OXFORD

human reproduction

Human Reproduction, 2025, **00(00)**, 1–10
https://doi.org/10.1093/humrep/deaf156
Original Article

Infertility

Stratifying IVF population endometria using a prognosis gradient independent of endometrial timing†

Josefa Maria Sanchez-Reyes [1,2,‡], Antonio Parraga-Leo [1,2,‡], Patricia Sebastian-Leon [1], Maria del Carmen Vidal[3], Diana Marti-Garcia [1], Katharina Spath[4], Imma Sanchez-Ribas[5], Francisco Jose Sanz [1], Nuria Pellicer[3], Jose Remohi[2,3], Dagan Wells[4,6], Antonio Pellicer [2,7], Patricia Diaz-Gimeno [1,*]

[1]IVIRMA Global Research Alliance, IVI Foundation, Instituto de Investigación Sanitaria La Fe, Valencia, Spain
[2]Department of Pediatrics, Obstetrics & Gynecology, University of Valencia, Valencia, Spain
[3]IVIRMA Global Research Alliance, IVIRMA IVI Valencia, Valencia, Spain
[4]JUNO Genetics, Oxford, UK
[5]IVIRMA Global Research Alliance, IVIRMA IVI Barcelona, Barcelona, Spain
[6]Nuffield Department of Women's & Reproductive Health, University of Oxford, Women's Centre John Radcliffe Hospital, Oxford, UK
[7]IVIRMA Global Research Alliance, IVIRMA IVI Rome, Rome, Italy

*Correspondence address. Instituto de Investigación Sanitaria La Fe, Biopolo La Fe, Av. Fernando Abril Martorell 106, Torre A, Planta 1ª, 46026 Valencia, Spain. Tel: +34-96-390-33-05. E-mail: patricia.diaz@ivirma.com https://orcid.org/0000-0003-0031-7545

†Some of the findings reported in this scientific article were presented at the *78th American Society for Reproductive Medicine Scientific Congress & Expo* (ASRM) held in Anaheim, CA, USA, October 22–26, 2022.

‡These authors contributed equally to this work and should be regarded as joint first authors.

ABSTRACT

STUDY QUESTION: Can the disrupted window of implantation (WOI) be stratified according to transcriptomic patterns associated with reproductive success in IVF patients undergoing HRT?

SUMMARY ANSWER: There are four transcriptomic patterns independent of endometrial timing associated with a gradient of reproductive prognosis underlying different molecular pathomechanisms.

WHAT IS KNOWN ALREADY: A molecular heterogeneous profile independent of endometrial timing has been discovered as a cause of implantation failure that disrupt the endometrial transcriptome in the mid-secretory phase. However, the molecular heterogeneous patterns underlying the disruption remain poorly identify and understood. Characterizing the molecular heterogeneity of this endometrial disruption is crucial to develop personalized and more accurate diagnostic tools for preventive medicine, particularly for patients with a high risk of endometrial failure.

STUDY DESIGN, SIZE, DURATION: In this multicenter prospective study, 195 IVF patients undergoing HRT with endometrial biopsy collection, during mid-secretory phase for endometrial progression evaluation, were recruited between January 2019 and August 2022. Out of 195 patients, 131 were finally included in the following analysis.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Endometrial biopsies were processed for whole endometrial transcriptome analysis using RNA-Sequencing. To identify disruptions in the WOI, the transcriptomic variation due to cyclic endometrial tissue changes was removed. Out of 195 biopsies sequenced, 131 were derived from patients that met the clinical criteria to be classified as implantation failure group (≥3 implantation failures, n = 32) or control group (<3 implantation failures, n = 99). An artificial intelligence (AI) model, based on two supervised learning algorithms: support vector machine (SVM) and k-nearest neighbors (kNN), was performed with 131 patients that were randomly allocated to training (n = 105) and test (n = 26) sets for biomarker signature discovery and assessment of predictive performance, respectively. The reproductive outcomes of the single embryo transfer immediately after biopsy collection were analyzed. Differential expression and functional analyses were performed to characterize molecular profiles. Finally, a quantitative PCR (qPCR) assay was used to corroborate the differential expression of six potential biomarkers.

MAIN RESULTS AND THE ROLE OF CHANCE: With the dichotomous clinical classification of poor or good reproductive prognosis, there was no transcriptomic distinction between patients with a history of implantation failures during HRT endometrial preparation. Alternatively, using an AI model to stratify IVF patients based on the probability of endometrial disruption revealed molecular and clinical differences between patterns. Patients were stratified into four reproductive prognosis-related profiles: p1 (n = 24), p2 (n = 14), c2 (n = 32) and c1 (n = 61). The highest pregnancy rate (PR) was associated with c1 (91%) and the highest ongoing pregnancy rate (OPR) was associated with c2 (78%), linking these profiles to good reproductive prognoses. On the other hand, p1 had the highest biochemical miscarriage rate (43%) while p2 had the highest clinical miscarriage rate (43%). Notably, both p1 and p2 were related to lower PR and OPR, supporting that these profiles were associated with poor prognoses. Regarding the functional characterization in the poor prognosis profiles that were linked to miscarriages, p1 was associated with an excessive immune response against the

Received: January 27, 2025. **Revised:** July 11, 2025. **Editorial decision:** July 22, 2025.

embryo during early pregnancy stages, while p2 was initially immune-tolerant but rejected the fetus in later stages due to the lack of metabolic response.

LIMITATIONS, REASONS FOR CAUTION: Due to the heterogeneous character of the disrupted WOI and the limited sample size of the different stratified groups, the AI model has limited population inference. However, our significant promising findings provide strong leads for further clinical studies with larger sample sizes.

WIDER IMPLICATIONS OF THE FINDINGS: This new transcriptomic taxonomy associated with distinct reproductive outcomes provides clues to design new and more accurate evaluation tools for endometrial-factor infertility. Furthermore, it enables tailoring therapeutic strategies to apply a personalized medicine to each patient suffering from endometrial-factor infertility, improving their odds of getting pregnant.

STUDY FUNDING/COMPETING INTEREST(S): This study was supported by the IVI Foundation (1706-FIVI-048-PD); Instituto de Salud Carlos III (ISCIII) and co-funded by the European Regional Development Fund "A way to make Europe" (PI19/00537 [P.D.-G.]) as well as Instituto Carlos III (ISCIII) through project (PI23/00806 [P.D.-G.]) and co-funded by European Union. Patricia Diaz-Gimeno is supported by Instituto de Salud Carlos III (ISCIII) through the Miguel Servet program (CP20/00118) co-funded by the European Union. Patricia Sebastian-Leon and Francisco Jose Sanz are funded by Instituto de Salud Carlos III (ISCIII) through the Sara Borrell postdoctoral program (CD21/00132 [P.S.-L.] and CD23/00032 [F.J.S.]) co-financed by the European Union. Josefa Maria Sanchez-Reyes was supported by a predoctoral fellowship program of the Generalitat Valenciana (ACIF/2018/072 and BEFPI/2020/028). Antonio Parraga-Leo (FPU18/01777) and Diana Marti-Garcia (FPU19/03247) were supported by predoctoral fellowship programs of the Spanish Ministry of Science, Innovation and Universities. The authors declare no conflicts of interest.

