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Transcriptomics of the human endometrium

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ABSTRACT During the mid-secretory phase, the endometrium acquires the receptive phenotype, which corresponds to the only period throughout the endometrial cycle in which embryo implantation is viable. Endometrial receptivity is a crucial process and even more important in Assisted ReproductiveTechnologies (ART) where embryo-endometrial synchronization is coordinated through embryo transfer timing. Over the last decade, transcriptomic analyses performed on the human endometrium have shown that specific genomic signatures can be used to successfully phenotype different phases of the menstrual cycle including the receptive stage, independently of the histological appereance of the endometrial tissue. In this paper, we review current evidence demonstrating that endometrial transcriptomics objectively identifies the implantation window in a personalized manner, opening the field for the diagnosis of the endometrial factor in ART and moving to stratified medicine at this level, using microarray technology and soon high-throughput next generation sequencing coupled with functional and systems genomics approach.

KEY WORDS: *Endometrial Receptivity Array, personalised embryo transfer, personalised window of implantation, clinical translation, systems biology*

Introduction

The human endometrium is a dynamic organ that undergoes cyclic changes in response to ovarian steroids, cytokines and chemokines (Wilcox *et al.,* 1999; Lessey 2000; Salamonsen *et al.,* 2007). In mamals, its main function is to allow the adhesion and invasion of the blastocyst in order to initiate successful placentation and pregnancy. In humans and primates, the endometrium is non-receptive to embryos for most of the menstrual cycle, but becomes receptive during a spatially and temporally restricted period during the secretory phase known as the window of implantation (WOI) (Harper 1992; Giudice 1999), which is regulated by the presence of exogenous or endogenous progesterone (P) after previous estradiol (E2) stimulation (Finn and Martin 1974; Martín *et al.,* 2002).

From anatomical medicine, with its use of histological dating methods (Noyes *et al.*, 1950, 1975), to the new '–omics' technologies, great effort has been put into understanding and characterising how endometrial receptivity functions, and how it can be used as a guide for diagnosing the endometrial factor in assited reproductive technologies (ART). Despite the historical relevance of traditional endometrial dating criteria, its accuracy, reproducibility, and functional relevance has been questioned in randomised studies (Coutifaris *et al.,* 2004; Murray *et al.,* 2004). However, the research effort and application of classical molecular technologies to objectively diagnose endometrial receptivity still remains insufficient (Lessey, 2011), especially given that it is obvious that it is a complex and multifactorial process. Therefore, we postulated that the application of an '–omics' approach might enable endometrial biomarker research and hold the key to its clinical application (Diaz-Gimeno *et al.,* 2011, 2013; Berlanga *et al.,* 2011; Altmäe *et al.,* 2012; Garrido-Gomez *et al.,* 2013; Ruíz-Alonso *et al.,* 2013; and recently reviewed by Altmäe *et al.,* 2013).

Twenty-first century biology is the era of '–omics' (Quackenbush, 2006), which refers to high-throughput techniques and massive data analysis, allowing molecular profiling and changes between groups or individuals to be investigated. The study of genomes and the complete collection of genes that they contain (genomics), epigenetic DNA modifications (epigenomics), gene expression or

Abbreviations used in this paper: ART, assisted reproductive technologies; COS, controlled ovarian stimulated; E2, estradiol; ERA, endometrial receptivity array; FDA, Food and Drug Administration; GSEA, gene set enrichment analysis; HRT, hormonal replacement therapy; IVF, *in vitro* fertilization; MAQC, microarray quality control; MIAME, minimum information about a microarray experiment; NGS, next generation sequencing; P, progesterone; P+, progesterone + days; PCA, principal component analysis; pET, personalised embryo transfer; RIF, recurrent implantation failure; RNA-seq, RNA sequencing; WOI, window of implantation.

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transcriptome profiling (transcriptomics), the presence and quantification of proteins i.e. the proteome (proteomics), or the composition and abundance of metabolites i.e. the metabolome (metabolomics), are examples of the different '–omic' approaches used to analyse biological samples in a physiological context. '–Omics' includes holistic perspectives that are being applied to create new types of biological data such as lipidomics (collection of lipids, i.e. the lipidome), secretomics (secreted proteins, i.e. the secretome), interactomics (the interactome, or a 'systems biology' approach) and a big list of derived '–omics' fields that consider other groups and subgroups of biological molecules and processes (omics.org http://omics.org/index.php/Omics_classification).

Personalised medicine uses genetic, or any other biomarker information, such as molecular profiles, along with it diagnostic, prognostic, and therapeutic strategies precisely tailored to each patient's requirements, including which therapies and what doses are required for an optimal outcome. The terms *genetic*, *personalised*, *stratified,* or *precision medicine*, *pharmacogenetics* and *pharmacogenomics* have all been used interchangeably to mean "the study of genetic variations and their influence on the way people respond to medications" (Mirnezami *et al.,* 2012; the Food and Drug Administration (FDA) organisation website http://www.fda.gov/Drugs/NewsEvents/ucm325475.htm)

In the last decade, a total of 269 relevant manuscripts have been published about '–omics' studies in the human endometrium. 164 of them (61%) were transcriptomic publications (for a review see Altmäe *et al.,* 2013, Fig. 1). Accumulated clinical evidence indicates that transcriptomics, based on microarray technology, is now the most mature and stable technology available for implementing personalised diagnosis of the endometrial factor in reproductive medicine.

