


The endometrial transcriptome of infertile women with and without implantation failure

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Abstract

Introduction: Implantation failure after transferring morphologically “good-quality” embryos in in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) may be explained by impaired endometrial receptivity. Analyzing the endometrial transcriptome analysis may reveal the underlying processes and could help in guiding prognosis and using targeted interventions for infertility. This exploratory study investigated whether the endometrial transcriptome profile was associated with short-term or long-term implantation outcomes (ie success or failure).

Material and methods: Mid-luteal phase endometrial biopsies of 107 infertile women with one full failed IVF/ICSI cycle, obtained within an endometrial scratching trial, were subjected to RNA-sequencing and differentially expressed genes analysis with covariate adjustment (age, body mass index, luteinizing hormone [LH]-day). Endometrial transcriptomes were compared between implantation failure and success groups in the short term (after the second fresh IVF/ICSI cycle) and long term (including all fresh and frozen cycles within 12 months). The short-term analysis included 85/107 women (33 ongoing pregnancy vs 52 no pregnancy), excluding 22/107 women. The long-term analysis included 46/107 women (23 ‘fertile’ group, ie infertile women with a live birth after ≤ 3 embryos transferred vs 23 recurrent implantation failure group, ie no live birth after ≥ 3 good quality embryos transferred), excluding 61/107 women not fitting these categories. As both analyses drew from the same pool of 107 samples,

Abbreviations: ART, assisted reproductive technology; BMI, body mass index; CPM, counts per million; DEGs, differentially expressed genes; ICSI, intracytoplasmic sperm injection; IVF, in vitro fertilization; LH, luteinizing hormone; mRNA, messenger RNA; OVGP1, oviductal glycoprotein 1; PCA, principal component analysis; PGT, preimplantation genetic testing; pLH, pseudo-timeline LH day; RCT, randomized controlled trial; RIF, recurrent implantation failure; RIN, RNA Integrity Number; RNA, ribonucleic acid; RNA-seq, RNA sequencing; TPM, transcripts per million; Δ LH, the difference between the pseudo-timeline LH day and the actual LH day of the endometrial biopsy; Δ WOI, the difference between the pseudo-timeline LH day and the optimum endometrial receptivity day by beREADY (LH+7).

For affiliations refer to page 1362.

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there was some sample overlap. Additionally, cell type enrichment scores and endometrial receptivity were analyzed, and an endometrial development pseudo-timeline was constructed to estimate transcriptomic deviations from the optimum receptivity day (LH + 7), denoted as Δ WOI (window of implantation).

Results: There were no significantly differentially expressed genes between implantation failure and success groups in either the short-term or long-term analyses. Principal component analysis initially showed two clusters in the long-term analysis, unrelated to clinical phenotype and no longer distinct following covariate adjustment. Cell type enrichment scores did not differ significantly between groups in both analyses. However, endometrial receptivity analysis demonstrated a potentially significant displacement of the WOI in the non-pregnant group compared with the ongoing pregnant group in the short-term analysis.

Conclusions: No distinct endometrial transcriptome profile was associated with either implantation failure or success in infertile women. However, there may be differences in the extent to which the WOI is displaced.

KEYWORDS

assisted reproduction, endometrial receptivity, endometrium, gene expression, implantation failure, IVF/ICSI outcome

1 | INTRODUCTION

Approximately one in six couples is affected by infertility,¹ a condition characterized by the failure to establish a clinical pregnancy after 12 months of regular, unprotected sexual intercourse.² Around 20% of these couples eventually need assisted reproductive technology (ART) to address their infertility.³ ART refers to the in vitro handling of both human oocytes and sperm and/or of embryos for the purpose of reproduction, including in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI).² Although many advances have been made over the last four decades to improve IVF protocols, including improved stimulation regimens and embryo culture and selection, the success rate remains around 30% per cycle.⁴ Despite the transfer of morphologically “good-quality” embryos, implantation failure often occurs.⁵ Critical for embryo implantation are both a competent blastocyst and a receptive endometrium.⁶ Chromosomal abnormalities in preimplantation embryos presumably account for up to 70% of implantation failures,⁷ leaving 30% that may be explained by impaired endometrial receptivity.

There are an increasing number of studies investigating molecular biomarkers for endometrial receptivity, since traditional histological dating is inaccurate in distinguishing between the endometrium of fertile and infertile women and suffers from a high interobserver variability.^{8,9} To date, endometrial transcriptome analysis has been the most commonly applied method to investigate molecular biomarkers.¹⁰ In addition, it may shed light on alterations in biological processes that could be responsible for the condition or provide potential therapeutic targets.^{11,12} Thousands

Key message

This exploratory study did not find a distinct endometrial transcriptome profile associated with either implantation failure or success in infertile women. However, our data may suggest differences in the extent to which the window of implantation is displaced.

of potential endometrial receptivity biomarkers have already been identified in transcriptome studies,¹⁰ and distinct gene expression profiles have been linked with either endometrial receptivity¹³⁻¹⁸ or implantation failure.¹⁹⁻²¹ Despite these efforts, there is poor concordance of biomarkers between studies, due to differences in experimental design and setting, sample size, selection criteria for study participants, timing of endometrial sampling, technology (eg microarray hybridization and sequencing methods), strategies for data processing and a lack of consistent standards for data presentation.^{10,22}

Nevertheless, distinct gene expression profiles, stratifying couples according to fertility prognosis, could be helpful in understanding the differences in prognosis based on underlying biology, as well as in optimizing assisted reproduction approaches, tailored to each couple's needs.

Therefore, this exploratory study describes the analysis of the endometrial transcriptome by RNA sequencing (RNA-seq), using endometrial tissue of women who had one full failed IVF/ICSI cycle. Our objective was to investigate whether the endometrial transcriptome

profile, assessed after a first full failed IVF/ICSI cycle, was associated with implantation outcome (ie success or failure) both in the short term and long term. In addition, we assessed whether endometrial receptivity profiles and cell type enrichment scored differed between these groups.

2 | MATERIAL AND METHODS

2.1 | Study population

Endometrial tissue, used for this exploratory substudy, was obtained from 141 infertile women participating in a previous randomized controlled trial (RCT) (ie the SCRaTCH trial) on endometrial scratching (ie biopsy),²³ who were allocated to endometrial scratching and consented to storage and future use of their tissue (see Figure 1 for the sample selection flow chart). All 141 participants underwent endometrial scratching just once during the study period. The full inclusion and exclusion criteria and study characteristics of the SCRaTCH trial have been described in detail elsewhere.²³ Briefly, women aged between 18 and 44 years, who had failed implantation after one full IVF/ICSI cycle (ie after transfer of fresh and all frozen embryos) and who were planning a new IVF/ICSI cycle, were eligible. Women undergoing oocyte donation cycles or pre-implantation genetic testing were excluded. Women were followed-up until 12 months after they had been randomized for endometrial scratching, or until delivery if an ongoing pregnancy was achieved within the 12-month follow-up period, either by spontaneous conception or resulting from ART treatments. The primary outcome of the SCRaTCH trial was live birth after a fresh embryo transfer during the second IVF/ICSI cycle (ie the first fresh cycle after randomization). Secondary outcomes included, among other outcomes, cumulative ongoing pregnancy leading to live birth within 12 months after randomization. Ongoing pregnancy was defined as a positive heartbeat on ultrasound at 10 weeks of gestational age, and live birth as the delivery of at least one live fetus after 24 weeks of gestation.²³

2.2 | Comparisons

As follow-up data up to 12 months after randomization was available for all 141 participants of whom endometrial tissue was stored, we investigated whether the endometrial transcriptome was associated with implantation outcome (ie success or failure) both in the short term and long term (Figure 1). However, of 141 biopsies, 107 samples had sufficient RNA quality to be sequenced and were used for further analysis.

In the short-term analysis, we considered only the implantation outcome after the second fresh embryo transfer (ie the first fresh embryo transfer following the endometrial biopsy), involving in total 85 women (Figure 1). Specifically, we compared the endometrial transcriptome profile of women with an ongoing pregnancy (Group 1, $n=33$) with that of women with no pregnancy (Group 2, $n=52$),

that is, negative urinary or serum human chorionic gonadotropin (hCG) levels 18 days after the ovum pickup, after the first fresh embryo transfer in the IVF/ICSI cycle following the endometrial biopsy. All women who did not actually undergo a fresh embryo transfer during that cycle ($n=13$) or had a biochemical or clinical pregnancy loss ($n=9$), were excluded from this analysis. The latter were excluded as women with a pregnancy loss may have a different endometrial transcriptome profile from women who had not been able to get pregnant.²⁴

In the long-term analysis, we considered the outcome of all IVF/ICSI cycles within the 12-month follow-up period after enrollment, involving 46 women in total (Figure 1). This includes outcomes after all fresh and frozen embryo transfer cycles. Specifically, we compared the endometrial transcriptome profile of women with implantation success after ART, hereafter referred to as "fertile" women (Group 3 $n=23$), with that of women with recurrent implantation failure (RIF) (Group 4, $n=23$). These samples also had been included in the short-term analysis. Both groups are defined below. Only samples meeting these definitions were included in the long-term analysis, resulting in the exclusion of 61 samples that did not fulfill the definition of either group (Figure 1).

