNKs possess ERβ and receptors for Il-15 (Il-15R), a crucial pathway for immunological regulation. Il-15 stimulates proliferation of uNK cells (pointing to a critical stromal cells-uNK cells interaction). Uterine NKs are cytolytic and cytotoxic, secrete other cytokines (LIF, TNFα, IFNγ, GM-CSF, Il-10), and angiogenic molecules (VEGF and angiopoietin). The epithelial cells lose PR receptors after ovulation, but decidualized cells maintain PR. Estradiol also may exert effects on uNK cells indirectly via cytokines secreted by stromal cells.

Robust angiogenesis takes place during the secretory phase with development of spiral arterioles and a subepithelial capillary plexus. At the time of this extensive neovascularization, endothelial cells exhibit ERβ and have abundant VEGF receptors (VEGFR). VEGF and angiopoietins are the major regulators of endometrial vessel formation, maintenance, stabilization, and regression. VEGF and its receptors (VEGFR) play a significant role in endometrial angiogenesis and participate in the regulation of other endometrial functions. VEGF mRNA and protein are present in glands and stroma; VEGF protein can be identified in neutrophils; and VEGF mRNA is present in uNK cells. VEGFR-1 and -2 are present on endothelial cells and stroma. VEGFR-3 is present on lymphatic cells[15].

Endometrial immunology has been extensively characterized. Immune cells include: uNK, macrophages, and other leukocytes. The uNKs are phenotypically unique ($CD56b_+$, CD16- and $CD3_+$ (as opposed to peripheral or systemic NK cells that are $CD56d_+$, $CD16b_+$ and CD3-). Their origin is still controversial, as it is unclear whether they are derived from in situ proliferation versus de novo recruitment and migration from leukocyte subtypes from blood. On the other hand, uterine macrophages act as oxygen sensors and secrete VEGF and angiopoietin. Neutrophils populate the endometrium before menstruation, and T cell lymphocytes constitute 45% of immune cells[14,15,16].

In normal pregnancy, the trophoblast invades the maternal endothelium releasing microvesicles and soluble mediators (such as TNFα) into the maternal circulation, leading to a low-level physiological inflammatory response that is a characteristic feature of trophoblast adhesion and invasion[1]. TNFα also induces expression of other cytokines, such as IL-6 and IL-8, with modulatory functions in angiogenesis, neutrophil migration, and differentiation. In support of this concept, it has been shown that abnormal inflammation due to elevated levels of TNFα is associated with miscarriages and adversely affects the viability and implantation competence of preimplantation embryos[19].

Endometrial secretions in the uterine cavity contain mediators important for endometrial receptivity and embryo implantation. Extracellular vesicles (exosomes and microvesicles) have been characterized in embryo-endometrium crosstalk[20,21]. Proteomic studies of the human endometrium and uterine fluid (secretome) suggested a pathway to biomarker discovery[20,21].

After ovulation, endometrial stromal cells and perhaps mesenchymal stem cells can be transformed into decidua stromal cells. Cytokines secreted by decidua stromal cells and by recruited leukocytes into the functional layer maintain a pro-inflammatory environment[1]. After the embryo implants, decidual stromal cells generate a ‘wave’ of decidualization by autocrine and paracrine cytokines that spread throughout the uterus[23]. Decidual stromal cells significantly induce uNK cells proliferation and differentiation by secreting IL-15[24]. Multiple cytokines and angiogenic factors secreted by decidua stroma cells, uNK cells, and macrophage cells induce uterine spiral arteries to remodel. Meanwhile, uNK cells and decidual stromal cells can control extra villous trophoblast cells invasion and “sense” embryo quality (see below)[25]. Single-cell sequencing showed that uNK cells in early pregnancy are divided into various subpopulations that differ in surface receptor expression profiles and cytokines secretion, with unique functions including cells destined to combat microbial infections, to determine immune tolerance, remodel spiral arteries. and stimulate fetal growth. It is speculated that functional alterations of one or more of the human uNK cells subpopulations may result in pregnancy complications such as miscarriage and preeclampsia[26].

In the absence of pregnancy, the endometrium enters the menstruation phase because of progesterone withdrawal. Progesterone withdrawal initially affects cells with PR resulting in extensive vasoconstriction and cytokine changes. Chemokine release and chemotaxis determine invasion and activation of neutrophils, with a cascade of events resulting in release of MMPs and tissue destruction. Vascular changes accentuate with hypoxia and secretion of VEGF is augmented. This cascade leads to activation of pro-MMP (MMP-1 and –7, and Il-1) and accentuation of hypoxia. Nevertheless, there is no certainty as to the origin of the MMPs and/or invading neutrophils[14].

As mentioned earlier, it has been postulated that stem cells present in the endometrial basalis may be at least partly responsible for initiating the regeneration process after menstruation. In a pioneer study, clonogenic cells or colony-forming units (CFUs) were identified in purified populations of human endometrial epithelial and stromal cells isolated from hysterectomy tissue[27]. These are stem cells located in the endometrial basalis, they represent <1% of cells, and are clonogenic cells, in both epithelial and stromal lineages. Growth is E2-dependent probably through EGF, TGF (transforming growth factor) and PDGF (platelet derived growth factor). These cells differentiate and transit into the endometrial functionalis 27). Novel “omics” approaches have now been characterized to identify and purify the endometrial mesenchymal stem/stromal cell (eMSC) population.

There is proof that migration of bone marrow mesenchymal stem cells bmMSCs to the human endometrium contributes to the endometrial stem cell pool and thereby endometrial renewal[13,28]. The change in “niche effect” (different local tissue environments in the endometrium layers) and the differentiation process towards endometrial fibroblasts will alter the migration properties and the cytokine secretion profile of these cells. The bmMSCs, the eMSCs possess high migration activity; during their differentiation process towards stromal fibroblasts there is loss of stem cell surface markers, decreased migration activity, and a subtler cytokine profile likely contributing to normal endometrial function. Progesterone and E2 withdrawal drive endometrial collapse and subsequent hypoxia during the late secretory phase of the menstrual cycle most likely triggering the homing signal for the bmMSCs for the subsequent cycle[28].

Importantly, new investigations are unveiling the potential cell-based therapeutic role(s) of bone marrow-derived and endogenous stem/progenitor cells in endometrial proliferative disorders, including endometriosis, adenomyosis, thin dysfunctional endometrium, and Asherman's syndrome.

Models for the study of human implantation

Ethical concerns have limited the use of in vivo approaches to study human embryo implantation. Since human implantation sites are not available for experimentation, and animal models may or not

represent human physiology, researchers have implemented in vitro culture systems with whole endometrial tissue, primary epithelial and stromal cells, and human established cell lines, to gain insight into human implantation[29].

While fixed human tissue enables identification of the in vivo cellular location of molecules, this approach cannot provide functional data. On the other hand, because of the very limited availability of fresh primary tissue, cell lines provide the tools for most functional studies. But these are far from perfect, and information gained with these models can be subsequently validated in primary tissue or animal models.

HESC). Time-dependent experiments demonstrated a high rate of attachment of Jar spheroids to the epithelium, and adhesion was strongly related to the various cell types present in the 3D culture. An architecturally and functionally competent 3D endometrial culture system was therefore established, that coupled with Jar spheroids mimicking trophoblast cells, provides a promising in vitro model for the study of certain aspects of human implantation[30].

Follow up studies[32] demonstrated that the attachment rate of Jar spheroids to the 3D was significantly increased by E2 plus MPA treatment. Analyses of Z-stack confocal and stained optic

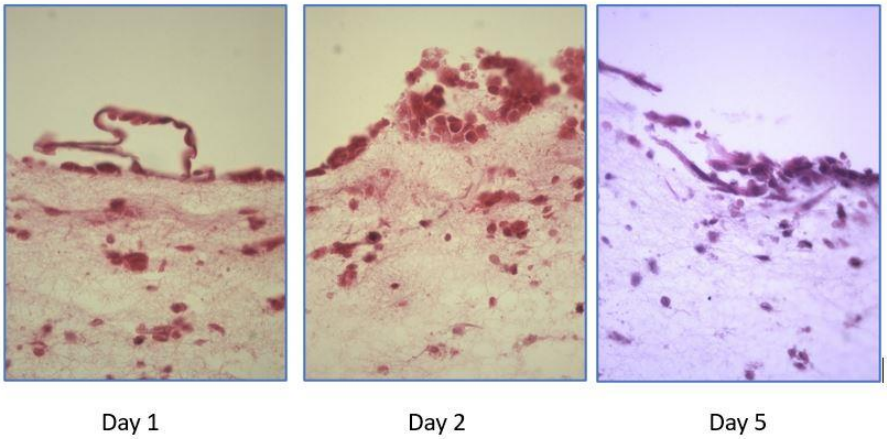

Figure 1. A novel heterologous model of human implantation: 3D endometrium-like culture system to study attachment and initial invasion of mouse blastocysts (Wang H, Oehninger S, and Horcajadas JA, unpublished observations). A 3D culture system was established with Ishikawa and HESC cells in agarose/fibrin/tranexamic acid/CaCl2 scaffold cultured in inserts as detailed in Wang et al[31,32]. Mouse blastocysts were grown from commercially available cryopreserved 2-cell murine embryos. Ten expanded blastocysts were seeded on top of the 3D construct per experiment. Day 1 of co-culture: attachment of a hatched blastocyst to the epithelial layer of the 3D construct. Days 2 and 5: initial invasion of the stroma by the trophoblast with breakage of epithelial layer (HE x400).

In vitro culture models of endometrium have been established from two-dimensional (2D) cell-based to three-dimensional (3D) extracellular matrix (ECM)/tissue-based culture systems[30]. Numerous human established cell lines have been used for examination of implantation. They include “receptive” endometrium (luminal epithelium): ECC1, Ishikawa, HES cells; “non-receptive” endometrium (luminal epithelium): HEC-1A cells; glandular epithelium: Ishikawa, RL 95-2; syncytiotrophoblast: BeWo; trophoblast adhesion and migration: AC 19-88, HTR-8/SVneo; trophoblast invasion: JEG 3, Jar, HTR-8/SVneo, BeWo cells; and stromal cells: T-HESC (immortalized)[29].

Wang et al[31] developed a novel model of human implantation consisting of a 3D endometrium-like culture system with fibrin-agarose as matrix scaffold, to study attachment and invasion of human trophoblast cells (Jar spheroids). The model uses either primary epithelial and stromal cells obtained from endometrial biopsies, or established cell lines (i.e., Ishikawa and

microscopic images showed that Jar spheroids breached the epithelial cell monolayer, invaded, and were embedded into the 3D matrix in response to decidualization signals. Further heterologous experiments, using mouse blastocysts as surrogates for human embryos, revealed a high degree of attachment (day 1 of co-culture) and embryonic cells breaking of epithelial layer and invasion of stroma (days 2 and 5) (**Figure 1**).

For long term 3D cultures systems, organoids have been generated from established human adult stem-cells. These organoids expand long-term, are genetically stable and differentiate following treatment with reproductive hormones. Transcript analysis confirmed great similarity between organoids and the primary tissue of origin, representing a novel system to recapitulate early pregnancy events[33].

However, all models described so far have their pros and cons, and there is no single ideal model to study the whole implantation process. Additional

studies are needed to establish a comprehensive in vitro model that can recapitulate the biology of trophoblast-endometrium interaction during early pregnancy.

Characterization of the WOI: from histology, through individual molecular markers, to genomics and proteomics, to the identification of the transcriptomic signatures of the window of implantation

The changes in the histologic appearance of the endometrium during the ovarian-menstrual cycle have been well characterized[34]. Measurement of mid luteal phase (day 21-22) serum P4 levels and a timed endometrial biopsy have been long used to confirm ovulatory status. However, the value of an endometrial biopsy to ascertain fertile status versus infertility using standard criteria defining an out-of-phase biopsy as a greater than 2-day delay in the histological maturation of the endometrium, has been challenged, leading to its abandonment. It has now been concluded that the histological dating of the endometrium does not discriminate between women of fertile and infertile couples and should not be used in the routine evaluation of infertility[35].

Since the early days of assisted reproductive technologies (ART), a key question has been whether the timing of nidation is dependent on the stage of embryonic development, endometrial maturation, or a possible dialogue between the two (synchrony). Based on an early IVF study of fresh embryo transfers of identical gestational age, it was concluded that the first embryonic signal detection (presumed window of implantation) extends between cycle days 20 and 24[36]. In subsequent studies, investigators examined women with ovarian failure, and induced histologically normal endometrial function during a preparatory cycle consisting of sequential administration of E2 and P4. During a subsequent cycle, endometrial stimulation was synchronized with surrogate-embryo transfers and pregnancies were achieved[37].

Moreover, taking advantage of the establishment of donor egg programs, elegant studies aimed to decipher the “window of transfer” in the human[38]. In these clinical studies, embryos were transferred on different days of the luteal phase. Embryos were transferred into a defined endometrial bed, characterized histologically as day 17 to 19 endometrium by the criteria of Noyes et al[34]. Results strongly suggested that 1- the window of transfer in the human for the 4- to 16-cell embryo extends to day 19 (perhaps day 20) of the idealized 28-day cycle, with the proximal width of the window yet undefined; and 2- that the WOI in the human does not extend beyond day 22 or 23 of the menstrual cycle.

The abandonment of the endometrial biopsy/histologic data as a diagnostic test led to an absence of any reliable diagnostic test to determine the endometrial status. Consequently, the standard workup for infertility in clinics worldwide no longer included endometrial status, beyond a limited use of ultrasound imaging to determine endometrial thickness and pattern. In a secondary approach, numerous authors reported on semiquantitative changes of a defined endometrial molecule known to participate in implantation, comparing its expression in the non-receptive and receptive days of the cycle. Included in this list are LIF, integrins, interleukins, CSF, glycodelin, MUC 1, and others, typically examined by immunohistochemistry[39]. Scanning microscopy analyzing presence of endometrial epithelial pinopds was also investigated[40]. But it was later agreed that no final conclusions could be drawn about the clinical value of these measurements in the assessment of endometrial function and prognosis for pregnancy after ART[39].

With the advent of the “omics” revolution, endometrial biologywas thoroughly re-examined/. Independent investigators simultaneously reported on wide genomic analysis of human endometrial receptivity (genomics) using high density microarrays and bioinformatics technology. Several gene candidates were identified that could segregate the secretory phase of the human endometrium in natural and ovarian stimulation cycles, as well as changes across the menstrual cycle[41,42,43].

The endometrial receptivity assay (ERA) was the first transcriptomic test developed to diagnose the endometrial receptivity status of infertile patients[44]. To identify genes involved in the human endometrial receptivity signature, the authors initially analyzed differences in genome-wide expression profiles between receptive and pre-receptive endometrium using raw expression data from three different models of endometrial receptivity: the natural cycle as the optimal model, the ovarian stimulation cycle as suboptimal, and the refractory endometrium induced by the insertion of an IUD as a negative control[44,45]. The ERA was devised as a customized array containing 238 differentially expressed genes that were coupled to a computational predictor able to identify the transcriptomic profiles of proliferative (PRO), pre-receptive (PRE), receptive (R) or post-receptive (POST) endometrial samples, regardless of their histological appearance.

Additional studies showed high specificity and sensitivity for endometrial dating, and the transcriptomic signature was further defined by 134 genes. Clinical algorithms were introduced for embryo transfer personalization during IVF cycles, derived from data suggestive of “displacement” of the WOI in cases

of failed implantation and others[45]. Over a decade of ERA clinical application there is still controversy as to the clinical significance of the test, populations to be applied to, and others[46,47,48,49]. Notwithstanding these caveats, the ERA has highlighted the significance of the transcriptomic endometrial receptivity status, and significantly deepened our basic knowledge. Other variations of this test have lately been introduced in the clinical scenario. As technology evolved, microarray and PCR-based clinical tests were replaced by next generation sequencing technology (NGS). Furthermore, it has been suggested that important clinical information may be obtained by combined analysis of the transcriptomic profiling (ERA-NGS) and uterine microbiota analysis by NGS. Using 16S rRNA gene sequencing it has been reported that the endometrial microbiota composition before embryo transfer is a useful biomarker to predict reproductive outcome in ART[50].

It has been discussed that endometrial receptivity is not an 'all or none phenomenon', nor does the analogy of a window indicate that the window opens at a certain point and then closes to any interaction with the embryo[51]. In 2020, Wang et al published a very elegant novel study aiming to characterize the human endometrial transcriptome at a single-cell level, revealing cell-specific expression signatures across the menstrual cycle[52]. The investigators applied single-cell RNA sequencing (scRNA-seq) to generate an RNA-seq library, followed by gene ontology functional enrichment. The authors were able to characterize the human endometrium across the menstrual cycle from both a static and a dynamic perspective with additional information being provided by constructing single-cell-resolution trajectories of the menstrual cycle.

Employing canonical markers and highly differentially expressed genes, Wang et al identified six endometrial cell types: epithelial and endothelial cells, stromal fibroblasts, macrophages, lymphocytes, and a novel ciliated epithelial cell type[52]. The ciliated epithelium is a distinct endometrial cell type with its own signature; these cells are consistently present in the healthy endometrium but dynamically changing in abundance across the menstrual cycle. Importantly, information was provided for the first time on gene expression modifications occurring at the estimated opening and closure of the WOI. Based on their data, the human WOI opens with an abrupt and discontinuous transcriptomic activation in unciliated epithelia, accompanied by a widespread decidualization feature in the stromal fibroblasts[52,53].

The term "endometrial receptivity" implies a passivity of function in implantation that recent discoveries have come to challenge[13,54]. Additional functions have been ascribed to the decidualized stromal compartment of the endometrium indicating that the decidua has a key role in directing the maternal response to the implanting embryo. It is speculated that migration of the decidualized stromal cells is controlled by transcription factors, chaperones, cytokines and trophoblast factors, and results in a regulatory system, which requires balancing of endometrial and embryonic phenotypes to modulate implantation[54,55].

The need for such "biosensor function" becomes clear when one considers the challenge that the implanting embryo presents to the endometrium. In contrast to other species, human embryos are characterized by their high rate of chromosomal abnormalities. Most aneuploidies will fail to establish an ongoing pregnancy, despite being invasive enough to initiate implantation. Although this may in part reflect incompetency, it has become evident that there is also an active maternal strategy to prevent investment in these invasive but poorly viable embryos. Aneuploidy is associated with proteotoxic stress, metabolic overdrive, and production of proteases, embryonic conditions that can be "sensed" by the decidua. If decidualization is suboptimal, then the biosensor function may be disrupted too. The consequence of this could be that rather than allowing early rejection of poor-quality embryos before the mother becomes aware that she may have conceived, the endometrium would allow poorly viable embryos to establish a clinical pregnancy, ultimately destined to fail, and present as a clinical miscarriage. Persistently impaired endometrial selectivity would result in recurrent early pregnancy loss. Conversely, an excessive decidual response would allow receptivity to dominate, reducing the incidence of miscarriage but increasing the likelihood of implantation delay or implantation failure after IVF[13,55,56,57].

At the current stage of knowledge, it appears that both concepts of receptivity (i.e., effective and timely opening and closure of the WOI under normal conditions, and pathological displacement of the WOI in groups of sub fertile women that can be corrected by modifying embryo transfer timing), and selectivity (power of decidua to accept/reject good/poor quality embryos, or function as sensor/driver of pregnancy health), may coexist.

CONCLUSIONS

We are witnessing an era of precise medicine, perhaps to be augmented by utilization of artificial intelligence, with incorporation of personalized medicine, versus a controversial philosophy that "one size (shoe) fits all". The data presented herein unequivocally highlights the complexity of the endometrium, the numerous cascades of control, and the fact that sophisticated mechanisms may coexist and indeed may be complementary to each other to determine embryo implantation.

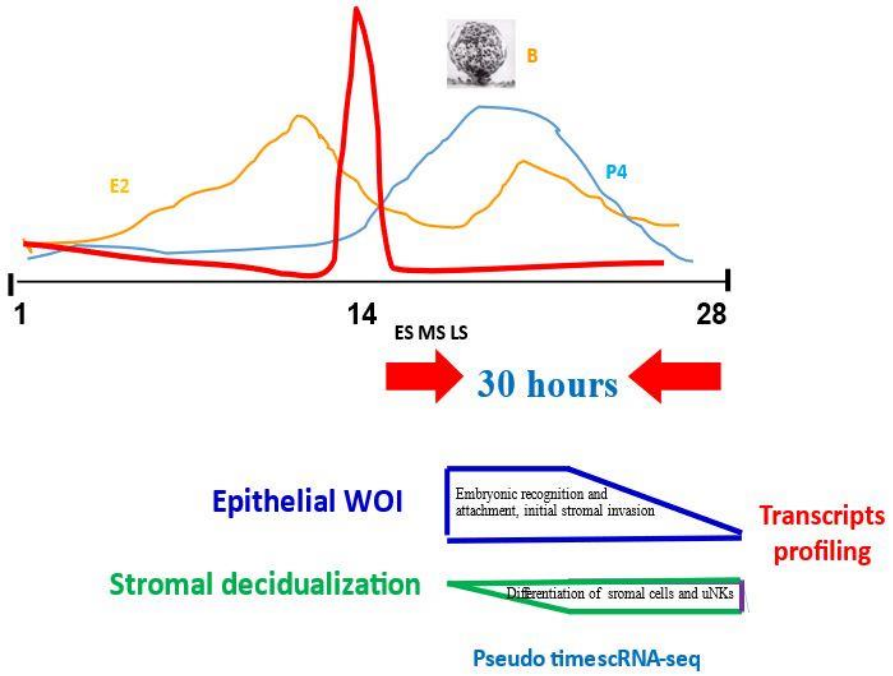

Figure 2. Endometrial receptivity and selectivity: characterization of the WOI. Diagram showing a panoramic view of endocrine events during the normal ovarian-menstual cycle, centered on the day of the LH surge, and the temporal transcriptome dynamics of endometrial transformation across the WOI obtained by single-cell RNA sequencing. Concomitant trasncriptomics changes during the WOI and the functional processes occurrung at the level of 1-epithelium (embryonic recognition and attachment, initial blastocyst stromal invasion); and 2-Stromal cells (decidualization with differentiation of sromal cells and uNKs, immunomodulation, neovascularization, embyonic controlled invasion) are depicted. Current evidence indicates that the WOI lasts 30–36 hours and, depending on the patient, occurs between LH + 6 to LH + 9 in natural cycles or from P + 4 to P + 7 in hormonal replacement therapy cycles[52,53] Note different timing and patterns of cellular and gene activation at opening and closure of the WOI. Receptivity[45] and selectivity[56] may represent two complemetaty mechanisms that regulate implantation of a healthy embryo. B: blastocyst. ES: early secretory; MS: mid secretory; LS: late secretory phase.