At a general level, transcriptomic and bioinformatic analyses are standardised and normalised (Brazma *et al.,* 2001; MAQC consortium 2006, 2010). Gene expression profiling has been used for tumor classification (Quackenbush, 2006), and machine-learning algorithms have been implemented as predictive models for the microarray signatures (Medina *et al.,* 2007) used in diagnostic and prognostic prediction (Simon, 2003). The FDA has already approved the Agendia MammaPrint microarray as a diagnostic test for the assessment and prognosis prediction of distant metastasis in patients with breast cancer (van´t Veer *et al.*, 2002). Following this line, the Endometrial Receptivity Array (ERA) test is a microarray-based machine-learning predictive model used to diagnose human endometrial receptivity status (Díaz-Gimeno *et al.,* 2011, 2013), guiding personalised embryo transfer (Ruiz-Alonso *et. al.*, 2013).

The aim of this manuscript is to review the use of transcriptomics in the human endometrium and its potential to be used as an objective tool to diagnose endometrial receptivity status and therefore to personalise embryo transfer according to endometrial status.

Transcriptomics

Transcriptomics attempts to analyse patterns of gene expression and to correlate them with their underlying biology. DNA microarray analysis was implemented to measure the expression of thousands of genes simultaneously and was quickly adopted by the scientific community for the study of a wide range of biological processes. Most of the early studies had a simple and powerful design: to compare two biological classes in order to identify the differential expression pattern between them. Genes with potential relevance to a wide range of biological processes, such as the progression of cancer, the causes of asthma, heart disease, and neuropsychiatric disorders, or factors associated with infertility have been identified and analysed (Schena, 1995; Quackenbush, 2006). Gene expression arrays are the ideal technology to analyse the levels of transcription in tissues, assessing functions that might be reversibly changeable.

Transcriptomics also allows gene expression characterisation at the mRNA level of a population, giving rise to a sample-specific molecular profile. This fact led to a new biomarker concept, that of transcriptomic signatures that define a biological process or disease, and thus represent new opportunities to characterise function or disease phenotypes (Nevins and Potti, 2007).

Several consortiums have produced guidelines on the quality of microarray analyses. Minimum information about a microarray experiment (MIAME) helps to increase the underlying standard of microarray data (Brazma *et al.*, 2001) and establish the basis of microarray procedures. The microarray quality control (MAQC)

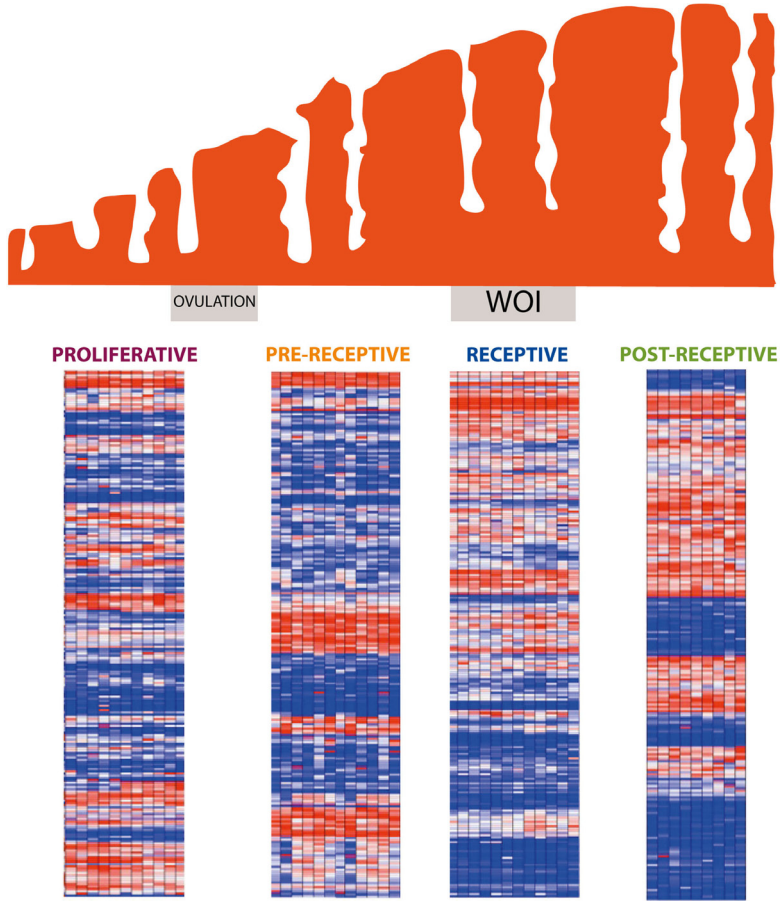

Fig. 1. Endometrial transcriptomics profile. *Evolution of endometrial tissue over time and the gene expression profile at each given stage. Heatmap showing the Endometrial Receptivity Array (ERA) gene expression profiles in each endometrial cycle stage (proliferative, pre-receptive, receptive, and post-receptive).*

phase I (MAQC Consortium, 2006) focused on the technical aspects of gene expression measurements and robust technology platforms. For clinical predictive phenotypes an additional requirement is the development of accurate and reproducible multivariate expression-based gene prediction models, also referred to as 'classifiers'. The second MAQC phase focused on these predictive models to establish a summary of observations and recommendations that could be used to develop and validate microarray-based predictive models. These are increasingly submitted by the industry to the FDA to support medical product development and testing applications (MAQC Consortium, 2010). In addition, the EMERALD project, based on MIAME standards, provides quality guidelines for further model validations and details about practical clinical applications for microarray data (Beisvag *et al.*, 2011).

An appropriate and reproducible protocol is needed in order to use any technology in a clinical setting, and so quality control and enabling data sharing, therefore the possibility of reanalysis and comparison is critical. All these studies and standardised microarray data analysis methods help to guarantee the efficacy of the clinical applicability of transcriptomics to phenotype diseases, and thereafter their application to precision medicine.