We defined RIF as no live birth after transfer of at least in total three good quality embryos regardless of whether they were involved in single or double embryo transfer cycles. Embryo quality was determined by morphology. For day two, day three and day four embryos, an embryo was considered good quality when the following morphological features were present: being at a 4-cell stage for a day two embryo, being at a ≥ 7 -cell stage with less than 20% fragmentation for a day three embryo and being at least at the morula stage for a day four embryo. For day five and day six embryos (ie blastocysts), the Gardner and Schoolcraft system was used.²⁵

We considered women "fertile" if they had a live birth after transfer of in total three or fewer embryos, in which the quality of the embryos was not considered, as implantation was successful. These "fertile" women were thus not fertile in the conventional sense, but were infertile women who achieved a pregnancy through ART. The criterion of fewer than three embryos was determined arbitrarily, considering the number of transferred embryos generally used in the definition of RIF.^{26,27}

2.3 | Study outcomes

The primary outcome of the current substudy was the assessment of differences in the endometrial transcriptome profile between implantation success and failure groups, as defined above, by differential gene expression analysis of RNA-seq data. Secondary outcomes included the analysis of cell type enrichment scores and endometrial receptivity gene profiles, along with the pseudo-timeline based estimations of the deviation of the window of implantation and luteinizing hormone (LH) day, using the endometrial transcriptome data. The methods used to study these outcomes will be discussed in detail below.

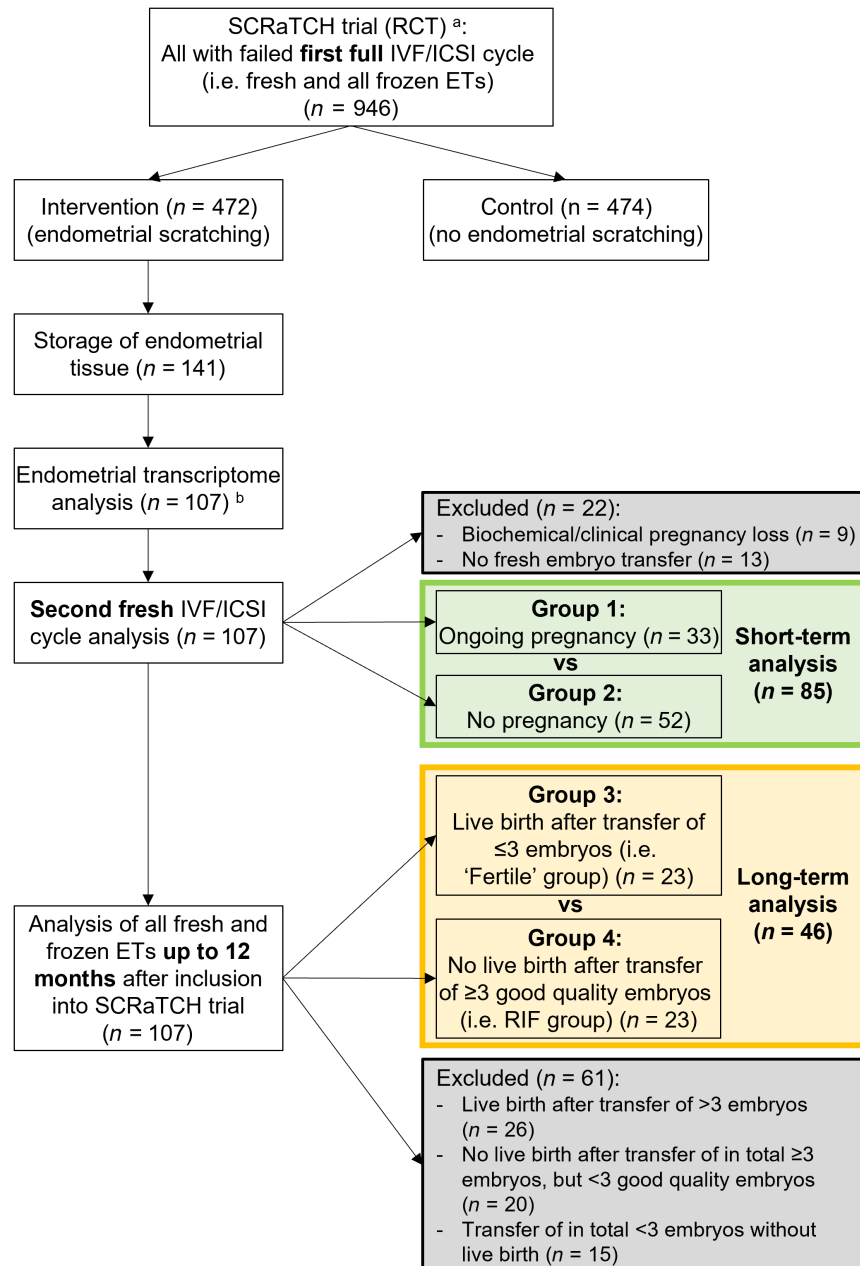


FIGURE 1 Flow chart of sample selection for the analyses of the current endometrial transcriptome study. In the short-term analysis, we considered only the implantation outcome after the second fresh embryo transfer (ie the first fresh cycle following endometrial biopsy), whereas in the long-term analysis, the outcome of all fresh and frozen embryo transfers within the 12-month follow-up period after enrollment were considered. Ongoing pregnancy was defined as a positive heartbeat on ultrasound at 10 weeks of gestational age, and no pregnancy as negative urinary or serum human chorionic gonadotropin levels 18 days after the ovum pickup. “Fertile” women were not fertile in the conventional sense but were infertile women who achieved a live birth through assisted reproductive technology after three or fewer embryos transferred. RIF was defined as no live birth after transfer of at least three good quality embryos as determined by morphology. Only the samples meeting these definitions were included in the respective analyses. ETs, embryo transfers; RCT, randomized controlled trial; RIF, recurrent implantation failure.^aEndometrial biopsies (n=141) were obtained from women undergoing endometrial scratching (biopsy) in the SCRaTCH trial, a randomized controlled trial on endometrial scratching in women with a first failed IVF/ICSI cycle (van Hoogenhuijze et al. *Hum Reprod* 2020;36:87–98).^bOf the 141 biopsies, 107 samples had sufficient RNA quality to be sequenced and were used for further analysis.

2.4 | Tissue sampling and storage

Endometrial tissue was obtained and stored in six hospitals in The Netherlands (Table S1). All hospitals followed the same strict

protocol for endometrial biopsy and tissue storage as previously reported,²⁸ which is described below.

Endometrial biopsy was performed in the mid-luteal phase of a natural cycle (5–8 days after detection of the LH surge by urinary

tests) prior to the second fresh IVF/ICSI cycle, using an endometrial biopsy catheter (eg Pipelle or other similar catheters).

To limit contamination of the samples with RNases as much as possible, a sterile gown, sterile gloves and a hair cap were worn by the physician who performed the endometrial biopsy. A sterile work field was created and all instruments and materials were unpacked from their sterile casing right before the procedure. The endometrial biopsy was performed as follows: after insertion of a speculum, the cervix was cleaned with sterile water. The catheter was introduced through the cervix up to the uterine fundus. The piston of the catheter was completely drawn back to create a vacuum, and the catheter was slowly retracted within 1–2 minutes while constantly rotating 360°. After the procedure, the physician changed gloves for new sterile gloves and divided the tissue over three tissue tubes (Brooks Life Sciences, USA) using a sterile scalpel and forceps. Within 3 minutes after taking the biopsy, the tubes were snap-frozen in liquid nitrogen and stored at -80°C as soon as possible. Tissue samples from all hospitals were transported on dry ice to the Central Biobank of the University Medical Center Utrecht for central storage.