TRIAL REGISTRATION NUMBER: Not applicable.

Keywords: gene expression signature / endometrial disruption / endometrial function / artificial intelligence / transcriptomic stratification / infertility / precision medicine / endometrial transcriptomics

Introduction

Maternal endometrial status is a key factor in successful embryo implantation and development in ARTs (Strowitzki *et al.*, 2006). Endometrium undergoes cyclical changes to enable the implantation, finding the maximum uterine receptivity during the window of implantation (WOI) in the mid-secretory phase (Noyes *et al.*, 1975; Harper, 1992; Wilcox *et al.*, 1999; Murphy, 2004). Synchronization between endometrium and embryo is essential during the WOI (Díaz-Gimeno *et al.*, 2011, 2013, 2017), as well as maintenance of an undisrupted endometrial function (Koot *et al.*, 2016; Sebastian-Leon *et al.*, 2018; Diaz-Gimeno *et al.*, 2024b). After successful embryo implantation, the endometrium must support placentation and provide an optimal equilibrium of embryo-maternal interactions as well as adequate vascularization for fetal growth (Duc-Goiran *et al.*, 1999).

Despite the development of ARTs, approximately 35% of transferred euploid embryos do not implant in anatomically normal uteri at the first attempt (Pirtea *et al.*, 2023). Patients experiencing successive implantation failures are clinically classified as having recurrent implantation failure (RIF); however, there is a lack of consensus on the definition of RIF (Cimadomo *et al.*, 2021; Pirtea *et al.*, 2023). The heterogeneous etiology and clinical symptoms of RIF do not provide sufficient criteria to stratify patients (Koot *et al.*, 2016; Devesa-Peiro *et al.*, 2021).

Artificial intelligence (AI) models using transcriptomic have been applied in endometrium to characterize WOI since 2011 (Díaz-Gimeno *et al.*, 2011, 2013, 2017). Nevertheless, the consideration of the endometrial failure as a condition independent of endometrial timing using AI models appeared more recently (Koot *et al.*, 2016; Macklon, 2017). This endometrial failure profile, independent of endometrial timing, has been characterized using transcriptomic in natural cycles (Koot *et al.*, 2016) and natural HRT cycles (Diaz-Gimeno *et al.*, 2024b), employing a binary classification model that categorizes samples as either control or pathology. Notably, this binary classification revealed molecular heterogeneity within each category, raising the question of whether different molecular profiles may exist within these binary classes. Therefore, based on the previous findings, further studies stratifying the endometrial failure independent of timing are required. These studies will enable to characterize the molecular profiles that contribute to the interpatient variability, discover new biomarkers and develop tailored treatments.

The combined use of transcriptomics and AI algorithms has significantly advanced the understanding of endometrial-factor infertility (Díaz-Gimeno *et al.*, 2011, 2017, 2022, 2024b; Koot *et al.*, 2016; Sebastian-Leon *et al.*, 2018). This work is laying the groundwork for new applications in precision medicine and ARTs, facilitating the characterization of reproductive diseases, patient diagnosis and prognosis. In this context, accurate patient stratification is necessary to match treatment to the right patient (Guttmacher and Collins, 2002; Mirnezami *et al.*, 2012; Bell, 2014).

Leveraging the use of AI algorithms, our group recently proposed a biomarker signature in luteal-phase endometrial biopsies that stratifies ART patients undergoing HRT into poor or good reproductive prognosis, independent of endometrial timing (Diaz-Gimeno *et al.*, 2024b). In contrast to previous studies performed in natural cycles (Koot *et al.*, 2016), this prospective study included clinical follow-up to investigate if the prognostic transcriptome-based groups were related to reproductive outcomes. Despite using a 404-gene panel, dichotomous patient classification into poor or good prognosis was limited by the molecular complexity of implantation failure that requires the identification of more subtypes (Diaz-Gimeno *et al.*, 2024b).

Hence, this current prospective study aims to use the whole transcriptomic profile to reproduce and refine our previous classification in a new cohort of patients undergoing HRT. Our new prognostic stratification elucidates the molecular heterogeneity of the endometrial disruptions in the mid-secretory phase, independent of endometrial timing.

Materials and methods

Ethical approval

This study was approved by the Ethics Committee of the Instituto Valenciano de Infertilidad (Valencia, Spain; 1706-FIVI-048-PD). Written informed consent was obtained from all participants.

Participants and clinical follow-up

Participants (n = 291) were recruited for a multicentric, prospective study between January 2019 and August 2022 at five private

fertility clinics in Spain. Participants were scheduled for endometrial evaluation before embryo transfer due to medical indications, and met the following inclusion criteria: 18–50 years old, with a BMI of 19–30 kg/m^2, undergoing HRT prior to single embryo transfer (SET) with a good-quality embryo (euploidy guaranteed by preimplantation genetic testing or oocytes from donors <35 years old), and presenting an endometrial thickness >6.5 mm with trilaminar structure in proliferative phase. Exclusion criteria were male-factor infertility (in cases with autologous sperm) as the only treatment indication, untreated reproductive pathologies that may compromise endometrial function, severe pre-menopausal symptoms, uncontrolled systemic or metabolic disorders, and co-administered medication that can interfere with ARTs.

Baseline participant characteristics were obtained from internal medical records, in accordance with data protection laws in Spain.

Study design

IVF patients undergoing HRT were clinically classified according to their history of implantation failures. Endometrial biopsies were collected during the mid-secretory phase for RNA-Sequencing (RNA-Seq) analysis. An AI probabilistic model was developed based on the transcriptome independent of endometrial timing, avoiding biases in transcriptomic variation due to cyclical changes in the endometrium (Supplementary Data File S1). The AI-determined probability of a poor prognosis was used to stratify the population. Finally, the clinical outcomes and molecular functions of the stratified profiles were studied (Figure 1).

Endometrial biopsy collection, sequencing and data processing

Endometrial biopsies were obtained from the uterine fundus, using a cannula (Pipelle Cornier, CCD Laboratories, Paris, France) under sterile conditions, after approximately 120 hours of progesterone treatment in the HRT cycles. Anonymized samples were stored in RNA-later® (Sigma-Aldrich, Madrid, Spain) at −80°C. RNA was extracted using the miRNeasy Mini Kit following manufacturer's instructions (Qiagen, Hilden, Germany). RNA quality was assessed using the NanoDrop™ One (AF-00342, ThermoFisher Scientific, Valencia, Spain) and 4200 TapeStation System® (Agilent, Valencia, Spain). Only samples that met the following RNA quality criteria were included in the study: 260/280 ratio ~2.0, 260/230 ratio = 1.8–2.2, RNA integrity number (RIN) ≥3 and RNA fragments with more than 200 nucleotides (DV200) ≥70%.