From a basic physiologic point of view, we have highlighted that endometrial receptivity has been defined at cellular and transcriptomic levels. Novel data confirm that the WOI clinically extends between days 20-24, and transcriptionally lasts 30–36 hours and, depending on the patient, occurs between LH + 6 to LH + 9 in natural cycles or from P + 4 to P + 7 in hormonal replacement therapy cycles[53]. The gene expression changes, and temporal patterns associated with opening and closure of the window, particularly at the level of the unciliated epithelial cells, are starting to be understood. Furthermore, the stromal decidual cells and uNK play a critical complementary regulatory role of embryo selection acting as biosensors that drive the normalcy of the implantation process and protect the health and growth of the embryo (**figure 2**).

From the point of view of novel technologies, the future appears to be bright. High throughput genomics methods allowed in-depth study of early implantation and development. RNA sequencing (transcriptomics) represents an average of gene expression patterns across thousands to millions of cells. On the other hand, scRNA-seq reveals RNA abundance. Single-cell RNA sequencing involves isolation of single cells, capturing their transcripts, and generating sequencing libraries in which the transcripts are mapped to individual cells at unprecedented resolution. However, it captures only static snapshots at a point in time. This information can now be extended by calculation of RNA velocity -the time derivative for dynamic biological systems such as endometrial differentiation and implantation, and embryo development, that are based on processes of cell differentiation and fate, transitions, and lineage[58].

RNA velocity, the time derivative of the gene expression state, can be directly estimated by distinguishing between unspliced and spliced mRNAs in common single-cell RNA sequencing protocols. Single-cell RNA sequencing successfully captures the heterogeneity that results from processes such as development, reprogramming, and regeneration, but it loses lineage relationships, since each cell can be measured only once. To mitigate this problem, scRNA-seq can be combined with lineage tracing methods or metabolic labeling methods that use the ratio of nascent to mature RNA molecules to link observed gene expression profiles over short time windows. Yet both strategies are mostly limited to in vitro applications, prompting the development of

computational approaches to reconstruct pseudotime trajectories[59].

In addition to transcriptomics, investigators have now been able to measure protein translation with spatially resolved, single-cell resolution. Recently, Zeng et al.[60] developed a highly multiplexed, ribosome-bound messenger RNA imaging technique called RIBOmap and applied it in single cells in situ to profile translation events with spatial coordinates. The pairwise spatial translatomic and transcriptomic mapping enabled the authors to systematically identify cell type- and tissue-region–specific translational regulation, paving the way for uncovering novel posttranscriptional gene regulation principles and mechanisms that shape the proteome for cellular and tissue functions. In the end, physiological processes and diseases need to be understood in terms of proteomics and metabolomics, which define individual phenotypes and functions in systems biology.

But unfortunately, this fast pace of discovery has not yet been matched by new clinical applications in infertility and ART, and much more work is needed to decipher endometrial pathologies that result in implantation failure and other challenging diseases. There is still no agreed test to study the WOI[47,48,51] and no novel management options have been introduced. But we remain enthusiastic that the new basic information, with surely more data yet to arrive, will lead to improved diagnostic tools and therapies for implantation and other gynecological disorders, and increased ART efficiency.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

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Non-Invasive Methodologies for Endometrial Evaluation in Medically Assisted Reproduction

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ABSTRACT

A conceptual synopsis of the state of non-invasive image-based endometrial receptivity assessment methods utilized within in vitro fertilization (IVF) cycles is presented in narrative review format. Many methods for assessing the endometrial contribution to cycle outcome have been posited over the decades since IVF became a mainstream approach for treatment of infertility. Yet, understanding of the endometrial component remains incomplete and most methods for assessing endometrium in the context of IVF are subject to significant divides within the literature. The need for non-invasive, per-cycle approaches to assess endometrial receptivity is being addressed with innovative methods; and, incremental progress is laying a foundation for quantitative assessment of the many factors that contribute to endometrial receptivity. Non-invasive image-based assessments of the endometrium align on two key factors: 1) they make quantifiable assessments of specific endpoints; and, 2) they are conducted on a per-cycle basis which enables real-time clinical decision making. Herein we summarize endometrial thickness, endometrial pattern, uterine biophysical profiles, endometrial scoring, Doppler approaches, uterine contractility, endometrial length and volume, endometrial compaction, the ultrasound-based endometrial receptivity test, and artificial intelligence and machine learning approaches to assessment of endometrium. We also note and discuss the importance of accounting for embryo quality when making decisions focused on endometrial assessment methods since the two factors are intimately intertwined in successful establishment of pregnancy.

KEYWORDS

Ultrasound, Endometrium, usER, Matris™, ER, IVF, MAR.

LIST OF ABBREVIATIONS

MAR	Medically assisted reproduction	**3D**	Three-dimensional
IVF	In vitro fertilization	**2D**	Two-dimensional
ART	Assisted reproductive technology	**ICSI**	Intercytoplasmic sperm injection
AI	Artificial intelligence	**usER**	Ultrasound based ER
FET	Frozen embryo transfer	**ET**	ET
PI	Pulsatility index	**ML**	Machine learning
RI	Resistance index	**NEQsi**	Numeric embryo quality scoring index
S/D ratio	Systolic to diastolic ratio		

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MANUSCRIPT

Introduction

The endometrium, and its ability to recognize, receive and implant a competent embryo, is a critical component to the success of in vitro fertilization (IVF) cycles. However, it is not clear which endometrial characteristics best predict implantation. Numerous approaches have been taken to better understand the endometrium and its contribution to IVF outcomes. Progress has been incremental and the literature is divided. Our objective in the present review is to provide a conceptual synopsis of the collection of non-invasive imaging-based approaches that have been proposed over time to assess endometrial receptivity (ER) in the context of IVF and guide those thought processes to the current state of the art.

The term 'endometrial receptivity' has multiple definitions within the literature. For example, ER has at times been defined as the thickness of the endometrium, circulating estrogen/progesterone levels, or as the goodness of fit of a particular gene profile. In contrast, poor or enhanced ER has been mentioned as the responsible factor for differences in pregnancy rates among various patient populations when no quantifiable metric was identified. The term ER also has been seemingly applied to several measurable and immeasurable factors. Herein, we have defined 'ER' as the state of readiness of the endometrium to support implantation of an embryo. We view ER as a continuum and expect ER in each patient cycle will fall at a unique point on the continuum. Importantly, we do not believe it reasonable to attribute ER to a single quantifiable factor. No single quantifiable metric of the endometrium has been conclusively demonstrated to predict cycle outcome.

The question then arises, is it possible to measure ER? Historically, there have been two types of approaches to assess the endometrium during IVF cycles: laboratory-based methods that rely on biopsies or tissue excisions, and non-invasive image-based approaches. Biopsy and image-based approaches each produce quantifiable metrics and provide data for guiding clinical decision-making. Histological examinations of endometrial tissues and RNA-sequencing based methods of determining gene expression profiles require surgical retrieval of tissues and cannot be conducted on live cycles in which embryo transfers are contemplated (mock cycles are required). These approaches are founded on the assumption that data acquired in one cycle will be representative of what will occur in future cycles for any given patient. However, inter-cycle variability is significant, even when medication protocols are equalized[1, 2]. In addition, tissue analysis methods are invasive, expensive, and time consuming. Non-invasive image-based approaches to assessing the endometrium may be utilized in each cycle for which embryo transfer is contemplated. Different image-based ER assessment methods have been proposed over time as developments in technologies arise. The methodologies reviewed here include measurement of endometrial thickness (ET); endometrial pattern; endometrial volume; endometrial compaction; uterine contractility; sub-endometrial blood flow quantitation; ultrasound based ER scoring systems; and, machine learning (ML) / artificial intelligence (AI) methods.

The need for non-invasive, per-cycle approaches to assess ER is driving innovation and laying a foundation for quantitative assessment of the many factors contributing to the ER continuum. In assessing ER, two key features are important: quantifiability and the ability to use the assessment in the cycle during which embryo transfer is contemplated. Patient-to-patient variability must be accounted for and intra-cycle variability within individual patients must be recognized. For brevity, topics which have been recently critically appraised are discussed succinctly and review articles have been referenced.

Methods

A literature search was conducted on July 13, 2023, for the years 1990-2023: search terms were (Endometrium or Endometrial or Uterus or Uterine) and (non-invasive or "non invasive"; or, noninvasive, diagnostic, test, assessment, evaluation, AI, artificial intelligence, usER, Matris, compaction, ultrasound or sonography or imaging) and (Infertility or IVF or reproductive or reproduction). Databases searched included: Academic search complete, Academic search elite, Alt Health Watch, CAB abstracts, Canadian Reference Centre, CINAHL Plus with full text, Health source – Nursing/Academic Edition, Pub Med. A total of 16,763 articles were identified. After duplicates were removed 15,780 articles remained. Articles eliminated at the title level, 14,669, left 1111 articles. A further 952 were eliminated at the abstract level, leaving 159. At the full text level, 17 articles were eliminated, leaving 142. An additional 12 articles were added through review of citations in the accepted articles. Criteria for elimination at title level included opinion pieces, conference abstracts, case studies, non-human focus, cancer focus, pathology not related to infertility, reviews, related to hysterectomy, post-partum and articles concerning technical advances for ultrasound equipment. Abstract exclusion criteria included fetal environment, drug preparations, uterine contractions at time of transfer, invasive tests such as biopsy, review or meta-analysis, uterine transplant technology, assessment of pathology. Abstract inclusion criteria were must be a non-invasive procedure and must have some measure of pregnancy as an outcome.

Endometrial Thickness and Pattern

ET and pattern assessments have been broadly adopted as the standard of care assessment of ER. The thickness of the endometrium is frequently measured at the day of oocyte pick up in fresh embryo transfer cycles or just prior to beginning progesterone supplementation in frozen embryo transfers (FET). Endometrial pattern refers to the relative echotextures of the stratum functionalis compared to the stratum basalis. Most often, endometrial patterns are described as “triple-line” or homogenous. Triple line patterns have discernible echotextural differences between stratum basalis and stratum functionalis and a well demarcated lumenal echo. Homogenous patterns do not demonstrate visually appreciable differences between the endometrial tissue layers. Endometrial pattern assessment have been interpreted broadly.

The focus of most imaging studies which measure ET has been to predict implantation; however, a clear understanding of the expected changes in the endometrial echoes during the ovarian cycle is critical to understanding the measures that would be expected at the time of implantation. One highly variable endpoint in measuring ET appears to be the locations at which the measurement were taken. To clarify, for consistency ET measurements should be taken using a line drawn from the endometrial–myometrial interface at the visually thickest superior and inferior aspects of the endometrial cavity, within 5 to 10 mm of the fundal aspect of the endometrium. Using this clearly defined location, ET changes over the ovarian cycle in a clinically typical population are demonstrable[3]. Current thinking is that implantation is more likely to occur when ET is greater than 7 mm; however, pregnancies are observed with significantly lower thicknesses[4]. An analysis of 96,000 embryo transfer cycles showed that in cycles with a fresh embryo transfer, live birth rates were higher until ET was 10-12 mm; in frozen embryo transfer cycles live birth rates plateaued after 7-10 mm[5]. ET less than 6 mm were associated with a reduction in live birth rates in fresh and frozen embryo transfer cycles; however, there did not appear to have been a focus on interpretation of the effects of confounding variables especially the quality of the embryos being transferred.

A recent critical appraisal of studies on ET and embryo transfer outcomes was performed with the intent of assessing the predictive of ET measurements in individual IVF cycles[6]. The extensive volume of contradictory reports and the apparent lack of correlation between ET and clinical outcomes in patients undergoing IVF was highlighted. The absence of consensus can be interpreted to mean that simple thickness measures are not sensitive enough to predict ER and the probability of implantation. The authors argued that patients should not be denied embryo transfer when their ET is below an arbitrary thickness threshold and found no evidence that ET played a clinically significant role. As such, ET would likely better be incorporated into a larger model to build analytic systems capable of identifying the mechanisms and confounding variables that collectively effect establishment of pregnancy.

The patterns displayed by the endometrium undergo predictable, quantifiable changes throughout the menstrual cycle under the influence of estradiol and progesterone[3]. However, the literature is equally divided on whether endometrial pattern, generally defined as triple-line versus homogenous, is a significant predictor of outcome. We located 17 articles that examined endometrial pattern as an outcome predictor. Approximately half found that endometrial pattern was not a significant predictor of outcome, while the others identified pattern as a significant predictor. Although the patterns associated with endometrial development during the ovarian cycle are well documented, there is considerable biological variability in the endometrial responses of individuals. It has become increasingly important that imaging technologies for evaluation of the endometrium not be limited to simple measurements of endometrial thickness and pattern, but include the full range of endometrial expression and reaction to reproductively active hormones.

Endometrial Biophysical Profile and Endometrial Scoring

A uterine biophysical profile system has been proposed[7]. The system was designed by assigning “points” for several noted criteria (including ET, pattern, PI, RI, contractility, and color Doppler) and then taking the sum as a result. Limited information was found concerning the biophysical profile system; however, one small study (n = 35) supported its use in assessing ER and a second study involving intrauterine insemination cycles (n = 85) reported contradictory results[8].

A recent study proposed a three-point grading system for endometrium on the day of □hCG and progesterone initiation[9]. The proposed method incorporated endometrial pattern, thickness and the proportion of the endometrium represented by the outermost tissue layer to create a collection of eight endometrial categorical grades. Higher pregnancy rates were reported when ET exceeded 7 mm and the external layer of the endometrium was greater than 50% of the total thickness.

Spectral Doppler and Color Flow Doppler Ultrasonography

Color flow Doppler and power flow Doppler imaging are means of turning motion, either toward or

away from the transducer (color flow Doppler) or motion in any direction (power flow Doppler) into a visually detectable color overlay on the two-dimensional ultrasound image[10-12]. Studies tend to be based upon color Doppler examinations which allow easy identification of uterine vessels and calculation of blood flow indices using pulsatility index (PI), resistance index (RI), Vmax, or the systolic to diastolic ratio (S/D ratio). Doppler assessments of vessels supplying the uterus are presumed to reflect downstream impedance of the blood flow towards the endometrium and thus endometrial perfusion[10, 11].

No differences were found between pregnant versus not-pregnant groups when uterine artery RI was investigated as a tool to assess ER. Data for PI grouped into low, medium, and high categories for evaluation of the predictive value of pregnancy showed no differences in pregnancy rates in the low and medium categories; however, no pregnancies were established in the women with high PI values[13]. Therefore, elevated PI was associated with a lower pregnancy rate leading to the conclusion of a high negative predictive value. A single recent study demonstrated differences in PI between pregnant and non-pregnant groups post-hoc and was interpreted to mean that PI may have positive predictive value, however, this observation stands in contrast with most reports[14]. Assessments of uterine artery RI have shown no positive predictive value, except that absent or low diastolic flow was associated with failure to conceive: reviewed in[10].

Measurements of uterine vascularity appear to have little relation to the probability of conception in ART cycles. It is important to note that it remains unlikely that measures of uterine vessels reflect the state of blood flow to the endometrium as most of the draw on vascular resources would be taken by myometrial tissues and there is significant collateral circulation among uterine and ovarian vasculature[10]. While some ultrasonographically detectable criteria have been observed to be associated with negative pregnancy outcomes; no prognostic value has been observed in any measurement of vascular perfusion. Power flow and 3D power Doppler assessments have not been able to provide a positive predictive index of pregnancy[10-12]. While it might be logical to infer that a high degree of endometrial perfusion would indicate a more favorable endometrium, we were unable to locate detailed studies supporting this hypothesis.

Sub-endometrial Contractility

Motion analysis, or direct measurement of sub-endometrial contractions, is a method of evaluating the endometrium based on the observation that the uterus and endometrium are in constant motion. Patients with higher frequency uterine contractions were found to have lower pregnancy rates[15]. The effects of progesterone on uterine contractions have been demonstrated by the observation that higher progesterone concentrations correlated with lower amplitude and frequency uterine contractions. Low amplitude and frequency of contractions is hypothesized to facilitate implantation. However, administration of a selective oxytocin antagonist to reduce the frequency and power of endometrial contractions did not affect pregnancy rates in a clinical trial[16, 17]. A single article using non-invasive imaging identified junctional zone thickness as a significant predictor of implantation in ICSI cycles[18]. No further exploration of junctional zone was conducted.

Endometrial Volume and Length

Evidence for 3D volumes as predictors of ER and implantation has been contradictory. When endometrial volumes were compared among patients who conceived and those who did not, pregnancy and implantation rates were significantly lower when volume was less than 2 mL, and no pregnancies were established when endometrial volume was less than 1 mL[11, 19]. We identified eight studies which evaluated the correlation between endometrial volume and pregnancy. Seven were prospective cohort studies and used similar stimulation protocols and embryo quality cutoffs. One study reported on endometrial volume as a stand-alone assessment[20], however, most also included either ET and pattern, or a various blood flow indices. In some, endometrial volume was significantly correlated with a positive pregnancy outcome[20-25]. However, others have found no relationship between 3D volume of the endometrium and conception[19, 26]. No correlations were found among estradiol levels, ET, or endometrial volume leading the authors to conclude that there was no positive predictive value in assessing endometrial volume.

Two small prospective cohort studies using a single stimulation protocol were identified and evaluated the relationship between ET, endometrial length and cycle outcome. Correlations between endometrial length and pregnancy outcomes were observed[27, 28]; however, neither found a significant relationship between outcome and ET. No data were presented regarding the biophysical height or torso length of the patients which could be correlated to organ dimensions. In addition, the relationships between endometrial length and endometrial volume were not explored.

Endometrial Compaction

Endometrial compaction as a method of assessing ER was originally proposed in 2019. The definition of compaction was noted as a decrease ". . . in ET between the end of the estrogen phase and the day of

embryo transfer"[29]. Ten additional publications focused on compaction were identified in our search of the literature and represent a mix of retrospective and prospective observational cohort analyses. All studies eliminated cycles in which the endometrium did not reach a minimum thickness of 7 millimeters. Some studies included multiple categories for the definition of compaction[29-31], while some studies assigned a single category of compaction. In most cases, the categories were defined by a decrease in thickness between 5% and 20%. Patient exclusion/inclusion criteria, cycle stimulation protocol, and number of embryos transferred varied among studies. Outcome measures ranged from ongoing pregnancy rate to live birth. Some studies reported multiple outcomes[32, 33]. The reports were conflicting in their conclusions. Significant correlation between compaction and the outcome was reported[29, 30, 32-34]; however, no connection between endometrial compaction and cycle outcome was also demonstrated[31, 35-39]. Studies with a significant correlation ranged in N from 71[32] to 1420[34] with a mean N of 454, and those that reported no significant correlation ranged in N from 107[36] to 3091[37] with a mean N of 1496. Limitations in the studies which identified a positive relationship between endometrial compaction and outcome[31, 33, 34] included that the first endometrial measurement was taken by transvaginal ultrasound and the second measurement was taken transabdominally. The difference in measurement methods has the potential to introduce significant variability and error into the assessment.

The Ultrasound-Based Endometrial Receptivity Test

Ultrasound-based Endometrial Receptivity (usER) testing was developed to provide a non-invasive method for assessing ER on a per-cycle basis (usER, Matris™, Synergyne Imaging Technology, Inc, Saskatoon, SK). Early clinical trials with the precursor to the usER test (40) and field trials with the commercialized usER test (41) demonstrated the proof of concept that an ultrasound based ER scoring system could correlate endometrial image attributes with IVF cycle outcomes.

The usER test is founded on a proprietary software system designed to quantify the state of glandular differentiation, glandular coiling, numerous typical and atypical anatomic features that have been demonstrated to effect IVF outcomes. usER testing evaluates the effects of reproductively active hormones on the endometrium using a virtual histology approach to extract image-based metrics and condense them into an ER score. The usER test is a 'real time' ER assessment implemented on each cycle in which embryo transfer is contemplated. Standardized transverse and mid-sagittal images of the endometrium are acquired ~48 hours prior to an anticipated day-5/day-6 embryo transfer. The image series are communicated to a secure central server, processed, scored, and the receptivity score is reported to the clinic. usER scores range from 0 – 10 (0 – poorest ER; 10 – optimal ER). Although the score is a numeric scale, the relationship between usER score and pregnancy rate is non-linear, leading to a threshold interpretation model. Endometria with scores >=7 or above are considered well – to – optimally prepared and ET is recommended. Scores of <= 6.5 or are recommended for deferral of embryo transfer[42].

