Modern Evaluation of Endometrial Receptivity

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Introduction

Endometrial receptivity is an essential component in human reproduction defined as a physiological status in which the endometrium acquires an adhesive phenotype that permits embryo implantation. Adequate proliferation and differentiation during the proliferative phase must be followed by timely secretory changes during the luteal phase with stromal decidualization. However, an impaired synchronization between embryo and endometrium will lead to implantation failure. The acquisition of endometrial receptivity occurs during a specific period of time known as the window of implantation (WOI) in the midsecretory phase of the menstrual cycle [1, 2].

During the WOI, the luminal epithelial cells suffer morphological remodelling leading to polarity loss, while apical microvilli known as pinopodes appear in the luminal surface while adhesive molecules as integrins and mucins, and some specific cytokines have been found to be overexpressed during the WOI. At the same time, the glandular epithelial cells increase in size and secrete the required factors to nurture the implanting embryo. Then, the endometrial stromal cells start a differentiation process referred to as decidualization characterized by acquisition of rounded phenotype, increased storage of nutrients, accumulation of uterine natural killer cells, and the vascular reorganization surrounding the site in which implantation is to occur.

Wilcox et al. [3] determined that the human embryo implants 8–10 days after ovulation. The methods they used to determine ovulation were never officially adopted; however, the clinical community has accepted their assertion that the endometrium in all patients becomes receptive during that time. Additionally, implantation has been believed to be equally successful over these 3 days, regardless of individual variations or hormonal treatment received (this is observed to occur within natural cycles, controlled ovarian stimulation, and hormonal replacement cycles). If the embryo does not implant, the decidualized endometrium is shed leading to menstruation, and a new functional endometrial layer is regenerated in the next menstrual cycle.

However, recent studies have demonstrated that the WOI varies between patients [4] and that endometrial microbiome plays a paramount role in implantation [5], leading the diagnosis of

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L. A. Stadtmauer, I. Tur-Kaspa (eds.), *Ultrasound Imaging in Reproductive Medicine*,
https://doi.org/10.1007/978-3-030-16699-1_22

endometrial receptivity to a crucial role in ART to avoid implantation failure and, consequently, improve pregnancy outcomes.

The aim of this chapter is to review the current methodologies used in evaluating the endometrial function.

A Quick Look Back at Endometrial Assessment Approaches

Several studies have composed a puzzle of endometrial factor where 360° must be considered. The pieces of this puzzle belong to diverse scientific approaches to find the proper moment for embryo implantation.

The Noyes criteria [6], based on the histological features of the different compartments of the endometrium across the menstrual cycle, reflect the differentiation of the endometrium each day of the luteal phase. However, the accuracy and functional relevance of these criteria as a predictor of endometrial receptivity have been questioned in randomized studies [7, 8], leading to the discontinuation of this diagnostic method.

The use of high-resolution ultrasonography as a cheap and noninvasive method of assessment of uterine receptivity arose as a necessity to the evaluation of the endometrial development. In the 1990s, magnetic resonance imaging (MRI) demonstrated significant differences in the relative MRI signal intensities of the myometrium between conception and non-conception cycles [9], but the translation of this technique to the clinic did not succeed due to practical obstacles such as availability and cost. Ultrasonography, color Doppler, and most recently 3D ultrasonography and power Doppler angiography can help to assess several markers of implantation in a quick, noninvasive and relatively low-cost way (Fig. 22.1). Such techniques have also been used to study reproductive disorders as the effects of hydrosalpinx in the regulation of endometrial receptivity [10] and to identify intrauterine adhesions in infertile women with Asherman's syndrome undergoing hysteroscopic adhesiolysis in order to help the improvement of endometrial receptivity [11]. However, data extracted from studies analyzing the role of ultrasound for predicting endometrial receptivity are controversial.