Samples were sequenced using the AmpliSeq for Illumina® Transcriptome Human Gene Expression Panel (Illumina, San Diego, USA). This panel targets amplicons in the whole transcriptome (20 802 amplicons of 104 base pairs) and was selected due to its compatibility with high- and low-quality RNA samples from human tissue. The sequencing process was carried out on a NextSeq500/550 system, using a paired-end design, with 150 cycles and sequencing 10 M reads per sample. Raw data were evaluated using FastQC (version 0.11.9) (Andrews *et al.*, 2012). STAR (version 2.7.3) (Dobin *et al.*, 2013) was employed to align high-quality data using GRCh37/hg19 as a reference. Gene counts obtained using featureCounts (version 2.0.3) (Liao *et al.*, 2014), excluding low-quality counts (Q < 30). Thereafter, counts were normalized using Voom transformation and quantile normalization in limma (version 3.46.0) (Ritchie *et al.*, 2015). Genes with low counts were filtered using EdgeR (version 3.32.1) (Chen *et al.*, 2016). Outliers and possible batch effects detected using principal component analysis (PCA) were corrected using limma linear models (Ritchie *et al.*, 2015). Finally, the transcriptomic variation due to endometrial luteal phase timing effects was detected using a transcriptomic endometrial dating (TED) model established by our group (Diaz-Gimeno *et al.*, 2022), then removed using limma as we previously described (Diaz-Gimeno *et al.*, 2024b). Additional details about the TED model are presented in Supplementary Data File S1.

Clinical classification of patients

Patients were initially classified based on their clinical history of implantation failures. Implantation failure was considered for patients who presented a negative beta chorionic gonadotropin (β-hCG) value (≤10 IU/L) 14–16 days following embryo transfer, or a biochemical miscarriage (defined by a positive serum β-hCG value, but absence of pregnancy within the first 10 weeks of gestation) (Pirtea *et al.*, 2023). Patients with at least three implantation failures were initially classified as implantation failure group whereas patients who achieved implantation success within the first three attempts, were initially classified as control group. Patients with insufficient attempts were excluded from further analyses (n = 62).

Patient stratification based on AI algorithms

A training set (80% of samples) was employed to identify a biomarker signature for endometrial disruption in the mid-secretory phase, stratify patients and develop an AI-based prediction model that was externally validated in an independent testing set (20% of samples).

The training set was used for endometrial gene signature discovery, as previously described (Diaz-Gimeno *et al.*, 2022) and used to develop a balanced probabilistic model (Supplementary Data File S1). The probabilistic model was internally evaluated through cross-validation (5-fold, 10 times) in 100 different balanced models and their performance was evaluated with the test set. The range of poor prognosis probabilities obtained by running the AI model in all samples was used to stratify the study cohort into the following good (c) and poor (p) prognosis profiles: c1 (probability ≤ 0.2), c2 (probability > 0.2 and probability < 0.5), p2 (probability ≥ 0.5 and probability < 0.8) or p1 (probability ≥ 0.8).

Molecular characterization of the different transcriptomic profiles

The differentially expressed genes (DEGs) [False Discovery Rate (FDR) < 0.05] between profiles were identified using limma. Next, the functional differences between the profiles were revealed with gene set enrichment analyses (GSEA) performed using ClusterProfiler (version 3.18.1) (Yu *et al.*, 2012). Biological functions were obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG; September 2021 version) (Kanehisa and Goto, 2000) while experimental annotated terms were obtained from Gene Ontology (GO; December 2021 version) (Ashburner *et al.*, 2000).

Validating the expression of selected potential biomarkers

The expression of six DEGs was evaluated in 20 samples (five samples per transcriptomic profile) with quantitative PCR (qPCR) using beta-actin (*ACTB*) as a housekeeping gene. RNA was reverse transcribed into cDNA using the PrimeScript Reagent Kit (Perfect Real Time, Takara, Japan) on a Thermocycler T3000 (Biometra, Ireland). The qPCR was carried out using Power-Up SYBR Green (Thermo Fisher Scientific, MA, USA) on a StepOnePlus System (Applied Biosystems, CA, USA). The specific primer sequences (Invitrogen, Thermo Fisher Scientific, MA, USA) are presented in

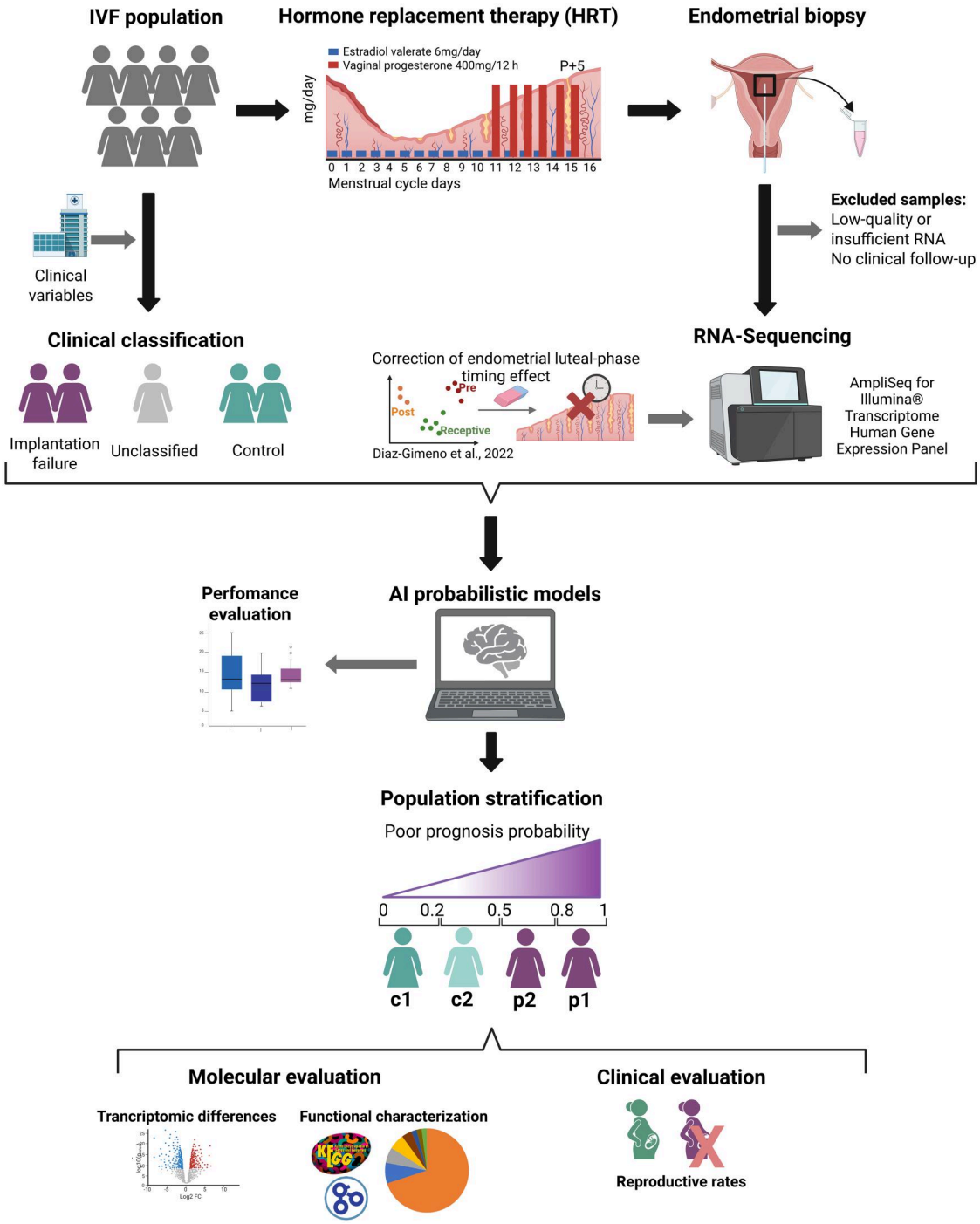

Figure 1. Study design. IVF patients undergoing HRT were classified as having good or bad endometrial prognosis profiles based on their reproductive histories. Endometrial biopsies were processed for whole-transcriptome RNA-Sequencing. Following RNA-Sequencing data normalization, the effect of endometrial luteal-phase timing was corrected. Subsequently, an artificial intelligence model was developed to stratify the population into four groups according to their probability of having a poor prognosis. Transcriptomic evaluation and functional characterization were followed by an analysis of clinical reproductive outcomes profiles that were clinically relevant.