Transcriptomics, functional analysis and the systems biology approach

To analyse transcriptomic profiles or signatures, exploratory methods and statistical tests are used. Exploratory methods such as trees, clustering, or principal component analysis (PCA) are used to discover the behavior of the profiling sample and its variability within the microarray data cohort. There are several different user-friendly software platforms available, such as the online *Babelomics* platform (Medina *et al.,* 2010; http://babelomics.bioinfo.cipf.es/), which make microarray data to analysis easy, by using visual heatmap representations to show differential expression patterns between different scenarios, genes, and samples.

Transcriptomics describes gene activity; therefore concurrent functional analysis is the key to understanding the underlying biology. Early functional analysis involved mapping expressed genes onto pathway maps such as the Kyoto encyclopedia of genes and genomes (KEGG pathways; Kanehisa and Goto, 2000) or functional ontologies such as gene ontology (GO; Ashburner, 2000). Nowadays, functional analysis has developed a new perspective, based on systems biology which considers the functional interdependency of the molecular components of human cells; therefore a disease is rarely a consequence of a single gene abnormality. Functional analysis involves genes in the genomic and cellular context and has recently expanded into three main approaches: *functional enrichment analysis* that highlights and ranks biologically relevant pathways, processes, disease markers, or other functionality in a gene list (Subramanian, 2005), *biological network reconstruction*, and *interactome analysis* (Vidal *et al.*, 2011).

Gene set enrichment analysis (GSEA), using a systems biology approach, analyses functions by considering genes either in a genomic or cellular context. The genes are ranked by any biological criteria (e.g. differential expression between experimental cohorts and healthy controls) and the algorithm searches for blocks of functionally-related genes without imposing any artificial thresholds (e.g *FatiScan* from *Babelomics*; for a tutorial see: http://bioinfo.cipf.es/babelomicstutorial/gene_set_analysis).

Network science models complexity by 'simplifying' complex systems into components (nodes: molecules) and interactions (edges) based on different biological parameters, and applies graph theory to generate systems properties (centrality, betweenness, the shortest paths etc. Vidal *et al.,* 2011). Depending on the relationship between these nodes, different types of networks emerge, e.g. metabolic, protein-protein interaction, gene regulatory, or interactome networks which integrate with other cellular networks such as the transcriptional profiling, phenotypic profiling, or genetic interaction networks (Vidal *et al.*, 2011).

In transcriptional profiling networks, nodes represent genes, and edges link pairs of genes that show co-expression above a set threshold. Common signalling cascades of gene products or protein complexes that function together are expected to show more similarities in their expression behavior than random sets of gene products (Stuart *et al.*, 2003).

Transcriptomics prediction

Predictors are used to classify new microarray data into specific classes (e.g. disease case samples or healthy controls) based on criteria previously constructed with a model dataset which contains the classes with which the samples will be phenotyped. This dataset is named the training set. The underlying predictor strategy is as follows: if the differences between the classes are as a consequence of measureable differences in gene expression levels, these differences can be identified and used to assign the classes for a new microarray profiling set.

Microarray data works well with several classification algorithms recommended by MAQC phase II (MAQC Consortium, 2010). The best algorithms for classifying microarray data are *support vector machines*, *nearest neighbour*, and *random forest*. The possibility of classification using transcriptomic profile data is a powerful tool in clinical applications and in personalised medicine, and is independent of the specific functional meaning of the transcriptomic signature (Shi *et al.*, 2010).

Transcriptomics of human endometrium

Evidence accumulated over the last decade (Table 1, for a review see Ruiz-Alonso *et al.,* 2012; Garrido-Gomez *et al.,* 2013; Altmäe *et al.,* 2013) indicates that human endometrial transcriptomics is reaching maturity. Different areas have been investigated, such as the transcriptomic expression throughout the menstrual cycle (for reviews see Horcajadas *et al.*, 2004; Giudice 2006; Horcajadas *et al.,* 2007; Aghajanova *et al.,* 2008 a, b; Haouzi *et al.,* 2012; Ruiz-Alonso *et al.,* 2012; Garrido-Gomez *et al.,* 2013), endometrial transcriptomics during different treatment protocols (reviewed in Martinez-Conejero *et al.,* 2007; Ruiz-Alonso *et al.*, 2012), endometriosis (reviewed in Matsuzaki, 2011), and endometrial cancer (reviewed in Doll *et al.*, 2008). The perceived limitations of this technology lie in the differences in experimental design, timing, the conditions of endometrial sampling, sample selection criteria, annotation versions used, pipelines for data processing, and the absence of consistent standards for data presentation (Horcajadas *et al.,* 2007; Ruiz-Alonso *et al.,* 2012; Ulbrich *et al.,* 2013). These different factors have made performing meta-analyses of similar studies on specific stages of endometrial development nearly impossible (Ulbrich *et al.*, 2013). Additionaly, the powerful value of transcriptomic data in pathologies such as endometriosis has been limited by the absence of uniformity in the validation of these

cumulative data. Multiple comparisons have been made, revealing distinct transcriptomic differences in a variety of biological processes and signalling pathways unique to ectopic versus eutopic endometrium (as is reviewed in Altmäe *et al*., 2013). However, clinically meaningfull biomarkers for the pathophysiology and/or aetiology of endometriosis are still to be identified (Matsuzaki 2011; Fassbender *et al.,* 2013).

Also different endometrial cellular compartments have been analysed, demonstrating specific cell-type gene expression profiles (Yanaihara *et al.,* 2005; Evans *et al.,* 2012; Ulbrich *et al.,* 2013). In all of these studies stromal and epithelial fractions were isolated by laser capture microdissection, and the mRNA signatures were related to the menstrual cycle day (Yanaihara *et al.,* 2005; Evans *et al.,* 2012). In addition, Evans' group compared Affymetrix and Agilent microarray platforms, demonstrating concordance in their results (Evans *et al*., 2012). An alternative approach to laser dissection for isolating tissues is fluorescence-activated cell sorting, which has also been used to demonstrate cell-type specific gene expression (Spitzer *et al*., 2012). However, the majority of transcriptomic studies have used random biopsies of endometrial tissue which contain all cell types. The transcriptomic profile of the diferent phases of the menstrual cycle is visualised using heatmaps (see Fig 1).

