Profiling the gene signature of endometrial receptivity: clinical results

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This article highlights the need for methods to objectively diagnose endometrial receptivity as a factor contributing to infertility in female patients. The correct identification of the appropriate window of implantation in a given patient, by using endometrial receptivity biomarkers, can help to prevent reproductive failure resulting from misplaced timing of the endometrial window of implantation (WOI). Although to date no single, clinically relevant morphologic, molecular, or histologic marker capable of indicating endometrial receptivity status has been identified, global transcriptomic analysis of human endometria performed in the last decade has given us insights into a genomic signature that is capable of identifying endometrial receptivity. As a consequence, a genomic tool named the Endometrial Receptivity Array (ERA), based on a customized microarray, was developed, and along with it a specially trained bioinformatic prediction computer algorithm was created to identify WOI timing in the endometrium. This tool has proven more accurate and consistent than histologic (Noyes) dating at identifying the personalized WOI day, thus leading to the new clinical concept of personalized ET on the optimum day of endometrial receptivity, identified individually case by case. (Fertil Steril® 2013;99:1078–85. ©2013 by American Society for Reproductive Medicine.)

Key Words: Endometrium, transcriptomic, infertility, Endometrial Receptivity Array (ERA), personalized embryo transfer (pET)

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Personalized medicine is a well-accepted concept in reproductive medicine and is implicated in diverse clinical situations, for example adjusting the hCG dosages used for ovarian stimulation according a patient's remaining ovarian reserve and body mass index; selecting specific fertilization techniques (e.g., intracytoplasmic sperm injection, IVF, or both according to sperm features and clinical background); monitoring embryo development in vitro; or ET based on both the number and quality of embryos as well as clinical background. Unfortunately, no single specific endometrial receptivity biomarker has been identified, meaning that objective diagnosis of endometrial receptivity remains neglected in the patient infertility workup, along with opportunities for personalized medical approaches to improving clinical success from this perspective.

Human endometrium is a complex and dynamic tissue that undergoes cyclical physiologic changes in response to steroid hormones. The embryo is unable to adhere to it through most of the menstrual cycle in humans, except during a short, self-limited period, in which the endometrial tissue acquires a functional and transient status that permits blastocyst adhesion (1) and is therefore receptive. This specific period, which is regulated by a combination of ovarian steroid hormones and genetic factors, is known as the window of implantation (WOI) and lasts 5 to 6 days after an exogenous or endogenous P impregnation (2, 3).

The luminal endometrial epithelium acquires a receptive phenotype through specific structural, functional, and morphologic changes, which include plasma membrane (4) and cytoskeletal (5, 6) modifications, known as the plasma membrane transformation (4), although in practical terms it cannot be used for diagnostic purposes. Indeed, despite the historical relevance of traditional histologic endometrial dating criteria defined by Noyes (7, 8), its accuracy,

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reproducibility, and clinical utility has been repeatedly questioned in randomized (9, 10) and prospective studies (11–17). Although it still aids endometrial research, histology is no longer used to guide clinical practice owing to its real and perceived limitations.

Nevertheless, it has been suggested that pinopodes, ectoplasmic projections on the surface of endometrial epithelial cells (18, 19), may be a good morphologic marker for diagnosing endometrial receptivity status, although their real function remains unknown. Pinopodes are generated by endocytosis of endometrial fluid from luminal epithelial cells, leaving a vacant endometrial cavity optimal for generating the required mechanical contact between the blastocyst and the endometrium during implantation. However, it has been reported that pinopodes are still present in the postreceptive period and therefore cannot be used as a reliable morphologic receptivity marker (20).

Biochemical markers are ideal as alternatives to classic Noyes criteria, and indeed many articles have documented the presence and regulation of a myriad of molecules in the human endometrium, found within different cellular compartments during the receptive phase. Among these, integrins (21), mucin 1 (MUC1) (22), calcitonin (23), leukemia inhibitory factor (LIF) (24), cyclo-oxygenase 2 (25), and homeobox A10 (HOXA10) (26) are the most notable examples. However, despite many of them being phenotypically implicated in murine models, none of them has been translated into clinical practice as an endometrial biomarker (27).