Immunohistochemical staining has been used to complement the analysis of endometrial dating by Noyes criteria. For this purpose, several markers of endometrial receptivity have been used to assess the abundance and localization of adhesion proteins, cell cycle progression of

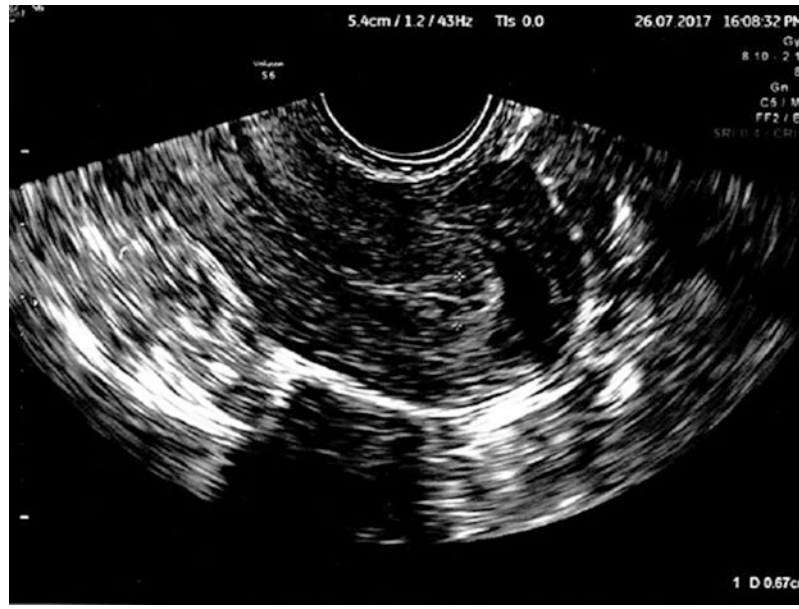

Fig. 22.1 Trilaminar endometrium assessed by ultrasound

endometrial cells, or the regulation of immune cells in endometrial specimens. Because endometrial receptivity involves an adhesive phenotype, the abnormal expression of adhesion proteins has been studied as potential markers of uterine receptivity. In this regard, alpha-1, alpha-4, and beta-3 integrins are observed in women with unexplained infertility [12] and constitute the basis of E-tegrity a clinical diagnosis test of endometrial receptivity (http://www.etegritytest.com). However, the association of beta-3 integrin with endometriosis is the main limitation of this test that may present cofounding results. Also, the expression and subcellular localization of two proteins involved in endometrial cell's mitotic cycle, cyclin E and cyclin-dependent kinase inhibitor p27, have been used to determine the endometrial receptivity in donor ovum recipients [13] and are the rationale of the endometrial function test® (EFT®) (http://klimanlabs.yale.edu/infertility/eft/).

Endometrial receptivity has been also analyzed by the immunohistochemical detection of immune cells involved in maternal adaptation to the semiallogenic developing embryo, especially uterine natural killer (uNK) cells. In this regard, it has been reported that high abundance of cytotoxic CD16(+) cells or the ratio NKp46(+):CD56(+) can be used as a marker of increased endometrial inflammation that correlates with implantation failure or pregnancy loss. However, the prognosis value of measuring total uNK cells or CD56(+) cells in endometrial specimens remains uncertain [14].

Using the single-molecule approach, many putative biochemical markers have been proposed as predictors of endometrial receptivity, but none of them have achieved the status of a diagnostic or predictive clinical tool [15]. More recently, the status of human endometrium has been more objectively classified by using transcriptomic profiling throughout the menstrual cycle [16, 17], as well as during the window of receptivity [18]. These pioneering diagnostic techniques, in conjunction with accumulated evidence that the endometrial molecular profile is unique during the WOI, prompted us to translate the molecular expression profile of the endometrium as it relates to endometrial function using transcriptomics.

Transcriptomic Assessment of Endometrial Receptivity

For more than 65 years, histologic evaluation has been the standard for clinical diagnosis based on morphological observations. The limitations of this method underscore a need to understand the genetic mechanisms underlying the observed histological changes. The possibility of classifying the endometrium using transcriptomic profiles offers an objective and powerful tool in clinical applications and is independent of the specific functional meaning of the transcriptomic signature [19].