Transcriptomics of endometrial receptivity

'Endometrial receptivity' describes the phenotype which allows embryo adhesion and placentation to occur. This concept was first suggested in the 1970s (Croxatto *et al*., 1978), but it was the pioneering work of Wilcox *et al.,* that initiated the field, demonstrating that in most successful pregnancies the "conceptus" implants 8 to 10 days after ovulation (Wilcox *et al*., 1999). Historically, it has always been accepted that the WOI is constant, always permitting embryo implantation, and so personalisation was never considered, especially because the objective diagnosis of the endometrial factor and therefore the WOI did not previously exist. Based on these grounds, an important scientific and clinical objective has been to find a molecular signature which characterises receptive endometrium in order to gain an objective insight into this crucial function (reviewed in Ruiz-Alonso *et al*., 2012).

Endometrial receptivity is the result of the synchronised and integrated interaction of ovarian hormones, growth factors, lipid

TABLE 1

ORIGINAL PAPERS ON ENDOMETRIAL TRANSCRIPTOMICS IN ASSISTED REPRODUCTIVE MEDICINE

Authors	Date	Time of Biopsy	Comparative	Array
Carson *et al*	2002	LH+(2-4) vs LH+(7-9)	ES vs MS	HG U95A (Affymetrix)
Kao *et al*	2002	CD 8-10 vs LH+(8-10)	LP vs MS	HG U95A (Affymetrix)
Borthwick *et al*	2003	CD 9-11 vs LH+(6-8)	LP vs MS	HG U95A-E (Affymetrix)
Riesewijck *et al*	2003	LH+2 vs LH+7	ES vs MS	HG U95A (Affymetrix)
Mirkin *et al*	2004	LH+8 vs hCG+9	Ag vs Atg vs NC	HG U95Av2 (Affymetrix)
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)
Horcajadas *et al*	2005	LH(+2;+7) vs hCG+7	NC vs COH	HG U133A (Affymetrix)
Mirkin *et al*	2005	LH+3 vs LH+8	ES vs MS	HG U95Av2 (Affymetrix)
Punyadeera *et al*	2005	CD 2-5 vs CD 11-14	M vs LP	HG U133A (Affymetrix)
Simon *et al*	2005	LH (+2;+7) vs hCG (+2;+7)	Ag vs Atg vs NC	HG U133A (Affymetrix)
Yanahaira *et al*	2005	CD 9-11	Epithelial vs Stromal cells in Proliferative phase	BD Atlas Nylon cDNA Expression Array; BD Biosciences (Clontech)
Critchley *et al*	2006	Dating by Noyes	MS vs LS	HG U133A (Affymetrix)
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)
Horcajadas *et al*	2008	LH+(1-9) vs hCG+ (1-9)	NC vs COS	HG U133A (Affymetrix)
Liu *et al*	2008	LH+7 vs hCG+7	NC vs COS	HG U133A (Affymetrix)
Macklon *et al*	2008	LH+5 vs hCG+2	NC vs COS	HG U133 Plus 2.0 (Affymetrix)
Haouzi *et al*	2009b	LH (+2;+7) vs hCG+(+2;+5)	NC vs COS	HG U133 Plus 2.0 (Affymetrix)
Haouzi *et al*	2009a	LH+2 vs LH+7	ES vs MS	HG U133 Plus 2.0 (Affymetrix)
Koler *et al*	2009	CD 21	Fertility vs Infertility	Array-Ready Oligo Set for the Human Genome Version 3.0 (Operon)
Altmae *et al*	2010	LH+7	Fertility vs Infertility	Whole Human Genome Oligo Microarray (Agilent Technologies),
Haouzi *et al*	2010	LH (+2;+7) vs hCG (+2;+5)	Ag vs Atg vs NC	HG U133 Plus 2.0 (Affymetrix)
Tseng *et al*	2010	Dating by Noyes	ES vs MS vs LS	HG U133 Plus 2.0 (Affymetrix)
Van Vaerenbergh *et al*	2010	LH+(5-7)	MS vs Pregnant	HG U133 Plus 2.0 (Affymetrix)
Blockeel *et al*	2011	Oocyte retrieval	rFSH vs low-dose hCG	HG U133 Plus 2.0 (Affymetrix)
Diaz-Gimeno *et al*	2011	(LH+1,+3,+5 vs LH+7) (LH+(1-5) vs LH+7 vs CD 8-12)	LP vs ES vs MS	HG U133A (Affymetrix) and Homemade "ERA"
Labarta *et al*	2011	rCG+7	Different serum progesterone level	Whole Human Genome Oligo Microarray (Agilent Technologies),
Van Vaerenbergh *et al*	2011	Oocyte retrieval	Different serum progesterone level	HG U133 Plus 2.0 (Affymetrix)
Evans *et al*	2012	LH+2 vs LH+7	Epithelial vs Stromal cells in Proliferative phase	Agilent 4x44K; HG U133 Plus 2.0 (Affymetrix)
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)
Díaz-Gimeno *et al*	2013	Dating by Noyes vs ERA prediction	MP vs ES vs MS vs LS	Homemade "ERA"
Ruiz-Alonso *et al*	2013	P+5/LH+7 RIF vs Controls	pWOI/pWOIdelayed/pWOIadvanced	Homemade "ERA"
Bermejo *et al*	2013	Oocyte retrieval COS	Comparing 4 GnRH-a protocols	Homemade "ERA"

Note that endometrial disorders such as cancer, endometriosis, and myomas are not considered in this table. Abbreviations: Ag: Agonist; Atg: Antagonist; CD: Cycle day; COH: Controlled Ovarian hyperstimulation; COS: Controlled ovarian stimulation; EP: Early-proliferative; ERA: Endometrial Receptivity Array; ES: Early-Secretory; GnRH-a: Gonadotropin Releasing Hormone-agonist; hCG+: hCG administration + days; LH+: LH surge + days; LP: Late-proliferative; LS: Late-secretory; M: Menstrual; MP: Mid-proliferative; MS: Mid-secretory; NC: Natural cycle; P+: Progesterone + days; pWOI: personalised window of implantation; rCG+: rCG administration + days; RIF: recurrent implantation failure.

mediators, transcription factors, and cytokines with paracrine signalling (reviewed by Cha *et al.*, 2013). Its objective identification using gene expression microarrays has been pursued since 2002.