As developments in microarray technologies now allow more reliable, quantifiable gene expression monitoring (28), these technologies have been used to investigate the transcriptomics of human endometria in the different phases of the menstrual cycle, including within the receptivity phase (29, 30). Importantly, these studies demonstrated that differential gene expression patterns exist in different phases, thus allowing the molecular status of the endometrium to be classified according to its transcriptomic signature regardless of its histologic appearance (31–33).

TRANSCRIPTOMICS OF THE HUMAN ENDOMETRIUM

Before the genomic era, researchers were limited to determining the molecular changes governing biological processes by studying one gene at a time. Advances in gene expression profiling, which has been facilitated by the development of DNA microarrays (28), represent major progress toward increasing the knowledge of global gene expression profiles. The transcriptome reflects the genes that are actively expressed at any given time within a specific cell population or tissue.

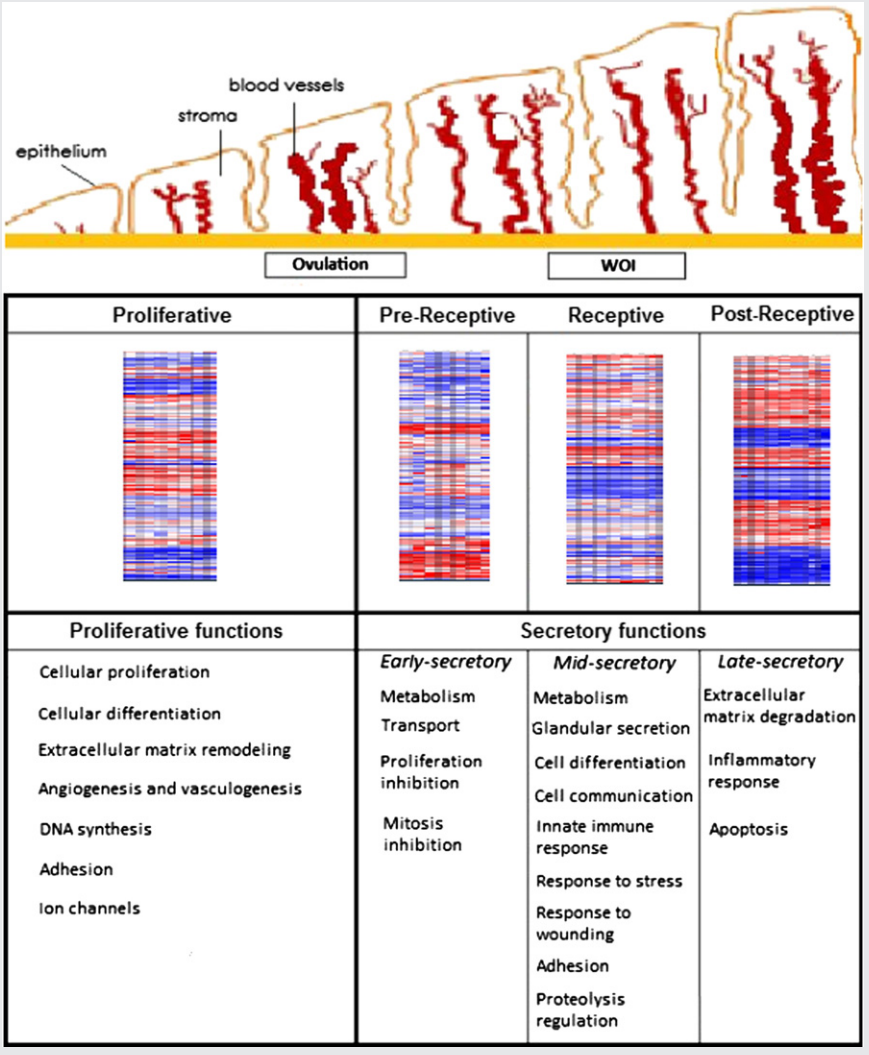
Knowledge of endometrial biology accumulated in the last decade has allowed human endometrial transcriptomics to be investigated from many different perspectives (Fig. 1). First, genomic profiles in the different phases of the menstrual cycle, and specifically during decidualization, have been studied (31, 34–49). Second, the endometrial transcriptome in patients with repetitive implantation failures has been analyzed and compared with that of fertile patients (33, 50, 51). Third, healthy patients and women with endometrial pathologies, such as endometrial cancer or endometriosis, have been compared (52, 53). Finally, gene expression pattern modifications during controlled ovarian stimulated (COS) and hormonal replacement (54–56) cycles have been investigated. These basic studies have led to the definition of a genomic signature of human endometrial receptivity that can be used as a strategy to overcome subjectivity problems caused by the inter- and intracycle variations in Noyes endometrial receptivity dating (Table 1).