2.5 | Total RNA extraction

RNA extraction was performed by the Utrecht Sequencing Facility (USEQ) (Utrecht, The Netherlands). Tissue was thawed and homogenized using TissueLyser II (Qiagen, Germany) after adding 50 μL nuclease-free water and stainless steel beads (\varnothing 5 mm, Qiagen). Each sample was incubated in 350 μL RLT Plus lysis buffer (Qiagen). Total RNA extraction, including a DNase treatment, was performed using the QIAasymphony SP and the QIAasymphony RNA Kit (Qiagen) following the manufacturer's protocol. RNA was quantified using the Qubit™ RNA BR Assay Kit (Thermo Fisher Scientific, USA) and the Qubit 4 Fluorometer (Thermo Fisher Scientific). RNA Integrity Number (RIN) was determined using the Agilent RNA 6000 Nano Kit (Agilent Technologies, USA) and the Agilent 2100 Bioanalyzer system (Agilent Technologies).

2.6 | RNA sequencing

RNA-seq was performed by USEQ. Endometrial samples with an RIN of ≥ 6.0 were used for library preparation with an input of 100 ng of total RNA. Libraries were prepared using the TruSeq Stranded mRNA Kit (Illumina, USA) following the manufacturer's protocol with xGen Dual Index UMI Adapters (Integrated DNA Technologies [IDT], USA) for indexing. Samples with a final library concentration of ≥ 1 ng/ μL were further processed for sequencing. Libraries were pooled equimolarly and paired-end (2×50 base pairs) sequenced on a NovaSeq 6000 S2 flow cell (Illumina).

Of 141 endometrial tissue samples, 107 samples (75.9%) had sufficient RNA quality to be sequenced. Substandard samples ($n=34$)

were not sequenced due to the following reasons: too low RIN ($n=24$), too low RNA concentration ($n=2$) or too low mRNA yield after poly(A) enrichment ($n=8$).

2.7 | RNA-seq data analysis

Two independent analysts performed data analysis in parallel to check for inconsistencies.

2.7.1 | Preprocessing

The dataset was handled in two ways: with and without preprocessing (trimming and quality filtering) before mapping. First, the raw reads were quality checked with FastQC tool version 0.11.9.²⁹ Raw fastqc files were then trimmed with TRIMMOMATIC 0.39.³⁰ For quality filtering, fastq_quality_filter from FASTX TOOLKIT 0.0.13 was used.³¹ Both the preprocessed dataset and the dataset without preprocessing showed a high number of duplicate reads (Figure S1).

2.7.2 | Alignment

Both datasets (preprocessed and not preprocessed) were mapped to human genome version 38 (GRCh38)³² with STAR v2.7.0.³³ The proportion of uniquely mapped reads for both datasets varied from 66% to 86%. The dataset without preprocessing had slightly more uniquely mapped reads than the preprocessed dataset. Therefore, the dataset without preprocessing was used thereafter. The datasets had a very high number of reads mapped to the mitochondrial chromosome (chrM) (Figure S2). This could explain the high number of duplicate reads in the quality control step.

A sensitivity analysis, excluding the mitochondrial transcripts, did not result in a significantly different number of differentially expressed genes (DEGs) when comparing gene expression data of the groups (data not shown). Additionally, we could not determine whether the high number of mitochondrial transcripts was due to technical issues (eg poor sample quality with a high fraction of apoptotic or lysing cells) or tissue biology. Therefore, we used the dataset with mitochondrial transcripts for our analyses.

Raw gene counts were generated with STAR during mapping with default parameters that were the same as HTSeq-count—"union" mode. The forward-stranded counts were used for further analysis.

2.7.3 | Quality control

Mapping stats were collected with PICARD TOOLS 2.0.1.³⁴ To aggregate all results of our samples together, we used MULTIQC TOOL v1.9.³⁵

2.7.4 | Normalization, differential gene expression analysis and covariates

Normalization and differential gene expression analysis were conducted using R v3.6.0 package edgeR v3.28.1.^{36–39} To visualize the high-dimensional transcriptome data, principal component analysis (PCA) was performed based on logarithm-transformed counts per million (CPM) normalized counts (logCPMs). Initially, several factors, such as age, body mass index (BMI), biopsy center, smoking, LH-timed biopsy day, infertility diagnosis, infertility type and number of previous failed embryo transfers were included in our data analysis as covariates (results not shown). However, in our final model, the covariates were narrowed down to age, BMI and LH-timed biopsy day, as the outcomes of our analysis were comparable when limited to these factors. In addition, these three factors have previously been identified as potential confounding variables in the context of endometrial gene expression.^{40–42} DEGs with a false discovery rate of <0.05 were considered significant.

2.8 | Enrichment analysis

We intended to subject significantly DEGs to enrichment analysis to annotate these genes to functional gene sets and pathways. However, as there were fewer than two DEGs in all analyses, we did not perform enrichment analysis.

2.9 | Analysis of receptivity gene profiles

Individual receptivity profiles of endometrial biopsies were analyzed with the beREADY receptivity dating model.⁴³ Simulating the original targeted RNA-seq method, 68 biomarkers with four housekeeping genes were selected from the quantified whole-genome sequencing data.⁴⁴ This filtered gene set was then further analyzed with the beREADY model. Briefly, biomarker genes were normalized using the geometric mean of the housekeeping genes. After clustering, distances to the reference receptivity groups were calculated. The two most similar receptivity profiles were found with a one-vs-rest exclusion method, and the input-sample's relative probabilities for either group were output.

The beREADY receptivity test was applied to the transcriptome data of all our sequenced endometrium samples to determine the proportion of samples classified as “pre-receptive”, “receptive” or “post-receptive”. In addition, beREADY distinguished two transitional groups as subgroups of the “receptive” classification, that is, “early-receptive” and “late-receptive”.

Due to the linear nature of the development of the endometrium as a tissue, these relative probabilities were interpreted as the transition of the endometrium from one phase to another. Based on this interpretation, we constructed an endometrial development pseudo-timeline by finding the location of the input

sample on a scale from -1 (pre-receptive) to $+1$ (post-receptive), based on endometrial gene expression data from reference samples taken on LH+2/3, LH+7/8 and LH+11/12.⁴³ Next, we divided this pseudo-timeline into 10 equal bins, representing LH days ranging from LH+2 to LH+12 of the pre-receptive and the post-receptive reference samples, respectively. We then estimated the location of our study samples on the pseudo-timeline by comparing the receptivity profile of these samples to those of the reference samples. To estimate more specifically the extent of deviation from the receptive period on the pseudo-timeline (ie where the reference samples taken on LH+7 are located, as LH+7 is considered the optimum endometrial receptivity day by beREADY), rather than just classifying the receptivity profile of a sample, we calculated the absolute difference between the estimated LH day of the study sample on the transcriptional pseudo-timeline (pLH) and LH+7 (Figure 2). This difference was termed ΔWOI and was expressed in number of days. Thus, the larger the ΔWOI , the greater the deviation from the most receptive period (LH+7), and can express either a delay or an advancement in endometrial development. We also assessed the difference between the estimated pLH of each study sample and the actual LH day of the biopsy, by calculating the absolute difference between the two, which we termed ΔLH and which was also expressed in number of days (Figure 2). Thus, the larger the ΔLH , the greater the deviation between the actual LH day of the biopsy and the pLH estimated by the beREADY model.

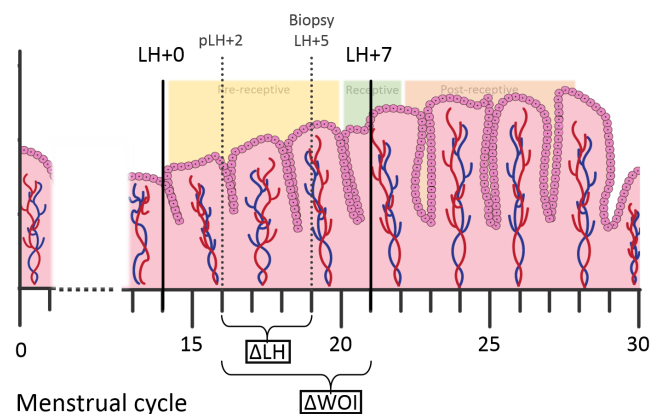


FIGURE 2 Visualization of ΔWOI and ΔLH . Based on the beREADY receptivity profiles of endometrium reference samples taken between LH+2 and LH+12 (Teder et al. *NPJ Genom Med* 2018;3:34. doi: 10.1038/s41525-018-0072-5), a pseudo-timeline was developed and divided into bins representing pseudo-LH days from the mid-cyclic LH-surge (LH+0). ΔWOI is calculated as the absolute difference in number of days between the estimated transcriptome-based pseudo-timeline LH day of the study sample (pLH) and LH+7, which is considered the optimum endometrial receptivity day by beREADY. ΔLH is calculated as the absolute difference in number of days between pLH and the LH day the biopsy was taken on (Biopsy LH). In this example, the biopsy was taken on LH+5, but the transcriptomic profile matches that of LH+2 on the pseudo-timeline (pLH+2). Thus, in this case, the ΔWOI is 5 and the ΔLH is 3.