Routine implementation of usER testing was demonstrated to improve pregnancy rates by 12% (when fresh and frozen ET cycles were considered in aggregate; N = 1521) and conserve embryo potential[42]. The improvements in pregnancy rates have been attributed to accurate identification of poorly prepared endometria and deferral of embryo transfer to a subsequent cycle with better ER[43]. An approximate 10% increase in pregnancy rate was observed in patients who proceeded with usER-based cycle selection during a frozen ET cycle. Accurate identification of poorly prepared endometria was particularly apparent when fresh ET cycles were considered, as ovarian stimulation protocols may have higher variability effects on the growth and development of the endometrium. The pregnancy rate for patients who had fresh ET cycles was 20% higher in the usER-based cycle selection group than standard of care ET group. We identified one report (N = 224) that conflicts with these findings[44] in which the authors stated that they failed to control for many of the factors which impact outcome. Inclusion and exclusion criteria for the patient case information included in the retrospective analysis comparing outcomes based on usER versus ET cut-off was not described. Negligible correlation between usER score and ET on a given IVF cycle has been demonstrated[45]. A pilot study has also provided proof of concept that usER testing may be implemented to optimize endometrial preparations over time by providing a standardized approach to quantifying overall quality of endometrial preparations as medication protocols are adjusted / standardized within the clinic[46].

Linking ER and Embryo Quality

The relative contributions of the embryo and the endometrial environment to IVF cycle outcome are not well understood. However, we cannot assess if a non-invasive approach to determining ER is truly competent if we do not consider the effect of embryo quality on the probability of conception. New tools like the numeric embryo quality scoring index (NEQsi) provide an opportunity to begin untangling the contribution of the embryo from that of the endometrium in a straightforward statistically driven way[47].

As a proof-of-concept, we integrated usER score and NEQsi score to determine how ER and embryo quality interact. We conducted a retrospective analysis of 1720 IVF cycles in which both usER scoring and Gardner embryo grading were utilized. The analysis was an assessment of all patients presenting for embryo transfer to approximate real world clinical practice. Inclusion into this analysis was based solely on the availability of data within the patient charts (patient demographics and date range for data collection for this cohort are published[47]). Multivariate statistical modelling was used to determine how embryo quality affected cycle outcome in both receptive and poor-receptivity endometrial environments, as assessed by the usER test (**Figure 1**). We made two key observations:

1) When the endometrium was identified as receptive (usER score of 7 to 10) and we applied the full range of NEQsi scores associated with the receptive endometria (n = 1574, NEQsi range 3-12), embryo quality was the outcome predictor ($p < 0.0001$); and,

2) When the endometrial environment was identified as poor-receptivity (usER score of 0 to 6.5) and the full range of NEQsi scores associated with the poor receptivity endometrial environments (n = 146, NEQsi range 4-12) was applied, the usER score was the primary outcome predictor ($p = 0.038$).

Figure 1: **Linking Embryo quality and ER**. The green circle (upper left) represents receptive endometria and red circle (lower left) represents poor-receptivity endometria, as assessed by usER. The range and distribution of NEQsi scores were comparable between the two groups. The outcome predictor for each subanalysis is shown on the right.

This initial analysis that merges embryo quality and usER scores provides evidence that receptive endometria would not be expected to offset low-quality embryos and that high-quality embryos are unlikely to overcome the effects of poor-receptivity endometria. A larger multi-center observational study to validate this proof-of-concept analysis is currently underway.

Artificial Intelligence and Machine Learning Approaches to Quantification of ER

Artificial intelligence (AI) and machine learning (ML) approaches are being integrated into many aspects of reproductive medicine. An AI/ML approach to understanding probability of pregnancy before an embryo transfer takes place would be desirable and have potential for significant improvement in clinical outcomes. New approaches utilizing AI/ML have been constructed to better assess and understand oocytes and embryos. Computational approaches to automatic identification and segmentation of endometrium on 2D ultrasound images have been developed[48-50] and describe automated methods for ET measurement with accuracy up to 90% with an error range of 4 mm (± 2mm when compared to human measurements). It is important to note however that these methods presuppose that ET measurements predict cycle outcome.

An AI algorithm was produced with the aim of predicting cycle outcomes by combining ultrasound image features and clinical case notes[51]. Like the other AI approaches, the authors implemented an automated approach to segmentation of the endometrium within 2D ultrasound images. Ultrasound based measurements of endometrial volume, blood flow, and contractility were assessed and entered the model in combination with clinical case information to produce an AI model with ~ 72% accuracy in outcome prediction. It is noted that many key reasons for infertility were excluded from development of this model and that further validation of the approach is needed to determine its utility.

Discussion

There is a high degree of variability in the results reported with most of the image-based approaches identified for assessing ER. It is probable that the conclusions of studies with contradictory findings are heavily influenced by the study designs, methodologies, patient cohorts examined, medication protocols, sample sizes, and statistical error levels. We also noted that there was significant variability in the timing of the various assessments within a given IVF treatment cycle. The contribution of embryo quality (whether morphologically or genetically assessed) has been approached with great variability across the studies that have evaluated ER. Due to this variability, a combined approach that simultaneously assesses multiple metrics is likely to be more successful than any one factor on its own.

Of the methodologies noted, few have been broadly adopted. The exception is the broad acceptance of ET measurement and pattern assessment. Each of these variables is subject to interpretation and clinical decisions are based upon the experience of individual practitioners, introducing considerable variability in interpretation. Although ET and pattern are widely utilized in clinical decision making, the literature is divided regarding their utility in predicting patient outcomes. In fact, there are concerns

that cancellation of an embryo transfer based solely on an arbitrary ET cut-off is unwarranted[6].

The present narrative review was intended to provide a synopsis of the state of the field of non-invasive ER assessment. We acknowledge that it is not a comprehensive critical appraisal of each method identified. Although we took a systematic approach to our literature search and followed a systematic method for inclusion of original research articles, other review articles have been cited due to restrictions to the number of references. Additionally, we acknowledge that methods of ER testing which involve swabs, aspirates, metabolites, or microbiome analysis may be considered non-invasive but have not been addressed.

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CONFLICT OF INTEREST

HP and JI are employees of Synergyne ART Analytics. RAP is Distinguished Professor of Obstetrics and Gynaecology at the University of Saskatchewan and President and CSO at Synergyne ART Technologies.

DISCLOSURES

HP and JI are employees of Synergyne ART Analytics. RAP is Distinguished Professor of Obstetrics and Gynecology at the University of Saskatchewan and President and CSO at Synergyne ART Technologies.

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The impact of the microbiome in the endometrial function: is it solely influenced by the local microbiome?

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ABSTRACT

The human microbiome plays an important role in the physiology of many organs and alterations in its composition can be accompanied by serious pathologies. At the reproductive level, dysbiosis of the endometrial microbiota has been related to subfertility, recurrent implantation failures (RIF), and complications during pregnancy, such as preterm birth and spontaneous abortions.

However, in recent years it has been observed that microbiome from other mucosal locations also influence women's fertility, ultimately affecting endometrial functionality.

For example, the intercommunication and colonization capacity of microorganisms present in the vagina towards the uterine cavity is well documented, evidencing the influence of the vaginal microbiome on endometrial health. Additionally, the gut microbiome also plays a critical role in regulating the female reproductive endocrine system and has a significant impact on female reproductive health and associated conditions.

This article aims to review recent findings on the influence of different microbiome locations on endometrial functionality.

KEYWORDS

Endometrial microbiome, vaginal microbiome, intestinal microbiome, eubiosis/dysbiosis, pathogens, endometrial functionality.

MANUSCRIPT

Introduction

Embryo implantation and a correct pregnancy development is the result of a combination of different factors, including an optimal anatomical structure of the uterus, and adequate hormonal and molecular signaling.

Firstly, for correct implantation to occur there must be good synchrony between the endometrium and blastocyst. The blastocyst should be competent for implantation, and the endometrium must be receptive, that is, it needs to be sufficiently mature for the trophectoderm to adhere to the endometrial epithelial cells and invade the endometrial stroma and vasculature. Among the factors that contribute to altering receptivity, inflammatory events seem to play a key role in the process(1).

Nevertheless, endometrial receptivity (ER) is not the only crucial factor for correct embryo implantation, elements affecting endometrial functionality also play a critical role. Endometrial functionality refers to

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physiological functions of the uterus, which include menstruation, preparation for implantation and maintenance of pregnancy if implantation occurs[2]. In this context, the immunological homeostasis of the uterus and the state of the local microbiome actively participate in the establishment of this correct physiological competence.

The endometrial mucosa has the peculiarity of being able to generate an immune response to infections and, in turn, tolerate external agents such as sperm and embryos. The superficial endometrium contains immunological cells that gradually mature during the menstrual cycle. These cells have a peripheral origin and respond to the expression of chemokines and cytokines regulated by sex hormones. An alteration in the composition of this cellular profile at the intrauterine level, such as the appearance of an inflammatory profile, can affect the endometrial function. In this context, it is known that inflammation can be triggered by an altered endometrial microbiome[3,4].

Advances in molecular biology make it possible to analyze the microbiome with high levels of identification and to design methods for its monitoring. Culture-based microbiological techniques are subjective and not sensitive enough, nor adequate in some cases, to identify microbiomes because some bacteria have no capacity to grow in culture. For instance, real-time quantitative Polymerase Chain Reaction provides a rapid and an efficient detection of target genes that allow microorganisms to be identified at the strain level, compensating for limitations of other techniques such as the time of operation and costs of Next Generation Sequencing (NGS) technology. Furthermore, NGS, which is based on the sequencing of the 16S rRNA gene, produces problems in identification at the species level, caused by high sequence similarity within this gene, and omits the detection of other non-bacterial microorganisms[5]. These techniques have made it possible to demonstrate that the uterus is not a sterile niche, as was traditionally believed. Both anaerobic and facultative aerobic microorganisms are found in the female tract, with a bacterial community dominated by *Lactobacillus* genus[6].

Furthermore, it has been possible to demonstrate that the uterine microbiome also appears to be influenced, under certain conditions, by other local microbiomes (**figure 1**). Dissemination through the bloodstream of microorganisms originating from other mucosal locations occurs due to ruptures of the epithelial barrier. In addition, there are several factors

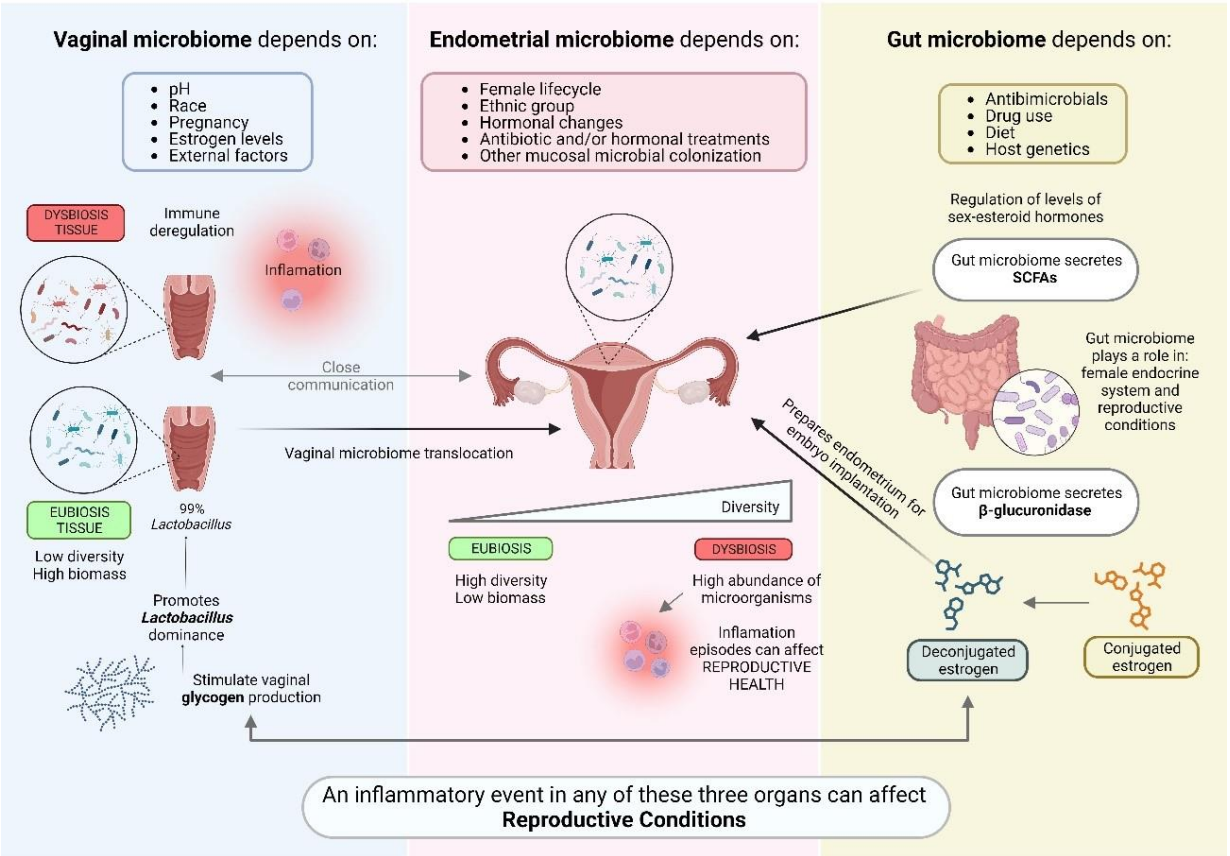

Figure 1. Interconnection between the endometrial microbiome and other mucosal tissues.

that promote the colonization of vaginal microorganisms towards the uterus, such as assisted reproductive technology (ART) procedures, the semen colonization itself, or the introduction of intrauterine devices, among others[3,7,8].

This review aims to describe the current state of art of the endometrial microbiome and its influence on endometrial functionality. In addition, the impact of other local microbiomes, such as the vaginal and intestinal ones, on the endometrial microbiome and in turn on its functionality will be addressed.

Methods

The bibliographic searches were conducted with a specific focus on the past decade (January 2013 to September 2023). These searches employed a carefully chosen set of keywords, such as "endometrium", "functionality", "immunology", "receptivity", "vaginal", "gut", "microbiome", "probiotics", "implantation", among others, to ensure alignment with the review's objectives. These keywords were combined using Boolean operators (AND, OR, and parentheses) primarily within several databases: Pubmed, SCOPUS, ScienceDirect and the online web of World Health Organization.

To be considered for inclusion in the review, the selected bibliography needed to meet specific criteria:

- It had to encompass reviews, observational studies, and randomized clinical trials.

- The publication date needed to be within the past decade.

- The documents were required to feature titles and/or abstracts containing information pertinent to the study objective.

Furthermore, exclusion criteria were applied to exclude articles that did not align with the scope of the review:

- Articles not written in English or Spanish.

- Articles devoid of relevance to the objectives or containing insufficient information.

After an exhaustive screening process, a total of 40 articles that fully satisfied the eligibility criteria were identified.

Impact of the microbiome in the endometrial function

Endometrial microbiome

The endometrium is the outermost layer of the uterus, and its main function is providing optimal conditions for a correct embryo implantation. This organ has traditionally been considered a sterile niche. However, thanks to the Human Microbiome Project and the development of precise and effective molecular biology techniques, this hypothesis changed; approximately 9% of the total human microbiome was found in the female reproductive system[9].

One of the main factors for embryo implantation is that there is a balance in the microbial composition of this tissue. Microbiota is defined as a group of living microorganisms within a particular environment, and the microbiome as whole, these microorganisms and their "theatre of activity". The latter involves the whole spectrum of molecules produced by the microorganisms, including their structural elements, metabolites, and molecules produced by coexisting hosts and structured by the surrounding environmental conditions[10]. Groups of microorganisms that colonize the endometrium include 85% bacteria, 10% fungi, 5% viruses and 0.3% archaea[11].

Numerous studies describe that woman uterine microbiota presents a low alpha diversity with a high abundance of species of the genus *Lactobacillus* such us *L. crispatus, L. iners, L. jensenii and L. gasseri*. These species are mainly responsible for maintaining optimal conditions in the tissues to increase the chances of embryo implantation, thanks to their properties that reduce pH and protect against the invasion of pathogens and the levels of pro-inflammatory parameters[9,12,13]. At the level of endometrial immunology, the production of lactic acid from *Lactobacillus* limits the toxicity of Natural Killer (NK) cells, promotes blood vessel regeneration processes and modulates the immune inflammatory response mediated by cytokines and other immune cells[14,15].

In addition to lactobacilli, other bacterial genera have been identified in endometrium thanks to metatranscriptomic analysis such us *Bifidobacterium, Corynebacterium, Gardnerella, Prevotella or Staphylococcus*, among others[16]. Unlike in other areas of the body, a high variety of microorganisms in the endometrium can result in a dysbiotic state and the subsequent development of some pathologies[16].

It is considered that endometrial tissue is in eubiosis state when the percentage of bacteria of the genus *Lactobacillus* is equal or greater than 90% of the total microorganisms abundance, being this condition responsible of maintaining a balanced tissue[9]. It is important to consider microbial typification when identifying endometrial microbiota. In general terms, obtaining a genomic index of the species *L. crispatus* and *L. gasseri* within its range, and a *Lactobacillus* percentage greater than 90% as a result, reports an optimal endometrial microbiota status; however, sometimes this percentage may also be due to the dominance of species such as *L. iners*, whose capacity

to produce beneficial substances is lower, being harmful and making tissue conditions not optimal for embryo implantation to occur[17]. Therefore, even though most research on reproductive microbiology determines endometrial status based on a percentage of *Lactobacillus* greater or less than 90%, the molecular technique applied need to be considered and the relationship between the concepts of eubiosis and *Lactobacillus* dominance should be re-examined[9].

Despite the predominance of bacteria of the genus *Lactobacillus* in the endometrial microbiota of most women, composition and individual microbial profile is highly dynamic and can change over time causing episodes of imbalance or dysbiosis. This balance can be altered by different causes including female lifecycle, ethnic group, hormonal changes, some pathologies, antibiotic treatment, and the use of intrauterine devices, among others[18]. During the menstrual cycle, natural hormonal fluctuations can have consequences on the microbial composition of endometrial tissue. For instance, the percentage of *Lactobacillus* increases during follicular development, reaching its peak in the luteal phase; and after menstruation, the proportion of these bacteria is decreased. Other species belonging to the *Prevotella* and *Sneathia* genus increase during the proliferative and secretory phase[19].

On the other hand, different studies have shown that women subjected to ART with exogenous hormones administration, suffer modifications in their endometrial microbiota. In particular, bacterial diversity boost while the proportion of bacteria of the genus *Atopobium* and *Prevotella* increase and the percentage of *Lactobacillus* decreases[20].

All these factors, can cause negative effects in endometrial functionality, producing inflammation episodes that can affect reproductive health, leading in turn to intra-amniotic infections, premature births, spontaneous miscarriages, and infertility through mechanisms such as alterations of vascular and immune cell functions[12]. Additionally, imbalance in the microbiome can trigger diseases such as chronic endometritis, which is most commonly caused by chronic bacterial infection at the uterine level[8].

From a clinical point of view, efforts are focused on the search for specific, complete and individualized treatments capable of improving pathologies caused by microbial imbalances, usually based on antibiotics, especially to treat infections such as bacterial vaginosis or prevent premature birth. However, the administration of these drugs to improve microbial balance before embryo transfer is controversial. The lack of specificity of broad-spectrum antibiotics could impair not only the growth of dysbiosis-causing microorganism, but also protective lactobacilli.

A possible strategy to modulate the reproductive microbiota is the combined use of antibiotics/antifungals with prebiotics and probiotics, which include, among others, live microorganisms of the genus *Lactobacillus*[21]. The use of these compounds could offer an interesting approach to restore the microbiota while avoiding the disadvantages of antimicrobials, such as resistance to them, the high rate of recurrent infections after treatment, and the side effects that could appear derived from the elimination of microbiota from other parts of the body. In addition, microbiota transplants are also gaining popularity to improve and maintain the optimal composition of the microbiota in order to benefit human health[22].

The role of the endometrial microbiota in the embryonic-maternal relationship during the beginning of pregnancy establishment is generating great interest in the field of reproductive medicine. A better understanding is needed of what optimal endometrial tissue means, how to achieve it, and what factors would improve the reproductive success of subfertile women with reproductive desires.

Vaginal microbiome

At vaginal level *Lactobacillus* is also the most dominant member of the local community in most healthy women of reproductive age. The most common species present vaginally include *L. crispatus, L. iners, L. gasseri, and L. jensenii*, with a community states (CST) described depending on the composition of these species to classify the vaginal microbiome[12].

The vaginal microbiota is also very dynamic during woman life and highly dependent on estrogen levels. The stability of this local microbial community depends on many other internal factors such as race, pH, pregnancy or menstruation, but also on external factors[6,12,23]. Furthermore, a good immune regulation is also necessary for vaginal microbiome maintenance. Disturbance of this immunological balance can lead to an acute inflammatory reaction or an insufficient immune response. In a normal state, commensal communities maintain a barrier and a stable mucosal environment and a correct interaction with the immune system through their metabolites. These mechanisms result in the activation of uNK cells and the regulated development of specific T cell subsets, essential steps for immunotolerance of the fetus[4].