With the introduction of microarrays four studies on human endometrial transcriptomics were initially published (36–38). One publication from our group (39) compared the whole-genome expression profiles of prereceptive endometrium 2 days after the LH peak (LH+2) vs. receptive endometrium 7 days after the LH peak (LH+7). Samples were obtained from the same fertile women during the same cycle, and microarrays containing 375 genes (67), including human cytokines, chemokines, and transcription factors related to them, were used. These results were contrasted with gene expression patterns in the highly adhesive cell line RL95-2 vs. a much less adhesive cell line, HEC-1A (67). This allowed us to identify 211 genes that were differently expressed in prereceptive (LH+2) endometrium vs. receptive (LH+7) endometrium, some of which were already known, including placental protein 14, osteopontin, integrin $\alpha 3$, and IL-1RtI. However, we also identified many others genes that had not been previously identified in the human endometrium and whose differential expression between the LH+2 and the LH+7 phases had not yet been described (56).

Many other studies that analyzed the whole-genome gene expression during different menstrual cycle phases have been published. Although a definitive genomic signature remains far from clear, osteopontin was consistently up-regulated in all the studies, and important enzymes and molecules involved in lipid metabolism, immune response, cell cycle regulation, and ion binding were identified in endometrial tissues at different receptivity stages (33–56). Thus, these findings indicate that accurate endometrial cataloguing at different cycle stages, based on endometrial tissue transcriptomic profiles, may be possible despite varying results in the literature.

Other studies have focused on specific cellular compartments, using laser capture microdissection to separate both stromal and epithelial fractions for analysis (43, 65). This analysis demonstrated that glands and stroma have distinct messenger RNA signatures, mainly related to cell cycle processes but also dependent on endometrial stage. As well as studying differences in cellular compartments, our laboratory also chose to investigate the effect of COS, a common practice in assisted reproductive treatments, on endometrial receptivity. Our first study assessed the endometrial impact of COS in a long protocol without P supplementation. The endometrial profiles obtained 7 days after hCG administration (hCG+7) were compared with those obtained on day LH+7 of the previous natural cycle in the same patient; more than 200 genes showed a greater than threefold differential expression (54). We also analyzed the impact of standard and high doses of a GnRH antagonist vs. treatment with a GnRH agonist (55, 56) in COS cycles. The natural-cycle endometrial genomic profile

FIGURE 1



Evolution of endometrial tissue over time and the gene expression profile at each given stage. Heat map showing ERA gene expression profiles in each endometrial cycle stage (proliferative, prereceptive, receptive, and postreceptive) and the major biological functions regulated in each of these phases.