2.10 | Analysis of tissue cellular heterogeneity

As our transcriptome data was generated by bulk RNA-seq, which does not provide any information on cell type-specific gene expression, we characterized the cellular signatures of the endometrial biopsies using xCell. xCell is a signature-based method in which signature genes are collected from diverse pure cell-type transcriptomes from different sources and gene expression data is converted to cell type enrichment scores.⁴⁵ We used the transcripts per million normalized gene counts as input to xCell and ran the deconvolution algorithm with additional expression data from GTEx (see GTEx⁴⁶ (diverse types of tissues) and GSE86491 and GSE119209^{47,48} (both endometrium)) as the program performs better with heterogeneous datasets.

2.11 | Statistical analyses

Continuous data of two independent groups were statistically compared using the independent *t*-test in the case of normally distributed data, and the Wilcoxon rank sum test in the case of non-normally distributed data. Categorical data were compared across groups using the Chi-square test or Fisher's exact test when the expected frequency was <5.

For statistical comparison of the mean and median Δ LH and Δ WOI measurements between two groups, we used both a one-sided and a two-sided Wilcoxon rank sum test. There was no difference in our null hypothesis in mean and median Δ LH and Δ WOI between our implantation failure and implantation success groups, either in the short term or long term. Based on the results of a previous study,⁴³ we tested our main alternative hypothesis of a larger Δ LH and Δ WOI in the implantation failure groups compared with the implantation success groups, using the one-sided Wilcoxon rank sum test. However, as there was a possibility that the difference in Δ LH and Δ WOI would be bidirectional (ie either smaller or larger) between the groups, we also tested the alternative hypothesis of a different Δ LH and Δ WOI, using the two-sided Wilcoxon rank sum test.

A *P*-value of <0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using SPSS Statistics v26 (IBM Corporation, USA) and R (v4.2.1).

3 | RESULTS

3.1 | Demographics

The baseline characteristics of the participants of the 107 sequenced endometrium samples are shown in Table S2; they did not differ from the characteristics of the participants of the initial 141 endometrium samples (Table S3).

3.2 | Short-term analysis: ongoing pregnancy (Group 1) vs no pregnancy (Group 2) after the first fresh embryo transfer in the IVF/ICSI cycle following endometrial biopsy

a. Clinical characteristics

The clinical baseline and cycle characteristics of the ongoing pregnancy (Group 1) and no pregnancy groups (Group 2) are shown in Table 1. A trend of a higher mean age was seen in Group 2 than in Group 1 (respectively 35.8 vs 33.9 years) but this difference was not statistically significant (*P*=0.050; Table 1). None of the other characteristics differed between the groups.

b. Differential gene expression analysis

The PCA did not reveal distinct separation of the groups (Figure 3A). Differential gene expression analysis resulted in only one significantly upregulated gene (Oviductal Glycoprotein 1: OVGP1) in Group 2 vs Group 1, and no significantly downregulated genes. This gene encodes a large, carbohydrate-rich epithelial glycoprotein that is secreted from non-ciliated oviductal epithelial cells and that associates with ovulated oocytes, blastomeres and spermatozoan acrosomal regions.⁴⁹ Adjustment of the gene expression data for age, BMI and LH-timed biopsy day as covariates, resulted in slightly better clustering of the samples (Figure 3B). However, no distinct clustering was seen based on the clinical outcome (ie Group 1 vs Group 2). After covariate adjustment, there were no significantly DEGs in Group 2 vs Group 1, suggesting that the covariates highly influenced OVGP1 gene expression.

c. Receptivity gene profile

There were no statistically significant differences in the proportions of receptivity gene profile classifications between both groups (Table 2). Furthermore, the difference between the pseudo-timeline LH day (pLH; based on the application of the beREADY receptivity test to the transcriptome data) and the actual LH day of the biopsy (Δ LH) or the optimal receptivity day of LH+7 (Δ WOI) were determined for the samples of both groups. We detected a significantly larger mean and median Δ WOI in Group 2 than in Group 1 (*P*=0.033; Figure S3A; Table 2) when using a one-sided Wilcoxon rank sum test, whereas with a two-sided test we did not find a statistically significant difference. There were no statistically significant differences in Δ LH between the two groups (Figure S3B; Table 2).

d. Cell type enrichment analysis

To check whether differences in cell type composition of the endometrial biopsies could have influenced the data, we estimated cell type enrichment scores based on the transcriptome data. Overall,

TABLE 1 Baseline characteristics of ongoing pregnancy vs no pregnancy after the first fresh embryo transfer in the IVF/ICSI cycle following endometrial biopsy.

	Ongoing pregnancy (n=33)	No pregnancy (n=52)	P-value
Baseline characteristics			
Female age, years	33.9 (\pm 4.2)	35.8 (\pm 4.5)	0.050
Female age groups			0.146
<35 years	18 (54.5%)	20 (38.5%)	
\geq 35 years	15 (45.5%)	32 (61.5%)	
Female BMI, kg/m ^{2a}	23.1 (21.4–25.5)	24.1 (21.7–26.8)	0.223
Duration of infertility, months	34.0 (21.0–44.0)	29.5 (23.0–43.0)	0.836
Female smokers ^a	4 (12.1%)	5 (9.6%)	0.733
Type of infertility of the female ^b			0.069
Primary	20 (60.6%)	21 (40.4%)	
Secondary	13 (39.4%)	31 (59.6%)	
Cause of infertility ^c			0.339
Idiopathic	15 (45.5%)	20 (38.5%)	
Male factor	18 (54.5%)	24 (46.2%)	
Tubal factor	0	5 (9.6%)	
Endometriosis grade I/II	0	1 (1.9%)	
Ovulatory disorder ^d	0	1 (1.9%)	
Mixed diagnosis ^e	0	1 (1.9%)	
No. of ETs prior to randomization per participant	2.5 (\pm 1.5)	2.2 (\pm 1.4)	0.531
Biopsy taken on LH+ days			0.064
5	4 (15.2%)	5 (9.6%)	
6	14 (42.4%)	17 (32.7%)	
7	10 (30.3%)	12 (23.1%)	
7.5	1 (3.0%)	0	
8	2 (6.1%)	15 (28.8%)	
Missing	2 (6.1%)	3 (5.8%)	
Cycle characteristics			
Treatment type			0.259
IVF	13 (39.4%)	27 (51.9%)	
ICSI	20 (60.6%)	25 (48.1%)	
Downregulation protocol ^a			0.697
Antagonist	5 (15.2%)	5 (9.6%)	
Agonist	28 (84.8%)	46 (88.5%)	
Starting dose FSH, IU	150 (150–225)	225 (150–225)	0.062
Total dose FSH, IU	1800 (1350–2362.5)	2025 (1500–2475)	0.278
Duration of stimulation (days)	11 (9–12)	10 (9–12)	0.606
No. of oocytes	10 (5–14)	7 (5–12)	0.431
ET performed on day			1.000
2	3 (9.1%)	4 (7.7%)	
3	30 (90.9%)	48 (92.3%)	
No. of embryos transferred			1.000
SET	28 (84.8%)	45 (86.5%)	
DET	5 (15.2%)	7 (13.5%)	
Total no. of embryos transferred	38	59	
Embryo quality ^f			1.000
Good	31 (81.6%)	47 (79.7%)	
Low/moderate	7 (18.4%)	12 (20.3%)	

Note: Data are presented as mean (\pm SD), median (IQR) or number (%).

Abbreviations: BMI, body mass index; DET, double embryo transfer; ET, embryo transfer; FSH, follicle stimulating hormone; ICSI, intracytoplasmic sperm injection; IU, international units; IVF, in vitro fertilization; SET, single embryo transfer.

^aData was missing for one participant in the No pregnancy group.

^bPrimary: female has never conceived before. Secondary: female has conceived before.

^cNo specific infertility diagnoses were excluded.

^dThe woman in the No pregnancy group with an ovulatory disorder had a WHO group III ovulatory disorder.

^eThe woman in the No pregnancy group with a mixed infertility diagnosis had both a male factor infertility and a WHO group II ovulatory disorder (unknown whether polycystic ovary syndrome).