Available data suggests that a 'transcriptional awakening process' takes place because most genes are upregulated compared to their expression in the pre-receptive phase (Riesewijk *et al.,* 2003; Borthwick *et al.,* 2003; Horcajadas *et al.,* 2008; Haouzi *et al.,* 2009 a, b; Díaz-Gimeno et. al. 2011). The early-secretory, or pre-receptive, phase is characterised by the predominance of products related to cell metabolism (fatty acids, lipids, eicosanoids, and amino alcohols), transport (with a large representation of transporters for the biological molecules involved in these metabolic processes), germ cell migration (which could facilitate sperm transportation and ensure an aseptic environment), and negative cell-proliferation regulation. An increase in metabolism is consistent with the fact that this phase is biosynthetically highly active, which probably represents tissue preparation for embryo implantation; inhibition of mitosis during this phase is supported by the downregulation of numerous growth factors (Talbi *et al.*, 2006).

Wnt pathway regulation during the secretory phase is very striking. Some, but not all ligands are downregulated, while some Wnt inhibitors, such as sFRP1 are repressed, but others such as DKK1 are highly upregulated compared to the proliferative phase (Talbi *et al.*, 2006), and these further increase in the mid-secretory phase (Carson *et al.*, 2002). The mid-secretory phase is characterised by its high level of metabolic and secretory activity, its non-proliferative phenotype, and increased sensitivity of the innate immune, stress, and wounding responses (Simmen and Simmen 2006; Giudice 2006; Talbi *et al.,* 2006).

Genes whose expression changes during the transition between the early- and the mid-secretory phases, and the mid- and the late-secretory phases, are potential candidates for regulation by progesterone (Kao *et al.,* 2002; Borthwick *et al.,* 2003; Talbi *et al.,* 2006). In fact, Ponnampalam *et al.,* (2004) detected a cluster of genes that follow a temporal regulation pattern during the endometrial cycle which is very similar to the increase in circulating progesterone during these phases. These genes have been identified amongst those participating in some of the major biological processes which take place during implantation, such as signalling, growth, differentiation, and cell adhesion. However, there are no significant gene changes associated with the oestrogen peak (reviewed by Ruiz-alonso *et al.*, 2012).

There are also genes that are overexpressed in the mid-secretory versus the early-secretory phases, and these are involved in processes related to cell adhesion, metabolism, response to external stimuli, signalling, immune responses, cell communication, and negative regulation of proliferation and development (Talbi *et al.,* 2006; Díaz-Gimeno *et al.,* 2011). The immune response plays an important role throughout the secretory phase. In the mid-secretory phase, the genes involved in the activation of the innate immune response are upregulated (including complements, antimicrobial peptides, and toll-like receptors), and there is also increased monocyte, T cell, and NK cell chemotaxis (CXCL14, granulysin, IL-15, carbohydrate sulfotransferase 2, and suppression of NK and T-cell activation, Talbi *et al.,* 2006).

Some overexpressed genes protect the endometrium and/or the embryo in this phase (Talbi *et al.*, 2006). GPX-3 is a selenium-dependent protein that has been associated with infertility in selenium-deficient women (Kingsley *et al.*, 1998). It protects the cell from oxidative damage by catalysing the reduction of hydrogen peroxide, lipid peroxides, and organic hydroxyperoxide by glutathione (Riesewijk *et al.*, 2003). DAF is a complement regulatory-protein with two postulated functions: protection of the embryo from maternal complement-mediated attack, and prevention of epithelial destruction by increased expression of complement at the time of implantation (Franchi *et al.*, 2008). This protein has been found in decreased levels in the endometrium of patients with recurrent abortion associated with antiphospholipid syndrome (Francis *et al.*, 2006).

A study by Tseng *et al.,* identified 126 upregulated genes in the mid- secretory phase compared to the late-secretory phase. Over-expressed processes included coagulation cascades and complex metabolism, including carbohydrates, glucose, lipids, cofactors, vitamins, xenobiotics, and amino acids, all of them suggesting that extracellular remodelling activity may occur in the mid-secretory phase (Tseng *et al.,* 2010).

During the late secretory phase, oestrogen and progesterone levels decrease and the main processes regulated are extracellular matrix degradation, inflammatory response, and apoptosis (Giudice 2006; Simmen and Simmen 2006). In the transition from the mid- to the late-secretory phase, changes in the extracellular matrix and cytoskeleton favour processes such as vasoconstriction, smooth muscle contraction, haemostasis, and the transition from an immune to an inflammatory response (Critchley *et al.,* 2001; Tseng *et al.,* 2010). The genes that are regulated in this transition mostly relate to innate or humoral and cellular immune responses (Talbi *et al.*, 2006), haemostasis, blood coagulation, steroid biosynthesis, and prostaglandin metabolism (Critchley *et al.*, 2001). The processes represented in this late-secretory stage, such as matrix degradation, inflammatory response, and cell apoptosis, do not favour implantation. Thus, the transition from the mid- to the late-secretory phase defines the closure of the WOI and a return to the non-receptive endometrial phenotype, and an intense immune system activation (Talbi *et al.*, 2006), which is consistent with the histological observation of leukocyte extravasation (Daly *et al.*, 1982).