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was more closely mimicked in women undergoing COS after daily treatment with a GnRH antagonist than in those treated with a GnRH agonist, thus highlighting the need for further efforts to optimize COS protocols. Finally, we also investigated the gene expression profile of refractory endometrium, induced by insertion of an intrauterine device (IUD) in fertile patients (68). Day LH+7 endometrial samples from five patients were obtained in the natural cycle before IUD insertion (month 1), in month 3 just before IUD removal, and in months 5 and 15. As a result, we identified 147 significantly dysregulated genes, 95 of which had not been previously implicated in the regulation of the WOI. This natural cycle indicated that IUDs prevent normal transition to a receptive genomic status, and moreover, identified a specific subset of genes responsible for the refractory status. Understanding both the normal transition into receptiveness as well as the reverse functional status, known as refractoriness, is likely to be important in improving receptivity in infertile female patients and as a contraceptive approach to prevent gestation.

ENDOMETRIAL DATABASE

Both clinical and traditional research professionals dedicated to the study of infertility, and specifically to the endometrial factors involved in it, must stay up to date with current knowledge, with the aim of continually improving their work. It is therefore imperative to stay informed about the rapid progress made by the scientific community in the field. To address this urgent need we created the Endometrial Database (EDB), a free online service and resource (www.endometrialdatabase.com) (69).

The EDB is an Instituto Valenciano de Infertilidad (IVI) Foundation service, sponsored by the University of Valencia

TABLE 1

Original articles on endometrial transcriptomics.

Authors	Date	Time of biopsy	Comparative	Array	Reference
Carson et al.	2002	LH+(2–4) vs. LH+(7–9)	ES vs. MS	HG U95A (Affymetrix)	37
Kao et al.	2002	CD 8–10 vs. LH+(8–10)	LP vs. MS	HG U95A (Affymetrix)	36
Borthwick et al.	2003	CD 9–11 vs. LH+(6–8)	LP vs. MS	HG U95A-E (Affymetrix)	38
Riesewijck et al.	2003	LH+2 vs. LH+7	ES vs. MS	HG U95A (Affymetrix)	39
Mirkin et al.	2004	LH+8 vs. hCG+9	Ag vs. Atg vs. NC	HG U95Av2 (Affymetrix)	55
Ponnampalam et al.	2004	Complete cycle, dating by Noyes	EP vs. MP vs. LP vs. ES vs. MS vs. LS vs. M	Homemade (Peter MacCallum Cancer Institute)	35
Horcajadas et al.	2005	LH(+2;+7) vs. hCG+7	NC vs. COH	HG U133A (Affymetrix)	57
Mirkin et al.	2005	LH+3 vs. LH+8	ES vs. MS	HG U95Av2 (Affymetrix)	44
Punyadeera et al.	2005	CD 2–5 vs. CD 11–14	M vs. LP	HG U133A (Affymetrix)	40
Simon et al.	2005	LH (+2;+7) vs. hCG (+2;+7)	Ag vs. Atg vs. NC	HG U133A (Affymetrix)	56
Yanahaira et al.	2005	CD 9–11	Epithelial vs. stromal cells in proliferative phase	BD Atlas Nylon cDNA Expression Array; BD Biosciences (Clontech)	43
Critchley et al.	2006	Dating by Noyes	MS vs. LS	HG U133A (Affymetrix)	46
Talbi et al.	2006	Complete cycle, dating by Noyes	EP vs. MP vs. LP vs. ES vs. MS vs. LS	HG U133 Plus 2.0 (Affymetrix)	45
Horcajadas et al.	2008	LH+(1–9) vs. hCG+ (1–9)	NC vs. COS	HG U133A (Affymetrix)	54
Liu et al.	2008	LH+7 vs. hCG+7	NC vs. COS	HG U133A (Affymetrix)	58
Macklon et al.	2008	LH+5 vs. hCG+2	NC vs. COS	HG U133 Plus 2.0 (Affymetrix)	59
Haouzi et al.	2009	LH (+2;+7) vs. hCG+(+2;+5)	NC vs. COS	HG U133 Plus 2.0 (Affymetrix)	60
Haouzi et al.	2009	LH+2 vs. LH+7	ES vs. MS	HG U133 Plus 2.0 (Affymetrix)	31
Koler et al.	2009	CD 21	Fertility vs. infertility	Array-Ready Oligo Set for the Human Genome version 3.0 (Operon)	51
Altmae et al.	2010	LH+7	Fertility vs. infertility	Whole Human Genome Oligo Microarray (Agilent Technologies)	50
Haouzi et al.	2010	LH (+2;+7) vs. hCG (+2;+5)	Ag vs. Atg vs. NC	HG U133 Plus 2.0 (Affymetrix)	61
Kuokkanen et al.	2010	CD 11–13 vs. CD 19–23	LP vs. MS with RNA and miRNA expression	HG U133 Plus 2.0 (Affymetrix) and miRCHIP V1 Array	47
Tseng et al.	2010	Dating by Noyes	ES vs. MS vs. LS	HG U133 Plus 2.0 (Affymetrix)	48
Van Vaerenbergh et al.	2010	LH+(5–7)	MS vs. pregnant	HG U133 Plus 2.0 (Affymetrix)	42
Blockeel et al.	2011	Oocyte retrieval	rFSH vs. low-dose hCG	HG U133 Plus 2.0 (Affymetrix)	62
Diaz-Gimeno et al.	2011	(LH+1 vs. +3 vs. +5 vs. +7) (LH+(1–5) vs. LH+7 vs. CD 8–12)	LP vs. ES vs. MS vs. LS	HG U133A (Affymetrix) and Homemade "ERA"	49
Labarta et al.	2011	rCG+7	Different serum P level	Whole Human Genome Oligo Microarray (Agilent Technologies)	63
Revel et al.	2011	CD 20–24	Fertility vs. infertility with microRNA	Taqman Human MiRNA Array Card A (Applied Biosystems)	41
Van Vaerenbergh et al.	2011	Oocyte retrieval	Different serum P level	HG U133 Plus 2.0 (Affymetrix)	64
Evans et al.	2012	LH+2 vs. LH+7	Epithelial vs. stromal cells in proliferative phase	Agilent 4x44K; HG U133 Plus 2.0 (Affymetrix)	65
Petracco et al.	2012	CD 1–3 vs. CD 5–8 vs. CD 11–13	EP vs. MP vs. LP	GeneChip Human Gene 1.0 ST Array (Affymetrix)	66