The microbiomes of the upper and lower sections of the reproductive tract are unique and specific. The total biomass is much lower at the endometrial level compared to the vagina. At the vaginal level, the lower pH makes the conditions unfavorable for most microorganisms and therefore there is less diversity compared to the endometrium. These differences

become smaller with circumstances such as aging, vaginal births and miscarriages. Furthermore, the existence of vaginal microbiome translocation to the endometrium due to different mechanisms and circumstances such as uterine contractions and instrumental manipulation is well known[6,8,12,24,25]. In fact, vaginal location is the most common source from which uterine colonization occurs, but the physiological significance of this translocation is still unclear[8,26].

It is important to note that, as well as at the endometrial level, at the vaginal location there are not exclusively bacterial microorganisms either. For instance, in the female reproductive tract vulvovaginal candidiasis accounts for 20-25% of all vaginitis. Several risk factors are known that can lead to the colonization of *Candida* into the uterine cavity, which may have an impact on a pregnancy, including the use of intrauterine devices, embryo transfers or underlying medical conditions[7,27].

On the other hand, a variety of vaginal DNA viruses have been also identified in generally healthy and asymptomatic women of reproductive age, such as the *Adenoviridae*, *Herpesviridae* or *Papillomaviridae* families. It is not yet clear if there is a core vaginal virome, since the function of this virome at the vaginal level is still unknown. What is likely is that viruses can influence the immunological profile present in the mucosa, and that their presence seems to be influenced by *Lactobacillus*[3,12,24]. In addition, the presence of phages has also been detected at the vaginal level. It is believed that they can influence the configuration of the local microbiota, protect pathogens from immune system and affect the health of the host to a certain extent[3].

All this complex and dynamic vaginal microbial community is highly important for reproductive outcomes, specially at the first stages of pregnancy. It plays a key role in preterm labor and spontaneous birth, being a stable microbiome, including viruses and fungi composition, more related to spontaneous births[3,12,28].

Vaginal microbiota in pregnant women with spontaneous births is reported to be less rich and less diverse compared to the non-pregnant vaginal microbiota[12]. Vaginal dysbiosis state, which is characterized by a non-*Lactobacillus* genus dominance, is linked to adverse outcomes such as premature birth and sexually transmitted diseases[6,12,23].The main contributing factors for these adverse clinical results include intra-amniotic infections, ascending infections, cervical insufficiency, stress, vascular disorders, etc. Ascending genital infections seems to alter the delicate maternal-fetal immune balance by releasing toxins and a series of enzymes that compromise the fetal membranes[12].

In addition, the predominance of anaerobic bacteria appears to have a negative impact on the outcome of ART. Patients who do not become pregnant have a higher abundance of pathogens such as *Gardnerella, Enterococcus, Streptococcus, Staphylococcus* and *Prevotella*, among others, while pregnant patients have a higher abundance of *Lactobacillus*[26,29,30]. Furthermore, a balanced vaginal microbiota seems to also modulate important metabolites for embryo implantation, such as glycerophospholipids[30]. However, there are studies which do not find significant differences between pregnant and not pregnant woman at vaginal microbiome level[25] probably due to the difficulty in microbiome profile characterization analyses and the fact that most studies focus on exclusively analyzing the bacteriome.

Ultimately, the close communication of the vagina with the endometrium and its influence on endometrial health make the vaginal microbiome the first essential barrier for a correct composition of the microbiome of the general reproductive tract, being key factor for the establishment and maintenance of pregnancy.

Gut microbiome

The gut microbiome encompasses the complex network of bacteria, viruses, fungi, archaea and protozoa residing within the gastrointestinal tract, as well as their genome and metabolites. This microbiome is recognized as an endocrine organ indeed, wielding the ability to exert influence over the intestinal environment and impacting distant organs, as well as several interconnected biological pathways. The preservation of a robust and balanced microbiota is essential for host well-being, since microbial community actively participates in digestive process, promotion of immune cell maturation, and detoxification[31].

However, the composition and dynamics of the gut microbiome are subject to a multitude of factors, which include dietary choices, host genetics, among others[31].

The gut microbiome plays a pivotal role in regulating the female reproductive endocrine system and significantly impacts female reproductive health and associated conditions. The human microbiome exerts its influence across all facets and stages of female reproduction, being involved in processes such as follicle and oocyte maturation within the ovary, fertilization, embryo migration, implantation, and throughout the entire duration of pregnancy and birth[32]. The impact of gut microbiota imbalances on such a huge list of conditions, that also involves infertility, polycystic ovary syndrome (PCOS), and endometriosis, has been extensively studied. In a

recent investigation[33], researchers compared the gut-vaginal microbiota axis in fertile women to that of women diagnosed with RIF. Findings revealed that the infertile group exhibited reduced gut α-diversity, indicating the presence of low-grade inflammatory disorders. Further analysis unveiled a species composition shifts related to a weakened mucosal protection mechanism. When the integrity of the mucus barrier is compromised, gut bacteria and other microbe-associated molecular patterns trigger an immune response that can lead to both localized and systemic inflammation[34]. In this intricate interplay, sex hormones serve as crucial mediators, facilitating communication between microorganisms and their host. Furthermore, the gut microbiome is recognized for its ability to modulate hormone levels, particularly influencing estrogen levels in females. Intestinal bacteria actively participate in estrogen metabolism through the secretion of β-glucuronidase (gmGUS), an enzyme that converts conjugated estrogen into its deconjugated form in the gastrointestinal tract. This conversion enables estrogen to bind to its receptors again, thereby initiating subsequent signaling cascades and estrogen-related physiological effects[35]. The ensemble of gut microbiota genes responsible for estrogen metabolism is collectively referred to as the «estrobolome». In this context, reduced gmGUS activity due to gut microbial dysbiosis can lead to a diminished deconjugation of estrogen, resulting in lower circulating levels. The alteration in this enzyme activity also contribute to health issues such as obesity, cardiovascular pathologies and other diseases such as endometriosis[35].Thus, eubiosis maintenance is a key factor for hormone signaling.

Gut microbiome may also impact the levels of sex-steroid hormones in females indirectly, for instance, throughout the production of SCFAs. SCFAs stand as the primary by-products resulting from the anaerobic fermentation of dietary fibers by intestinal microbiome[36]. This scenario shows a plausible mechanism through which dietary components and metabolites derived from the microbiota might contribute to the regulation of estrogen and progesterone levels in females. However, the precise molecular mechanisms underlying these interactions remain to be fully elucidated.

Inflammation exerts its influence on key events such as ovulation, menstruation, implantation, placentation, and pregnancy. Consequently, any disruption in the scale or duration of inflammatory events becomes a significant contributor to the pathophysiology of infertility[37], through mechanisms discussed in previous sections. However, the precise mechanism through which chronic low-grade inflammation hampers reproduction remains an area that requires further elucidation. On the contrary, it has been clearly depicted that chronic inflammation has the potential to disrupt the process of folliculogenesis by triggering oxidative stress[38]. Moreover, chronic low-grade inflammation can also compromise ER. Notably, inflammatory conditions such as endometriosis, adenomyosis, and chronic endometritis rank among the leading causes of recurrent pregnancy loss. In patients with endometriosis, increased levels of inflammatory cytokines are found in the peritoneal fluid. This overexpression leads to heightened local estrogen production, ultimately disrupting ER[39].

Furthermore, oral probiotics can modulate the composition of the intestinal microbiota, improve intestinal integrity, and have an impact on the maintenance and recovery of the normal reproductive microbiota[27].

Future research efforts should prioritize uncovering the precise molecular mechanisms that underlie the connections between gut microbiota and reproductive diseases. Gaining a comprehensive understanding of this mutually influential relationship holds the potential to pave the way for the creation of innovative and impactful approaches for the prevention, early diagnosis, and treatment of female reproductive disorders.

Discussion

In recent years, the study of human endometrial microbiome has become a growing field of knowledge. IF can be due to various factors, such as the maternal immune system, the embryo genetics, anatomical factors, thrombotic factors or the reproductive microbiome, among others[33]. A balance between immune response, resistance and immunogenic tolerance is important for embryo implantation and establishing a viable pregnancy. However, the extent of interactions between the microbiome and these immune responses remains unknown[29].

Alteration of the endometrial microbiome can affect the implantation process through different vias. On the one hand, the integrity of the endometrial mucosal barrier can be weakened. This weakness allows pathogens to colonize and an immune response to occur with an imbalance in the production of cytokines in favor of pro-inflammatory types. Furthermore, an aberration in the maturation of uNK cells and the alteration of macrophages balance may lead to an incomplete remodeling of the maternal spiral arteries[4,7]. These events could alter ER, impair implantation process and the onset of a successful pregnancy[40]. In addition, reproductive microbiome and its influence on immune response may play a fundamental role in highly prevalent diseases such as endometriosis[6].

The known interaction between other mucosal locations and endometrium includes translocations of metabolites, immune signaling and/or inflammation without knowing what the real impact is on endometrial functionality and eproductive pathologies[3,7,8]. In this sense, and in order to delve deeper into the search for biomarkers at the reproductive microbiome level that are related to reproductive pathologies and clinical outcomes, it is necessary to reach a consensus on what is considered a normal or healthy microbiome in a multiethnic, global world. It is necessary to standardize different aspects such as sampling contamination, the molecular technique used, the data analysis for characterization, and the classification of "control" individuals based on their characteristics and medical clinical history[7].

Another important aspect to improve is the design of appropriate therapeutic strategies to treat the alteration of local microbiomes. For instance, vaginal microbiota transplantation has been shown to reduce the recurrence of bacterial vaginosis, opening a door to the possible success of uterine microbiota transplantation. However, it is still essential to consider the different aspects, ethical and technical ones, that will allow the clinical application of microbiome transplantation.

Furthermore, the use of probiotics, alone or in combination with antimicrobials, is a promising strategy. However, it is essential to determine which strains have the most therapeutic potential in each case. Different strains of *Lactobacillus* and their properties have been tested regarding their ability to restore bacterial balance at a reproductive level. However, it is necessary that the probiotic strain has the ability to colonize the uterus and that it be isolated from healthy fertile women for the achievement of future accurate clinical trials[21]. The use of these probiotics can avoid the drawbacks of antibiotic use, such as antibiotic resistance or the elimination of microbiota from other locations[7].

Furthermore, many other questions remain to be resolved, such as the role of specific microorganisms on clinical reproductive outcomes and the interaction of the microbiome with endocrine regulation[7]. In addition, establishing what is considered a eubiotic microenvironment taking into account the analysis technique used, among other aspects, will allow a better understanding of the physiological profile of the endometrium and will reduce the overconsideration of the dysbiotic state and the treatment failure rates[12].

In short, it is essential to carry out a joint study of the reproductive microbiome and the immunological profile for the management of infertility in patients with an indication for endometrial evaluation. Personalized treatment of the microbiota of the reproductive tract with more and more specific treatments could improve the clinical success. Current advances in research even allow us to study the presence of antibiotic resistance genes to facilitate the management of recurrent infections or the treatment of microorganisms with high levels of resistance to wide ranges of antibiotic groups[10].

Well-designed clinical studies on the importance of certain microorganisms, including those less studied such as fungi and viruses, in reproductive results and on the effectiveness of different therapeutic strategies will allow the resolution of infertility in numerous clinical cases and the improve in reproductive health.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

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Disorders affecting endometrial receptivity

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ABSTRACT

Uterine receptivity is the ability of the endometrium to allow normal embryo implantation. Abnormal uterine receptivity leads to a range of reproductive problems, from infertility or defective implantation (miscarriage) to recurrent implantation failure after IVF procedures. The best management for our couples would be to identify in advance the possible disorders that could lead to implantation failure.

Most uterine malformations and acquired abnormalities of the uterine cavity are relevant to reproductive outcomes. However, the impact of some abnormalities remains controversial, such as adenomyosis and chronic endometritis.

External factors can also affect the receptivity of the endometrium, even if they are not located inside the uterine cavity. The possible effects of endometriosis, hydrosalpinx and obesity are factors to consider when considering assisted reproductive technology.

KEYWORDS

Endometrial receptivity, Müllerian malformations, chronic endometritis, adenomyosis, endometriosis, obesity.

MANUSCRIPT

Brief Introduction

Embryo transfer is the culmination and conclusion of in vitro fertilization (IVF). Once the embryo has been transferred, its future depends on its ability to implant, but also on the ability of the endometrium to host it. Therefore, the key to optimizing outcomes is to transfer an embryo of the highest possible quality, ideally euploid, to a suitably receptive uterus and endometrium. In this sense, the factors that determine the probability of implantation and pregnancy are dual: the quality of the embryo and the state of the uterus and endometrium.

The study of uterine receptivity involves aspects that are not exclusive but rather complementary; namely, morphology, functionality and synchronization of the endometrium with the embryo. All are key factors that indicate the global state of the endometrium and its receptivity, information that allows us to optimize reproductive results.

Recurrent implantation failure (RIF) refers to a scenario in which the transfer of optimal embryos fails with sufficient frequency to warrant further tests and/or interventions. This scenario can be avoided if disorders that lead to implantation failure are previously identified(1).

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In this chapter we will address the different conditions that can affect endometrial receptivity and reduce the chance of pregnancy.

1. Congenital uterine abnormalities – Müllerian malformations

Female genital malformations are deviations from a normal anatomy that occur during intrauterine development and in which the Müllerian ducts fail to form, canalize, fuse or absorb[2].

Mullerian malformations are associated with a significant decrease in implantation and pregnancy rates and an increase in miscarriage and preterm birth rates. The definition and significance of an arcuate uterus were a matter of debate for some time, in part due to a lack of consistency in classification. Both the ESHRE-ESGE and ASRM classification systems now consider this condition to be a minor anomaly[2,3].

In a prospective comparative study, reproductive outcomes, including clinical pregnancy, live birth, and preterm birth, were compared in women with a normal uterus and in those with a congenital uterine anomaly. A total of 2,375 women were included in the study, of whom 1943 (81.8%) had a normal uterus and 432 (18.2%) had a congenital uterine anomaly. Patients with an arcuate uterus presented similar clinical pregnancy rate (P = 0.78) and live birth rate (P = 0.91) to those with a normal uterus. However, women with major uterine anomalies presented statistically lower clinical pregnancy (P = 0.048) and live birth (P = 0.042) rates than controls. These results highlight the importance of accurate and reliable classification of uterine morphology prior to any assisted reproductive technique[4].

Transvaginal ultrasound is considered part of the fertility work-up, and objective documentation of abnormalities of the female genital tract is vital when evaluating infertile couples/individuals. 3D ultrasound is a non-invasive and safe method for the diagnosis and classification of Müllerian malformations. In fact, according to ESHRE guidelines, it is the "gold standard" test and should be supplemented by magnetic resonance imaging (MRI), Hysteroscopy (HSC) and Laparoscopy when a diagnosis is not completely clear. To date, no studies have evaluate whether 3D transvaginal ultrasound improves outcomes in patients with RIF; however, given the limited cost involved and its non-invasiveness, it would be logical to apply it as a routine diagnostic tool in the work-up of RIF, when available[1, 2].

Insofar as congenital uterine anomalies, good clinical practice guidelines indicate that surgical hysteroscopy should only be offered in the management of morphological malformations of the uterus if they are detrimental and can be resolved, such as uterine septal resection or metroplasty of T-shaped uterus[1, 2].

2. Acquired uterine anomalies

Assessment of the status of the endometrium and uterine cavity is an essential part of the initial evaluation infertile women or couples. Acquired intrauterine pathology is reported to be the cause of IVF failure in approximately 10-15% of patients. Indeed, some series describe a diagnosis of intrauterine pathology in up to 50% of women with RIF. Moreover, 85% of clinicians take anatomical and gynecological investigations into account when attempting to diagnose the cause of RIF[4, 5].

Hysteroscopy is the most accurate technique for diagnosing intrauterine or endometrial pathologies. In fact, in some cases, these pathologies cannot be detected by gynecological ultrasound. This has led several some professionals to include diagnostic hysteroscopy in the routine assessment of couples undergoing their first IVF attempt. There is evidence that performing hysteroscopy before IVF treatment significantly increases the probability of pregnancy in the subsequent IVF cycle of women with one or more failed IVF cycles. However, the importance of routine hysteroscopy prior to initiation of a first cycle of IVF has not been demonstrated. Diagnostic hysteroscopy to examine the uterine cavity should only be recommended in couples with a history of previous implantation failure, or when a uterine pathology has been detected by transvaginal ultrasound and further diagnosis is required. The purpose of this test is to exclude the existence of synechiae, Asherman's syndrome, submucous fibroids, endometrial polyps, adenomyosis and chronic endometritis[4,6].

Most acquired abnormalities of the uterine cavity are considered to be relevant to reproductive outcome and can be treated with well-established procedures such as endometrial polypectomy, surgical removal of submucous fibroids or intrauterine adhesions[4]. However, the impact of some abnormalities remains controversial.

Refractory or thin endometrium

The definition of a thin or refractory endometrium varies widely among authors, but is generally defined as an endometrium thickness of less than 7 or 8 mm on the day of human chorionic gonadotrophin injection in fresh IVF cycles or the day on which progesterone is initiated prior to frozen-thawed embryo transfer. In endometrial atrophy, which is considered the maximum expression of this pathology, there is a partial or complete absence of the functional endometrium.

In the past, research evaluating the effect of endometrial thickness on IVF outcomes was inconsistent. However, in recent years, large series have been published showing that clinical pregnancy rates and live birth rates decrease if embryo transfer is performed when endometrial thickness is below 7 mm. A retrospective cohort analysis of the Canadian database analyzed over 40,000 embryo transfer cycles and found that clinical pregnancy and live birth rates decreased ($P < 0.0001$) and pregnancy loss rates increased ($P = 0.01$) with each millimeter of reduction of endometrial thickness below 8 mm. In frozen-thawed embryo transfer cycles, clinical pregnancy ($P = 0.007$) and live birth rates ($P = 0.002$) decreased with each millimeter of decrease in endometrial thickness below 7 mm, with no significant difference observed in rates of pregnancy loss. The likelihood of achieving endometrial thickness ≥8 mm decreased with age[7].

Recent evidence endorses platelet-rich plasma (PRP) therapy as a promising treatment for patients with refractory endometrium[8].

Adenomyosis

Adenomyosis is defined as the presence of ectopic endometrial tissue (endometrial stroma and glands) within the myometrium, but it is not considered a form or subtype of endometriosis[9]. Given that adenomyosis can be associated with changes in the junctional zone close to the embryo implantation site, there may be a causal relationship between adenomyosis and subfertility. However, it is difficult to quantify the effect of adenomyosis on infertility and relevant data are limited. Infertility may arise in women with adenomyosis, mostly due to local endometrial inflammation, at least when lesions infiltrate the internal myometrium[10].

In contrast, numerous studies have attempted to determine the impact of adenomyosis on the reproductive outcomes of IVF. Benaglia et al. conducted a study in which women scheduled for IVF were prospectively screened for the presence of adenomyosis, and found that implantation rates were not affected in asymptomatic women diagnosed with adenomyosis. More recently, the results of systematic reviews suggest that adenomyosis has a negative effect on endometrial receptivity[10,11]. The effect of treatment for adenomyosis on pregnancy or live birth rates in women with RIF has not been evaluated[1]. Further research should aim to clarify the relationship between adenomyosis and infertility in order to refine treatment strategies.

Endometritis

Chronic endometritis (CE) is a controversial issue due to a complicated diagnosis and a lack of consensus on its impact on fertility. It is defined as persistent inflammation of the endometrial mucosa caused by bacterial pathogens, and is traditionally diagnosed by anatomopathology. To this end, an endometrial biopsy is required, ideally during a hysteroscopy, to identify plasma cells by hematoxylin and eosin staining or CD138-labelling. This method is nonspecific and may delay a definitive diagnosis, which will depend on the expertise of the pathologist in charge. Besides endometrial histology, macroscopic inspection of the uterine cavity via hysteroscopy is also employed to diagnose CE. The criteria for a positive diagnosis are the presence of mucosal oedema, focal or diffuse endometrial hyperemia and/or isolated or diffuse micropolyps. Some series have found concordance between hysteroscopic findings and histological diagnosis, though others have shown it to be as low as 20%. In fact, diagnosis by this technique can be complicated by the physiological changes that the endometrium undergoes during the cycle and should, therefore, be carried out by an experienced doctor during the initial proliferative phase of the menstrual cycle[12,13,14].

More recently, new molecular techniques have shown potential as tools for a reliable diagnosis of CE, such as next generation sequencing (NGS), but there are remain essential questions to be answered[14].

The limited data currently available suggest that CE evaluation is not necessary as part of the initial evaluation of infertile patients/couples, and women suffering from recurrent early pregnancy loss and RIF patients are likely to benefit most from screening and treatment of CE. ESHRE good practice recommendations for RIF include assessment of chronic endometritis (CE) and treatment with antibiotics in the case of a positive diagnosis[13, 15, 1].

Other studies have investigated adjuvant therapies as alternative treatment options, such as anti-inflammatory drugs, probiotics to regulate the female reproductive tract microbiome, and progestogens; however, there is not yet sufficient evidence to apply them in daily practice[16].

3. Communicating hydrosalpinx

Hydrosalpinx is defined as a distally occluded, dilated, fluid-filled Fallopian tube.

Tubal occlusion is a cause of infertility; in fact, the original indication for IVF treatments was a tubal pathology, assuming that pregnancy could be achieved by bypassing the damaged tube. However, the adverse effects of a hydrosalpinx persist even after IVF, and its negative effects on IVF outcomes are well documented. Many retrospective studies and some meta-analyses have highlighted a detrimental effect on implantation and pregnancy rates after fresh or cryopreserved-thawed embryos, and even after oocyte

donation. Moreover, there are several reports demonstrating an increased rate of spontaneous miscarriage[17, 18].

