Clinical application of the endometrial receptivity array

Application clinique de l’array de réceptivité endométriale

Abstract. The endometrial factor is one of the main factors to be considered in reproductive treatments, since it is needed a healthy and receptive endometrium to allow the blastocyst implantation. During the last decade, the human endometrial transcriptome has been broadly studied as the holistic perspective that could be able to assess if the endometrium is receptive and ready for the embryo implantation. Following this line, our group have developed a molecular tool named the Endometrial Receptivity Array (ERA), able to diagnose the endometrial receptivity status. This tool consists of a customized array that analyzes 238 genes related to receptivity coupled to a computational predictor. Until now, the stage of embryo development has been the main factor that guides the timing of embryo transfer (ET) in assisted reproductive treatments, while the window of implantation (WOI) has been considered constant in the general population. Now the ERA test allows us to know when the endometrium has a receptive profile and if there are a displacement of the WOI, which could be advanced or delayed regarding the standard timing. Therefore, we have the ability to analyze when the real WOI opens for each individual patient and to perform a personalized embryo transfer (pET) according to this timing. To prove the efficiency of pET, a pilot study was performed with 17 patients undergoing ovum donation (OD). The results showed higher implantation rates (IR) and pregnancy rates (PR) with pET compared to conventional ET: 40% IR and 60% PR with pET and 11% IR and 19% PR for ET, and of note, the ongoing pregnancy rate with pET was 75% while it was 0% for ET. On the other hand, one in four patients with recurrent implantation failure (RIF) have shown a displaced WOI in one of our studies, and when pET was performed their IR and PR rose to levels similar to patients without displaced WOI. Thus, given our findings, the personalized diagnosis of endometrial receptivity is now possible, and it can help to recover the necessary synchronization between embryo and endometrial development.

Key words: Receptive, window of implantation, personalized embryo transfer, endometrium

Résumé. Le facteur endométrial est l’un des principaux facteurs qui doit être pris en considération en aide médicale à la procréation (AMP) étant donné qu’un blastocyte a besoin d’un endomètre sain et réceptif pour s’implanter. Durant la dernière décennie, le transcriptome de l’endomètre humain a été largement étudié comme une perspective holistique capable d’évaluer si l’endomètre est réceptif et prêt pour l’implantation embryonnaire. Dans cette lignée, notre groupe a développé un outil moléculaire appelé l’Endometrial receptivity array (ERA), capable de diagnostiquer l’état de réceptivité endométriale. Cet outil consiste en l’analyse personnalisée par array de 238 gènes de réceptivité endométriale, couplée à un prédicteur informatisé. Jusqu’ici le stade de développement embryonnaire était le principal facteur qui guidait le timing du transfert embryonnaire (ET) en AMP, la fenêtre d’implantation (WOI) étant considérée comme un paramètre constant dans la population générale. Actuellement le test ERA nous permet de savoir quand l’endomètre a un profil réceptif et s’il existe un déplacement de la fenêtre d’implantation, cette dernière pouvant être avancée ou retardée par rapport au timing standard.

Nous avons donc la possibilité d’analyser le moment d’ouverture de la fenêtre d’implantation réelle pour chaque patient de manière individuelle et de réaliser un transfert d’embryons personnalisé (pET) en fonction de ce timing. Afin de prouver l’efficacité du pET, une étude pilote a été menée chez 17 patients en don d’ovocytes.

Les résultats ont montré des taux d’implantation (IR) et des taux de grossesse (PR) plus élevés avec le pET en comparaison aux ET conventionnels : 40% IR et 60% PR avec pET versus 11% IR et 19% PR avec ET. De plus, le taux de grossesse évolutive avec pET était de 75% alors qu’il était de 0% pour ET. Par ailleurs, dans l’une de nos études, un déplacement de la fenêtre d’implantation était constaté chez 1 patient sur 4 ayant des échecs répétés d’implantation, et quand pET était réalisé chez ces patients, IR et PR augmentaient à des niveaux similaires au groupe sans déplacement de fenêtre d’implantation. Donc, selon nos résultats, le diagnostic personnalisé de la réceptivité endométriale est maintenant possible et peut aider à rétablir la nécessaire synchronisation entre l’embryon et le développement de l’endomètre.