Supplementary Table S1. Relative gene expression was calculated using the ΔΔCt method (Livak and Schmittgen, 2001) and *ACTB* as a housekeeping gene.

Statistical analyses

Rates for reproductive outcomes [i.e. pregnancy (PR), cumulative pregnancy (CPR), live birth (LBR), biochemical miscarriage (BMR) and clinical miscarriage (CMR)] were calculated as described in the Supplementary Data File S1. Descriptive statistics were used to ensure homogeny of the patients' baseline clinical characteristics. Continuous variables were presented as an overall mean ± standard deviation, whereas discrete variables were presented as counts and percentages. Statistical differences between groups were compared using the Wilcoxon rank-sum test for continuous variables and the Fisher's exact test for discrete variables. All statistical analyses were conducted in R (version 4.0.5, 2021-03-31) (R Core Team, 2021). Graphical results were generated with ggplot2 (version 3.5.1) (Wickham, 2016). In all cases, $P < 0.05$ was considered statistically significant.

Results

Transcriptomic data and clinical characterization of patients

After quality control and analysis of available clinical information (Supplementary Figure S1A), 131 samples and 14 674 genes qualified for evaluation. Patients were clinically classified as having an endometrium associated with a poor (n = 32) or good (n = 99) prognosis based on the number of previous implantation failures. Both groups were homogeneous in terms of main clinical variables (e.g. number of patients, age, BMI, infertility type and duration, endometrial dating) (Supplementary Table S2), ensuring that there were no potential biases in endometrial-factor transcriptomic differences. As expected, the number of transfers and implantation failures were significantly different between groups (*P*-value = 2.20e-16) due to the clinical classification criteria used for this study. However, pairwise comparison revealed there were no significant differences between the good prognosis groups (c1 and c2). Batch effects were corrected to ensure the transcriptomic differences were related to endometrial disruption independent of endometrial timing (Supplementary Figures S1B and C and S2). For more details, see Supplementary Data File S1.

Four new prognostic stratification groups for endometrial function

Participants were stratified into four profiles using a 236-gene signature and a probabilistic model that predicts endometrial profiles with 77% accuracy, 67% sensitivity and 80% specificity (Supplementary Figure S3, Supplementary Tables S3 and S4). The four profiles were established as poor (p) or good (c) according to the probability of presenting an endometrium associated with a poor prognosis: p1 (n = 24) and p2 (n = 14), c2 (n = 32) and c1 (n = 61). Clinical variables were homogenous for all stratified profiles, including age, BMI, infertility type and duration, endometrial dating, number of embryo transfers, number of implantation failures and embryo origin (Table 1).

As expected, the number of transfers and implantation failures were significantly different between the four profiles. Profiles associated with a poor prognosis showed lower PR (29.17%) and LBR (50.00%) coupled with higher CMR (42.86%) and BMR (42.68%) compared to the good prognosis profiles (Figure 2A). These differences were statistically significant (p-value ≤ 0.05) for all outcomes except CMR (Figure 2A). Interestingly, the p1 profile was related to a higher rate of biochemical miscarriages, the p2 profile was related to clinical miscarriages and the c1 profile was associated with the best reproductive outcomes. When groups were compared, pairwise significant differences were found between the p1 and c1 groups in terms of PR (*P*-value = 0.0011), LBR (*P*-value = 0.0478) and BMR (*P*-value = 0.0018); between p2 and c1 groups in terms of LBR (*P*-value = 0.0147) and CMR (*P*-value = 0.0478), and finally, between the p1 and c2 groups in terms of PR (*P*-value = 0.004) (Figure 2A). Considering all embryo transfer attempts, the cumulative PR reached 38% for p1, 76% for p2, 81% for c2 and 93% for c1 (Figure 2B).

Molecular characterization of the transcriptomically defined profiles

With respect to c1, there were 47 DEGs identified in p2, 3 DEGs in p1 and 1 DEG in c2 (Supplementary Table S5). Only one transcript, mitogen-activated protein kinase 8 interacting protein 1 pseudogene (*LOC644172* or *MAPK8IP1P2*), was shared between p1 and c1 profiles, as well as c2 and c1 profiles (Supplementary Table S6).

The p2 and p1 profiles showed the highest number of functional dysregulations compared to c1 (54 and 38 dysregulations, respectively). The downregulated functions (30/54, 55.6%) in p2 were mainly related to immune response (n = 10) or metabolism and energy production (n = 12). The upregulated functions (24/54, 44.4%) in p2 were mainly related with nervous system and sensory perception (n = 6), gene expression and protein degradation (n = 6). Compared to the c1 profile, the p1 profile had mainly upregulated functions (27/38, 71.1%), which were related to immune responses (n = 9), nervous system and sensory perception (n = 6). Notably, most of the downregulated functions (11/38, 28.9%) in p1 were related to cellular movement and ciliary processes (n = 6) (Figure 3, Supplementary Figure S4).

Both poor-prognosis-related profiles had a noticeable dysregulation of immune responses compared to the c1 profiles. However, the p1 was characterized by nine upregulated functions while the p2 was characterized by ten downregulated

Table 1. Baseline characteristics and reproductive history of the stratified groups.

	c1	c2	p2	p1	P-value
No. patients	61	32	14	24	N/A
Age (years)	40.77 ± 4.41	39.84 ± 4.97	40.14 ± 4.44	42.5 ± 3.56	0.1686
BMI (kg/m^2)	23.62 ± 3.97	22.53 ± 3.47	22.53 ± 3.34	22.22 ± 3.68	0.3324
Infertility type	P = 48 (82.8%) S = 10 (17.2%) N/A = 3	P = 23 (79.3%) S = 6 (20.7%) N/A = 3	P = 12 (92.3%) S = 1 (7.7%) N/A = 1	P = 18 (85.7%) S = 3 (14.3%) N/A = 3	0.8501
Infertility duration (years)	2.95 ± 2.86	3.31 ± 3.10	3.58 ± 1.87	2.8 ± 1.7	0.7943
Endometrial dating (TED)	Ds = 41 (67.2%) Ot = 20 (32.8%)	Ds = 18 (56.3%) Ot = 14 (43.7%)	Ds = 7 (50.0%) Ot = 7 (50.0%)	Ds = 17 (70.8%) Ot = 7 (29.2%)	0.4354
No. embryo transfers	1.75 ± 0.85	1.78 ± 0.91	2.71 ± 1.44	4.21 ± 1.32	5E-17*** [a, b, c, d, e]
No. implantation failures	0.75 ± 0.85	0.81 ± 1.00	2.00 ± 1.75	3.79 ± 1.14	4E-22*** [a, b, c, d, e]
Embryo origin	Donated = 39 (66.1%); Own = 20 (33.9%) N/A = 2	Donated = 22 (68.8%); Own = 10 (31.2%)	Donated = 22 (91.7%); Own = 2 (8.3%)	Donated = 10 (71.4%); Own = 4 (28.6%)	0.0972