The negative effects of a hydrosalpinx have been attributed to different reasons. The strongest theory is that of a mechanical effect of hydrosalpinx fluid, whose leakage into the cavity can flush out the transferred embryo. A second theory, demonstrated in animal models, is based on the gametotoxicity of the hydrosalpinx fluid. Finally, it has also been suggested that the hydrosalpinx fluid is rich in cytokines and inflammatory response materials, which result in disordered and/or impaired endometrial receptivity[18].

Several studies have demonstrated that treatment of hydrosalpinx is mandatory if higher success rates are desired. Surgical interventions, such as salpingectomy, tubal occlusion or aspiration of hydrosalpinx fluid (if the patient is at high risk prior of surgery), should be considered in all women with hydrosalpinx who are due to undergo IVF treatment. In this context, the Cochrane review summarized the current evidence on the effectiveness of tubal surgery prior to IVF. Laparoscopic salpingectomy increased the odds of ongoing pregnancy and clinical pregnancy versus no treatment; to be specific, ongoing pregnancy rates in the intervention and control groups were 27-52% and 19%, respectively. Laparoscopic tubal occlusion in some studies increased clinical pregnancy rates, but there was very low-quality evidence that it is a reliable alternative to salpingectomy. Randomized controlled trials were needed to assess the effectiveness of other alternative treatments, such as ultrasound-guided aspiration. Unfortunately, none of the trials included reported live birth as an outcome, and no conclusions could be drawn about the adverse effects of interventions, as data on ectopic pregnancy, miscarriage, or surgical complications were not provided[19, 20].

Despite the fact that IVF outcomes are improved by salpingectomy when a hydrosalpinx is identified, some concerns have been raised about the potential negative effect of surgical intervention on ovarian function and vascularization. That said, current data suggest that salpingectomy does not compromise ovarian response to subsequent stimulation[19].

4. Endometriosis

Endometriosis is a highly prevalent chronic inflammatory disease defined as the presence of endometrium-like tissue outside the uterus. It affects about 10% of women of reproductive age and is one of the major causes of female infertility. It has a serious impact on quality of life due to the pain it provokes and the aforementioned reproductive problems[9].

Endometriosis is not completely understood, though the mechanisms involved in endometriosis-related infertility are known to be multifactorial and to include anatomical changes, reduction of ovarian reserve, endocrine abnormalities, genetic profile, immunity markers, inflammatory mediators, and altered endometrial receptivity[9].

The effect of chronic endometriosis on endometrial receptivity after IVF is undetermined due to a lack of relevant data, the main limitation being that factors associated with the disease are known to lead to lower implantation rates[15]. The effects of intrapelvic inflammatory processes (cytokines, growth factors, prostaglandins and reactive oxygen species, which are found in high levels in the peritoneal fluid) can interfere with ovulation, sperm function, gamete fertilization and embryo quality and migration. Assisted reproductive technology has been able to overcome some of these adverse phenomena, but they continue to have effects on oocyte and embryo quality[21].

When eutopic endometria from women with endometriosis are analyzed, several molecular aberrations can be observed, and it is hypothesized that these changes cause defects in endometrial receptivity. For example, levels of endometrial proteins that are essential for normal implantation are reported to be lower in patients with endometriosis, such as leukemia inhibitor factor, HOXA-10 and some cell adhesion molecules (called CAMs). In addition, inflammation is known to alter endometrial receptivity and has been specifically associated with endometriosis. Several immunological abnormalities, particularly those involving uterine natural killer cells, have been described in the endometrium of women with endometriosis[22, 23].

Garcia-Velasco et al. evaluated the expression of 238 specific genes directly related to endometrial receptivity by using the Endometrial Receptivity Array (ERA) to assess endometrial receptivity in patients with different stages of endometriosis and in healthy controls. No differences in gene expression were detected, suggesting that endometrial function is similar among women with and without endometriosis, and across the different stages of endometriosis[24].

Since implantation is a complex procedure in which the embryo is obviously a crucial factor, egg donation is the best way to rule out all the factors that can affect embryo implantation, apart from endometrial receptivity. Our group conducted a study in which healthy egg donors were shared out to 25 women with stage III-IV endometriosis and 33 healthy control women. There were no significant differences between the groups in pregnancy, implantation or miscarriage rates. Similarly, cumulative pregnancy rates in our oocyte donation program over a 10-year period were

similarly successful in women with a variety of reproductive disorders, including endometriosis[25, 26].

Clinical findings regarding donation support the idea that oocyte and embryo quality are the main determinants of IVF success, and seem to indicate that endometrial receptivity is similar in women with and without endometriosis. New prospective, randomized, and controlled studies are necessary to improve our knowledge of the enigmatic changes that occur in the uteruses of patients with endometriosis[25, 26].

5. Obesity and endometrial receptivity

Worldwide obesity has almost tripled in the last 50 years. Increased body mass index (BMI; kg/m2) is a major risk factor for many diseases, including cardiovascular disease, type II diabetes, musculoskeletal disorders, and some types of cancer. Furthermore, female obesity is considered to be a relevant risk factor for subfertility and infertility, with a significant reduction in implantation, pregnancy and live birth rates after IVF demonstrated in proportion to an increase in BMI[27,28,29].

Although most studies suggest that obesity does not significantly affect embryo quality, the role of BMI in oocyte and embryo quality cannot be ruled out[30,31,32].

In contrast, data regarding the detrimental effect of female obesity on endometrial receptivity are more consistent. In fact, studies using an oocyte donation model and including large patient samples have shown a reduction in implantation, pregnancy and live birth rates among obese recipients, demonstrating that outcomes are compromised even when embryo quality is good and suggesting a reduction in endometrial receptivity in obese women[33,34].

The mechanisms responsible for this detrimental receptivity are not well understood and constitute a hot topic for the field. Metwally et al. employed proteomic analysis to examine potential endometrial defects in obese and overweight women with recurrent miscarriage. Their studies described a negative correlation between endometrial glandular leukemia inhibitory factor (LIF) concentration and BMI, and endometrial protein profiles varied with an increased expression of haptoglobin in overweight/obese women[35,36].

On the other hand, systemic metabolism alterations induced by obesity are associated with impaired endometrial receptivity; for example, the disruption of insulin signaling has been closely related to endometrial dysfunction. Our group demonstrated that there is a linear increase in glycaemia, insulinemia, TSH, LDL cholesterol, triglycerides, and systolic and diastolic blood pressure and a reduction in HDL cholesterol in line with a rise in BMI[37,38]. We also designed a study in which we used endometrial receptivity analysis (ERA) to determine prospectively whether increased BMI affects endometrial receptivity by displacing the window of implantation (dWOI). We recruited a population of 170 infertile women with normal uteruses and no clinical history of recurrent miscarriage or implantation failure. These women were divided into four groups according to BMI. Endometrial receptivity assessed by ERA during a hormonally prepared cycle revealed that dWOI increased in a BMI-dependent manner. The pattern of displacement was generally delayed, as most of the endometria of the obese women were pre-receptive after 120 hours of progesterone administration. Such evidence allows us to conclude that metabolic disorders associated with obesity have a negative effect on endometrial receptivity, probably by delaying the dWOI[38].

6. Conclusions

Embryo implantation requires an adequate dialogue between a good quality embryo and a receptive endometrium. Implantation is still considered the enigma of reproductive medicine, and further research is needed to shed more light on the process.

The following conclusions can be highlighted (**Table 1**):

- The arcuate uterus does not appear to be associated with poor prognosis in ART.

- Surgical hysteroscopy should be offered to treat morphological uterine abnormalities that are major but can be resolved.

- If endometrial thickness is less than 7 mm on the day on which embryo transfer is scheduled, the patient should be advised that outcomes may be compromised. Nowadays, new therapies involving PRP are obtaining promising results.

- Some authors have suggested that adenomyosis can affect endometrial receptivity, though there is no consensus with respect to the matter.

- Chronic endometritis may be a detrimental factor for embryo implantation, but more studies are needed to standardize methods and the criteria for diagnosis, and to facilitate a consensus on treatment criteria and on the benefits of antibiotic therapy administered to improve reproductive outcome.

- When a hydrosalpinx is diagnosed prior to IVF, salpingectomy is the recommended approach.

- There is no evidence that endometriosis affects endometrial receptivity.

- Obesity has a negative effect on endometrial receptivity and can directly affect the endometrial

environment, leading to a delayed implantation window and, subsequently, worse ART outcomes.

Disorder	Management suggested
Arcuate uterus	No particular intervention
Major and reparable uterine malformations: - Class U1: Dysmorphic uterus - Class U2: Septate uterus	Surgical HSC: - Uterine septal resection - Metroplasty
Acquired intrauterine pathologies: - Endometrial polyp - Submucosal fibroids - Asherman's syndrome and synechiaes	Surgical HSC: - Polypectomy - Myomectomy - Adhesions resection
Refractory endometrium	PRP therapies is a prosing treatment
Adenomyosis affecting cavity	Hysteroscopic resection is suggested
Chronic endometritis	Antibiotic therapy
Hydrosalpinx	Salpingectomy by LPS
Endometriosis	No effect on implantation
Obesity	Diet and exercise for weight loss

HSC: hysteroscopy; **LPS**: Laparoscopy.

Table 1. Summary of conclusions on the management of disorders affecting endometrial receptivity.

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Endometrial receptivity: The omics revolution

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ABSTRACT

Infertility, affecting millions globally, remains a significant challenge despite advances in Assisted Reproductive Technologies (ART) over the past four decades. While the success rates have improved, many couples still face challenges in conceiving. A major contributing factor is the limited understanding of infertility causes and the inefficiency of available treatment solutions. This article delves into the intricate processes of embryonic implantation and endometrial receptivity, crucial aspects of successful pregnancy.

The emphasis is placed on the frequently overlooked endometrial environment in the context of *In Vitro* Fertilization (IVF) procedures. Traditional methods of assessing endometrial receptivity, such as histological examination and hormonal level monitoring, have proven insufficient.

In the last years, omics approaches have generated a vast amount of highly valuable information, contributing significantly to the comprehension of the endometrial environment necessary for successful embryonic implantation. Genomic studies have unveiled specific loci related to endometrial disorders, epigenetic regulation studies have identified distinctive signatures associated with receptivity and endometrial pathologies, proteomic analysis has revealed protein expression changes during the menstrual cycle and metabolomic signatures in endometrial fluid may offer a direct insight into tissue function. Among these methodologies, transcriptomic analysis stands out as the most advanced. Particularly, high-throughput methods have proven to be robust in assessing functional states and pathologies leading to the development of commercial tests that evaluate endometrial receptivity. Recent advancements in single-cell and spatial transcriptomics provide great potential for the advanced study of endometrial function.

Current and emerging technologies employed in the exploration of the uterine compartment exhibit significant promise for advancing diagnostic methodologies and therapies addressing infertility. Despite existing challenges, we believe that the key to optimizing ART success rates lies in advanced assessments of the endometrial environment. As technology continues to evolve, the possibilities of precision medicine and personalized approaches offer hope and new opportunities for couples pursuing parenthood.

KEYWORDS

Endometrium, Gene Expression Profiling, Reproduction, Pregnancy.

MANUSCRIPT

Infertility is a disease of the male or female reproductive system defined by the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse. Infertility impacts millions of people worldwide. The World Health Organisation estimates that 1 out of every 6 people are affected by the inability to have a child at some point in their life; this is regardless of where they live and what resources they have.

The advent of ART technology and the improvements in reproductive medicine in the last 40

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years have made possible the birth of more than 8 million babies. Although pregnancy rates have improved significantly, still the efficacy of the cycles is quite low and many couples struggle to conceive. Out of the 2.5 million cycles performed annually, only one fifth result in the delivery of a baby. The main reasons behind this ineffectiveness are that the infertility causes are currently understudied and that the variety of treatment solutions available is still reduced and quite inefficient[1].

The causes of infertility are varied and often complex, affecting both, men and women. One of the most challenging situations is the treatment of couples experiencing repetitive implantation failure (RIF) and recurrent pregnancy loss (RPL). RIF refers to as the failure to achieve a clinical pregnancy after two to three transfers with good-quality embryos[2]. RPL is referred to when having two or more consecutive pregnancy losses[3].

It is well known that one of the key processes for the success of the treatment is the implantation of the embryo into the uterine lining, the endometrium. Embryonic implantation is a pivotal process in human reproduction, it occurs when the developing embryo attaches and embeds itself into the endometrium, marking the initiation of pregnancy. The significance of this process lies in its fundamental role not only in the establishment of pregnancy but also enabling its successful progression[4,5].

For successful embryonic implantation, two essential elements are required: a competent embryo and an endometrium ready to receive it. Implantation involves a complex molecular and cellular dialog between the embryo and the endometrial matrix. This interaction is essential for the apposition, adhesion and invasion of the blastocyst in the human endometrium[6].

The synchronization of embryo development with endometrial preparation is a critical aspect of implantation, this precise coordination is fundamental to the success of the pregnancy. Numerous morphological and functional changes must take place in these two structures so that implantation can occur. Both, embryo and endometrium, have to reach states of development that allow the molecular signalling and communication required for implantation success[7]. On one side, the embryo has to be at the blastocyst stage of development, as this stage is most conducive to implantation. On the other side, the endometrium needs to reach a status that warrants an optimal environment for embryo implantation. This period of time where the endometrium is receptive to the embryo is called the window of implantation[5].

In an IVF treatment, most of the attention is focussed on the selection of the most competent embryo to be transferred to the uterus, and little relevance is given to the study of the endometrial environment. Uterine ability to receive the embryo is not routinely assessed.

Numerous investigations have reported that endometrial receptivity plays a crucial role in implantation. However, the accurate identification of the window of implantation is challenging. The process of endometrial receptivity acquisition is extraordinarily complex and tightly regulated, it involves a finely tuned interplay of hormonal fluctuations, structural modifications and molecular changes. The influence of hormones, particularly estrogen and progesterone, is critical for preparing the endometrium and transitioning it into a receptive state. Additionally, local factors like cytokines and growth factors also play a role in communication between the embryo and the endometrium[6].

Traditional approaches to endometrial evaluation, such as the histological examination of morphological changes in an endometrial biopsy or the monitoring of hormonal level changes in serum, do not seem to be accurate enough for the precise identification of the window of implantation. Other diagnostic methods like ultrasound, MRI, or advanced high-resolution ultrasound have been suggested as potent tools for evaluating endometrial tissue. Nevertheless, the predictive value of these techniques in anticipating endometrial receptivity or IVF outcomes remains unclear[8]. Hence, more sophisticated tests are necessary to ascertain the optimal timing for embryo transfer. Monitoring molecular alterations in the endometrium throughout the implantation window could offer a more dependable option compared to traditional morphological methods.

In the last years there has been a remarkable advancement in the techniques available for the study of the molecular mechanisms that underlie cell function, tissue physiology and hence the origin of disease. New tools generally grouped under the term of “omics” have been developed for the study of the genome, the transcriptome, the proteome, the epigenome and the metabolome, in some cases at the single cell level, as individual profiles or in combination (multiomic approaches)[9]. The advancements on bioinformatics analysis, artificial intelligence (AI) and algorithms for prediction, are very valuable tools for the analysis, interpretation and description of all data produced by these approaches, and also for its integration for the study of the interactome, the complete set of interactions of a particular tissue or system. The knowledge generated by these technologies is crucial for the advancement in our understanding of the tissues and the development of better diagnostic methods and treatments.

These powerful tools are being applied in many areas of medicine. In the field of reproduction, the study of the endometrium and the process of embryo implantation is starting to be deciphered by the application of these omics tools. Several groups have studied how the changes in morphology and function associated to endometrial pathologies or the process of endometrial receptivity acquisition are also reflected in changes in the genes, proteins and metabolites[10].

Genomic studies of the human endometrium are mainly focussed on GWAS approaches and have revealed specific loci or genomic alterations related to endometrial disorders such as endometriosis or endometrial cancer[11]. No genomic studies have been published so far for the prediction of endometrial receptivity.

Epigenetic regulation studies, however, have shown specific epigenetic signatures associated with variations in the expression of genes critical for the establishment of endometrial receptivity and also some associated with endometrial pathologies[12]. Other gene expression regulatory elements such as lncRNA, sncRNA and miRNA have also been found to be differentially expressed at different endometrial stages (secretory vs proliferative), receptivity status or reproductive conditions. It has been suggested that the identification of aberrant miRNA expression levels in serum may allow for clinical diagnosis of fertility and receptivity[13,14].

Proteomics has also been applied for the study of endometrial receptivity. Early research observed notable protein expression changes between the proliferative and secretory phases in fertile women. Despite some controversy due to differences in the proteins identified, certain proteins consistently found in various studies, which align with transcriptomic data, are deemed significant for endometrial receptivity[15].

In addition, the analysis of endometrial secretions has also been explored as an approach to study the uterine compartment. Analyses of the endometrial fluid composition though different spectrometric approaches along the menstrual cycle, in fertile and infertile women, or in different receptivity stages, have also shown differential profiles of secreted proteins[16]. Recently, Azkargota et al.[17] confirmed different protein composition of the endometrial fluid from implanting versus non-implanting IVF cycles, indicating the existence of endometrial status more favourable to implantation.

Metabolomics analyses have also been performed in endometrial samples in search for new biomarkers of endometrial receptivity with potential usefulness in the clinical context. The metabolome provides a more direct view of cellular function compared to the genome, transcriptome, or proteome. Despite its potential, its utilization in discovering new biomarkers for endometrial receptivity is not as advanced as other omics approaches. Unlike analysing tissues, the more commonly conducted metabolic analysis are based on biofluids, such as endometrial fluid. Endometrial fluid not only encompasses proteins but also includes lipids and other released metabolites within the uterine cavity. Lipidomic studies have shown variable levels of prostaglandins across the menstrual cycle, during the WOI, in endometrial disorders or RIF patients[18].

The transcriptomic analysis of the endometrium has been widely applied for the characterization of the molecular changes that underlie the morphological and functional modifications that this tissue experiments during the menstrual cycle, beginning initially with microarray studies[19], then applying high throughput RT-qPCR[20], RNA-sequencing[21] or microRNA sequencing[22]. Differential gene expression profiling has been described by many authors in different endometrial stages, pathologies, or treatments[21, 23–25].

Variations in the number and list of regulated genes exist among studies, mainly due to differences in experimental design, technology and sample processing. Despite these disparities, several studies and literature reviews aim to identify common regulated genes as potential biomarkers for receptivity[26].

Most studies report upregulation in the mid-secretory phase of numerous genes linked to implantation, indicating the need for transcriptional activation in the receptive endometrium. Overexpressed genes play crucial roles in implantation-related functions, including cell adhesion, lipid metabolism, ECM remodelling, immune response, intracellular signalling and response to external stimuli. Conversely, downregulated genes often encode DNA binding proteins, transcription factors, DNA-modifying enzymes or genes with unknown functions[15, 20].

The transcriptomic approach to the study of the endometrial tissue has been shown to be quite robust for the evaluation of functional states. A few diagnostic tools based on the application of transcriptomic technologies have been developed. Five molecular methods based on the transcriptomic analysis of the tissue have been published in peer reviewed papers and are currently commercially available for assessing endometrial receptivity. They allow the diagnosis of endometrial alterations as a cause of infertility diagnosis and the personalised treatment of the disorder identified:

1. Win-Test. Analyses by RT- qPCR the transcriptomic signature of 11 genes specifically modulated during the WOI coupled with an algorithm to identify the receptive state[27].

2. ERA, endometrial receptivity array is a molecular predictive tool that identifies the gene expression of 238 genes and employs artificial intelligence to determine the receptivity status of an endometrium. It was initially implemented using microarray technology and has been currently modified to apply NGS[28].

3. ER Map uses high throughput RT-qPCR combined with a predictive modelling algorithm for the accurate determination of the WOI. This test evaluates the expression of 40 genes involved in the development and acquisition of endometrial receptivity[20].

4. BeREady Test utilises target allele counting by sequencing (TAC-seq) to estimate the original molecule counts of mRNAs of 57 endometrial receptivity genes that have been identified as biomarkers of the different phases of the menstrual cycle[29].

5. The rsERT, combines the evaluation by RNA-Seq and machine learning of 175 biomarker genes to predict the WOI period and guide embryo transfer. This test has shown to improve the pregnancy outcomes of patients with RIF[30].

These tests offer valuable insights into the molecular status of the endometrium and its receptivity. By tailoring the timing of embryo transfer to the individual's receptivity profile, clinicians can optimize the chances of successful implantation and pregnancy in assisted reproductive treatments. The use of transcriptomics in endometrial receptivity assessments has proven more objective and concordant than traditional methods. The application of these tools has shown improvements in the results of the assisted reproduction treatments; some studies have emphasized the importance of precise and personalised analysis of the endometrium to achieve better implantation and pregnancy rates and also to reduce miscarriage rates[4,27,28,30]. This precision enables clinicians to tailor the timing of embryo transfer to an individual's receptivity profile, thereby optimizing the prospects of successful implantation and pregnancy in assisted reproductive treatments.

Endometrial receptivity tests have also been proven instrumental in the characterization of pathologies and the identification of specific molecular signatures associated with infertility complications such as RIF, endometriosis and RM, showing the power of these approaches for the understanding of infertility cases, its adequate diagnosis and further treatment[21]. Nevertheless, some controversial findings in the application of these tests have also been published[31].