Mots clés : réceptif, fenêtre d’implantation, transfert personnalisé d’embryons, endomètre
The endometrium is the tissue that lines the inside of the uterus cavity and is morphologically divided into the functional and the basal layers. The functional layer is responsible for proliferation, secretion, and tissue degeneration, while the regenerative capacity of this organ lies in the basal layer [1, 2]. The human endometrium is a dynamic organ, with the capacity to undergo physiological changes in response to ovarian steroid hormones, cytokines, and chemokines, among other factors [3, 4]. These changes are cyclic and comprise the menstrual cycle in women; it can be simply divided into the proliferative, secretory, and menstrual phases. The first day of the cycle corresponds to the beginning of the menstrual phase, and after this and up to the point of ovulation, the proliferative phase takes place. In this phase, estrogen levels gradually increase, leading to the proliferation of stromal cells and glands, and to the elongation of spiral arteries. Finally, the secretory phase occurs between ovulation and menstruation, and its onset is characterized by an increase in progesterone levels while estrogen remains at basal levels [2]. If there is no implantation progesterone and estrogen levels decrease at the end of the secretory phase leading to involution of the endometrium, and the restart of the cycle [2].

During the menstrual cycle in humans and primates, the endometrium is non-adhesive to embryos most of the time, and only becomes receptive during a spatially and temporally restricted period when the endometrium acquires a functional and transient status that allows blastocyst implantation [5]. This concept was first suggested in the 1970s [6], and was subsequently strengthened by the work of Wilcox et al., demonstrating that in most successful pregnancies the “conceptus” implants 8 to 10 days after ovulation [3]. This receptive period corresponds to the mid-secretory phase, known as the window of implantation (WOI) [7] and it is the result of the synchronized and integrated interaction of paracrine signaling with ovarian hormones, growth factors, lipid mediators, transcription factors, and cytokines [8].

Since the last century, the endometrium has been studied from the morphological, biochemical, molecular, and cellular points of view, in order to understand how endometrial receptivity works. The results of this work resulted in several endometrial receptivity markers being suggested as diagnosis criteria, and all parameters or characteristics that allow a receptive endometrium to be identified as endometrial receptivity markers were considered [9].

Anatomical criteria have classically been used to date the endometrium, mainly using the histological dating methods defined by Noyes [10, 11]. These criteria consider changes in endometrial tissue morphology described by optical microscopy studies on histological endometrial sections. This method was developed according to criteria that were obtained from 8,000 endometrial biopsies from infertile patients [10, 11]. However, despite its historical importance, its accuracy, reproducibility, and functional relevance have been questioned in retrospective clinical studies [12-14], prospective studies [15-17], and randomized trials [18, 19]. Therefore, although it is still useful in endometrial research, histology is no longer used to guide clinical practice due to its real limitations. On the other hand, several biochemical markers have been proposed and are supported by many publications documenting the presence and regulation of different molecules in the human endometrium during the receptive phase [20-25]; however, none of them have been translated into clinical practice [26]. Therefore, for the reasons described above the objective diagnosis of the endometrial factor had remained neglected until recently.

In recent years, the focus of this research has changed from a single molecule or specific families of molecules to the concept that receptivity is a very complex and multifactorial process that requires a multitude of molecules to adequately describe it [27, 28]. Therefore several researchers have postulated that the present era of ‘Omics’, which refers to high-throughput techniques and massive data analysis, may lead to the design of a molecular tool that can be used for this type of diagnosis which has real clinical applicability [29-35].

Following in this line of thought, our group used transcriptomics to develop a molecular tool able to diagnose the endometrial receptivity status. This tool is named the Endometrial Receptivity Array (ERA), and consists of a microarray-based learning-machine predictive model [29]. The accuracy and consistency of this test has been proven [30], it is more accurate than the classical histological criteria, and is reproducible in different cycles from the same woman. The most relevant contribution of this test is its potential to be used as an objective tool to diagnose endometrial receptivity status, thus guiding personalized embryo transfer (pET) in assisted reproductive treatments [35].