^fBased on morphology of the embryo on the day of the embryo transfer.

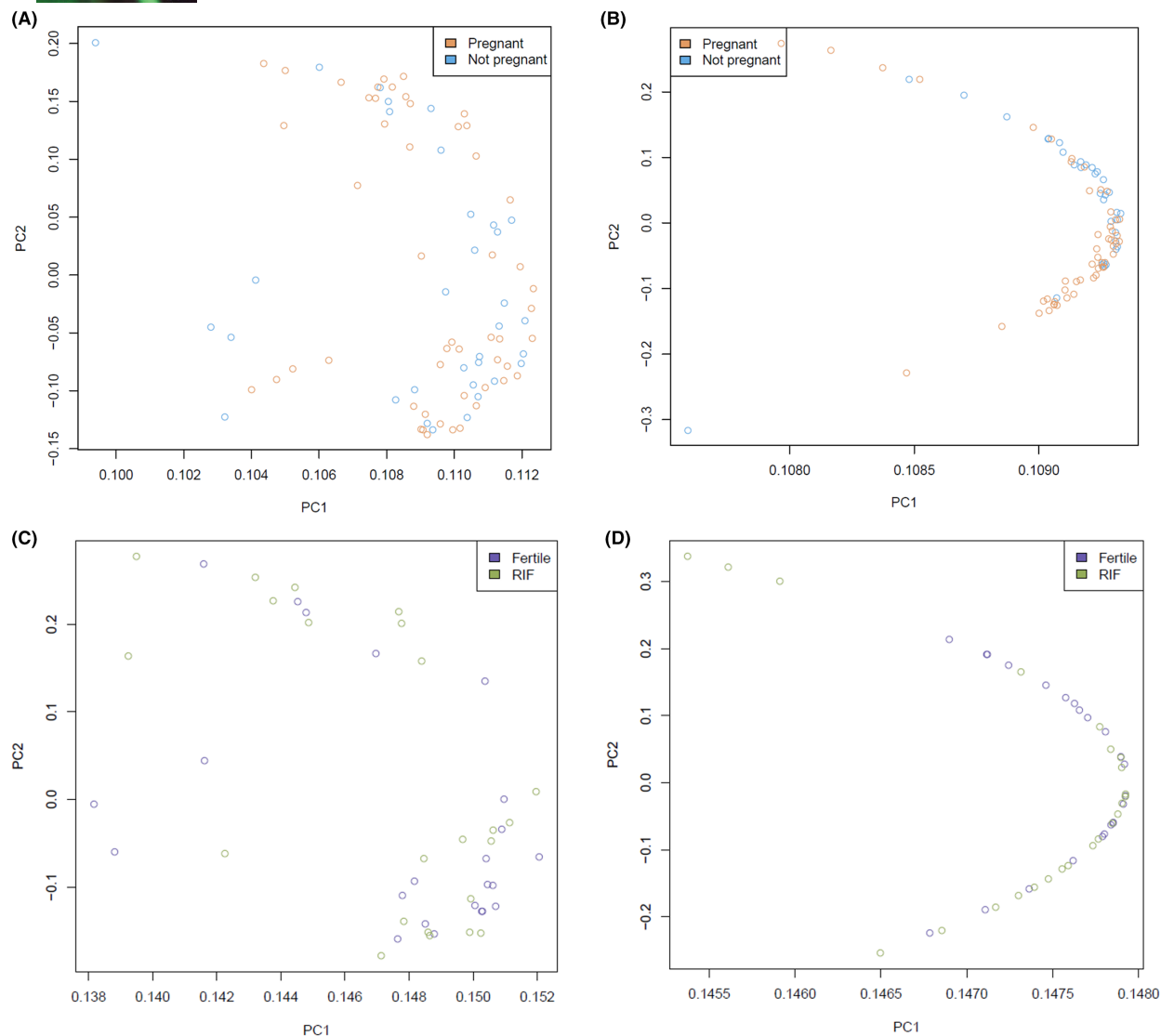


FIGURE 3 Principal component analysis (PCA) of the RNA-sequencing data of the endometrium samples. Plots displaying the samples of the short-term (A,B) and long-term (C,D) analysis groups, before (A,C) and after (B,D) adjustment of the gene expression data for age, body mass index (BMI) and LH-timed biopsy day as covariates. Two distinct clusters of samples were seen in the long-term analysis before adjustment of the data (C), which were not explained by the clinical phenotype of the samples, whereas in the short-term analysis no distinct clusters were seen (A). After adjustment of the data, samples clustered more in a crescent shape in both the short-term and long-term analyses (B,D), but no distinct clusters were present.

the endometrium samples in both groups displayed the highest cell type enrichment values for smooth muscle cells (0.54–0.56), followed by epithelial cells (0.44–0.45) (Figure S4A; Table S4). There were no significant differences in the cell type enrichment scores between the two groups (Figure S4A,B; Table S4).

3.3 | Long-term analysis: fertile group (Group 3) vs recurrent implantation failure (RIF) group (Group 4)

a. Clinical characteristics

The clinical and cycle characteristics of the participants in the fertile group (Group 3) and the RIF group (Group 4) are shown in Table 3

and Table S5. Besides a significant difference in the number of embryo transfers per participant, there was also a statistically significant difference in the days on which the embryo transfers were performed ($P < 0.001$), the number of fresh and frozen-thawed embryo transfers ($P < 0.001$) and the embryonic stage of transferred embryos ($P = 0.005$) between the groups (Table 3). A higher proportion of day three embryo transfers were performed in Group 3 than Group 4 (respectively 85.0% vs 51.0%), and a higher proportion of day four and day five embryo transfers were performed in Group 4 than Group 3 (respectively 27.5% vs 8.3% for day four, and 19.0% vs 6.7% for day five) (Table 3). More fresh embryo transfers were performed in Group 3 than Group 4, (respectively 86.7% vs 48.4%), whereas in Group 4, more frozen-thawed embryo transfers were performed compared with Group 3 (respectively 51.6% vs 13.3%) (Table 3). We also noted a higher proportion of

TABLE 2 Receptivity profile analysis of samples of women with ongoing pregnancy vs no pregnancy after the first fresh embryo transfer in the IVF/ICSI cycle following endometrial biopsy.

	Ongoing pregnancy (n = 33)	No pregnancy (n = 52)	P-value	
Receptivity profile			0.236	
Pre-receptive	11 (33.3%)	15 (28.8%)		
Early-receptive	1 (3.0%)	8 (15.4%)		
Receptive	20 (60.6%)	27 (51.9%)		
Late-receptive	0	1 (1.9%)		
Post-receptive	0	1 (1.9%)		
Not classifiable	1 (3.0%)	0		
Median Δ WOI ^a	2.0 (0–3.0)	3.0 (1.0–4.0)	0.082 ^b	0.033 ^c
Mean Δ WOI ^a	1.8 (\pm 1.5)	2.4 (\pm 1.4)		
Δ WOI ^a			0.472	
0	8 (25.8%)	7 (14.3%)		
1	6 (19.4%)	6 (12.2%)		
2	6 (19.4%)	9 (18.4%)		
3	5 (16.1%)	13 (26.5%)		
4	6 (19.4%)	14 (28.6%)		
Median Δ LH ^a	2.0 (1.0–2.0)	2.0 (1.0–3.0)	0.143 ^b	0.200 ^c
Mean Δ LH ^a	1.7 (\pm 1.1)	2.0 (\pm 1.0)		
Δ LH ^a			0.603	
0	4 (12.9%)	3 (6.1%)		
1	11 (35.5%)	12 (24.5%)		
2	9 (29.0%)	19 (38.8%)		
3	5 (16.1%)	11 (22.4%)		
4	2 (6.5%)	4 (8.2%)		

Note: Data are presented as mean (\pm SD), median (IQR) or number (%).

^aThe difference between the pseudo-timeline LH day and the actual LH day (Δ LH) or LH + 7 (Δ WOI). Due to missing data on actual LH day, two samples in the Ongoing pregnancy group and three samples in the No pregnancy group were excluded, leaving 31 and 49 samples, respectively.

^bTwo-sided Wilcoxon rank sum test.