Endometrial receptivity tests present a revolutionary stride in understanding endometrial receptivity, however, their clinical application is not without challenges. A more profound characterization of the menstrual cycle and of the molecular mechanisms behind endometrial receptivity are crucial for understanding how the normal endometrium is regulated and synchronised with the developing implanting embryo. This knowledge together with the ongoing evolution of technology is key for refining these tests, enhancing their accuracy and minimizing limitations. A nuanced approach, evidence-based guidelines, and a focus on refining methodologies will contribute to realizing the full potential of endometrial receptivity tests in improving fertility treatments and increasing success rates in implantation.

In recent years several groups are focussing on applying the latest technologies for the study of the human endometrium both in vivo and in vitro in an aim to understand tissues structure and physiology. The advancements in single cell transcriptomics have allowed high resolution transcriptomic-based molecular and cellular characterization of human endometrial transformation across the menstrual cycle providing insights into this essential physiological process. Wang et al.[32] studying endometrial biopsies from healthy ovum donors after the onset of their menstruation by single cell RNA-seq analysis have identified the specific profile of six cell types and the definition of 4 major transcriptomic phases across the cycle, one of them corresponding to the WOI. Their data suggest interesting functions of different cell types in the process of embryonic implantation such as immune cells in the decidualisation period.

Other high resolution approaches for the study of the physiology and disease of tissues include spatial transcriptomics. This technique opens up a unique opportunity to understand how tissues are structured and how cells interact with each other. The definition of the spatial arrangement of cells and their interaction in tissues as complex as the endometrium is key to define its function and physiology.

The Human Cell Atlas initiative aims to map all cells in the human body using genomic technologies[33]. In the case of the endometrium, the group of Roser Vento-Tormo have generated a cellular map of the human endometrium that account for the temporal and spatial changes of this tissue during the menstrual cycle [34]. They have used single cell and spatial transcriptional profiling to study endometrial biopsies for donors screened for potential endometrial disorders and the whole endometrium and myometrium of donors with non-gynaecological disorders. They reported spatio-temporal changes in gene expression characteristic of cell types and menstrual cycle stages as well as differential expression associated with specific endometrial disorders such as endometriosis or endometrial cancer. This specific and in depth

profiling of the uterus of healthy women and the establishment of the normal endometrium signature will serve as a reference for the study of endometrial disorders.

In addition to deepening our understanding of the molecular mechanisms underlying endometrial function, emerging technologies may offer exciting opportunities for the development of therapies for enhancing endometrial receptivity.

One promising approach explored recently involves the transplantation of mesenchymal stem cells (MSCs) into the endometrium as a strategy to address endometrial dysfunction. MSCs have the ability to differentiate into endometrial cells. Studies in animal models and clinical trials have demonstrated that this approach can augment endometrial thickness and receptivity[35]. The use of growth factors and cytokines has also been shown to have a significant impact in endometrial function and implantation[36]. Another very interesting approach that is being the focus of many research studies is the utilization of exosomes for the treatment of endometrial dysfunction. Exosomes are minute, membrane-bound vesicles released by various cell types, containing a diverse array of biomolecules, including proteins, lipids, and nucleic acids. Exosomes play pivotal roles in intercellular communication and the regulation of cellular functions, which can be used for therapeutic purposes. Current findings propose that exosomes may hold diagnostic and therapeutic promise in addressing endometrial dysfunction[37].

In the decades to come, new high-throughput omics technologies and therapies will enable a better understanding of the complex and dynamic changes involved in receptivity and implantation. Omics approaches are expected to innovate further, leading to a deeper and more holistic description of cell and tissue biology and transforming our understanding of health and disease. Progress is expected in various areas, such as enhancements in efficiency, cost reduction and the integration of several testing methods into a single assay. Improvements in computational approaches that allow integrated analyses across various modalities are also envisaged and will be crucial for uncovering interdependencies within and among each molecular layer. Additionally, making more commercially available high resolution methods such as single-cell and spatial multi-omics assays will enhance their accessibility and application by a broader research community. These advancements in technology and computation will ultimately lead to an improved comprehension of cell and tissue function. This will be key in deciphering the origins of pathogenesis and disease, establishing more effective diagnosis methods and therapeutic approaches and providing guidance for precision medicine.

The field of ART needs to increase its success rate, patients deserve to be offered effective treatments, established after adequate diagnosis of the origin of their difficulty to conceive. It would be advisable for health professionals, and scientists, to explore new ways of improving success rates, by applying new approaches to increase implantation and thus reproductive outcomes, especially in complex cases such as RIF patients, unexplained infertility or RM. These couples are exposed to repeated cycles of assisted reproduction techniques with small success rates, producing in them not only high levels of psychological stress but also intense financial pressure. Advancements in the study of uterine environment and personalised approaches resulting from the knowledge that is expected to be generated in the coming years will enhance the chances of implantation success, offering hope and new possibilities to couples on their journey to parenthood.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

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Evaluation of the Endometrium in the XXI Century

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ABSTRACT

The endometrium is a fundamental layer of the uterus that is relevant to female reproductive function. This specialized tissue lines the inner wall of the uterus and its condition and health are essential to the process of conception and subsequent embryo implantation.

Since the early days of reproductive medicine, the maternal endometrium has been considered a passive part of the reproductive process; a "good quality embryo" was all that mattered.

This fact requires revision since the efficacy of in vitro fertilization remains low despite considerable improvements in embryology and embryo transfer technologies in recent decades. We now know that human implantation is a very complex and multifactorial process. Successful implantation requires the presence of a healthy embryo, a receptive endometrium and a synchronized molecular dialogue between the two, as well as host immune tolerance/protection.

The study of the endometrium is one of the fundamental aspects to be taken into account in assisted reproduction treatments. It is a necessary practice that is routinely performed in assisted reproduction clinics, specifically in cases of implantation failure and repeated miscarriage.

KEYWORDS

Preimplantation genetic testing for aneuploidy, Recurrent implantation failure, Endometrial receptivity, Window of implantation, Embryo transfer.

ABBREVIATIONS

PRP	Platelet-rich plasma
RIF	Recurrent implantation failure
CE	Chronic endometritis
PGT-A	Preimplantation genetic testing for aneuploidy
BV	Bacterial vaginosis

MANUSCRIPT

Introduction

The endometrium is an essential layer of the uterus that is relevant to the female reproductive function. This specific tissue lines the inner wall of the uterus and its condition and health are essential to the process of conception and subsequent embryo implantation.

Nowadays, we know that human implantation is a very complex and multifactorial process.

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Successful implantation requires the presence of a healthy embryo, a receptive endometrium and a synchronized molecular dialogue between the two, as well as the host immune tolerance /protection.

Implantation is initiated by adhesion of the blastocyst to the epithelial layer of the endometrium[1].

Preimplantation genetic testing for aneuploidy (PGT-A) reports the transfer of normal embryos based on chromosomal analysis[2] to avoid chromosomal aneuploidies, which can cause early pregnancy lost and implantation failure.

Despite of many publications on recurrent implantation failure (RIF)[3, 4], there is no universal accepted definition yet.

According to the study published by Pirtea in 2021, the incidence of RIF in women who have a normal uterus (anatomy) and undergo 3 consecutive transfers of single euploid embryos is less than 5%[5].

There are other publications that consider endometrial abnormalitiy the cause of implantation failure in assisted reproduction treatments. Ledee et al.[6] studied the endometrial immune function by measuring NK cells and other markers of inflammation in endometrial biopsies from the luteal phase. Excessive or insufficient immune responses, assessed by biopsies, are given as an explanation for implantation failure[7]. Unfortunately, there are no validated and available diagnostic tests to confirm immune-mediated implantation failure[8]. These investigators recommend different therapeutic options to treat the abnormal uterine immune response, from increasing the dosage of vaginal progesterone to intravenous intralipid administration and corticosteroid therapies. Therefore, the immune therapies are often initiated empirically without solid evidence of efficacy[8].

Abnormal results in the study of blood coagulation may also be a condition for implantation failure and pregnancy loss[9].

In the recent years, the timing of progesterone-induced endometrial changes has been assessed by gene expression panels in endometrial tissue[6,7,8] rather than histological changes. Gene assessments performed on luteal endometrial findings are reported as prereceptive, receptive, or postreceptive. Recommendations made by proponents of these tests are that adjustments need to be made in the timing of embryo transfers to achieve a synchronous window of implantation[10]. More recently, endometrial assessment strategies based solely on hormonal changes in the endometrium have been questioned, recognizing that the endometrium may simply be pathologic (or altered).

Refractory Endometrium

Another important factor to take into consideration is the endometrial thickness. It is a prognostic parameter for an embryo transfer. Refractory endometrium is considered endometrium of less than 7mm on the day of ovulation or on the day of human chorionic gonadotrophin (HCG) injection in fresh in vitro fertilization (IVF) cycles, or when progesterone starts in frozen and thawed embryo transfer cycles[11]. Endometrial thickness is directly correlated with levels of estrogens[12].

This is a rare finding, present in only 2-3% of patients undergoing assisted reproductive technology. Multiple therapeutic approaches have been described with questionable results such as high doses of estrogens, different methods of estrogen administration (oral, vaginal, transdermal, intramuscular), adjuvant treatment with vasoactive agents such as aspirin, vitamin E, pentoxifylline, sildenafil citrate, granulocyte colony stimulating factor (G-CSF) IU infusion, stem cell therapy and platelet-rich plasma instillation.

Garcia-Velasco, showed that platelet-rich plasma and sildenafil citrate could have a beneficial effect in this type of patients[13].

Xin Li et al[14] described that the endometrial thickness in patients who received sildenafil citrate was significantly greater than in the control group (placebo or no treatment); the radial artery resistance index was significantly lower and the clinical and biochemical pregnancy rate were significantly higher in the sildenafil citrate group compared to the control group.

Uterine PRP is an experimental treatment consisting of a concentration of protein derived from blood, free of red blood cells, containing severe cytokines, as well as a large group of growth factors, such as interleukin 8 (IL-8), insulin-like growth factor I, II (IGF-I, II), vascular endothelial growth factor (VGEF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor (TGF) and connective tissue growth factor (CTGF); and it is supposed to increase endometrial growth and receptivity. The regulatory effect of PRP on the expression of growth factors and cytokines in the endometrium is due to its anti-inflammatory and pro-regenerative functions[12, 15,16]. Although PRP is widely used in other fields, its clinical efficacy in the field of obstetrics and gynecology is still very limited.

During the implantation window, around days 19-23 of each cycle, a molecular cascade leads to the creation of implantation and pregnancy proteins; cytokines, growth factors, prostaglandins and adhesion molecules are among these proteins, and their deficiency has been shown to be related to implantation

failure[17]. The hypothesis is that since PRP contains various growth factors and cytokines, it could stimulate proliferation and regeneration, enhance endometrial receptivity and improve implantation. Therefore, intrauterine infusion of PRP could have a positive effect on implantation and pregnancy[18].

Agarwal described the beneficial effect of hysteroscopic injection of PRP in the subendometrial region, showing an improvement in endometrial thickness and higher pregnancy rates in patients with previous thin endometrium[19].

Endometrial Microbiota in Human Reproduction and Chronic Endomeritis

The community of microorganisms (bacteria, fungi, archaea, viruses and parasites) that coexist with every human being inside or on the external surface of the body is called the "human microbiota and its genomic constitution is called the microbiome"[20].

The term microbiome "comprises the entire habitat, including the microorganisms, their genes and their environmental conditions"[21].

In the last few years, there is a great interest in the study of the endometrial microbiota and its impact on the success of embryo transfer.

The uterine microbiota of women of reproductive age is mainly composed of five types of "community states; four of them are lactic acid-producing Lactobacillus and the fifth are mainly aerobes and strict anaerobes[22].

Lactic acid production has been associated with contributing to the overall health of the vagina due to its direct and indirect effects on pathogens and host defense. Some bacterial species outside the Lactobacillus family can trigger immune responses and degrade the host mucosa, processes that increase susceptibility to infection and contribute to negative reproductive outcomes such as infertility and preterm delivery[23].

In general, the microbes that exist in the uterus present a mutualistic relationship with the host, that is beneficial for both, and they work as a first barrier of defense against the colonization of opportunistic pathogenic organisms.

As mentioned above the microorganisms found in the highest percentage in the vagina and endometrium belong to the Lactobacillus family, and these are: L.crispatus(CST-I), L.iners(cst-III), L.gasseri(CST-II) and L.jensenii(CST-V). These microbial communities are associated with healthy women of reproductive age and allow the production of large amounts of lactic acid achieving an acid pH < 4.5. Such an acidic environment is protective against infections or colonization of the endometrium by non-native pathogens and microbes[23].

The increased presence of Gardnerella, Atopobium, Mobiluncus, Prevotella and Clostridiales[24], means a decrease in Lactobacillus which is associated with clinical symptoms resulting in discharge, foul odor and irritation. Usually, this symptomatology translates into bacterial vaginosis (BV).

In general, the microbes that exist in the uterus present a mutualistic relationship with the host, that is, both benefit and function as a first barrier of defense against the colonization of opportunistic pathogenic organisms. As mentioned above the microorganisms found in the highest percentage in the vagina and endometrium belong to the Lactobacillus family, and these are: L.crispatus(CST-I), L.iners(cst-III), L.gasseri(CST-II) and L.jensenii(CST-V). These microbial communities are associated with healthy women of reproductive age and allow the production of large amounts of lactic acid achieving an acid pH < 4.5. Such an acidic environment is protective against infections or colonization of the endometrium by non-native pathogens and microbes[23]. The increased presence of Gardnerella, Atopobium, Mobiluncus, Prevotella and Clostridiales[24], means a decrease in Lactobacillus which is associated with clinical symptoms resulting in discharge, foul odor and irritation. Usually this symptomatology translates into bacterial vaginosis (BV).

Bacterial vaginosis is usually caused by the presence of aerobes such as group B Streptococcus, Staphylococcus aureus, Escherichia coli and Enterococcus. These organisms produce an inflammatory response and destroy the acidic environment generated by the other community groups, meaning an increase in pH>4, which is associated with a diseased vagina.

Women with BV have an increased risk of adverse outcomes during childbirth, in addition to an increased likelihood of contracting sexually transmitted diseases such as HIV. It can also induce associated complications such as "chorioamnionitis, endometritis, salpingitis[24].

Currently for the study of human microbiota, 16S rRNA gene sequencing is used in the identification of complex microbial communities due to its feasibility to infer the representation of certain disease-causing microbial communities[20].

The 16S rRNA gene is a commonly used gene for the detection of microorganisms because it is present in the DNA of bacteria. It is transcribed and translated in the 16S rRNA, i.e. in the small subunit of the bacterial ribosome. It is a conserved sequence in all bacteria so

that there will be specific regions of the gene in all bacteria and other unique regions. The specific regions are used for the design of primers for subsequent PCR amplification of the gene. That is why the sequencing of the amplified 16S rRNA gene can serve as a marker to identify the bacteria in a given sample. For all these reasons, it is a very recurrent technique for the identification of the vaginal and endometrial microbiota in order to evaluate the presence of microorganisms that may be positively or negatively affecting the health of the female genital tract and to predict the success of IVF.

In the study by Chen et al, transcriptome and 16s rRNA sequencing technology was combined to analyze the interaction between endometrial microbial disorder caused by chronic endometritis and immune cells in the endometrium of patients with recurrent implantation failure. It was observed that the composition of endometrial microorganisms of patients with chronic endometritis and those without endometritis was significantly different. Phyllobacterium and Sphingomonas mainly regulated immune cells by interfering with the process of carbohydrate and/or fat metabolism in the endometrium[25].

We are currently having the question if it its necessary to perform a preliminary study of chronic endometritis (CE) in patients undergoing assisted reproduction treatments.

In most cases, chronic endometritis is caused by an alteration of the normal endometrial microbiome by bacterial pathogens. It is a possible origin of cronic endometritis the infection because antibiotic therapy normalizes the endometrium and improves clinical outcomes[26]. However, non-infectious forms of chronic endometritis may also exist. Clinically, it is silent or asymptomatic in most cases, which makes it difficult to diagnose. Symptoms are usually mild and nonspecific, with abnormal uterine bleeding, pelvic pain and dyspareunia.

Chronic endometritis is characterized by an alteration in the quantity and quality of leukocyte infiltration. Leukocytes are usually organized in nodular aggregates that infiltrate glands and vesicles, leading to structural alterations. In addition, abnormal leukocyte subpopulations (increased B cells and plasma cells, reduced NK cells cause alterations in the expression of inflammatory mediators and cytokines. The inflammatory milieu may alter the expression of genes involved in cell replication, the implantation process and also the immune tolerance of the endometrium to enhance embryo implantation[27]. Finally, an alteration of autophagy observed in chronic endometritis may affect endometrial cell function and impair endometrial decidualization[28].

Inflammatory mediators can alter uterine contractility during the mid-luteal phase, preventing fertilization and transuterine migration of the embryo before implantation[29].

Cicinelli's group considers a possible routine screening for chronic endometritis in all patients undergoing assisted reproductive technology or women with an adverse obstetric history (such as miscarriage) because of the deleterious effect of this pathology on reproductive outcomes[30].

However, the ASMR (American Society for Reproductive Medicine) does not recommend routine endometrial biopsy for screening for cronic endometritis prior to ART. Also, they say that there are not clear diagnostic criteria.

There are two methods to diagnose EC: hysteroscopic visualization of endometrial lesions with moderate specificity and sensitivity[31] and endometrial biopsy with histology.

Endometrial biopsy is considered the gold standard for the diagnosis of EC, based on the identification and counting of plasma cells in the endometrial stroma[32]. For this purpose, IHC staining with CD138 is routinely used, which allows a simple and reliable identification of plasma cells (PC) in endometrial tissues, thus gaining popularity over the classical hematoxylin-eosin analysis. However, there is no clear evidence for the number of plasma cells required for the diagnosis of CD; we note that it varies among investigators. Some studies use 1 to 5 PC per HPF, while others use 1 PC per 10 HPF, and still others use different criteria[33]. These levels were arbitrarily selected without having a clear reference population. The dilemma of the Plasma cells originates from the fact that PCs are not evenly distributed throughout the endometrial stroma and may even be concentrated in patches or scattered throughout the stroma.

In Pirtea's work, they describe that the clinical impact of CE is of short duration and usually resolves without treatment or is very infrequent[5], so they do not support the idea of the systematic study of CE in patients prior to ART.

On the other hand, patients diagnosed with endometriosis have a higher incidence of chronic endometritis. This does not mean that it has an impact on the final results of assisted reproduction treatments and, in particular, on live birth rates, since deferred transfer protocols are currently preferred in endometriosis[30].

Another factor to be taken into account is uterine disorders. Uterine pathology is a risk factor for the development of chronic endometritis[34]. Endometrial polyps and uterine synechiae are the two pathologies

most significantly associated with CE. The diagnosis of CE is less frequent in patients with a septate uterus. Hysteroscopic surgery can cure most cases of CE without antibiotherapy simply by surgically correcting the pathology, regardless of the type of intrauterine abnormalities. This type of surgery should be indicated before the administration of antibiotics[34].

Endometrial Receptivity Test

Methods to explore endometrial receptivity have been carried out by genomic studies. This is the case of the endometrial receptivity microarray (ERA) developed in 2008 and whose objective is to "define the transcriptomic of the endometrial receptivity"[35]. For this purpose, a cohort study was performed, including three groups of subjects: one to select the genes to be included in the array, another for endometrial dating and a last one with pathological endometrial samples to train the predictor. In total, 95 patients were included in the this study. For the selection of genes that were related to endometrial receptivity, expression profiles of the entire human genome were analyzed, focusing on the receptive and pre-receptive endometrium. The inclusion criteria for these were those showing a fold change > 3 and a p value < 0.05. Agilent technology was used to fabricate the microarray and it underwent a training process to train the predictor and locate the receptivity genes. The results showed that there were 238 genes that were differentially expressed in the transition between the pre-receptive and the receptive phase. The objective was to demonstrate the importance of studying the state of the endometrium in reproductive medicine during the WOI (window of implantation).

In 2018, another study was published. The objective was to demonstrate the existence of another test called ER Map/ ER Grade that could predict endometrial receptivity status by RT-qPCR using a new panel of genes involved in endometrial proliferation and maternal immune response associated with embryo implantation. For this purpose, a cohort of 216 patients with endometrial samples including fertile women and patients who had undergone fertility treatments was analyzed for the expression of 184 genes involved in endometrial receptivity. The results in this case showed that 85 of the 184 genes had significant differential expression and showed that these genes were associated with aspects such as cell division and proliferation, vascular proliferation, embryo implantation. In addition, 40 of these genes allowed the classification of the endometrium according to its state of receptivity into pre-receptive, receptive and post-receptive. The conclusions of this new study were similar to the one published in 2008 and establish that identifying the optimal time to perform embryo transfer is essential to increase the success rate in assisted reproduction[36]. According to Simón et al. personalized embryo transfers statistically significantly improved pregnancy, implantation and cumulative live birth rates[37]. In general, 62.5% of the population have receptive implantation windows with 5 days of progesterone impregnation[37].

However, and despite what has been published, the efficiency of this type of test is increasingly controversial and there are multiple publications that affirm that personalized transfer according to the ERA protocol does not provide any benefit in patients undergoing euploid embryo transfer[38,40].

In patients who did obtain an euploid blastocyst as an outcome after IVF treatment, the use of receptivity testing to guide the timing of frozen embryo transfer compared to the standard timing of transfer did not significantly improve the live birth rate[39,40].

The evidence does not support the routine use of endometrial receptivity tests to guide the timing of embryo transfer during in vitro fertilization[40].