Note: LH+ = LH surge + days; ES = early secretory; CD = cycle day; LP = late proliferative; MS = mid-secretory; hCG+ = hCG administration + days; Ag = agonist; Atg = antagonist; EP = early proliferative; MP = mid-proliferative; LS = late secretory; M = menstrual; NC = natural cycle; COH = controlled ovarian hyperstimulation; rCG+ = rCG administration + days.

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(Spain) that includes thousands of data from scientific publications, providing comprehensive information regarding the biological function, expression pattern, and regulation of genes expressed in the human endometrium. It contains a complete list of all the articles indexed in the literature that describe any gene involved in any aspect of human endometrium regulation. It includes links to PubMed and other life science journals for biomedical articles dating back to the 1950s, and it contains links to full-text articles and other related resources. It is a great tool for anyone interested in learning more about the implication of specific genes in endometrial molecular mechanisms, or those looking for genes involved in specific biological functions or diseases (e.g., embryo implantation or endometriosis).

Gene classifications in the EDB have been organized into eleven fundamental biological categories in reproductive medicine: natural cycle, stimulated cycle, contraception, endometriosis, endometrial cancer, in vitro models, animal models, preeclampsia, decidualization, implantation, and others. This aids the speed and efficiency of searches, providing access to the latest publications in each of these categories with a single mouse click. The Web site works on a search engine format: typing the name of the specific gene of interest produces a list of all the literature related to that gene, classified by the biological categories highlighted above. The search can be also performed directly via these categories, resulting in a bibliographic list of all the genes involved in reproductive medicine within the class of interest. To aid accessibility of the EDB to professionals, there is a monthly e-mail newsletter that provides subscribers with all the new references added, as well as an really simple syndication (RSS) system to read the news in real time using standard feed program readers on any smart phone or tablet.