Regarding immune activation, the expression of Fc receptors, MHC molecules, and molecules secreted by T and NK cells are upregulated. This corresponds to the preparation of innate and adaptive immune responses: monocytes and granulocytes are primed to respond to antibodies because of Fc-receptor upregulation, and by expressing MHC-II molecules (Talbi *et al.*, 2006). TNF alpha and IL beta are secreted by white blood cells present in the stromal cell compartment at the end of the cycle, and stimulate the release of matrix-degrading enzymes which contribute to degradation of the vascular basal membrane and connective tissue (Salamonsen and Woolley 1999). The above describes the predominant activities of the late-secretory phase and corresponds to decidualisation and preparation of the endometrium for the next menstrual phase, when the process starts again.

The effect of the embryo on endometrial transcriptomics has been investigated *in vitro* using a co-culture system involving human blastocysts and endometrial epithelial cells (De los Santos *et al.,* 1998, Simón *et al.,* 1997; Caballero Campo *et al.,* 2002, Meseguer *et al.,* 2001, Galan *et al.,* 2013) which has also been translated to the clinic (Simón *et al.,* 1999; Mercader *et al.,* 2003). The transcriptomics of the endometrium during implantation has been analysed *in vivo* in a unique case report study in which an

endometrial biopsy was taken in the mid-secretory phase from a patient who was later found to be pregnant at the time of sampling. The transcriptomics of this valuable sample were compared with samples from non-pregnant patients on the same day of the cycle, highlighting a total of 394 differentially expressed genes (Van Vaerenbergh *et al.,* 2010). The major networks represented by these genes included post-translational modification, cell signalling, cell movement, cell development, and hematological function. These networks and canonical pathways form part of the molecular mechanisms known to be involved in an embryo-endometrial dialogue, and in implantation in both mice and humans. However, this study did not define the location of the endometrium analysed in relation to the embryo implantation site, and therefore some doubt remains as to whether the observed changes were due to embryo paracrine and/or maternal endocrine effects.

Clinical translation of endometrial transcriptomics and personalised medicine

A great challenge in biomedicine is understanding the relationship between the genotype and phenotype. The clinical objective is to visualise data that objectively links molecular profiles or gene signatures to function or disease phenotypes. The most widely used data exploration method is hierarchical clustering with visual heatmap representations (Fig. 1, 2A) and PCAs (Fig. 2B). With these types of analyses, the similarities of the various types of samples can be detected based on their transcriptomic profiles or signatures. In all the studies conducted (Riesewijk *et al.,* 2003; Ponnampalam *et al.,* 2004; Talbi *et al.,* 2006; Tseng *et al.,* 2010; Critchley *et al.,* 2006; Diaz-Gimeno *et al.,* 2011, 2013), samples clearly group according to the stage to which they belong. In general, the phase assignment is made based on previous histological dating in these studies, and is usually performed by at least two independent pathologists (Díaz-Gimeno *et al.*, 2013).

Sample grouping, according to the endometrial cycle phase is clearly observable using hierarchical clustering methods, especially where the entire menstrual cycle is analysed. Two important studies grouped samples into two main branches, which were further divided into other sub-branches (Ponnampalam *et al.,* 2004; Talbi *et al.,* 2006). One major branch contains the proliferative and early-secretory phases, as well as the menstrual phase (Ponnampalam *et al.,* 2004), while the other main branch groups include samples from the mid-secretory and the late-secretory phases (Talbi *et al.,* 2006). Subsequent PCA analysis on both data sets detected four clusters of samples, corresponding to the predominant proliferative, early-secretory, mid-secretory, or late-secretory phases. Despite the sets of genes used for PCA and hierarchical clustering in these studies being different, the same clusters were found by both methods. In addition, although six samples were dated histologically as 'ambiguous', both PCA and hierarchical clustering categorised them in the same phase. These facts provide evidence for the existence of well-defined transcriptomic profiles for each phase. Our group also defined the transcriptomic transition between the pre-receptive to receptive status in natural and controlled ovarian stimulated cycles (COS) (Horcajadas *et al.*, 2008). These studies all conclude that it is possible to accurately catalogue the endometrium at different stages based on its transcriptomic profiles, thus facilitating the transition from anatomical to molecular medicine for the human endometrium.

Personalised, or stratified, medicine has been used in reproductive medicine in diverse clinical situations, including adjusting FSH/LH dosages using the body mass index and according to the ovarian reserve as assessed by the presence or absence of specific biomarkers, the selection of specific fertilisation techniques (e.g., intracytoplasmic sperm injection, *in vitro* fertilization (IVF) or both according to sperm features and the clinical background), and monitoring embryo development *in vitro*.

Since the last century, endometrial receptivity has been studied from the morphological, biochemical, molecular, and cellular points of view, however, none of the proposed markers have become diagnostic tools because they offer a low predictive value