The transcriptome reflects the genes that are being actively expressed at any given time in a specific cell population. Transcriptomics also allows gene expression characterization at the messenger RNA (mRNA) level of a population, leading to a sample-specific molecular profile. Several areas have been covered, from the transcriptomic expression throughout the menstrual cycle to the changes identified under different treatments or gynecological conditions. However, the main interest has been the identification of the specific transcriptomic signature that can diagnose the receptive function to develop a mathematical function based on the expression profiles that can accurately predict the biologic group, diagnostic category, or prognostic stage and improve the effectiveness of reproductive treatments.

Based on this research, in 2011 our group identified the transcriptomic signature of endometrial receptivity, characterized by the expression of 238 genes unique to the WOI [4]. This led to the launch of the endometrial receptivity analysis (ERA) (https://www.igenomix.com/tests/endometrial-receptivity-test-era/).

The original design of the ERA test was based on microarray data. Following the accumulation of data after 7 years from the analysis of more than 35,000 transcriptomic profiles, algorithms have been developed to provide a new

computational predictor based on next-generation sequencing (NGS) technology. The new ERA predictor defines a shorter, optimal WOI frame. To define this receptivity signature, the training of the new predictor was performed by selecting well-defined and curated endometrial profiles. Only receptive profiles from patients that were receptive and became pregnant in this cycle were used. For the non-receptive stages, training was performed using only samples in which receptivity was reached after following the specific recommendation associated with that profile. This technique has been refined and improved such that the predictor potency provides more detailed insights into the use of gene signature profiles for patient stratification.

To perform ERA, mRNA is extracted from an endometrial sample. After determining its quantity and quality, the sample is analyzed using NGS coupled with a computational predictor and an algorithm able to identify the receptivity of the endometrial sample (Fig. 22.2).

Although it has been classically considered that the WOI opened the same "standard" day of the menstrual cycle for all the women, it is possible that a displacement of the WOI occurs in some women. In these cases, the assay provides the personalized WOI of a specific patient independent of endometrial histology (Fig. 22.3). This strategy allows performing a personalized embryo transfer (pET) on the day in which the endometrium is receptive [20] (Fig. 22.4).

Interpretation of Era Results

Receptive

A receptive endometrial profile is divided into three sub-signatures: optimal receptive, early receptive, and late receptive.

- An optimal receptive profile indicates an optimally receptive endometrium. In this case, it is recommended to proceed with the embryo transfer in the same type of cycle and on the same day in which the endometrial biopsy was performed.
- An early receptive profile indicates that the endometrium is entering the receptive phase but needs 12 more hours of progesterone administration in a hormone replacement therapy (HRT) cycle to acquire an optimally receptive profile.
- A late receptive profile indicates that progesterone administration should be reduced by 12 hours in a further cycle to achieve optimal receptivity.

The early and late receptive profiles are considered transitional profiles, and it is recommended that personalized embryo transfer be performed after following the indicated treatment with progesterone (12 more or less hours) without need of further verification.