This new free online database (69) fills a major gap in the resources available to endometrial researchers. Together with existing databases such as the Endometrium Database Resource, which focuses on genes reported in the literature as regulated in the uterus of human, mouse, rat, cow, guinea pig, pig, and sheep, it is a key tool for the detection of new molecules that might be possible biomarkers for endometrial receptivity, and it covers all current scientific knowledge on the endometrium.

ENDOMETRIAL RECEPTIVITY ARRAY

Genome-wide technology, coupled with sophisticated bioinformatics tools, has revolutionized the classification and ability to predict the prognosis for various pathologic conditions. A prominent example is in cancer, for which diverse origins and complex molecular mechanisms come into play between and within individual tumor pathologies (69–74). We applied similar genome-wide bioinformatic prediction techniques to the classification and diagnosis of endometrial receptivity (49).

The Endometrial Receptivity Array (ERA) is a customised array based on the transcriptomic signature of human endometrial receptivity, specifically when human endometrium is receptive to blastocyst adhesion (49). It has been designed to identify endometrial receptivity by comparing the genetic profile of a test sample with those of LH+7 controls in a natural cycle, or on day 5 of P administration (P+5) after E_2 priming in a hormonal replacement therapy (HRT) cycle. It consists of a customized array, containing 238 genes that are differentially expressed between these profiles, which is coupled to a computational predictor that can diagnose the personalized endometrial WOI of a given patient regardless of their endometrial histology (49). To select the genes for inclusion in the ERA platform, our group analyzed the expression profile of endometrial samples obtained on day LH+7 in a natural cycle compared with the prereceptive phase (LH+1, +3, +5) (68). Using stringent criteria of a 3.0-fold change increase and false discovery rate of <0.5, we selected 238 genes that were incorporated into a customized Agilent gene expression microarray using the 569 probes already existing on the array. The ERA expression values for the training set were used to train the bionformatic predictor to classify an endometrial sample as "receptive" or "nonreceptive." Once the array and the predictor were designed, a cohort of samples obtained in the prereceptive (LH+3, +5), receptive (LH+7), and proliferative phases (days 8–12 of the menstrual cycle) were used to validate this transcriptomic signature. We obtained specificity and sensitivity figures of 0.8857 and 0.99758, respectively (49).

The accuracy and consistency of the ERA test has recently been demonstrated (75); both histologic and ERA dating, related to the LH peak as a reference, were in agreement, and the interobserver variability between pathologists, which was statistically analyzed by the quadratic weighted κ index, also showed concordance. The reproducibility of the ERA was tested by analyzing a second biopsy obtained from the same patient, on the same day of the menstrual cycle, 2 to 3 years after the first one. Paired-sample gene expression analysis by principal component analysis and clustering showed the reproducibility of the tool and demonstrated that the transcriptomic profile of the mid-secretory phase endometrium did not substantially change between cycles or over relatively long periods of the women's reproductive life. The results obtained indicate that for each pathologist, concordance against the LH peak yielded median (±SEM) values of 0.618 (0.446–0.791) and 0.685 (0.545–0.824), respectively, and interobserver variability between pathologists yielded a κ index of 0.622 (0.435–0.839). Concordance for ERA endometrial receptivity dating against the LH peak showed a value of 0.922 (0.815-1.000), and the reproducibility of the ERA test was 100% consistent (75).

Therefore, the ERA is more accurate than histologic dating and is a highly reproducible method for endometrial dating and diagnosis of endometrial receptivity status. Hence, for the first time, a molecular tool based on the expression of a cluster of endometrial biomarker genes can be clinically used in reproductive medicine to assess the endometrial receptivity factor with proven accuracy and consistency. This molecular signature can now be used in research to investigate the effect of different treatments or conditions on the receptivity status of the human endometrium, or in the search for new, less-invasive methods to evaluate receptiveness.