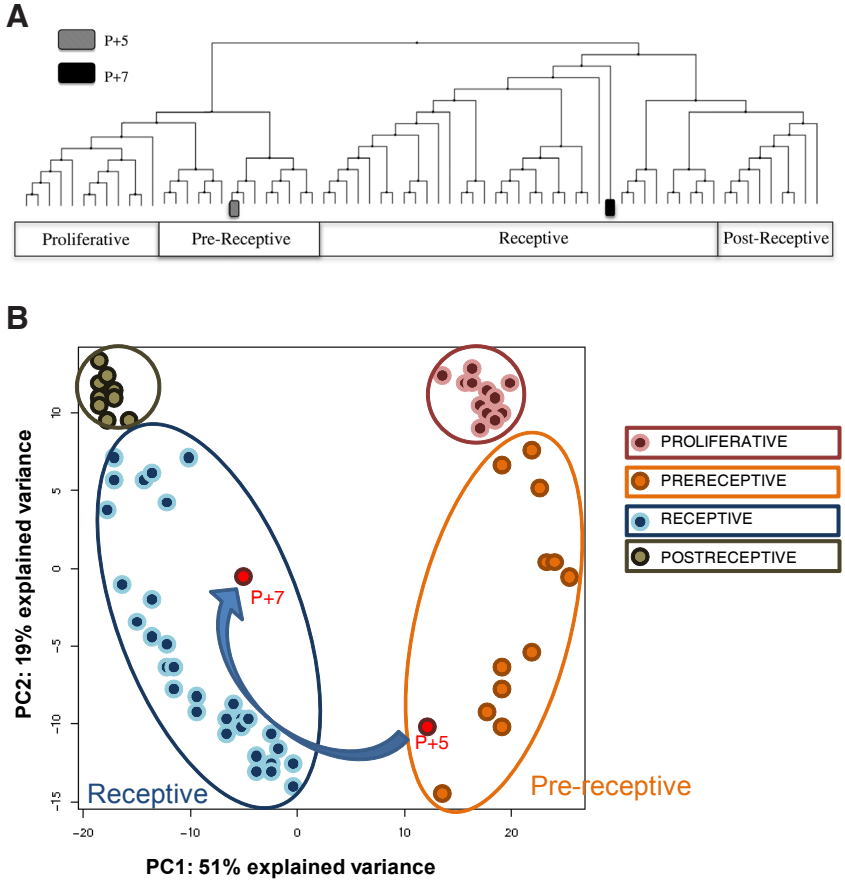

Fig. 2. Personalised Window Of Implantation (pWOI). *Clustering* **(A)** *and Principal Component Analysis (PCA)* **(B)** *of genes identified in two samples from the same patient, taken at different times, cluster with the Endometrial Receptivity Array (ERA) test training set genes. The first sample was taken after five days with progesterone (P+5) and shows a pre-receptive profile, but a sample taken two days later, at P+7, shows a receptive endometrial profile.*

(Haouzi *et al.,* 2012; Aghajanova *et al.,* 2008 a,b; Martin *et al.,* 2002; Lessey 2011). Many studies searching for endometrial markers have focused on single molecules or on specific families of molecules (Hynes 1992; Lessey *et al.,* 1992; Meseguer *et al.,* 2001; Dubowy *et al.,* 2003) reaching the conclusion that a single molecule is not sufficient to describe such a complex phenomenon. By acknowledging this fact, transcriptomic profiles may prove to be complex enough to classify the different endometrial cycle phases, including the window of endometrial receptivity (Fig. 1).

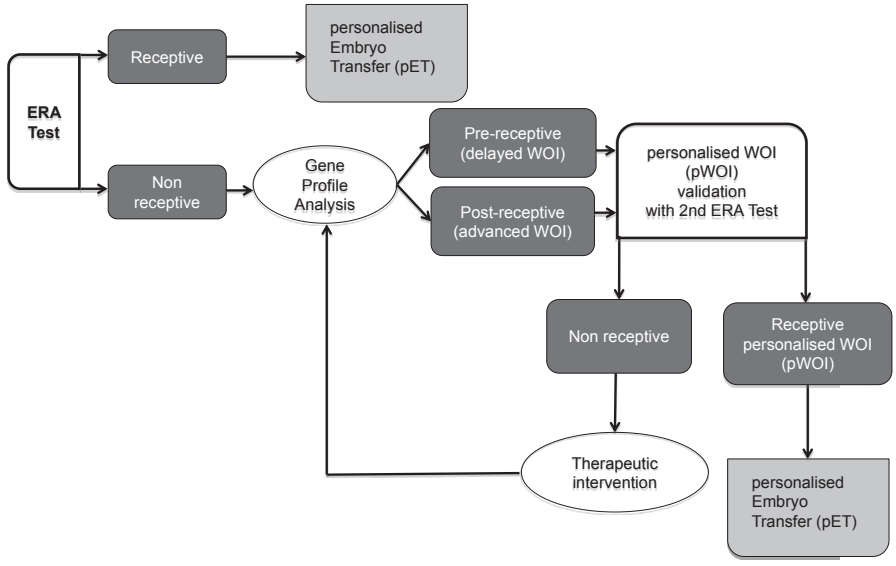

Fig. 3. **Clinical algorithm for embryo transfer personalisation.** *It consists of a decision making tree approach for embryo transfer based on the diagnosis of the endometrial factor. ERA: Endometrial Receptivity Array; WOI: Window Of Implantation.*

The Endometrial Receptivity Array (ERA)

Given the need for reliable, objective, molecular dating methods for the endometrium, our group developed a specific tool to identify the transcriptomic signature of the window of endometrial receptivity, called ERA (Díaz-Gimeno *et al.,* 2011, 2013).

The ERA is a customised array that has been designed to identify the endometrial receptivity status by comparing the transcriptomic profile of a test sample with those of control samples from 7 days after the luteinising hormone peak (LH+7) in a natural cycle, or five days after P administration (P+5) after E2 priming in a hormonal replacement therapy (HRT) cycle. It contains 238 genes that are differentially expressed between these profiles, which are coupled to a computational predictor that can diagnose the personalised endometrial WOI of a given patient regardless of their endometrial histology (Díaz-Gimeno *et al.*, 2013). The predictor was trained with gene expression profiles obtained from samples at different stages of the menstrual cycle (proliferative, pre-receptive, receptive, and post-receptive) in order to be able to classify a test sample according to the gene expression values obtained with the array. This classification has a specificity and sensitivity of 0.8857 and 0.99758 respectively (Díaz-Gimeno *et al.*, 2011). The ERA is more accurate than histological dating and is a highly reproducible method, even up to 40 months after first diagnosing the personalised WOI (Díaz-Gimeno *et al.*, 2013). Hence, for the first time, a molecular tool based on the expression of a cluster of endometrial biomarker genes has been clinically used in reproductive medicine to assess the endometrial factor with proven accuracy and consistency. This molecular signature can now be used to personalise the definition of patients' WOI and 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.