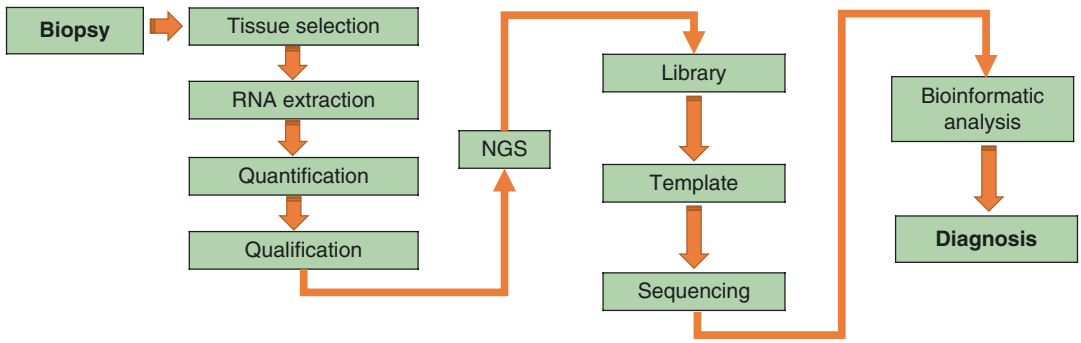

Fig. 22.2 Flow chart of the ERA laboratory and data analysis procedure

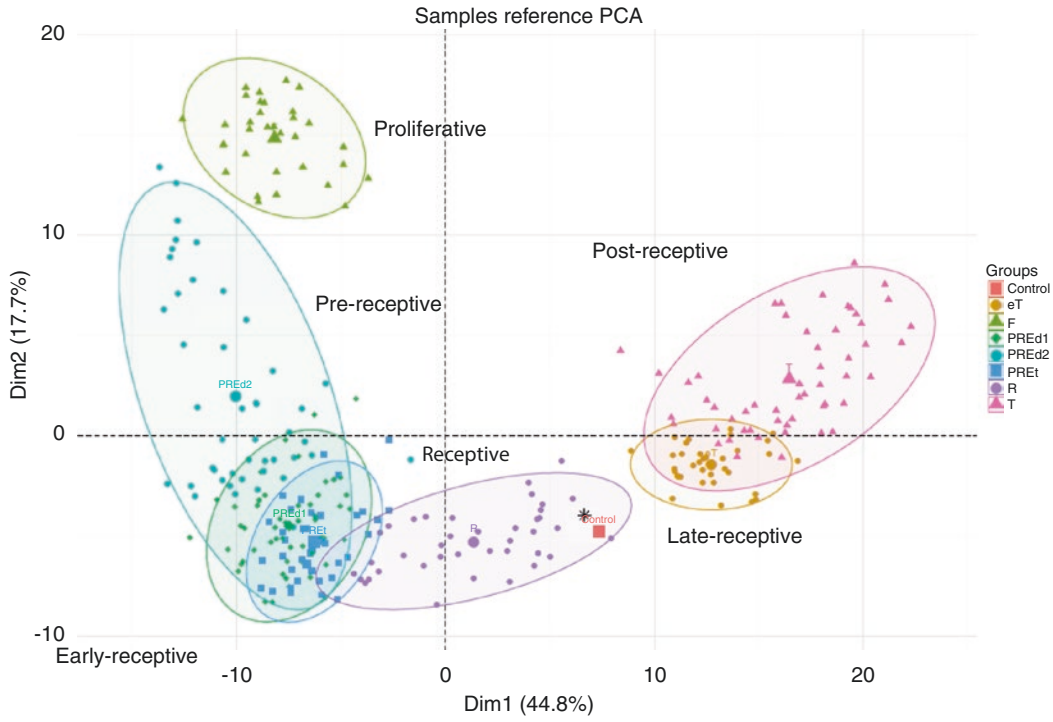

Fig. 22.3 Individual variations of the window of implantation and personal embryo transfer