^cOne-sided Wilcoxon rank sum test.

cleavage stage embryos transferred in Group 3 (respectively 91.7% vs 71.2%) and a higher proportion of morulas and blastocysts transferred in Group 4 (respectively 9.2% vs 1.7% and 19.6% vs 6.7%) (Table 3).

b. Differential gene expression analysis

Differential gene expression analysis resulted in no significantly DEGs in Group 4 compared with Group 3. The PCA revealed two distinct groups that were not explained by clinical phenotype (ie RIF vs fertile), as samples of both groups appeared evenly in the two clusters (Figure 3C). After adjustment of the transcriptome data for age, BMI and LH-timed biopsy day as covariates, the two clusters were no longer distinct, but samples of both groups remained intermingled in the PCA plot, with no significantly DEGs in Group 4 vs Group 3 (Figure 3D).

c. Receptivity gene profile

There were no statistically significant differences in the proportions of different receptivity gene profile classifications between the both groups (Table 4). The mean, median and proportions of Δ LH

and Δ WOI were comparable between both groups (Figure S3A,B; Table 4).

d. Cell type enrichment analysis

Cell type enrichment score analysis showed that samples of both groups had the highest enrichment values for smooth muscle cells (0.54–0.55) and epithelial cells (0.43–0.45) (Figure S4C; Table S6). There were no significant differences in the cell type enrichment scores between the samples of the two groups (Figure S4C,D; Table S6).

4 | DISCUSSION

This study compared the RNA-seq endometrial transcriptome profiles of women with at least one full failed IVF/ICSI cycle, who either had implantation success or implantation failure in the short and long term. Differential gene expression analysis did not result in any important differences in the endometrial transcriptome profile of implantation

TABLE 3 Clinical characteristics of the fertile group and recurrent implantation failure (RIF) group.

	Fertile (n = 23)	RIF (n = 23)	P-value
Female age, years	34.2 (\pm 4.4)	36.3 (\pm 3.9)	0.095
Female age groups			0.074
<35 years	13 (56.5%)	7 (30.4%)	
\geq 35 years	10 (43.5%)	16 (69.6%)	
Female BMI, kg/m ²	23.0 (20.7–25.9)	23.7 (21.6–28.3)	0.560
Duration of infertility, months	26.0 (20.0–38.0)	29.0 (23.0–39.0)	0.403
Female smokers ^a	1 (4.3%)	1 (4.3%)	1.000
Type of infertility of the female ^b			0.552
Primary	14 (60.9%)	12 (52.2%)	
Secondary	9 (39.1%)	11 (47.8%)	
Cause of infertility ^c			1.000
Idiopathic	9 (39.1%)	8 (34.8%)	
Male factor	13 (56.5%)	12 (52.2%)	
Tubal factor	1 (4.3%)	2 (8.7%)	
Ovulatory disorder ^d	0	1 (4.3%)	
No. of embryo transfers per participant	2.4 (\pm 0.5)	5.7 (\pm 2.5)	<0.001
1	15 (65.2%)	0	
2	8 (34.8%)	0	
3	0	5 (21.7%)	
4	0	4 (17.4%)	
5	0	3 (13.0%)	
6	0	4 (17.4%)	
7	0	1 (4.3%)	
8	0	3 (13.0%)	
9	0	2 (8.7%)	
13	0	1 (4.3%)	
Total no. of embryos transferred, all participants	60	153	
No. of SET and DET			0.379
SET	46 (86.8%)	105 (81.4%)	
DET	7 (13.2%)	24 (18.6%)	
ET performed on day			<0.001
2	0	2 (1.3%)	
3	51 (85.0%)	78 (51.0%)	
4	5 (8.3%)	42 (27.5%)	
5	4 (6.7%)	29 (19.0%)	
6	0	2 (1.3%)	
No. of fresh and frozen-thawed ETs			<0.001
Fresh	52 (86.7%)	74 (48.4%)	
Frozen-thawed	8 (13.3%)	79 (51.6%)	
Embryonic stage			0.005
Cleavage stage embryo	55 (91.7%)	109 (71.2%)	
Morula	1 (1.7%)	14 (9.2%)	
Blastocyst	4 (6.7%)	30 (19.6%)	

Note: Data are presented as mean (\pm SD), median (IQR) or number (%). The P-value indicating the difference between mean number of embryo transfers per participant was determined by Independent Samples T-test. All other P-values were determined by Chi square test.

Abbreviations: BMI, body mass index; DET, double embryo transfer; ET, embryo transfer; SET, single embryo transfer.

^aData was missing for one participant in the RIF group.

^bPrimary: female has never conceived before. Secondary: female has conceived before.

^cNo specific infertility diagnoses were excluded.

^dThe woman in the RIF group with an ovulatory disorder had a WHO group III ovulatory disorder.

TABLE 4 Receptivity profile analysis of samples of fertile women vs women with recurrent implantation failure (RIF).

	Fertile (n = 23)	RIF (n = 23)	P-value	
Receptivity profile			0.377	
Pre-receptive	11 (47.8%)	6 (26.1%)		
Early-receptive	2 (8.7%)	4 (17.4%)		
Receptive	10 (43.5%)	11 (47.8%)		
Late-receptive	0	1 (4.3%)		
Post-receptive	0	1 (4.3%)		
Median Δ WOI ^a	3.0 (1.0–4.0)	3.0 (1.0–4.0)	0.894 ^b	0.584 ^c
Mean Δ WOI ^a	2.4 (\pm 1.5)	2.4 (\pm 1.6)		
Δ WOI ^a			1.000	
0	3 (13.6%)	4 (18.2%)		
1	5 (22.7%)	4 (18.2%)		
2	2 (9.1%)	1 (4.5%)		
3	5 (22.7%)	5 (22.7%)		
4	7 (31.8%)	8 (36.4%)		
Median Δ LH ^a	2.0 (1.0–3.0)	2.0 (1.0–3.0)	0.577 ^b	0.502 ^c
Mean Δ LH ^a	2.0 (\pm 1.1)	2.2 (\pm 1.3)		
Δ LH ^a			0.767	
0	2 (4.6%)	1 (4.5%)		
1	5 (27.3%)	7 (31.8%)		
2	9 (40.9%)	5 (22.7%)		
3	4 (18.2%)	5 (22.7%)		
4	2 (9.1%)	3 (13.6%)		
5	0	1 (4.5%)		

Note: Data are presented as mean (\pm SD), median (IQR) or number (%).

^aThe difference between the pseudo-timeline LH day and the actual LH day (Δ LH) or LH + 7 (Δ WOI). Due to missing data on actual LH day, one sample in each group was excluded, leaving 22 samples in both groups.

^bTwo-sided Wilcoxon rank sum test.

^cOne-sided Wilcoxon rank sum test.

failure and success groups, either in the short or long term, indicating no association of an endometrial transcriptome profile with implantation outcome. Moreover, we did not observe a higher proportion of a receptive profile among samples of women with implantation success compared with samples of women with implantation failure, either in the short term or long term. We also determined the difference between the transcriptome data-based pseudo-timeline LH day (pLH) and the actual LH day of the biopsies, termed as Δ LH, as well as the difference between pLH and LH + 7, termed Δ WOI. Although we did see a significantly larger mean and median Δ WOI in the implantation failure group than in the implantation success group in the short term with a one-sided test, there was no significant difference between both groups when using a two-sided test. In the long term, no differences were seen in either Δ LH or Δ WOI between both groups. Lastly, cell type enrichment analysis showed that our endometrial biopsies were mostly enriched for smooth muscle cell and epithelial cell gene signatures. No differences in cell type enrichment scores were seen between the implantation failure and success groups in either the short-term or long-term analyses.

Previous studies comparing the endometrial transcriptome of women with (recurrent) implantation failure with those of women with

implantation success reported a considerably larger amount of significantly DEGs compared with our study.^{20,21,50–60} Although quite some heterogeneity in DEGs and enriched pathways was observed across these studies, annotations of DEGs to processes involving the immune system and cellular functions occurred in the majority of the studies.

The difference in the number of significantly DEGs between our study and previous studies can most likely be attributed to differences in study population selection criteria, as well as differences in study methodology, such as the timing of endometrial biopsies (eg natural cycle vs artificial or stimulated cycle, and differences in LH timing when performed during the mid-luteal phase of a natural cycle), sequencing methods (eg RNA-seq vs microarray hybridization) and data processing and analysis. For instance, there is no consensus on the definition of RIF,²⁶ which contributes to substantial heterogeneity across studies. Pirtea et al. reported that RIF after three euploid embryo transfers is actually rare (<5%), suggesting that ART failures mainly result from embryonic factors.⁶¹ In our study, we assessed embryo quality based on morphologic characteristics to determine the RIF group and did not perform preimplantation genetic testing for aneuploidy (PGT-A). Therefore, it is likely that we included women with aneuploid embryo transfers in the RIF

group, which may have affected implantation chances and therefore, the selection of the study participants for the comparisons in our study. However, it is worth noting that even with PGT-A, embryonic mosaicism can complicate the interpretation of the test results.⁶²⁻⁶⁴

Our study showed that endometrial gene expression changed after covariate adjustment (ie age, BMI and LH-timed biopsy day) and that samples clustered better. This finding supports that these factors can influence endometrial gene expression data, as also observed in previous studies,⁴⁰⁻⁴² and should be taken into consideration when attempting to identify true biological differences between implantation failure and success groups.