Endometrial profiles (c1, c2, p2 and p1), corresponding to good prognosis profile 1 and 2 (c1, c2) and poor prognosis profile 1 and 2 (p1, p2), respectively, are compared according to the main clinical variables related to reproductive outcomes. Using the transcriptomic endometrial dating (TED) model, endometria in early and late secretory classes were grouped as displaced (Ds) while early and late mid-secretory classes were grouped as on time (Ot). BMI, body mass index; N/A, not available; No., number of.; P, primary; S, secondary.

*** P-value < 0.001.

[a] P-value < 0.05 in p1 vs. c1.

[b] P-value < 0.05 in p1 vs. c2.

[c] P-value < 0.05 in p2 vs. c1.

[d] P-value < 0.05 in p2 vs. c2.

[e] P-value < 0.05 in p2 vs. p1.

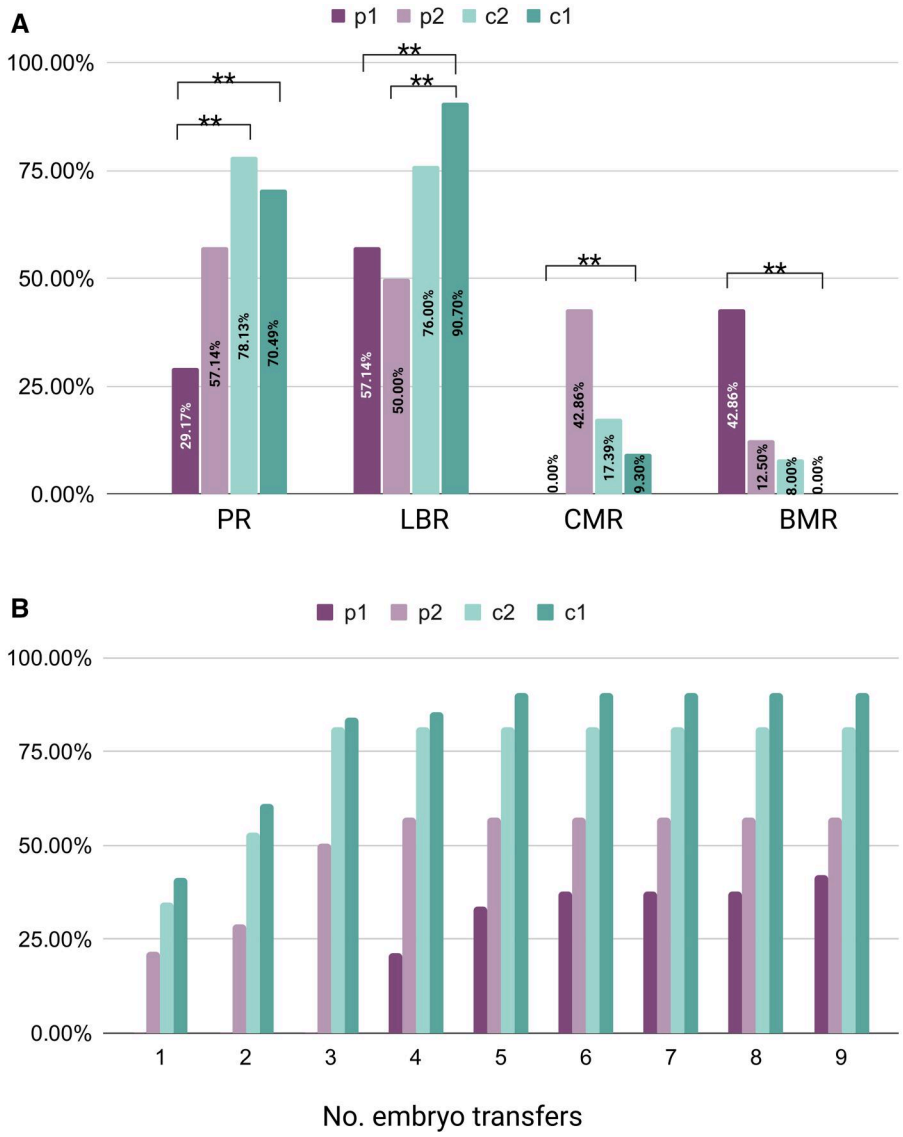

Figure 2. Clinical evaluation of transcriptomic profiles. (**A**) Bar plot showing the significant differences obtained through pairwise comparisons of different profiles. PR, pregnancy rate; LBR, live birth rate; CMR, clinical miscarriage rate; BMR, biochemical miscarriage rate; p1, poor prognosis profile 1; p2, poor prognosis profile 2; c1, good prognosis profile 1; c2, good prognosis profile 2. (**B**) Bar plot showing the cumulative pregnancy rate from multiple embryo transfers. *P-value < 0.05; **P-value < 0.01; ***P-value < 0.001.

functions. The p2 profile also presented 12 downregulated functions related to metabolism and energy production (Figure 3).

There were 22 functional dysregulations between the control profiles, with the c2 presenting a dysregulated profile similar to p2. Specifically, the c2 profile presented a downregulation of nine functions related to immune responses and an upregulation of five functions related to the nervous system and sensory perception (Figure 3).

Validating expression of potential biomarkers of endometrial disruption

The expression of six DEGs was validated by qPCR. Three of these DEGs were identified in the p1 vs. c1 comparison [DND microRNA-mediated repression inhibitor 1 (*DND1*), synaptotagmin 10 (*SYT10*) and mitogen-activated protein kinase 8 interacting protein 1 pseudogene (*MAPK8IP1P2*)] or c2 vs. c1 comparison (*MAPK8IP1P2*). The remaining three DEGs were selected for having the highest absolute fold change between p2 and c1 [CF transmembrane conductance regulator (*CFTR*), V-set domain containing T cell activation inhibitor 1 (*VTCN1*) and solute carrier family 17 member 8 (*SLC17A8*)]. Except for *SLC17A8* (Supplementary Figure S5), all genes showed the same gene expression trends in qPCR and RNA-Seq, reinforcing their role as potential biomarkers of endometrial disruption.

Discussion

This study is the first to stratify endometrial function into four transcriptomic profiles, independent of endometrial timing. The four transcriptomic profiles corresponded with significantly different reproductive rates, showing a gradient of prognoses and highlighting the complex nature of endometrial disruption in the mid-secretory phase.