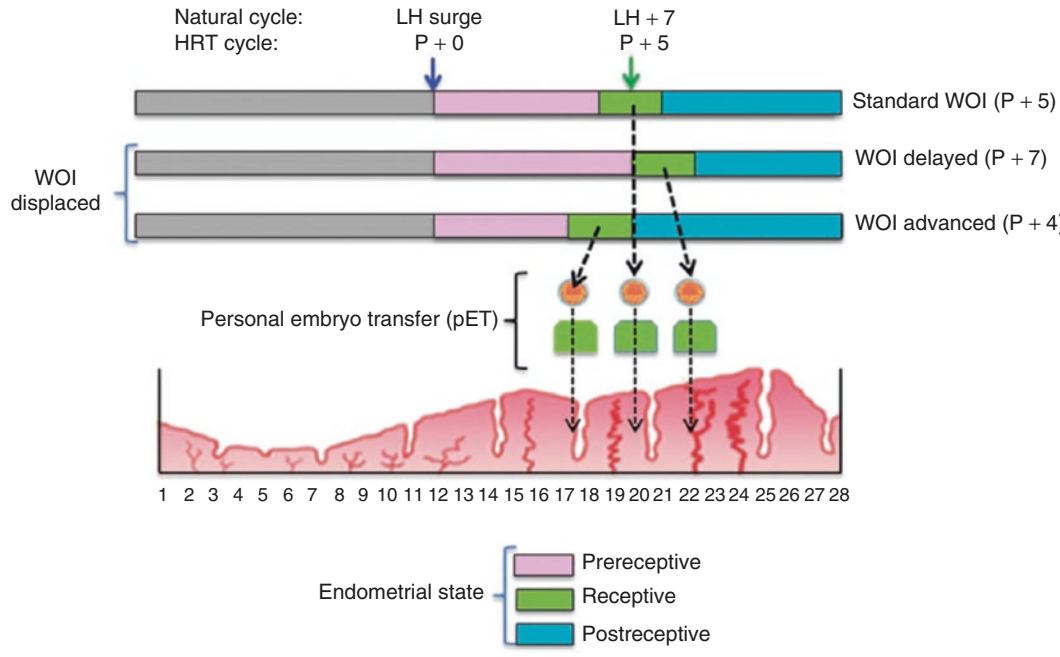

Fig. 22.4 Principal component analysis of the ERA predictor set and the classification parameters for all transcriptomic profiles

Non-receptive

Our algorithm revealed that the gene expression profile in a non-receptive endometrium is usually due to a physiological displacement of the WOI. In addition to a proliferative profile, which generally indicates that the endometrium has not been exposed to endogenous or exogenous progesterone, a non-receptive patient can also show a pre-receptive or a post-receptive transcriptomic profile.

- A pre-receptive diagnosis indicates that the transcriptional activation necessary to achieve receptivity has not yet occurred. The patient needs 1 or 2 more days of progesterone administration from the day of cycle in which the biopsy was taken to reach the receptive state.
- A post-receptive diagnosis indicates that the endometrium has already passed the ideal window for embryo implantation in the day of the cycle when the biopsy was performed, so 1 or 2 days less of progesterone administration is required to achieve receptive status.

A recent study [21] investigated whether the contribution of the endometrial factor could be identified with the ERA test and if actionable results can lead to improved outcomes. In this study 88 patients with a history of euploid blastocyst implantation failure underwent ERA testing between 2014 and 2017. Reproductive outcomes were compared for patients undergoing FET using a standard progesterone protocol versus those with non-receptive results by ERA and subsequent FET according to a personalized embryo transfer (pET) protocol. Results show that 22.5% of patients with at least one previously failed euploid FET had a displaced WOI diagnosed by ERA and qualified for pET. After pET, implantation and ongoing pregnancy rates were higher (73.7 vs 54.2% and 63.2 vs 41.7%, respectively) compared to patients without pET, supporting the optimal results obtained by ERA.

An international randomized controlled study is underway to perform endometrial assessment during fertility screening at the beginning of reproductive care (the ERA as a diagnostic guide for personalized embryo transfer. ClinicalTrials.gov Identifier: NCT01954758). An ERA RCT consortium was created to include 28 clinics worldwide. This randomized study included patients undergoing transfer at the blastocyst stage (day 5 or day 6) in their first IVF/ICSI cycle with a body mass index (BMI) between 18.5 and 30, younger than 37 years old, and a normal ovarian reserve. If any pathology affecting the endometrial cavity existed, patients were previously operated. Exclusion criteria were recurrent pregnancy loss and/or severe male factor.