CONCLUSIONS

The study of the endometrium is one of the fundamental aspects to be considered in assisted reproduction treatments. It is a necessary practice that is routinely performed in assisted reproduction clinics, specifically in cases of implantation failure and repeated miscarriage.

The impact and management of thin endometrium and RIF are a common challenge for patients undergoing assisted reproduction. Both are an infrequent but challenging occurrence in assisted reproduction.

Currently, there is minimal evidence to support any specific protocols or adjuvants to significantly improve pregnancy outcomes in patients with thin endometrium.

PGT-A may be beneficial for patients with recurrent implantation failure. However, endometrial receptivity test does not appear to be clinically useful for patients with RIF.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

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Advancements in Artificial Intelligence in the study of Endometrium

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ABSTRACT

Objective

Findings on the application of artificial intelligence (AI), particularly convolutional neural networks (CNNs), in enhancing diagnostic and prognostic capabilities in gynecological health were synthesized.

Design

Recent technological advancements, particularly AI and machine learning, in the study and management of endometrial conditions were reviewed.

Subjects

Various studies exploring the role of AI in diagnosing and managing endometrial conditions such as endometriosis, endometrial receptivity, and endometrial cancer were examined.

Intervention

The development and implementation of CNNs, radiomics models, and integration of omics data (proteomics, metabolomics, transcriptomics), ultrasonographic imaging, in endometrial studies were analyzed.

Main Outcomes

Diagnostic accuracy, prognostic assessments, early detection, personalized treatment, and clinical management of endometrial conditions were evaluated.

Results

It was found that AI technologies, surpassing manual methods in accuracy, enhance the classification of endometrial patterns and analysis of uterine peristalsis. The quantitative assessment of endometrial vascularization and blood supply is improved by AI, leading to better predictions for pregnancy outcomes. Traditional challenges, such as time-consuming manual measurements and significant inter-observer variability, are mitigated by AI-assisted ultrasound, which provides automated detection and measurement of follicles, reducing examination time and improving reproducibility. Diagnostic accuracy in follicular monitoring and endometrial receptivity (ER) assessment is enhanced by AI models, though challenges remain, including the need for robust AI models and validation across diverse populations. The integration of AI with transcriptomic testing and biomarkers in assisted reproductive technology (ART) shows promise in improving embryo transfer timing and personalized treatment strategies. In endometrial cancer and hyperplasia, AI models significantly enhance diagnostic accuracy, sensitivity, and specificity, improving

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preoperative risk classification and prognostication. Non-invasive diagnostic methods like proteomic profiling and AI models demonstrate high sensitivity and specificity for endometriosis, potentially reducing the need for invasive procedures.

Conclusions

It has been demonstrated that AI models, particularly those leveraging deep learning, show promise in enhancing diagnostic efficiency, predicting molecular subtypes, and improving clinical outcomes in gynecological cancers and reproductive health. However, challenges such as model generalization, data standardization, and interpretability need to be addressed. Future research should focus on validating these models and integrating them into clinical workflows to optimize patient care.

KEYWORDS

Endometrial Receptivity, Endometrial Cancer, Endometriosis, PCOS, Recurrent Implantation Failure, Ultrasound, Artificial Intelligence, Convolutional Neural Networks, Machine Learning.

MANUSCRIPT

Introduction

The endometrium is a complex and dynamic tissue composed of epithelial cells, both luminal and glandular, surrounded by supporting stromal cells, together comprising the innermost layer of the uterus. The primary function of the uterus is supporting fertility, and the endometrium is the layer critically involved in receiving an embryo, facilitating implantation and decidualization, and supporting embryo growth and development until placentation. The adequate development of the endometrium is considered essential for the window of implantation (WOI)[1], and adverse reproductive outcomes, such as implantation failure[2,3] and miscarriage, may be caused by altered proportions during the WOI.

The abnormalities of the endometrium, such as endometriosis[4,5,6,7], hyperplasia[9] and endometrial cancer[9,10,11,12] are traditionally diagnosed using transvaginal ultrasound (TVUS)[13,14] and endometrial histologic[15,16,17,13,2,18]. However, the manual segmentation of the endometrium is subject to subjectivity and prone to errors, especially by inexperienced sonographers.

The study of the endometrium plays a critical role in women's health and reproductive medicine, offering insights into various gynecological conditions and enhancing fertility treatments. Recent advancements in technology, particularly in artificial intelligence (AI)[19,20,14] have revolutionized how endometrial studies are conducted and interpreted.

The important role of ultrasound in female reproductive function is evaluation of ovarian reserve (OR)[21,4] and endometrial receptivity (ER)[19,22,21,2]. In the assessment of OR, serial ultrasound examinations can provide reliable markers to follicular monitoring[21], the diagnosis of Polycystic Ovary Syndrome (PCOS)[21,2,3], and prediction of oocyte quality and pregnancy outcomes, such as ovarian follicular diameter and volume, number of follicles, ovarian stromal blood flow index, etc.

For ER, endometrial thickness[19,22,3] and volume, endometrial morphology[21], and spiral arterial blood flow index[21] are effective evaluation indicators.

Materials and Methods

This section provides an overview of the methodologies and materials employed in this comprehensive analysis on various aspects of endometrial health and disease, with a focus on advanced AI and machine learning techniques. Detailed methodologies are described for five key areas of analysis: Endometrial Hyperplasia and Endometrial Cancer, Endometriosis, Endometrial Receptivity, AI for Identifying Endometrial CD138+ Cells in PCOS and Recurrent Implantation Failure, and the overall Role of AI in Endometrial Studies. In each subsection, the application of convolutional neural networks (CNNs) and other AI models is detailed, along with data sources, patient cohorts, and validation techniques. Diagnostic accuracy is enhanced, treatment outcomes are improved, and the complex molecular and structural features of the endometrium are understood through these methodologies. Various data types, including histopathological images, MRI scans, and transcriptomic profiles, are integrated to offer comprehensive insights into endometrial conditions, support non-invasive diagnostics, and facilitate personalized treatment strategies. Technological advancements and techniques, diverse datasets and features, and rigorous classification and validation criteria are highlighted to ensure the reliability and robustness of the findings.

Endometrial Hyperplasia and Endometrial Cancer

Recent advancements in AI and ML have revolutionized the diagnosis of endometrial hyperplasia and endometrial intraepithelial neoplasia. Convolutional neural networks (CNNs) developed from histopathological images are used to screen and

diagnose these conditions, improving diagnostic accuracy and reducing subjective interpretation variability. The interpretable deep learning pipeline, im4MEC[23], predicts molecular classifications of endometrial cancer from whole-slide images, further enhancing diagnostic capabilities.

Artificial intelligence (AI) in medicine encompasses the application of knowledge- and data-intensive computer-based solutions for disease prevention, diagnosis, and treatment. One of its pivotal domains is medical imaging, which has seen continuous evolution since its inception in the mid-twentieth century.

Efforts to improve diagnostic accuracy and automation in medical image analysis, particularly for the diagnosis and classification of endometrial cancer using convolutional neural networks (CNNs) and other advanced models, have been concentrated on.

Role of CNNs in Medical Image Analysis:

CNNs extract complex features from medical images, particularly whole-slide images (WSIs) in endometrial cancer diagnosis. Morphological features correlating with molecular subtypes are identified by CNNs, reducing pathologist workload. Advanced models like G2LNet[8] and im4MEC[23] facilitate precise lesion identification through feature extraction, fusion, and predictive analysis. Integration of radiomics and deep learning enables non-invasive prediction of tumor characteristics, showcasing AI's potential in gynecological healthcare.

Technological Advances and Techniques:

Models such as DeepLab v3[10], ResNet-50[10], HIENet[17], YOLOv3[11], and MoCo-v2[24] are employed for tasks such as lesion detection, self-supervised learning, and enhancing diagnostic performance. Spectroscopic techniques and DNA methylation analysis[25] combined with machine learning identify biomarkers critical for diagnostics.

Data Sources and Features:

Datasets include diverse medical imaging (histopathological images[17,26,13], WSIs[27,10,23], MRI scans[9,28,29,30] and clinical data. Key features for training CNN models include textural patterns, shape, intensity variations, and morphometric characteristics. Cellular structures, tissue architecture, glandular morphology, nuclear atypia, and radiomic features are commonly analyzed.

Patient Cohorts and Clinical Context:

Patients encompass various stages and subtypes of endometrial conditions, providing comprehensive training and validation contexts. Clinical features such as age, CA125 levels[18], tumor size, grade, menopausal status[30], BMI[29,25,31], and family history are considered. Specific diagnostic procedures and annotations by expert pathologists enrich the clinical relevance of the imaging data.

Classification and Validation:

Endometrial hyperplasia and neoplasia are classified based on image features, while molecular subtypes guide endometrial cancer classification. Risk groups are determined using ESGO/ESMO/ESP guidelines, integrating whole-lesion segmentation on MRI images and statistical methods like random forest[28,31] and LASSO[9,29,18]. Survival predictions (5-year PFS)[29] utilize radiomics features from MRI scans combined with clinical and MRI risk factors. Validation metrics include accuracy, sensitivity, specificity, AUC-ROC, precision rate, positive and negative predictive values, and clinical outcome comparisons. Techniques like cross-validation[28,23,17,26,13,31], ROC analysis[12], Kaplan-Meier analysis[9], Cox models[9], and biomarker validation[2,18,32,31] ensure robust model validation and reliability.

Endometriosis

Studies related to endometriosis were analyzed to determine correlations, classification criteria, models applied, data quality, quantity and specific features, cohorts, validation criteria, and clinician validation.

Diverse AI and Machine Learning Approaches in Medical Diagnostics for Endometriosis:

The ability of Convolutional Neural Networks (CNNs) to analyze medical images and support non-invasive diagnostics for endometriosis is the focus of many studies. A variety of models and techniques are explored in other research, including Artificial Neural Networks (ANNs) for proteomic data analysis[33], logistic regression[6,20,14], decision trees[34], Random Forest[6,20,34,14], eXtreme Gradient Boosting[6, 20], and Support Vector Machines (SVMs)[14] for classification and predictive modeling. Genetic algorithms[7] and mass spectrometry[5,7] are also utilized for diagnostics, with peptide profiling[5] and protein expression[7] analysis being leveraged. The broader landscape of AI and machine learning in medical diagnostics is highlighted by these diverse methodologies, where imaging tasks are excelled in by CNNs, and significant benefits for data analysis and pattern recognition in molecular and cellular biology are offered by other models.

Technological Advances and Techniques:

A range of AI and machine learning techniques for endometriosis diagnostics have been employed, including CNNs for imaging, ANNs for proteomic data analysis, logistic regression, decision trees, Random

Forest, eXtreme Gradient Boosting, SVMs for classification and predictive modeling, and genetic algorithms and mass spectrometry for diagnostics, with peptide profiling and protein expression analysis highlighted.

Data Sources and Features:

A range of datasets, including urine, plasma, serum samples, MRI, and histopathological images, is utilized in various studies. Peptide profiles[5] are analyzed from urine samples, while proteomic[5,7] and metabolomic[35] data are processed from serum samples. MRI datasets[6,35], annotated for endometriosis, emphasize tissue characteristics and lesion specifics. Numerical, categorical, and text data focusing on symptoms, diagnosis, and treatment are collected from clinical records. RNA-seq and methylomics data[34,36] are included in some datasets, while digital histopathological images[37] are used in others, with textural, structural, and morphometric features extracted by CNN models. The diversity of data features highlights the need for standardized data collection and analysis protocols, encompassing hormone profiles[4], miRNA[6], metabolite levels[38,35], and genetic variables[14].

Patient Cohorts and Clinical Context:

Individuals with dysmenorrhea, pelvic pain, and infertility, including those diagnosed with endometriosis, were included in the patient cohorts. Clinical data such as age, symptoms, and surgical history were incorporated into MRI datasets. Plasma samples were collected from women with chronic pelvic pain, confirmed by laparoscopy or MRI. Hormonal profiles and ovarian reserve markers characterized subfertile women. Increased metabolite levels in endometriosis patients were indicated by serum samples. Endometriosis stages, age ranges, and symptoms like dysmenorrhea and infertility were included in proteomic studies. Comprehensive demographic, symptom, and treatment data were provided by the Ziwig Health[20] platform.

Classification and Validation:

Various methods have been used to classify endometriosis in studies, including peptide diagnosis, CNN-classified images, sensitivity, specificity, AUC, hormone levels, and spectra analysis. Protein expression[7], spectral shifts[35], serum protein patterns[7], clinical features[14], age, colorectal involvement, likelihood of live birth, microbiota composition[3], and genetic data[14] have also been utilized. Models were validated using metrics such as sensitivity, specificity, F1-score, and AUC. Cross-validation methods included random datasets, 10-fold splits, and 70/30 training-validation divisions. Statistical methods like t-tests, ROC analysis, and biomarker assessment were employed. Specific validation results included 90.9% sensitivity and 92.9% specificity from urine samples, and 86% classification accuracy with 100% specificity and 58.3% sensitivity using an 80/20 cross-validation.

Endometrial Receptivity:

AI models predict success rates of fertility treatments and assess endometrial receptivity, addressing challenges in assisted reproductive technology. Integration of proteomics, metabolomics, and transcriptomics with AI provides novel insights into disease mechanisms and improves clinical outcomes in gynecological healthcare.

Studies related to endometrial receptivity were analyzed to determine correlations, classification criteria, applied models, data quality, quantity, specific features, cohorts, validation criteria, and clinician validation.

The application of convolutional neural networks (CNNs) and other AI models in endometrial studies is varied based on the study's focus and data type. While CNNs are not specifically mentioned in some studies, various AI models, including machine learning algorithms, are often used to analyze transcriptomic[39,16] and gene expression data[39,1]. A crucial role in studies involving image analysis, such as histological image[15] segmentation and classification, where models like Attention U-Net and GoogLeNet Inception[19] are used to significantly enhance accuracy. In studies where transcriptomic analysis of gene expression profiling is the primary focus, traditional AI and machine learning techniques are more commonly used to interpret complex data and identify patterns related to endometrial receptivity and fertility treatment outcomes.

Endometrial images were classified as "good" or "bad" based on features such as external layer thickness and echogenicity, following the Asch endometrial grading system[22]. The endometrium was classified into six categories by this novel system, which were then used to train our AI model, Endoclassify. The endometrial structural features were assessed, images were classified, and endometrial receptivity was predicted by this AI model without clinical intervention, achieving a high degree of correlation with clinical outcomes.[**Figure 1**].

In our previous study[19], clinical outcomes of two categories—fresh and frozen embryo transfer cycles—were analyzed, demonstrating significant pregnancy success rates within these groups. Building on this foundation, the current analysis has been expanded to introduce two new categories related to assisted reproductive technology (ART): OD (Oocyte Donation cycles) and GS (Gestational Surrogacy cycles) [**Table**

1]. The inclusion of these categories is intended to enrich the understanding of pregnancy outcomes and further validate the efficacy of different ART methods.

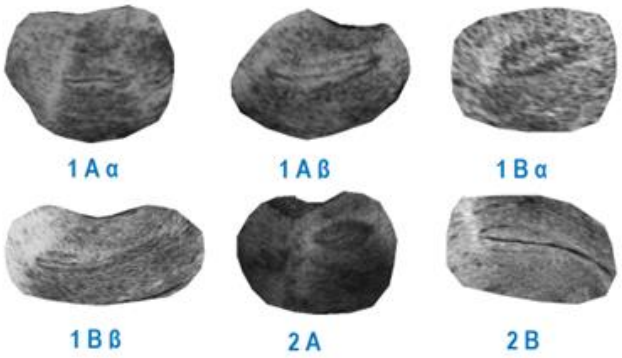

Figure 1. Asch Endometrial Grading System

Oocyte donation (OD) and gestational surrogacy (GS) are increasingly utilized in reproductive medicine, providing viable options for individuals and couples facing infertility.

patients). The number of cases according to endometrial classification and the corresponding pregnancy rates were assessed. The goal was to demonstrate that the extended categories (OD and GS) not only reach but also excel in pregnancy outcomes, similar to the previous findings with fresh and frozen eggs.

The new data presented in this study underscore the importance of expanding the analysis to include OD and GS cycles. By doing so, a more comprehensive understanding of ART success rates is sought, providing valuable insights into the effectiveness of these reproductive technologies [**Table 2**].

It is suggested that the inclusion of OD and GS categories enhances pregnancy outcomes, further supporting their integration into clinical practice. Our Endoclassify AI model[19] has been instrumental in accurately identifying and classifying these new categories, ensuring that the right outcomes are achieved. This advanced AI technology has

	1 A α	1 B α	1 A β	1 B β	Patients (n)
FRESH	80	40	14	7	141
FROZEN	93	27	6	4	130
OD	100	30	14	9	153
GS	80	10	4	2	96
TOTAL	353	107	38	22	520

Number of cases according to endometrial classification
(Fresh cycles / Frozen cycles / OD Oocyte Donation cycles / GS: Gestational Surrogacy cycles).

Table 1

	1 A α		1 B α		1 A β		1 B β		Pregnancies (%)	
FRESH	59	74%	20	50%	1	7%	0	0%	80	57%
FROZEN	70	75%	13	48%	1	17%	0	0%	84	65%
OD	75	75%	20	67%	7	50%	0	0%	102	67%
GS	62	78%	7	70%	2	50%	0	0%	71	74%
TOTAL	266		60		11		0		337	

The pregnancy rates in patients of the four groups (Fresh-Frozen-Oocyte Donation and Gestational Surrogacy cycles) that belong to Asch classification 1Aα and 1Bα are significantly higher than of all other groups in the study ($p <= than 0.05$).

Table 2

In this expanded study, clinical data from 520 patients were analyzed, categorized into four groups: fresh cycles (141 patients), frozen cycles (130 patients), OD cycles (153 patients), and GS cycles (96

demonstrated its capability to effectively analyze and predict successful pregnancies across diverse ART methods, thereby reinforcing the reliability and

applicability of these innovative reproductive techniques.

The normalization of data management across the studies reveals that the datasets are primarily composed of either transcriptomic data or high-resolution images of endometrial samples, with some studies utilizing augmented ultrasound images. Gene expression profiles for transcriptomic datasets and segmentation or histological markers for imaging datasets are generally included as data features. An understanding of molecular environments is emphasized by the studies focusing on transcriptomic data, while structural and classification features of the endometrium are concentrated on by imaging studies. Where applicable, image features include the segmentation of endometrial layers, glandular structures, and stromal density.

Regarding patient features, women of reproductive age undergoing fertility treatments are involved in most studies, with specific inclusion criteria such as age ranges (typically up to 40 years old) and clinical backgrounds (e.g., history of infertility, recurrent implantation failure, or specific outcomes of previous ART cycles). Demographic details, clinical histories, and treatment outcomes are commonly included to correlate with the molecular or structural data being analyzed. Homogeneity in patient populations is generally ensured through detailed inclusion and exclusion criteria, often focusing on patients without known endometrial pathologies or systemic diseases.

The classification criteria are based on differentiating between receptive and non-receptive endometrium using transcriptomic profiles[39] to determine the optimal timing for embryo transfer; endometrial images are classified as 'good' or 'bad' based on segmentation and classification models[19]; histological features[15] relevant to endometrial receptivity and fertility outcomes are classified by the CNN model; specific gene expression profiles are identified to distinguish between receptive and non-receptive endometrium states for personalized embryo transfer protocols; gene expression profiles are compared between natural and stimulated cycles during the window of implantation.

The validation criteria include metrics such as accuracy, sensitivity, specificity, and other relevant metrics to ensure the reliability of the AI-based transcriptomic testing; accuracy (95%), loss (10%), sensitivity (93%), and specificity (93%) are included; model performance is validated using a hold-out dataset to ensure reliability and robustness; gene expression profiles are compared between patients with successful and unsuccessful implantation outcomes using statistical methods to validate the predictive power of identified biomarkers; differential expression of selected genes is confirmed using quantitative real-time PCR (qRT-PCR).

AI for Identifying Endometrial CD138+ Cells in PCOS and Recurrent Implantation Failure:

The anovulatory PCOS samples exhibited significantly lower CD138+ cell percentages than PE PCOS samples, with phenotype A PCOS showing higher percentages than phenotype D. Interestingly, CD138+ cell percentages were unaffected by endometrial receptivity status in RIF samples[2,3].

The estradiol-mediated recruitment of CD138+ cells from systemic circulation into the PE is suggested, potentially explaining the higher concentrations observed. However, further investigation into estrogen receptor expression and sensitivity dynamics across the menstrual cycle is warranted, due to lower CD138+ cell percentages in anovulatory PCOS despite comparable endometrial thickness.

Despite limitations such as a lack of confirmed chronic endometritis cases and incomplete clinical data for RIF patients, the AI-algorithm training and validation for identification of endometrial CD138+ cells in infertility-associated conditions, including polycystic ovary syndrome (PCOS) and recurrent implantation failure (RIF), demonstrates the AI's efficacy in rapid, reproducible assessment of endometrial CD138+ plasma cells. Larger datasets and prospective study designs should be explored in future research to validate AI's role in assessing endometrial inflammation and optimizing reproductive health outcomes.

Role of AI in Endometrial Study:

Artificial intelligence (AI) technology has made significant strides in the medical field, yet its application in assessing female reproductive function remains at an early stage. Ethical concerns surrounding AI, including issues of responsibility and transparency in AI processes and human-machine interaction, pose challenges such as ethical, liability, and legal risks. These issues can contribute to patient and clinician distrust of AI, especially concerning data privacy and security, with many patients hesitant to consent to data uploading for intelligent analysis.