Disrupted endometrial function, independent of luteal phase endometrial timing, was associated with a heterogeneous transcriptomic behavior among IVF patients undergoing HRT, as previously reported in patients undergoing natural cycles (Koot *et al.*, 2016). Our binary prediction model (good vs. poor prognosis) was

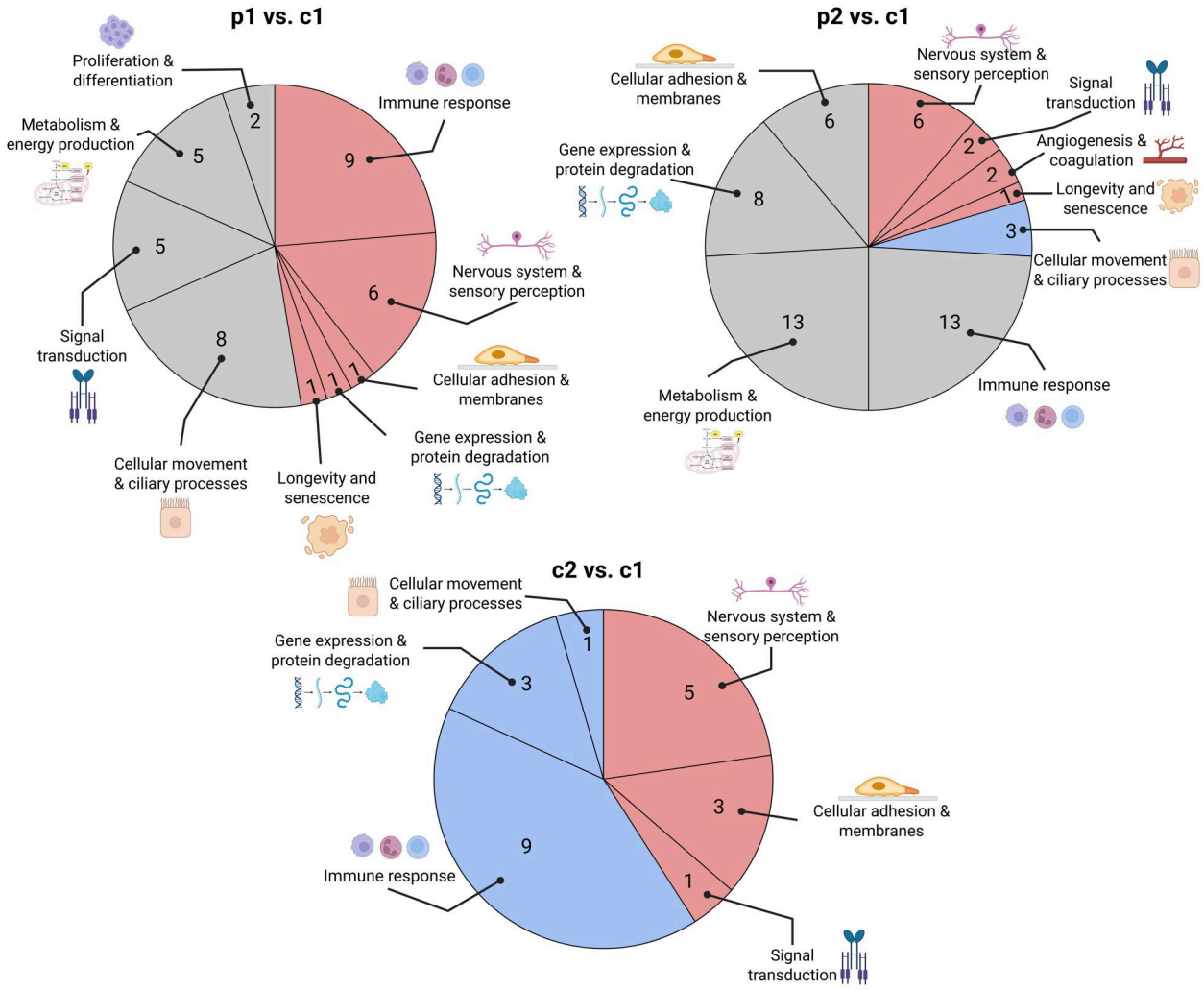

Figure 3. Functional differences between endometrial profiles. The pie charts show significantly upregulated (red sections), downregulated (blue sections) or both up- and downregulated (grey sections) biological functions [False Discovery Rate (FDR) < 0.05], identified through a gene set enrichment analysis (GSEA). These results correspond to the comparisons that showed differentially-expressed genes (p1 vs. c1, p2 vs. c1 and c2 vs. c1), where p1 and p2 represent poor prognosis profiles 1 and 2, and c1 and c2 represent good prognosis profiles 1 and 2, respectively.

based on a signature of 236 genes that characterized the transcriptomic behavior with 67% sensitivity. In addition, this study proposed a novel stratification based for first time on four whole-transcriptome-based profiles with a gradient of reproductive prognosis for IVF patients. This approach leverages AI-computed probabilities which are more objective and robust than traditional clinical parameters to classify patients with endometrial-factor infertility based on the number of implantation failures. Furthermore, potential biomarkers were validated using a qRT-PCR, where all of them showed the expected tendency except for SLC17A8, which showed the lowest fold change in RNA-seq analysis among the selected potential biomarkers. Notably, a pseudogene, MAPK8IP1P2, was also selected, which is involved in hippo signaling pathway (Liu *et al.*, 2021), molecular pathway with an important role in the endometrium processes such as angiogenesis, cell proliferation or differentiation (Moon *et al.*, 2022).

Although previous studies have also developed predictive models to identify the endometrial disruption, we observed that our model's predictive performance exceeds that of Koot's binary model, which was based on a larger signature of 301 genes and reached a 58.3% sensitivity when used to compare control and RIF patients in natural cycles (Koot *et al.*, 2016). On the other hand, our last study in patients undergoing HRT showed that stratifying patients using a 404-gene panel instead of clinical criteria significantly enhanced the transcriptomic and clinical differences between poor and good prognosis profiles (Diaz-Gimeno *et al.*, 2024b). However, the probabilistic model was not tested in an independent test set, it was based on a small number of biomarkers and designed to obtain a binary classification, impeding comparison of the model's performance and the study of the variability of endometrial disruption.

It is worth mentioning that due to the stratification into four groups and the limited sample size by group, the AI model needs further optimization. Further studies with larger patient cohorts are required to boost the statistical power of the model for population inference, assess inter-cycle reproducibility and conduct rigorous prospective clinical trials prior to clinical implementation (Diaz-Gimeno *et al.*, 2024a). Moreover, *in vitro* and animal experiments are needed to elucidate the biological pathways underlying the identified transcriptomic profiles. The development of new animal models able to replicate these transcriptomic profiles or specific phenotypes of such profiles would be valuable to study underlying molecular mechanisms. Finally, our model was developed using transcriptomic profiles from patients

undergoing HRT. Although, HRT cycles reduce the hormone variability among patients, further analyses should be conducted to determine whether the same pathological gradient could be observed in patients undergoing a natural cycle.