The study consists of three arms comparing fresh embryo transfer under stimulation protocol, frozen embryo transfer at P + 5 in HRT cycles, and pET guided by ERA with frozen embryos in HRT cycles. At the midpoint of recruitment, results show significant differences between pregnancy rate (PR) for pET arm (85.7%) versus fresh embryo transfer (FET) (61.7%) and deferred embryo transfer (DET) (60.8%). Although not yet significant, there are also differences in implantation rate (IR) (47.8% for pET, 35.3% for FET, and 41.4% for DET) and in ongoing pregnancy rate (OPR) per embryo transfer (55.1% for pET, 43.3% for FET, and 44.6% for DET). These interim results were published in the American Society of Reproductive Medicine (ASRM) 2016 scientific congress [22] and show that 14% of patients have a displaced WOI whose correction would likely result in an effective cost-benefit strategy at the first clinical appointment.

Other studies have attempted to describe the transcriptomic profile of endometrial receptivity [23]. A lately meta-analysis found that 57 genes, including genes present in the ERA (i.e., SPP1, ANXA4, CLDN4, DPP4, GPX3, MAOA, and PAEP), were identified as potential receptivity biomarkers in multiple studies and are the most representative panel for predicting the WOI [24]. However, these findings have not been translated to the clinic.

Endometrial Microbiome: The New Kid on the Block

Humans are inhabited by trillions of microbes, residing in different body sites. The advent of highly sensitive molecular techniques, especially next-generation sequencing, has opened up new possibilities to explore the microbiota of body sites that were previously unexplored or considered sterile and how they participate in our physiology. In fact, a recent study has reported the microbiota across the female reproductive tract [25], showing that there is a continuum of slightly different microbiota expanding gradually from the vagina to the ovaries.

According to recent publications [26, 27], up to 40% of patients undergoing IVF treatments present abnormal vaginal microbiota, being bacterial vaginosis the most common vaginal disorder in reproductive age women and resulting in millions of health care visits per year. It is associated with infertility, endometritis, pelvic inflammatory disease, and increased risk of acquiring HIV, which implies a decrease in reproductive outcomes.

Aiming to find out if there is a specific endometrial microbiota and its putative role in endometrial receptivity and pregnancy outcomes, our group carried out three separate prospective studies which were published in 2016 [5]. In this study, the species-specific sequences of the variable regions of the 16S rRNA gene were analyzed by NGS to evaluate the relative abundances of each microorganism present in the microbial population.

In the first part of the study, it was compared the microbiota of paired samples of endometrial fluid and vaginal aspirates from 13 healthy and fertile subjects in pre-receptive (LH + 2) and receptive phase (LH + 7) in natural cycles. From all the samples, nine were colonized only by *Lactobacillus* spp., while the rest showed a combination of different operational taxonomic units (OTUs) in addition to *Lactobacillus*. In 24 out of 26 paired of samples, there were found slight differences between endometrial and vaginal microbiota, but in 6 of them the bacterial communities were completely different with a high proportion of potential pathogens in the endometrium or in the vagina; the same bacterial OTUs were present in only two pair of samples. The conclusion was that the uterine cavity is not sterile and endometrial and vaginal microbiomes are different in asymptomatic women.

The second part of the study consisted in investigating the hormonal regulation of the endometrial microbiota. For this purpose, the endometrial fluid from 22 healthy and fertile women in natural cycle was taken in LH + 2 and LH + 7 in the same cycle. The bacterial communities found were clustered according to the bacterial different OTUs identified and their abundances. The resulting heatmap showed two sets of samples classifying depending on the percentage of *Lactobacillus* OTUs identified. The first set of samples included those with a high abundance of *Lactobacillus* (over 90%) and very low or nonexistent other OTUs. The second set of samples was formed by lower *Lactobacillus* abundances that coexisted with bacteria represented by other OTUs. Clustering of individual samples showed two groups depending on the abundance of *Lactobacillus* OTUs. This part of the study concluded that endometrial microbiome is not regulated by hormones during the acquisition of endometrial receptivity.