Moreover, the effectiveness of AI models is heavily influenced by data quantity, quality, and diversity. Small sample sizes, inadequate sample diversity, or imbalanced data ratios can introduce bias into models, limiting their generalization and practical applicability, particularly in the complex realm of female reproductive evaluation. Ensuring high-quality images and accurate data collection methods are crucial prerequisites for achieving accurate AI evaluations. Multicenter studies are essential to expand sample

sizes and enhance model robustness and generalizability across different institutions and machine types.

Standardization of evaluation criteria and data storage methods among institutions is paramount to address variability in assessing female reproductive function. While AI has demonstrated potential to match or surpass expert performance in some areas, its role should be viewed as complementing rather than replacing clinicians. AI's strength lies in screening and early warning systems, augmenting clinical decision-making rather than supplanting it. Clinicians must critically evaluate AI model construction and ensure alignment with real-world clinical scenarios to optimize patient care.

Results

In the domain of artificial intelligence models, advancements in endometrial studies are facilitated through the proposal of multi-level frameworks tailored to specific areas of research. This paper explores the integration of diverse technologies to enhance the analysis of endometrial receptivity during Assisted Reproductive Technology (ART) procedures. By combining transcriptomic data, segmented ultrasound images, high-resolution histopathological images, and comprehensive patient clinical data, a multi-omics approach is advocated. This integration enables correlation of clinical outcomes with molecular and structural features, thereby improving the understanding of endometrial receptivity and facilitating biomarker discovery for enhanced predictive capabilities in ART outcomes.

Furthermore, this study introduces Endoclassify[19], a novel method utilizing transvaginal ultrasound images to objectively evaluate endometrial conditions. Employing rigorous criteria, Endoclassify categorizes images into ‘Good’ or ‘Bad’ and quantifies the likelihood of pregnancy for each classification. These insights equip clinicians with essential information for informed decision-making regarding embryo transfer or cycle postponement.

Moreover, for enhancing endometrial cancer analysis, the integration of convolutional neural networks (CNNs) such as G2LNet, im4MEC, and HIENet with radiomics-based nomograms and traditional machine learning algorithms is proposed. Advanced detection techniques and self-supervised learning models complement these approaches, aiming to improve feature extraction, biomarker identification, and predictive modeling accuracy.

In the context of endometriosis, a multi-modal AI approach is recommended, leveraging CNNs for imaging data analysis, machine learning algorithms for clinical and molecular data integration, and ensemble methods for robust predictive outcomes. Logistic regression and partial least squares discriminant analysis play roles in initial variable selection and relationship understanding, contributing to comprehensive insights into this complex condition.

These integrated solutions illustrate the potential of artificial intelligence to advance understanding and management across diverse facets of endometrial studies, from reproductive health and cancer analysis to complex conditions like endometriosis.

Discussion

The best analysis, decision-making, and preparation of the data are required for artificial intelligence models to develop solutions. For these reasons, datasets, data features, image features, and patient cohorts need to be defined. Additionally, classification and validation criteria must be established. Finally, validation by clinical researchers is essential.

For endometrial receptivity, integration of transcriptomic data from ART procedures, alongside high-resolution ultrasound and histopathological images, aims to correlate gene expression and imaging features with clinical outcomes.

In endometrial cancer, a multi-modal approach utilizing histopathological images, MRI scans, radiomics, and proteomic/metabolomic profiles enhances diagnostic accuracy and personalized treatment strategies.

For endometriosis, AI models integrate clinical, imaging, molecular, and histopathological data to predict stages and outcomes, utilizing biomarkers and advanced imaging techniques. Validation across these domains involves rigorous statistical analysis and diverse validation cohorts to ensure robustness and clinical applicability.

Conclusions

This review synthesizes findings on the application of artificial intelligence (AI), particularly convolutional neural networks (CNNs), in enhancing diagnostic and prognostic capabilities across various aspects of gynecological health.

The classification of endometrial patterns (trilinear, semi-trilinear, unilinear) and the analysis of uterine peristalsis are enhanced by AI technologies, surpassing manual methods in accuracy. Quantitative assessment of endometrial vascularization and blood supply is improved using AI, leading to better predictions for pregnancy outcomes. Traditional challenges, such as time-consuming manual measurements and significant inter-observer variability, are mitigated by AI-assisted ultrasound,

which provides automated detection and measurement of follicles. Accuracy is further enhanced, examination time reduced, and reproducibility improved in follicular monitoring through segmentation algorithms (e.g., optimal thresholding, edge-based methods, active contour methods) and three-dimensional (3D) ultrasound techniques.

Variability is reduced and diagnostic accuracy in follicular monitoring and endometrial receptivity (ER) assessment is improved by AI models in ultrasound imaging. However, challenges remain, including the need for robust AI models that generalize well across different imaging conditions and equipment. Technical issues must be addressed and model validation across diverse patient populations ensured for AI to be integrated into clinical practice. Future research focuses on enhancing AI algorithms for real-time follicular monitoring and improving ER assessment, with clinical adoption dependent on overcoming current limitations and demonstrating practical benefits in improving ART outcomes.

For endometrial receptivity in assisted reproductive technology (ART), integration of AI with transcriptomic testing and biomarkers shows promise in improving embryo transfer timing and personalized treatment strategies, particularly in patients with recurrent implantation failure. However, challenges such as retrospective study designs and the impact of controlled ovarian stimulation on gene expression profiles are acknowledged.

In endometrial cancer and hyperplasia, AI models, including CNNs and radiomics-powered machine learning, significantly enhance diagnostic accuracy, sensitivity, and specificity. These models improve preoperative risk classification, prognostication, and facilitate personalized treatment planning. Challenges such as data standardization and model interpretability need addressing to maximize clinical utility.

For endometriosis, non-invasive diagnostic methods like proteomic profiling and AI models demonstrate high sensitivity and specificity, potentially reducing the need for invasive procedures. CNNs and machine learning algorithms enhance diagnostic accuracy but require further research to improve specificity and reproducibility.

Collectively, AI models, particularly those leveraging deep learning, show promise in enhancing diagnostic efficiency, predicting molecular subtypes, and improving clinical outcomes across gynecological cancers and reproductive health. Future research should focus on validating these models and integrating them into clinical workflows to optimize patient care.

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CONFLICT OF INTEREST

No conflict of interest exists among authors.

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Tumor necrosis factor alpha and milk fat globule-epidermal growth factor 8: Novel biomarkers to predict implantation failure and pregnancy loss

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ABSTRACT

Objective

To determinate whether implantation failure (IF) and recurrent pregnancy loss (RPL) can be predicted in serum prior to in vitro fertilization (IVF)?.

Design

Multicentric prospective controlled pilot clinical study from January 2016 to January 2020.

Material and methods

Thirty women ages 21-35 years were recruited from 3 groups: fertile controls (C), unexplained IF (following 3 failed good quality embryo transfers), and RPL (at least 2 unexplained first trimester miscarriages) in their natural cycle in which serum tumor necrosis factor (TNFα) and milk fat globule-epidermal growth factor 8 (MFG-E8) estradiol (E2) and progesterone (P4) levels were quantified in the early proliferative (cycle day 2) and secretory phases (urinary luteinizing hormone (LH)+7 days). Additionally, an endometrial biopsy was obtained on urinary LH+7 days for MFG-E8 and TNF α protein and gene expression analysis.

Results

Ten women were assigned to each group. No statistical differences were found in age, body mass index, antimullerian hormone, baseline follicle stimulating hormone and baseline antral follicle count among cohorts. Mean serum E2 and P4 levels were similar among all groups in both the proliferative and secretory phases: E2 proliferative (C 69.19±26.64 pg/ml, IF 64.19±32.56 pg/ml, RPL proliferative 57.44±38.51; p= 0.55), E2 secretory (C 164.10±52.57 pg/ml, IF 172.57±121, RPL 173.81±.97.35; p=0.25), P4 proliferative (C 0.45±0.15 ng/ml, IF 0.45±0.19 ng/ml, RPL 0.53±0.18 ng/ml; p=0.85), P4 secretory (C 7.42±4.06 ng/ml, IF 7.8±4.56 ng/ml, RPL 8.05±4.38 ng/ml; p= 0.74). Mean serum TNFα levels were significantly higher in both, the proliferative and secretory phases for the RPL group (proliferative RPL 9.98±4.47 pg/ml, IF 4.73±2.56 pg/ml, C 3.42±1.01 pg/ml; p=0.001 vs secretory RPL 8.67±4.45 pg/ml, C 3.35±0.94 pg/ml, IF 3.85±1.01 pg/ml; p= 0.03). Mean serum MFG-E8 levels were significantly higher in the IF group during the proliferative phase (IF 373±201 pg/ml, RPL 201±115 pg/ml, C 225.58±109.73pg/ml; p=0.03), but not in the secretory phase (IF 237±101 pg/ml, RPL 189±116 pg/ml, C 199.41±112.43 pg/ml; p=0.15). Endometrial MFG-E8 mRNA levels were significantly lower in the IF and RPL group compared to C (p=0.03). TNFα mRNA levels were not statistically significant among groups (p=0.12).

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Conclusions

TNFα and MFG-E8 serum levels may serve as serum markers to predict IF and RPL.

KEYWORDS

Tumor necrosis factor (TNFα) and milk fat globule-epidermal growth factor 8 (MFG-E8), implantation failure (IF), recurrent pregnancy loss (RPL).

MANUSCRIPT

Introduction

Successful implantation is dependent on both endometrial receptivity and the development of good quality embryos with implantation potential[1]. The uterus plays an indispensable role in the initiation and termination of the pregnancy. As host to the embryo, it is crucial to maintain a homeostatic relationship between the endometrium and the embryo. Endometrial receptivity in humans can be defined as a temporal maturation of the epithelium (primed by progesterone and estrogen) during which the trophectoderm attaches and invades the stroma[1]. Several cellular, hormonal and molecular pathways are involved in this orchestra. A synchronous embryo and endometrial development are indispensable[1-3].

Implantation begins when the trophectoderm cells contact the uterine wall, also known as apposition (the first stage of implantation). This stage is followed by adhesion in which the contact of the trophectoderm with the uterine epithelium increases. Finally, in the third stage, the syncytiotrophoblast and cytotrophoblasts penetrate and invade the vasculature and myometrium. In response to the implanted embryo, the uterine stroma undergoes decidualization[4]. Little is known as to the cellular and molecular changes that define the window of implantation of the human endometrium. Understanding the molecular events underlying the development and maintenance of a receptive endometrium is fundamental if we are to further improve the success of embryo implantation during in vitro fertilization (IVF) therapy.

In normal pregnancy, the trophoblast invades the endometrial layers releasing soluble mediators (such as tumor necrosis factor alpha, TNFα) into the maternal circulation, leading to a low-level physiological inflammatory response that is a characteristic feature of normal trophoblast adhesion and controlled embryonic invasion. On the other hand, exaggerated inflammation due to excessive levels of TNFα has been associated with clinical miscarriages and an up-regulation of inflammatory factors, such as interleukins (IL) 10, IL-8, IL-6. A disrupt equilibrium in these factors may account for the failure in implantation (IF)[5,6].

The endometrial development is controlled by sex steroids, which regulate the secretion of growth factors and cytokines and the establishment of the window of implantation. Among these factors, a novel gene/protein, milk fat globule-epidermal growth factor 8 (MFG-E8). In extra-uterine tissues, this secreted protein has been reported to have functions in apoptosis control, neovascularization, cell remodeling, and immunomodulation. Recent studies have shown that MGF-E8 is up-regulated over 2-fold during the receptive phase in the endometrium[7,8]. Also, it is highly expressed in human chorionic villi at all trimesters of gestation and it is up regulated in vitro by Human chorionic gonadotrophin (hCG)of trophoblast origin[9]. MFG-E8 and its integrin receptor participate in trophoblast adhesion in an in vitro model of human implantation[10,11]. Furthermore, it has been demonstrated that endometrial MFG-E8 gene expression, is significantly up-regulated by TNFα[12]. Also, MFG-E8 protein secretion has been associated with microvesicles (MV) from human endometrial epithelial cells and demonstrated that TNFα significantly up-regulated MFG-E8 expression in the secreted MV[13].

These in vitro data therefore strongly suggest that MFG-E8, either soluble and/or in MV, can be used as a detectable biomarker from serum under excessive inflammatory conditions. However, no studies published to date address the possible association between MFG-E8 excess or deficiency and IF, or its relationship to TNFα secretion.

We hypothesize that TNFα and MFG-E8 cooperatively maintain the integrity of the normal endometrium, and that in patients with IF, or recurrent pregnancy loss (RPL) of unexplained origin, excessive TNFα increases the maternal shedding of MFG-E8, disrupting the normal protective effect of this protein, resulting in damage of the endometrial epithelium and impairing trophoblast invasion. We propose that TNFα is up-regulated in serum of women with implantation defects, and this causes perturbation of MFG-E8 secretion. The basis for this hypothesis is found by precedent in human tissue and murine models as well as by way of our largely unpublished preliminary data.

Materials and Methods

Study design and patient populations

This is a multicenter, prospective controlled clinical pilot study, from December 2015 through January 2020 included three groups of patients between 18 and 35 years of age. Fertile controls (C): women who participated in the donor egg program as egg donors with regular menstrual cycles, previously confirmed ovulation, and who were of proven fertility (n=10), patients with unexplained IF: patients who have failed implantation following 2 or more frozen embryo transfers of good quality blastocysts (n=10), and patients with recurrent unexplained first trimester miscarriages: at least 2 consecutive miscarriages under 10 weeks of gestation of unexplained origin, after spontaneous or IVF conceptions (n=10).

Patients with history of uterine surgery, abnormal uterine cavity (fibroids, endometrial polyps, adhesions, adenomyosis and congenital uterine abnormalities), hydrosalpinx, diminished ovarian reserve, harboring chromosomal rearrangements, thrombophilia, or autoimmune diseases were excluded.

Intervention

Participants were asked to come in their natural cycle in which serum MFG-E8, TNFα, estradiol (E2) and progesterone (P4) levels were quantified in the early proliferative (cycle day 2) and secretory phases (urinary LH+7 days). Additionally, an endometrial biopsy was obtained on urinary LH+7 days for MFG-E8 and TNF α protein and gene expression analysis. A clinician performed the endometrial biopsy procedures using a pipelle, a plastic biopsy catheter approximately 3 mm in diameter (e.g., Pipelle de Cornier, Laboratoire CCD, France). Participants were advised to attend with a full bladder and to take pain medication before the procedure, according to clinic protocols. The procedure was carried out as described previously[14]. If it was not possible to insert the pipelle into the uterus, a tenaculum, local anesthetic, and cervical dilatation were permitted. All women provided written informed consent.

The following kits were used: ELISA Kit for Milk Fat Globule EGF Factor 8 from USCN Life Science Inc, with the cat. No. E91286Hu-96 tests, and TNFα, Life Technology Inc., No. KHC3011 to measure MFG-E8 and TNFα respectively. A preliminary study was performed under IRB approval (EVMS IRB# 14-05-WC-0078) to validate the technical usefulness of these commercial kits. Immulite Immunoassay System (Siemens, NY) was used to measure estradiol and progesterone. Manufacturer's recommendations were followed to perform the tests. Positive and negative controls as well as serial dilutions were tested. A standard curve was created by plotting the mean optical densities (OD). Samples were run in duplicates. Endometrial biopsies were placed in sterile normal saline and immediately processed for mRNA (quantitative RT-PCR) and protein extraction (Western blot) for MFG-E8 and TNFα, in order to correlate their levels of endometrial expression with the serum levels of these biomarkers.

Outcome measures

The primary outcome was serum and endometrial MFG-E8 and TNFα. Secondary outcome measures included serum estradiol and progesterone levels.

Statistical analysis

Continuous data was reported as mean+-SD with Clopper-Pearson binomial 95% confidence intervals (95% CI). Groups were compared using ANOVA for continuous normally distributed data and Kruskal-Wallis when the conditions of normality were not met. Categorical data was analyzed using Fisher exact or Chi squared tests as appropriate. Statistical analyses were performed using SAS version 9.4 (SAS institute Inc., Cary, NC, USA). All p-values were two-sided and were considered significant if less than 0.05.

Regulatory approval

This study was approved by EVMS IRB committee (#15-01-FB-004).

RESULTS

Ten women were assigned into each group. Patient demographic and cycle characteristics are described in **Table 1**. No statistical differences were found in age, body mass index (BMI), antimullerian hormone (AMH), baseline follicle stimulating hormone (FSH) among cohorts.

Mean serum E2 and P4 levels were similar among all groups in both the proliferative and secretory phases: E2 proliferative (C 69.19±26.64 pg/ml, IF 64.19±32.56 pg/ml, RPL proliferative 57.44±38.51; p= 0.55), E2 secretory (C 164.10±52.57 pg/ml, IF 172.57±121, RPL 173.81±.97.35; p=0.25), P4 proliferative (C 0.45±0.15 ng/ml, IF 0.45±0.19 ng/ml, RPL 0.53±0.18 ng/ml; p=0.85), P4 secretory (C 7.42±4.06 ng/ml, IF 7.8±4.56 ng/ml, RPL 8.05±4.38 ng/ml; p= 0.74).

Mean serum TNFα levels were significantly higher in both, the proliferative and secretory phases for the RPL group (proliferative RPL 9.98±4.47 pg/ml, IF 4.73±2.56 pg/ml, C 3.42±1.01 pg/ml; p=0.001 vs secretory RPL 8.67±4.45 pg/ml, C 3.35±0.94 pg/ml, IF 3.85±1.01 pg/ml; p= 0.03).

Mean serum MFG-E8 levels were significantly higher in the IF group during the proliferative phase (IF 373±201 pg/ml, RPL 201±115 pg/ml, C 225.58±109.73pg/ml; p=0.03), but not in the secretory phase (IF 237±101 pg/ml, RPL 189±116 pg/ml, C 199.41±112.43 pg/ml; p=0.15).

	Controls		Implantation failure		Recurrent pregnancy loss		P value
	N=10		N=10		N=10		
	Mean	SD	Mean	SD	Mean	SD	
Patient age (years)	26.8	4.1	27.2	6.1	27.4	4.8	0.44
BMI (kg/m^2)	21.6	4.2	22.9	2.3	22	3.5	0.53
Baseline FSH (IU/mL)	6.4	3.1	6.7	4.2	6.5	4.8	0.68
Antimullerian hormone (ng/ml)	3.1	2.4	3.2	2.3	3.2	3	0.35
Baseline Antral Follicle count	16.3	5.1	14.7	5	15.8	4.8	0.70

Demographic characteristics among cohorts

Table 1

Note: Data presented as mean, percentages and standard deviation, unless stated otherwise.

Abbreviations: BMI, body mass index; FSH, follicle-stimulating hormone. Significance established at p < .05.

Endometrial MFG-E8 mRNA levels were significantly lower in the IF and RPL group compared to C (p=0.03). TNFα mRNA levels were not statistically significant among groups (p=0.12).

DISCUSSION

To our knowledge, this is the first study to determine whether IF and RPL can be predicted in serum prior IVF by measuring MFG-E8 and TNFα. In a normal pregnancy, mediators such as TNFα are released creating a physiological inflammatory response. However, an exaggerated release of TNFα has been associated with IF and recurrent pregnancy loss (RPL). Recent studies demonstrated that TNFα up-regulates the expression of inflammatory factors such as MFG-E8[11]. MFG-E8 is known to modulate implantation by acting at various levels of the trophoblast and endometrial compartments[12]. Hence an overexpression of this protein may result in apoptosis, endometrial damage, and impaired implantation. Our results showed that mean serum MFG-E8 levels were significantly higher in the IF group during the proliferative phase but not in the secretory phase and that.

TNF-α, an important pro-inflammatory and pro-apoptotic cytokine, may have both physiological and pathological roles in endometrial homeostasis. Numerous studies have shown that TNF-α induces cells to undergo apoptosis and have suggested that local TNF-α production is critically involved in placental trophoblast invasion[15]. During normal implantation, the inflammation caused by TNF-α may improve embryo implantation and assist with endometrial repair in response to injury[16,17]. However, TNF-α levels could be deregulated in certain pathological conditions, such as when maternal and fetal vascular perfusion are reduced. Our results demonstrated that mean serum TNFα levels were significantly higher in both the proliferative and secretory phases for the RPL group.

Serum estradiol levels have not been reported to discriminate between fertile and infertile patients. Our results are in accordance with these findings showing that serum estradiol levels did not significantly differ between groups. And as expected, mean serum progesterone levels were significantly higher in the secretory phase compared to the proliferative phase in all groups.

This new concept could lead to the discovery of novel mechanisms and holds strong potential for diagnostic and therapeutic alternatives. The findings may have a significant clinical impact, providing the basis for the potential therapeutic use of MFG-E8 and TNFα antagonists[18-21]. Recent studies have shown that MFG-E8 offers therapeutic benefits by mitigating inflammation and tissue injury after hemorrhagic stroke and aiding in the healing of injured intestinal mucosa[21,22]. Additionally, TNFα inhibitors have been demonstrated to significantly increase IVF success rates in infertile patients. By understanding the physiology and pathophysiology underlying implantation, we can continue to develop innovative research ideas to improve IVF outcomes and prevent IF and RPL.

CONCLUSION

These novels differentially expressed serum and endometrial markers may provide information on the physiology of implantation and could generate the basis for non-invasive diagnostic tools and therapeutic use of MFG-E8/TNFα antagonists in women with IF and RPL.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

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