The four new prognostic profiles characterized in this work can be used to predict reproductive outcomes in IVF patients with a history of implantation failures. Specifically, the p1 transcriptomic profile was related to the worst prognosis, characterized by the highest BMR and the lowest PR. On a molecular level, the p1 was associated with an excessive immune response. While a local inflammatory environment is required during implantation and placentation (Mor *et al.*, 2017; Förger and Villiger, 2020), the excessive immune response observed in p1 profile suggests that a poor feto-maternal tolerance could be driving miscarriages in the early stages of pregnancy (Sargent *et al.*, 1988; Colucci, 2019). Indeed, functional analyses revealed an upregulation of natural killer cell activity, which has been strongly linked to high rates of recurrent miscarriage (Guerrero *et al.*, 2020). Moreover, the overall downregulation of ciliary processes in this cohort of patients with RIF reinforces their role in uterine disorders (Devesa-Peiro *et al.*, 2020). More specifically, in the context of recurrent miscarriages, abnormal ciliogenesis in decidua stromal cells has been proposed as a potential explanatory factor of this condition (Hassan *et al.*, 2022). Alternatively, the p2 profile was related to the highest CMR and a lack of immune and metabolic responses. Functional analyses highlighted the dysregulation of important metabolic processes such as insulin pathway, carbohydrate metabolism or cellular respiration, which have previously reported to be involved in pregnancy loss (Craig *et al.*, 2002; Cai *et al.*, 2022; Zhu *et al.*, 2023). These findings suggest that implantation is facilitated by an initial immune tolerance but miscarriage occurs in subsequent pregnancy stages due to impaired cellular energy metabolism. This novel hypothesis about the relevance of the endometrial factor in clinical miscarriages requires further investigation. Finally, there were two good prognosis profiles characterized by high PRs and LBRs. The c1 profile was related to the highest LBR and lowest BMR. It is interesting to highlight despite being considered healthy profiles, these results are showing transcriptomic differences between c1 and c2 were found, which could explain the trend toward lower LBRs of c2 compared to c1 although not significant, possibly due to limited statistical power. For instance, alterations in immune system, ciliary processes and cellular adhesion were observed in functional analyses. Further investigation is required to elucidate the difference between both profiles and understand the main causes of these transcriptomic differences and uncover clinical implications for preventive medicine.

Overall, this new taxonomy can help improve the precision of diagnosis and treatment of infertile women with the discovery of new biomarkers and/or tailored therapeutic targets for each profile. It lays the groundwork for a new generation of tools for evaluating patients with suspected endometrial-factor infertility or stratifying types of miscarriages.

Conclusion

Regardless of endometrial timing, the heterogeneous endometrial function can be leveraged to stratify IVF patients undergoing HRT. This study uncovers four distinct prognostic groups reflecting disrupted molecular profiles related with the highest BMR and CMR (p1 and p2, respectively) or less disrupted profiles associated with the highest LBR (c1) and the highest PR (c2). These molecular findings were linked to functional differences, highlighting overactive immune responses in p1 and decreased metabolism in p2, and revealing potential mechanisms of actions underlying the biochemical and clinical miscarriages in this cohort. Taken together, our results support that good and poor endometrial prognoses have evident molecular and clinical differences. These findings advance the research in personalized diagnostic and therapeutic strategies in reproductive medicine, particularly for patients with endometrial-factor infertility.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Acknowledgements

The authors thank the patients who participated in the study and the clinical staff who contributed to their recruitment, especially Elena Labarta, Juan Antonio García Velasco, Juan Giles, Ernesto Bosch, Agustín Ballesteros, Gema Castillón, Marcos Ferrando, Graciela Kohls, Francesca Gelosi, Laura Caracena, Marga Esbert, Isabel Llorens, Cristina Gaya, Mónica Toribio and Fernando Quintana. The authors would also like to thank Ester Castillo and Lourdes Fernandez from Illumina® Spain for their technical support for sequencing. Finally, the authors acknowledge Raquel Amigo and Cristina Cardona from the Biobank and Genomics Unit of the IIS La Fe, respectively for assisting biopsy storing and processing.

Authors' roles

J.M.S.-R.: investigation (RNA-Seq and qPCR), visualization, formal analysis, writing—original draft; A.P.-L.: formal analysis, visualization, software, writing—original draft; P.S.-L.: formal analysis, visualization, writing—review & editing; M.C.V., I.S-R., N.P.: patient recruitment and sample collection; D.M.-G: investigation (qPCR), K.S.: investigation (RNA-Seq); F.J.S.: visualization, writing—review & editing; J.R., A.P.: patient recruitment, sample collection and supervision; D.W.; conceptualization; P.D.-G.: conceptualization, methodology, formal analysis, writing—review & editing, supervision, project administration, funding acquisition. All authors have read and approved the final version of the manuscript.

Funding

This study was supported by the IVI Foundation (1706-FIVI-048-PD); Instituto de Salud Carlos III (ISCIII) and co-funded by the European Regional Development Fund "A way to make Europe" (PI19/00537 [P.D.-G.]) as well as Instituto Carlos III (ISCIII) through project (PI23/00806 [P.D.-G.]) and co-funded by European Union. P.D.-G. is supported by Instituto de Salud Carlos III (ISCIII) through the Miguel Servet program (CP20/00118) co-funded by the European Union. P.S.-L. and F.J.S. are funded by Instituto de Salud Carlos III (ISCIII) through the Sara Borrell postdoctoral program (CD21/00132 [P.S.-L.] and CD23/00032 [F.J.S.]) co-financed by the European Union. J.M.S.-R. was supported by a predoctoral fellowship program of the Generalitat Valenciana (ACIF/2018/072 and BEFPI/2020/028). A.P.-L. (FPU18/01777) and D.M.-G. (FPU19/

03247) were supported by predoctoral fellowship programs of the Spanish Ministry of Science, Innovation and Universities.

Conflict of interest

The authors declare no conflicts of interest.

References

Andrews S, Krueger F, Segonds-Pichon A, Biggins L, Krueger C, Wingett S. *FastQC: A Quality Control Tool for High Throughput Sequence Data*. 2012. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (15 July 2024, date last accessed).

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT *et al.* Gene ontology: tool for the unification of biology. *Nat Genet* 2000;**25**:25–29.

Bell J. Stratified medicines: towards better treatment for disease. *Lancet* 2014;**383**:S3–S5.

Cai W-Y, Luo X, Lv H-Y, Fu K-Y, Xu J. Insulin resistance in women with recurrent miscarriage: a systematic review and meta-analysis. *BMC Pregnancy Childbirth* 2022;**22**:916.

Chen Y, Lun ATL, Smyth GK. From reads to genes to pathways: differential expression analysis of RNA-Seq experiments using Rsubread and the edgeR quasi-likelihood pipeline. *F1000Res* 2016;**5**:1438.

Cimadomo D, Craciunas L, Vermeulen N, Vomstein K, Toth B. Definition, diagnostic and therapeutic options in recurrent implantation failure: an international survey of clinicians and embryologists. *Hum Reprod* 2021;**36**:305–317.

Colucci F. The immunological code of pregnancy. *Science* 2019;**365**:862–863.

Craig LB, Ke RW, Kutteh WH. Increased prevalence of insulin resistance in women with a history of recurrent pregnancy loss. *Fertil Steril* 2002;**78**:487–490.