**Transcriptomics for endometrial diagnosis**

The transcriptome reflects the genes that are being actively expressed at any given time in a specific cell population or tissue, therefore it gives the holistic perspective that using single endometrial biomarkers is unable to. During the last decade, the human endometrial transcriptome has been broadly studied resulting in 164 relevant publications [28-33]. The main goal of these first publications was to find a specific gene expression profile for each phase of the endometrial cycle [27-29, 36-40]. The differences between endometrial transcriptomes under diverse treatments [41], between fertile patients and those with
recurrent implantation failure (RIF) [42], between healthy patients and patients with endometriosis [43], and in endometrial cancer [44] have also been studied. Although some of these studies have focused only on one cellular compartment [40], most of them used endometrial tissue biopsies containing all cell types.

Despite the differences in the studies which compared gene expression between different stages of the endometrium, they all found specific transcriptomic signatures, which are commonly visualized with heatmaps [27, 28]. From all of these endometrial profiles, the most interesting are those of the receptive and mid-secretory phases, because embryo implantation is only possible during these stages. These stages are regulated by increasing progesterone levels and are characterized by a ‘transcriptional awakening process’, since most genes are upregulated compared to their expression in the pre-receptive phase [27]. The objective identification of this stage using gene expression microarrays has been pursued since 2002 [28].

In spite of the differing results from publications describing the receptive profile, which is mostly due to protocol and study design differences, all of them conclude that it is possible to accurately catalogue the endometrium at different stages based on its transcriptomic profiles. This finding implies that it is possible to overcome the subjectivity of histological dating by moving towards more objective and accurate molecular endometrial dating. Indeed, microarray-based transcriptomics is already proven to be a robust technology for the classification of several pathologies at the clinical level [28].

**Endometrial receptivity-array development**

Because of the need for an objective and reliable endometrial dating tool, our group started to investigate endometrial transcriptomics. In one of the first publications from our group in this field [39] we used the peak in luteinizing hormone (LH) as a reference to compare endometrial biopsies taken in a natural cycle from the same fertile women, taken both in the pre-receptive and receptive stage, two and seven days after the LH surge respectively (LH+2 and LH+7). The results showed 211 genes which were differentially expressed between these stages and, moreover, some of these genes had been previously related to receptivity. We then started the process of developing a specific tool to identify the transcriptomic signature of the WOI.

To select genes that were putatively involved in receptivity we analyzed the gene expression profile of endometrial biopsies obtained in a natural cycle at LH+1, +3, and +5 (the pre-receptive stage) and at LH+7 (the receptive stage). Using the stringent criteria of a 3.0-fold change increase and a false discovery rate of less than 0.5, we selected 238 genes with differential expression between these stages. A customized Agilent gene expression microarray was then designed by incorporating 569 existing probes including these 238 genes along with several probes for positive and negative hybridization controls [29].

In order to perform the transcriptomic analysis a bioinformatic tool able to classify samples according to their gene expression profile was also needed. Predictors are used to classify microarray data into specific classes based on criteria previously constructed with a model dataset containing the classes used to phenotype the samples [28]. To set this up, a set of endometrial biopsies were taken from fertile women in the proliferative (days 9-12 of the menstrual cycle), pre-receptive (LH+1 to LH+5), receptive (LH+7), and post-receptive (LH+9 to LH+11) stages, which were hybridized with the customized array and used as a training set. The gene expression profile for each sample was used to train the bioinformatic predictor in order to classify samples into each different stage. Once the array and the predictor were designed, a set of samples (proliferative, pre-receptive, receptive, and post-receptive) were used to validate each transcriptomic signature. The results that we obtained indicated an ACC of 95 % and an AUC of 0.94, with a specificity and sensitivity of 0.8857 and 0.99758 respectively [29]. The final tool developed, comprising this customized array and the computational predictor, was named the ERA. By analyzing an endometrial biopsy, the ERA is able to diagnose the endometrial stage of an endometrial sample on the specific day on which that biopsy was taken.