The diagnostic and clinical value of the ERA test in patients with recurrent implantation failure (RIF) has been tested in a prospective interventional, multicentre, clinical trial (Ruiz-Alonso *et al.*, 2013). Patients with at least three previous failed ovum donation cycles, and IVF patients less than 40 years old with at least three failed IVF cycles, composed the RIF group. Patients with no failed ART cycles composed the control group.

In this trial, RIF and control patients underwent ERA-based 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 (Ruiz-Alonso *et al.*, 2013). 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. In other words one in four patients with RIF has a displaced WOI and therefore their incapability to implant can be attributed to the endometrial factor.

The 'non-receptive' diagnosis, not only indicates that the endometrium is not ready for embryo adhesion, therefore making embryo transfer futile at this moment, but also gives us information about their profile of pre-receptivity or post-receptivity status (Fig. 2 A,B). Although it has been assumed that the WOI is constant in time in all women, now with the information obtained from this transcriptomical tool, we learned that the capability to diagnose a displacement of the WOI and to personalised embryo transfer (pET) in each patient. Then, pET was applied in patients who had an initial non-receptive result at the first biopsy, who then achieved a 50.0% pregnancy rate and a 38.5% implantation rate. This is very close to that of patients who had a receptive result at their first biopsy: 51.7% and 33.9% respectively (Ruiz-Alonso *et al.*, 2013).

Until now, the stage of embryo development has been the primary factor guiding the timing of embryo transfer in ART because it was generally accepted that the timing and duration of the WOI was constant in all women. However, our work has shown that with the information obtained from the ERA transcriptomic tool it is possible to identify the status of the endometrium using the transcriptomic profile of a selected group of genes to identify a delayed or advanced WOI (Fig. 3). Therefore, we now have the ability to diagnose displacement of the WOI and to personalise embryo transfer in each patient as necessary (Ruiz-Alonso *et al.*, 2013), and so helping to improve clinical success from the endometrial perspective using this novel approach (Fig. 3). This highlights the need to synchronize embryonic and endometrial development, personalising the timing of embryo transfer.

Although this molecular tool has already been demonstrated as effective in RIF patients, a prospective, randomised clinical trial on the effectiveness of the ERA test in the infertility work-up

is also ongoing and is registered at U.S. National Institutes of Health (NCT:01954758 http://clinicaltrials.gov/).

Future directions in the transcriptomics of human endometrium

In addition to gene expression microarrays, technology to measure gene expression called RNA sequencing (RNA-seq), based on next generation sequencing (NGS), is also emerging. This new technology is capable of true genome-wide analysis, sequencing all the mRNAs present in a sample. 25% of genes with low expression remain undetected with standard microarray technologies but are detected in RNA-seq reads (Wang *et al.,* 2009; Mane *et al.,* 2009). Studies that compare results derived from an Affymetrix microarray study and an RNA-seq study of bovine endometrium revealed a consistent overlap between the results but there were many more differentially expressed genes in the sequencing data set (Mane *et al.,* 2009; Ulbrich *et al.,* 2013).

The development and popularisation of the high-throughput tecnologies in the post-genomic era (microarrays, GWAS, NGS, etc.) have increased both the volume and the accuracy of data processing and have revolutionised medical diagnoses and treatments. However, this situation does not yet correspond to the expectation of an improved ability to diagnose disease-associated genes, therefore, whether these technological improvements will translate into clinical diagnostic advances, remains to be seen.

Functional analysis methods operate best on large (preferably whole genome) data sets, which are nonstringently filtered by fold change, *p*-values or false discovery rate thresholds, (Chen *et al*., 2007), or in data sets not limited in size at all (GSEA; Subramanian *et al.,* 2005): i.e. the opposite of the trend for reducing the size of data sets used in transcriptomics analysis.

Transcriptomics in the human endometrium remains a research focus, not only from the perspective of NGS analysis, but much more for its potential in the analysis of gene expression data from functional genomics analysis from different biological perspectives. Moreover, the systemic approach of systems biology analysis, via networks and other statistical and mathematical analysis methods remains to be developed. New functional analysis, based on the systems biology approach has been applied to endometrial physiology by some groups already (van Vaerenbergh *et al.,* 2010; Altmäe *et al.,* 2012). For example, Bourgain's group, performed network analysis on differentially expressed genes to reveal 30 networks involved in implantation (van Vaerenbergh *et al*., 2010) and Altmäe *et al.,* described the complex molecular network of the implantation process in humans in which embryonic and endometrial transcriptomic profiles were integrated with protein-protein interactions (Altmäe *et al*., 2012). Nevertheless, this new systems approach must still be further developed in order to integrate and mathematically model the data so that information about functional genomics in normal and disease endometrial physiology can be deduced (Wang *et al*., 2010).

The new highthroughput technologies and their associated computational analysis have to evolve and develop much more before they can be considered cutting-edge technologies. Transcriptomics, based on microarray technology, currently has sufficiently standarised procedures to allow them to be applied clinically, although their functional relevance still remains unclear. However, the current standard in RNA-seq, the definition of the transcriptome using NGS platforms is likely to be challenged by newer global gene-expression analysis technologies, as reported by the MAQC consortium (Mane *et al*., 2009). While RNA-seq technology is improving the precision of our knowledge of the transcriptome by detecting previously undiscovered alternative splicing variants, this improved-sensitivity NGS method and systems biology approach combined together could be used to elucidate even more about embryo implantation using transcriptomics.

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