Devesa-Peiro A, Sebastian-Leon P, Garcia-Garcia F, Arnau V, Aleman A, Pellicer A, Diaz-Gimeno P. Uterine disorders affecting female fertility: what are the molecular functions altered in endometrium? *Fertil Steril* 2020;**113**:1261–1274.

Devesa-Peiro A, Sebastian-Leon P, Pellicer A, Diaz-Gimeno P. Guidelines for biomarker discovery in endometrium: correcting for menstrual cycle bias reveals new genes associated with uterine disorders. *Mol Hum Reprod* 2021;**27**:gaab011.

Díaz-Gimeno P, Horcajadas JA, Martínez-Conejero JA, Esteban FJ, Alamá P, Pellicer A, Simón C. A genomic diagnostic tool for human endometrial receptivity based on the transcriptomic signature. *Fertil Steril* 2011;**95**:50–60.e15.

Díaz-Gimeno P, Ruiz-Alonso M, Blesa D, Bosch N, Martínez-Conejero JA, Alamá P, Garrido N, Pellicer A, Simón C. The accuracy and reproducibility of the endometrial receptivity array is superior to histology as a diagnostic method for endometrial receptivity. *Fertil Steril* 2013;**99**:508–517.

Díaz-Gimeno P, Ruiz-Alonso M, Sebastian-Leon P, Pellicer A, Valbuena D, Simón C. Window of implantation transcriptomic stratification reveals different endometrial subsignatures associated with live birth and biochemical pregnancy. *Fertil Steril* 2017;**108**:703–710.e3.

Diaz-Gimeno P, Sebastian-Leon P, Pellicer A. Reply of the authors: altered endometrial receptivity: walking across the long path of precision medicine. *Fertil Steril* 2024a;**122**:551.

Diaz-Gimeno P, Sebastian-Leon P, Sanchez-Reyes JM, Spath K, Aleman A, Vidal C, Devesa-Peiro A, Labarta E, Sánchez-Ribas I, Ferrando M *et al.* Identifying and optimizing human endometrial gene expression signatures for endometrial dating. *Hum Reprod* 2022;**37**:284–296.

Diaz-Gimeno P, Sebastian-Leon P, Spath K, Marti-Garcia D, Sanchez-Reyes JM, Vidal MDC, Devesa-Peiro A, Sanchez-Ribas I, Martinez-Martinez A, Pellicer N *et al.* Predicting risk of endometrial failure: a biomarker signature that identifies a novel disruption independent of endometrial timing in patients undergoing hormonal replacement cycles. *Fertil Steril* 2024b;**122**:352–364.

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 2013;**29**:15–21.

Duc-Goiran P, Mignot TM, Bourgeois C, Ferré F. Embryo–maternal interactions at the implantation site: a delicate equilibrium. *Eur J Obstetr Gynecol Reprod Biol* 1999;**83**:85–100.

Förger F, Villiger PM. Immunological adaptations in pregnancy that modulate rheumatoid arthritis disease activity. *Nat Rev Rheumatol* 2020;**16**:113–122.

Guerrero B, Hassouneh F, Delgado E, Casado JG, Tarazona R. Natural killer cells in recurrent miscarriage: an overview. *J Reprod Immunol* 2020;**142**:103209.

Guttmacher AE, Collins FS. Genomic medicine–a primer. *N Engl J Med* 2002;**347**:1512–1520.

Harper MJK. 10 The implantation window. *Baillière's Clin Obstetr Gynaecol* 1992;**6**:351–371.

Hassan E, Kojima R, Ozawa F, Yoshihara H, Goto S, Kitaori T, Inagaki H, Kato Y, Sugiura-Ogasawara M. Abnormal ciliogenesis in decidual stromal cells in recurrent miscarriage. *J Reprod Immunol* 2022;**150**:103486.

Kanehisa M, Goto S. KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* 2000;**28**:27–30.

Koot YEM, van Hooff SR, Boomsma CM, van Leenen D, Groot Koerkamp MJA, Goddijn M, Eijkemans MJC, Fauser BCJM, Holstege FCP, Macklon NS. NS. An endometrial gene expression signature accurately predicts recurrent implantation failure after IVF. *Sci Rep* 2016;**6**:19411.

Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 2014;**30**:923–930.

Liu X, Fu Q, Bian X, Fu Y, Xin J, Liang N, Li S, Zhao Y, Fang L, Li C *et al.* Long non-coding RNA MAPK8IP1P2 inhibits lymphatic metastasis of thyroid cancer by activating hippo signaling via sponging miR-146b-3p. *Front Oncol* 2021;**10**:600927.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 2001;**25**:402–408.

Macklon N. Recurrent implantation failure is a pathology with a specific transcriptomic signature. *Fertil Steril* 2017;**108**:9–14.

Mirnezami R, Nicholson J, Darzi A. Preparing for precision medicine. *N Engl J Med* 2012;**366**:489–491.

Moon S, Hwang S, Kim B, Lee S, Kim H, Lee G, Hong K, Song H, Choi Y. Hippo signaling in the endometrium. *Int J Mol Sci* 2022;**23**:3852.

Mor G, Aldo P, Alvero AB. The unique immunological and microbial aspects of pregnancy. *Nat Rev Immunol* 2017;**17**:469–482.

Murphy CR. Uterine receptivity and the plasma membrane transformation. *Cell Res* 2004;**14**:259–267.

Noyes RW, Hertig AT, Rock J. Dating the endometrial biopsy. *Am J Obstetr Gynecol* 1975;**122**:262–263.

Pirtea P, Cedars MI, Devine K, Ata B, Franasiak J, Racowsky C, Toner J, Scott RT, De Ziegler D, Barnhart KT. Recurrent implantation failure: reality or a statistical mirage? *Fertil Steril* 2023;**120**:45–59.

R Core Team. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing, 2021. https://www.R-project.org/ (15 July 2024, date last accessed).

Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 2015;**43**:e47.

Sargent IL, Wilkins T, Redman CWG. Maternal immune responses to the fetus in early pregnancy and recurrent miscarriage. *Lancet* 1988;**2**:1099–1104.

Sebastian-Leon P, Garrido N, Remohí J, Pellicer A, Diaz-Gimeno P. Asynchronous and pathological windows of implantation: two causes of recurrent implantation failure. *Hum Reprod* 2018; **33**:626–635.

Strowitzki T, Germeyer A, Popovici R, Von Wolff M. The human endometrium as a fertility-determining factor. *Hum Reprod Update* 2006;**12**:617–630.

Wickham H. *ggplot2: Elegant Graphics for Data Analysis*. New York: Springer-Verlag, 2016.

Wilcox AJ, Baird DD, Weinberg CR. Time of implantation of the conceptus and loss of pregnancy. *New Engl J Med* 1999;**340**:1796–1799.

Yu G, Wang LG, Han Y, He QY. ClusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS J Integr Biol* 2012;**16**:284–287.

Zhu X-Z, Deng Z-M, Dai F-F, Liu H, Cheng Y-X. The impact of early pregnancy metabolic disorders on pregnancy outcome and the specific mechanism. *Eur J Med Res* 2023;**28**:197.