**Endometrial receptivity array accuracy and consistency**

Historically the histological dating method was the gold standard used to evaluate the endometrial stage. Therefore we evaluated the accuracy of both the ERA and the histological Noyes methods in order to check if our molecular dating tool was more reliable. To do this a dating set, composed of 49 endometrial biopsies, was analyzed by the ERA microarray and by two independent pathologists and the concordance between each method was statistically analyzed by the quadratic weighted Kappa index [30]. The 49 samples were collected in a natural cycle at different stages (receptive, pre-receptive, proliferative, and post-receptive) and these samples were classified according to the day of the cycle in which they were taken (for the proliferative samples) or by taking the day of the LH surge as a reference (for receptive, pre- and post-receptive samples). The histological study involved
Clinical applicability of the ERA test and personalized embryo transfer

Until now, the stage of embryo development has been the main factor that guides the timing of ET in assisted reproductive treatments. This is based on a set of assumptions that: the WOI opens on day 19 or 20 of the cycle, remains open for 4 or 5 days [45], and that this timing is constant in the general population, therefore personalization of the endometrial factor was never considered in previous studies. Now, with an objective and reliable test to precisely diagnose which day the endometrium shows a receptive profile, we have the ability to analyze when the real WOI opens for each individual patient.

The ERA has been designed to identify endometrial receptivity by comparing the genetic profile of a test sample with those of controls collected on LH+7 in a natural cycle, or on P+5 (on day 5 of progesterone administration after proper estradiol priming) in hormone replacement therapy (HRT) cycles. Days LH+7 and P+5 are considered as the standard days of receptivity in women because they match with the timing of blastocyst implantation. To evaluate the endometrial factor of a patient for a subsequent ART, an endometrial biopsy must be taken in the same kind of cycle (natural or HRT) in which an ET will be performed in the subsequent cycle and on these standard days (according to the kind of cycle selected).

A non-receptive ERA diagnosis on these days indicates that the endometrium is not ready for embryo implantation, making any ET on this day unproductive. However, the ERA analysis not only obtains this information, it also defines what stage the endometrial sample is in (proliferative, pre-receptive, or post-receptive). Therefore, a pre-receptive result could imply a delayed WOI, meaning that one or two more days of exposure to progesterone are required for the endometrium of this patient to enter into the receptive stage. In the same way, a post-receptive result may imply an advanced WOI, meaning that the endometrium of this patient requires fewer days of progesterone exposure in order to reach the receptivity stage. This highlights the necessity to synchronize embryonic and endometrial development, and the personalization of the timing of ET. In these cases a second biopsy to validate the displaced WOI is needed, for which the predictive computer recommends a specific day for the biopsy according to the result of the first analysis. If a receptive result is obtained with this second biopsy, then it is recommended that a pET is performed in the same conditions (type of cycle and day) in the subsequent cycle.

To prove the efficiency of pET, a pilot study was performed with 17 patients undergoing ovum donation (OD) [46]; in all these patients, the same approach to diagnose their WOI was performed and then pET instead of routine ET was implemented resulting in normalization of their reproductive outcome. In order to compare the pET clinical outcome with the previous cycles, we only considered as ET those previous cycles in which the conditions were the same as for pET (i.e. OD and HRT cycle) but with different day for the embryo transfer. The ET day was later diagnosed as non-receptive by the ERA and pET was performed on the day indicated as receptive by the ERA. The results showed higher implantation and pregnancy rates (IR and PR respectively) with pET compared to conventional ET: 40 % IR and 60 % PR with pET and 11 % IR and 19 % PR for ET, and of note, the ongoing pregnancy rate with pET was 75 % while it was 0 % for ET [47].

This pilot study shows that we can now diagnose displacements of the WOI and personalize ET for each patient as necessary, helping to improve clinical success from the endometrial perspective using this novel approach.
Clinical applicability of the ERA in recurrent implantation failure patients

RIF is a major cause of infertility in otherwise healthy women and is a problem that remains largely unaddressed and is still poorly characterized [47]. Among other factors implantation failure may be caused by embryo quality, the endometrium, or the dialogue between them. Now, with the ERA tool, it is possible to evaluate if RIF in individual patients has its origin in a displacement of the WOI: an important development because the WOI had previously been considered constant for all patients, including those with RIF. In fact, some published data, e.g. delayed endometrial cyclical timing in RIF patients revealed by classical morphometric analysis [48], or deregulation of the gene expression cycle in RIF versus fertile women [42], suggest that transcriptomic modification of the endometrium may be a cause of RIF. Thus, the diagnostic and clinical value of the ERA test and pET in RIF patients was tested in a prospective interventional, multicenter, clinical trial [35].