Finally, the functional impact of the endometrial microbiota composition on reproductive outcome in patients undergoing IVF was studied, concluding that low abundance of *Lactobacillus* in endometrial microbiota is associated with poor reproductive outcomes in IVF patients. In fact, subjects with a non-*Lactobacillus* dominant microbiota had significantly lower implantation (60.7% vs 23.1%, $p = 0.02$), pregnancy (70.6% vs 33.3%, $p = 0.03$), ongoing pregnancy (58.8% vs 13.3%, $p = 0.02$), and live birth (58.8% vs 6.7%, $p = 0.002$) rates, as well as higher miscarriage rates (16.7% vs 60%, $p = 0.007$), although this was not statistically significant, compared to those with a *Lactobacillus* dominant microbiota.

In conclusion, the uterine cavity is not sterile. A human endometrial microbiota exists, and it is different from the vaginal microbiomes in asymptomatic women. Furthermore, the endometrial microbiome is not hormonally regulated during the acquisition of endometrial receptivity, and the existence of non-*Lactobacillus* bacteria is related to negative impacts in reproduction.

The molecular microbiology method has also been used to identify culturable and nonculturable endometrial pathogens associated with chronic endometritis such as *Enterobacteriaceae*, *Enterococcus*, *Streptococcus*, *Staphylococcus*, *Mycoplasma*, and *Ureaplasma* [28].

Chronic endometritis is a persistent inflammation of the endometrial mucosa that can be asymptomatic, but it is found in up to 40% of infertile patients and is responsible for repeated implantation failure and recurrent miscarriage.

With this aim, the classical methods used to diagnosis of chronic endometritis (hysteroscopy of the uterine cavity, endometrial biopsy with plasma cells being identified histologically, and microbial culture) were compared to the molecular method by evaluating 113 endometrial samples from patients assessed for chronic endometritis by real-time PCR. The results were lately confirmed by the microbiome assessed by next-generation sequencing. In the endometrial samples with concordant results in the three classic methods, the molecular microbiology diagnosis demonstrates 75% sensitivity, 100% specificity, 100% positive and 25% negative predictive values, and 0% false-positive and 25% false-negative rates, concluding that the molecular microbiology method is a fast and inexpensive diagnostic tool that allows for the identification of culturable and nonculturable endometrial pathogens associated with chronic endometritis.

Improving Endometrial Receptivity Assessment

Despite careful embryo selection, reproductive outcomes resulting from ART remain lower than optimal. Among the multiple factors implied in effective IVF treatment, the primary limiting factor is successful embryo implantation. Implantation failures are caused primarily by poor endometrial receptivity, defects in the embryo, diseases or disorders in the endometrium, and unbalance endometrial microbiome. It is accepted that two-thirds of these implantation failures have their origin in low endometrial receptivity or in a defective endometrium-embryo dialogue.

The functional genomics of endometrial receptivity has been extensively investigated to find transcriptomic markers of endometrial receptivity during the implantation window, with the vision of using this information in diagnosing endometrial receptivity. This advance implies the substitution of other classic biochemical and morphological markers, whose effectiveness has been frequently questioned. The ERA has become the gold standard for the diagnosis of WOI displacement in patients with RIF based on the transcriptomic profile of endometrial samples and has been used for clinical and academic research in endometrial receptivity. Currently, our group is validating a noninvasive test to provide consistent results and make it easier for clinicians to obtain samples and avoid unnecessary pain and discomfort to the patients.

Furthermore, technological advances in genetics have enabled the association of single-nucleotide polymorphisms or genetic variants with several traits and diseases. Genome-wide association studies (GWAS) would be helpful to identify genetic variants in non-receptive patients that are causative of a displacement of the WOI. If such association is found, this information could be finally used for the development of less-invasive test in blood samples for endometrial receptivity assessment, and the genes identified can be target for new research lines oriented to the clinical management of infertile patients with endometrial factor.

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List of Relevant Websites

E-tegrity: http://www.etegritytest.com/
EFT®: http://klimanlabs.yale.edu/infertility/eft/
ERA: https://www.igenomix.com/tests/endometrial-receptivity-test-era/