When considering the causes of infertility among the participants in our study, a relatively large proportion had male factor infertility (46.7%), followed by idiopathic infertility (38.3%) (Table S2). Notably, an endometrial factor as cause of the infertility is less likely in male factor infertility compared with idiopathic infertility, potentially introducing heterogeneity into our endometrial transcriptome findings. However, the proportions of male factor infertility were comparable between the implantation failure and success groups (Tables 1 and 3). Consequently, differences in the endometrial transcriptome between these groups are less likely attributable to differences in the underlying causes of infertility.

A relatively large percentage (13.1%) of our total study population were smokers (Table S2), though similar incidences were previously reported among women undergoing ART treatment.⁶⁵ Cigarette smoking has been widely associated with poor reproductive outcome. However, the effect of female smoking on ART outcome remains unclear, as it is difficult to study its precise effect due to multiple confounding factors, resulting in conflicting results from previous retrospective observational studies.⁶⁵⁻⁶⁷ To our knowledge, it is unknown whether smoking affects the endometrial transcriptome. In our study, we did not observe differences in the proportions of female smokers between the comparison groups (Tables 1 and 3), therefore it is unlikely that smoking would have resulted in differences in the endometrial transcriptome profile between groups.

Furthermore, we noted a difference in the days on which the embryo transfers were performed, the number of fresh and frozen-thawed embryo transfers and the embryonic stage of the transferred embryos between the RIF and the fertile group. We observed that more day three, fresh and cleavage stage embryos were transferred in the fertile group, whereas more day four and day five, frozen-thawed and morula and blastocyst embryos were transferred in the RIF group (Table 3). Due to the way the fertile and RIF groups were defined in our study, there were more embryo transfers in the RIF group, which also explains the higher proportion of frozen-thawed embryo transfers in this group. In the Netherlands, fresh embryo transfers are generally performed on day three,⁶⁸ and embryos are usually cryopreserved on day three or four, and, after thawing, transferred on day four or five. The higher proportion of fresh embryo transfers in the fertile group, and the higher proportion of frozen-thawed embryo transfers in the RIF group explain the differences in embryo transfer days and embryonic stage of transferred embryos between the fertile and RIF group. To our knowledge, it

is not recognized yet whether the stage of the embryo influences endometrial gene expression. However, we cannot rule out potential differences in the extent to which “embryonic factors” vs “endometrial factors” were involved in the fertile and the RIF group, which may have affected the endometrial transcriptome profile.

Additionally, we did not see any differences in the proportion of different endometrium receptivity profiles between samples of implantation failure and implantation success groups, either in the short term or long term. However, in the short term, we observed a significantly larger mean and median Δ WOI in the not pregnant group than in the ongoing pregnant group with one-sided testing ($P=0.033$); two-sided testing did not result in a significant difference, albeit with a P -value with a trend towards statistical significance ($P=0.082$). This difference in statistical significance is most likely related to the small sample size of the groups in the short-term analysis. Nevertheless, the inconclusive statistical analysis may still indicate a biologically relevant difference and may suggest that delayed endometrial development could be a potential cause of implantation failure. Previously, a displaced window of implantation (WOI), detected by endometrial transcriptome analysis, was identified as a cause of RIF.⁶⁹ A previous study that also used the beREADY model to determine receptivity profiles of endometrium samples, detected a significantly higher proportion of displaced WOI in the RIF group than in the fertile group.⁴³ The results of our study, however, do not indicate a displacement of the WOI in the RIF group compared with the fertile group (ie our long-term analysis). A potential explanation for this difference could be that the endometrium samples in our study were taken prospectively. The women in our RIF group were not classified as having experienced RIF yet at the time of the biopsy and could potentially have a different endometrial gene profile than if the biopsy had been taken after RIF was confirmed. Though endometrial gene expression has been shown to remain relatively stable between natural cycles in fertile women,⁷⁰ it is unknown whether this also applies to infertile women or women with RIF. A study in which endometrial biopsies were subjected to endometrial receptivity array (ERA) testing, failed to observe a larger proportion of WOI displacement in samples of women with RIF than controls with no RIF.⁷¹ A recent study also demonstrated no differences in the proportion of receptive or non-receptive profiles according to ERA testing between women with and without a history of unsuccessful frozen embryo transfers.⁷² However, in these studies, samples were taken in a hormone replacement therapy (HRT) cycle as opposed to in a natural cycle in our study. Although we assessed the endometrial receptivity profiles of our samples during a natural cycle, our groups (ie pregnant vs not pregnant and fertile vs RIF) were based on clinical outcomes after stimulated cycles in the short-term analysis and a mix of stimulated and natural and/or artificial cycles in the long-term analysis. To our knowledge, it is yet unknown whether endometrial receptivity assessment during a natural cycle similarly represents the receptivity profile during subsequent stimulated or artificial cycles. Endometrial gene expression during the mid-secretory phase of an artificial cycle (ie on the 6th day of progesterone supplementation) has been shown to be different to that of a natural cycle (ie LH + 7).⁷³

Therefore, it is deemed likely that the expression of endometrial receptivity genes is altered likewise by exogenous hormones, with potentially different receptivity profiles and different clinical outcomes. In addition, endometrial gene expression in women with RIF was more similar to that in fertile controls during a natural cycle than during an artificial cycle,⁷³ suggesting that exogenous hormones may negatively influence endometrial gene expression towards an implantation failure profile. It could therefore be possible that our prospective natural cycle endometrial receptivity assessments were not fully representative of the participants in our study groups, who were selected based on their outcomes after stimulated, HRT and/or natural cycles. However, given all the above, evaluation of endometrial receptivity during a natural cycle presumably provides a more physiologic view on the window of implantation and its potential deviations or disruptions.

In our study, all women had a failed first full IVF/ICSI cycle, which introduced variability in the number of prior failed embryo transfers. This variation could affect subsequent pregnancy prospects and the selection of the comparison groups, which in turn could have influenced differential gene expression, representing the difference in gene expression between two different groups. However, the incorporation of the number of previous failed embryo transfers as a covariate did not yield significant alterations in the analysis outcomes (data not shown).

Additionally, all patients underwent endometrial scratching after the initial failed IVF/ICSI cycle but before the second fresh cycle to obtain tissue samples. In selecting samples for the fertile and RIF groups in our long-term analysis, we considered the total number of successfully transferred good quality embryos after 12 months of follow-up. Importantly, a recently published individual participant data meta-analysis⁷⁴ confirmed that the number of prior failed embryo transfers does not impact the effectiveness of endometrial scratching. Therefore, it is reasonable to conclude that endometrial scratching did not affect the prognosis of our fertile and RIF groups, and thus did not influence the selection of patient samples for these groups.

Our endometrial biopsies showed high enrichment for smooth muscle cell and epithelial cell signatures. Although not classified as such, it seems plausible that the smooth muscle cell signature is considered part of an “endometrial stromal cell signature”. However, in xCell there is no specific signature for endometrial stromal cells. The xCell smooth muscle cell signature, along with those of chondrocytes, astrocytes and mesenchymal stem cells, has been shown to exhibit high enrichment for genes of the endometrial stromal fibroblast cluster based on single-cell sequencing data.⁷⁵ Previous single-cell RNA sequencing of endometrium demonstrated smooth muscle cells as a separate cell entity, but these cells expressed a similar gene profile to that of the endometrial stromal cells.⁷⁶ Finally, the estimated cellular enrichment of endometrium samples did not differ between implantation failure and implantation success groups in our study, suggesting no alterations of endometrial tissue composition in implantation failure.

Strengths of the present study are the prospective and multi-center sampling of endometrial tissue, the availability of clinical follow-up data of the participants up to 12 months after sampling, and the large number of samples included in the RNA-seq.

As for limitations of the study, while we included a large number of samples for RNA-seq, our comparison groups consisted of a relatively small number of samples, which may have been insufficient to detect significant differences.