CLINICAL APPLICATIONS

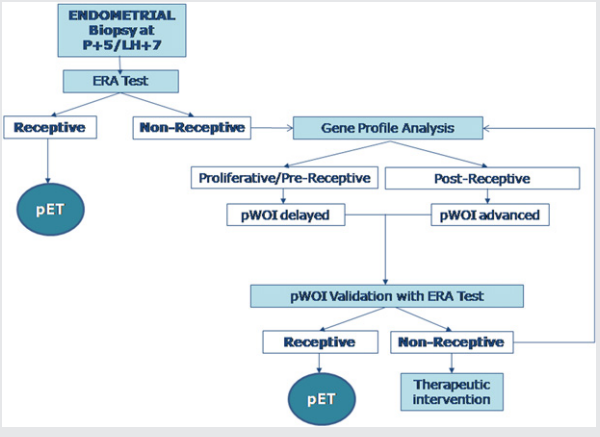
The diagnostic and clinical value of the ERA test has been tested in a prospective, interventional, multicenter, clinical trial in which patients with recurrent implantation failures (RIFs) and controls underwent endometrial receptivity diagnosis using an endometrial biopsy obtained either on day LH+7 in a natural cycle or on day P+5 in an HRT cycle (75). Patients with at least three previous failed ovum donation cycles, and IVF patients aged <40 years, with at least three failed IVF cycles, made up this group. The ERA test identified 73.7% of the samples as receptive and 26.3% of them as nonreceptive. Patients with a receptive ERA diagnosis achieved a 62.8% pregnancy rate and a 37.9% implantation rate, when transferred the day after the receptive ERA diagnosis, which was similar to controls for whom the embryos were transferred in a subsequent cycle.

At the clinical level, the most important contribution of the ERA test is the objective diagnosis of the window of implantation, thus leading to the creation of the concept of personalized ET (pET) (Fig. 2). Personalised medicine is a well-accepted concept in reproductive medicine. We all agree that patients must be treated differently at different stages of assisted reproductive technology application, according to their personal phenotype and characteristics. However, the medical community has always considered that all infertile patients must be equally treated in terms of the day of ET, which is guided by the embryo development stage and supported by the administration of P/hCG in the luteal phase. Thus the possibility of personalized and unique treatment modifications guided by endometrial biomarkers has never been considered before.

Given our findings that personalized endometrial receptivity diagnosis is now possible, we consider it of utmost importance that a personalized approach to improving clinical success from the endometrial perspective be used. The ERA test informs us whether the endometrial biopsy obtained during the expected WOI is really in a receptive state or whether it is nonreceptive at the time of testing. In the first case, ET must be performed in a subsequent natural or HRT cycle on the designated day. In case the result is nonreceptive, it can then be classified by our predictor as pre- or postreceptive, and a second ERA test following this guideline can be performed to validate a personalised WOI resulting from displacement caused by some intrinsic genomic alteration inherent in the patient, an observation we have made in one in four RIF patients (76). This new concept has been functionally proven by applying pET, following ERA results indicating a displaced WOI, in RIF patients with a previously non-receptive endometrium, either on days LH+9 or P+7; their implantation rate and pregnancy rate rose to similar levels as those in normally receptive control patients (76).

Although this molecular tool currently focuses on RIF patients, research is underway to test the ERA in patients with endometriosis and hydrosalpinx. Additionally, a prospective, randomized study on the effectiveness of the ERA test in the infertility workup, to guide pET in patients receiving assisted reproductive technology treatments, is ongoing. Moreover, this molecular tool could be useful not only for clinical diagnosis but also for research based on the analysis of variations in receptive expression profiles due to different treatments or conditions.

FIGURE 2



Clinical algorithm for ET personalization. This consists of a decision tree approach to health care treatment.

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