This trial compared a RIF group, comprising a total of 85 patients either 51 years old or younger and with at least three previous failed OD cycles, or IVF patients younger than 40 years old and with at least three failed IVF cycles, to a comparison group. Implantation failed in all the RIF patients after having had at least four morphologically high-grade embryos transferred, and there was no other explanation for RIF. The control group consisted of 25 IVF and OD patients with the same age inclusion criteria, but with only one or no failed ART cycles [35]. RIF and control patients underwent ERA diagnosis using an endometrial biopsy obtained either on day LH+7 in a natural cycle or on day P+5 in an HRT cycle. One of the most significant results was that the ERA test identified 88% of the samples as receptive versus 12% as non-receptive in the control group, while in the RIF group 74.1% of the samples were receptive versus 25.9% which were non-receptive. The proportion of RIF patients undergoing IVF versus OD was 61/39 versus 40/60 in the comparison group patients, which indicates that most implantation failure problems related to the embryo had been solved by implementing the ERA test results: in other words one in four patients with RIF has a displaced WOI.

Most endometrial samples classified by the ERA predictor as non-receptive were pre-receptive, and a few were post-receptive. In these patients, a second ERA test was suggested (and was performed in 18 cases) to confirm the suspected WOI displacement. In patients with a pre-receptive diagnosis for the first biopsy, the second one was recommended on LH+9 in natural cycles or on P+6 or P+7 in HRT cycles, depending on the specific expression profile of each sample. In the post-receptive ones, the second test was recommended on P+4 or P+3. A receptive result was obtained for 15 of these second biopsies, thus validating the suspected WOI displacement.

Because of the large proportion of RIF patients with a displaced WOI we suspected that the origin of their RIF may lie in the incapacity of their endometrium to implant on the standard ET day. Functional proof of this concept comes from the pET results for RIF patients previously diagnosed as non-receptive following application of their individual ERA test results indicating receptivity with a displaced WOI. Clinical follow-up was possible for 8 of these patients: their implantation rates (IR) and pregnancy rates (PR) rose to levels similar to patients with a receptive result with the first ERA test, achieving a 38.5% IR and 50.0% PR compared to a 33.9% IR and 51.7% PR in the comparison group. It is also interesting that the ERA test also identified a notable proportion of control patients (first visit patients) with an out-of-phase endometrium in which no embryo implantation could have been expected.

We showed that there is an increased percentage of WOI displacement in RIF patients compared to control group patients, leading to the concept of pET as a therapeutic strategy. However, although this molecular tool has already been demonstrated as effective in RIF patients, a prospective randomized clinical trial on the effectiveness of the ERA test in the infertility work-up is also ongoing (‘The ERA as a diagnostic guide for personalized embryo transfer’, ClinicalTrials.gov Identifier: NCT01954758).

Conclusions

Until now, personal and unique treatment modifications guided by endometrial biomarkers have never been considered. However, with the development of the ERA we have learned that a woman can have a delayed or advanced WOI which is not open on the day generally considered as standard for receptivity. Moreover, we have shown that sampling the same woman a few days (or even hours) sooner or later than the first non-receptive ERA test can be sufficient to find her personal WOI. This diagnosis has been proven to consistently diagnose the timing of the WOI and is more accurate than histological criteria. Thus, given our findings, the personalized diagnosis of endometrial receptivity is now possible, and it can help to recover the necessary synchronization between embryo and endometrial development. Indeed, patients have a higher PR and IR with pET compared to routine ET, and when pET is performed in RIF patients their IR and PR levels normalize to non-RIF patients. In conclusion, the most important contribution of the ERA test at the clinical level is its objective WOI diagnosis which has led to the creation of the concept of pET.
Disclosures: Ruiz-Alonso M, Gómez E and Rincón-Bertolín A, are employers of IVIOMICS S.L.
Simon C, is co-inventor of the patent Gene expression profile as an endometrial receptivity marker licensed to IVIOMICS S.L.

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