Additionally, we noticed that LH-timed biopsy day, age and BMI affected endometrial gene expression. Our endometrial biopsies were taken between LH+5 to LH+8. Adhering to a stricter window (eg LH+6 and LH+7 only) may have resulted in less variability in gene expression profiles. However, this was not possible due to logistic constraints.

Bulk RNA-seq was performed in this study, which provides mixed gene expression profiles of all cell types present in the endometrial tissue, rather than cell type-specific gene expression profiles, such as is the case with single-cell RNA-seq.⁷⁷ Therefore, to estimate the proportions of the cell types present in the tissue, we determined cell type enrichment scores, based on gene expression data of our samples. However, as we did not perform a validation of these cell type fractions in a separate cohort, we could not adjust the transcriptome data for endometrial tissue cellular composition.

To our knowledge, this is the first study comparing the optimal receptivity LH day and actual LH day of the biopsy with the estimated LH day, based on a pseudo-timeline constructed by applying the beREADY model on endometrial transcriptome data of reference samples taken on LH+2/3, LH+7/8 and LH+11/12. This pseudo-timeline was divided into 10 equal bins, representing the inferred transcriptome profile of each LH day. However, there could be both intra-individual (cycle-related) and inter-individual variation in transcriptome data at each LH day bin; thus a bin may not represent the “true” endometrial transcriptome of a sample of an individual taken at that same LH day. This limits the use of the Δ LH only to quantifying the relative difference of the actual LH day and the LH day based on endometrial transcriptomic dating. In addition, our endometrial biopsies were taken between 5 and 8 days after the LH surge, meaning that samples taken on LH+5/6 were compared with an ‘inferred transcriptome profile’, as compared with samples taken on LH+7/8, which could be directly compared with the profile of reference samples taken on LH+7/8. As for Δ WOI, the calculations do not take into account the actual LH day of the endometrial biopsy, relying only on the transcriptome profile of sample and thus its position on the pseudo-timeline. However, we were aware that the sampling day of the biopsy does affect endometrial gene expression. In addition, the Δ WOI values depend heavily on the chosen reference profiles, which in our study were set at LH+7, the most well-established endometrial receptivity day by beREADY. Thus, samples taken at LH+5 were also compared with this reference profile of LH+7, which could already have resulted in some deviation. Taken all the above in consideration, there may be some uncertainties in both the Δ WOI and Δ LH calculations. Although valuable to provide more insight into endometrial

development in respect of receptivity, the Δ WOI measurements as well as the concordance between the actual LH day and pseudo-timeline LH-day do require further validation.

Lastly, endometrial tissue was obtained by Pipelle biopsy. A potential limitation of this method is the inadvertent collection of endometrial tissue from the lower part of the uterus, which might not accurately represent the tissue where implantation occurs. However, in our approach, we introduced the catheter up to the fundus and conducted the biopsy from that point onwards, primarily targeting endometrial tissue within the fundus area. Additionally, as tissue was obtained within an RCT by endometrial scratching with a Pipelle catheter, we opted for this method to simplify the process of obtaining tissue and in alignment with the pragmatic nature of the study.

Our study has been unable to find a specific gene expression profile for either implantation failure or implantation success, either in the short term or long term, contrary to many previous studies. When focusing on the expression of endometrial receptivity genes only, there may be a difference between both groups, which we detected only in the short term. Therefore, we cannot yet exclude a role of impaired endometrial function in implantation failure, and further research is required. In addition, multiple cell types in the endometrium are known to be involved in the process of implantation.⁷⁸ It may therefore be possible that impairment of the function of a single cell type contributes to the occurrence of implantation failure. As bulk RNA-seq data mask gene expression levels of individual cell types present in the whole endometrial tissue, single-cell RNA-seq studies are required to provide more information on function per cell type and exclude a role of impairment of specific cell types in implantation failure.

Despite the multitude of omics studies in the past two decades that performed a molecular characterization of endometrial receptivity, pregnancy rates in ART have not improved.⁷⁹ Due to a lack of concordance in molecular markers between studies based on differences in study methodology (eg sampling, processing and analysis), it is difficult to compare results and to study relevant pathways in more depth.

Future omics studies with a standardized methodology, following a set of universal guidelines regarding experimental design, sample collection, preparation and analysis, data validation and data presentation,¹⁰ are required to obtain consistencies in relevant biomarkers and pathways. To enhance reproducibility, the phenotype of our participants has been meticulously defined, furnishing all available participant data, including age, BMI, smoking status and cycle characteristics. Furthermore, a detailed description of the tissue sampling method, sample storage, sample processing and data analysis was offered, as well as a thorough examination of the study's strengths and limitations.

For transcriptome studies, single-cell RNA-seq studies are highly recommended, as they provide more information on cell type-specific functions and their potential role in implantation. In addition, validation of promising biomarker sets for endometrial receptivity in RCTs will need to be undertaken. Subsequently, more studies on endometrial omics downstream of the transcriptome, such as proteomics and metabolomics, are warranted, as they are the main executors

of most physiological processes.^{80,81} Although proteins are synthesized from mRNA templates, not all mRNAs are translated to proteins, and tightly regulated post-transcriptional to post-translational modifications add complexity to these processes, resulting in a poor correlation between mRNA and protein expression.⁸²

Only with all the above can we improve our understanding of human endometrial (patho)physiology, determine whether the endometrium really plays a role in implantation failure or success, and create possibilities to improve care for infertile couples.

5 | CONCLUSION

Our exploratory study did not find an endometrial transcriptome profile associated with either implantation failure or success either in the short term or long term. In the short term, a larger deviation from the WOI may potentially be an underlying cause for implantation failure; however, in the long term, our study was unable to link WOI displacement to implantation failure. Future studies with standardized methodology are required to obtain more consistencies in relevant biomarkers, which help improve our understanding of the role of the endometrium in implantation failure, and in due course potentially provide targets to improve ART outcome.

AUTHOR CONTRIBUTIONS

Frank Broekmans designed the SCRaTCH trial and obtained funding. Nienke van Hoogenhuijze, Femke Mol, Gijs Teklenburg, Jan-Peter de Bruin, Dagmar Besselink, Linda Stevens Brentjens collected clinical data of the SCRaTCH trial participants. Nienke van Hoogenhuijze, Frank Broekmans, Femke Mol, Gijs Teklenburg, Jan-Peter de Bruin, Dagmar Besselink, Linda Stevens Brentjens, Ron van Golde obtained endometrial biopsies. Frank Broekmans, Shari Mackens, Andres Salumets, Gaby Steba, Triin Laisk initiated this international collaborative transcriptome project. Bich Ngoc Bui, Viktorija Kukushkina, Alvin Meltsov, Catharina Olsen, Gaby Steba, Triin Laisk, Signe Altmäe, Shari Mackens, Linda Stevens Brentjens, Darina Obukhova, Masoud Zamani Esteki, Ron van Golde, Andrea Romano, Andres Salumets, Frank Broekmans actively participated in online meetings to work out ideas and discuss results of data analyses. Bich Ngoc Bui, Viktorija Kukushkina, Alvin Meltsov, Catharina Olsen analyzed the data. Bich Ngoc Bui, Viktorija Kukushkina, Alvin Meltsov drafted the manuscript. All authors critically appraised the pre-final manuscript, after which Bich Ngoc Bui and Alvin Meltsov adjusted the manuscript for submission. All authors approved submission of the final version.

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CONFLICT OF INTEREST STATEMENT

The SCRATCH trial and the current endometrial transcriptome sub-study received funding from public sources and Merck, but none of these sources had input in the study design and analysis. In addition, several authors report having received personal fees for consultations, conference attendance and travel as follows: BB from Gedeon Richter Benelux and Guerbet; NvH from Organon; GT from Merck; GS from Guerbet; SM from Abbott Pharmaceuticals, Ferring, Abbott Pharmaceuticals, and IBSA. FB is a paid member of the advisory boards of Merck and Ferring, and also received a speakers' fee from Besins Healthcare. All of these personal fees were unrelated to the study reported in this manuscript. The remaining authors declare no conflicts of interest relevant to this study.

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ETHICS STATEMENT

This transcriptome study is a substudy of the SCRATCH trial, a Dutch multicenter randomized controlled trial in which endometrial tissue was obtained. The RCT was approved by the Institutional Review Board of the University Medical Center Utrecht (15-495) on November 30, 2015²³ and registered under trial registration number NTR5342 (<https://trialsearch.who.int/Trial2.aspx?TrialID=NTR5342>). For this substudy, ethical approval was obtained from the Biobank Research Ethics Committee of the University Medical Center Utrecht (19-520) on October 31, 2019. Written informed consent was obtained from all study participants.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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