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Uterine factors associated with fertility impairment

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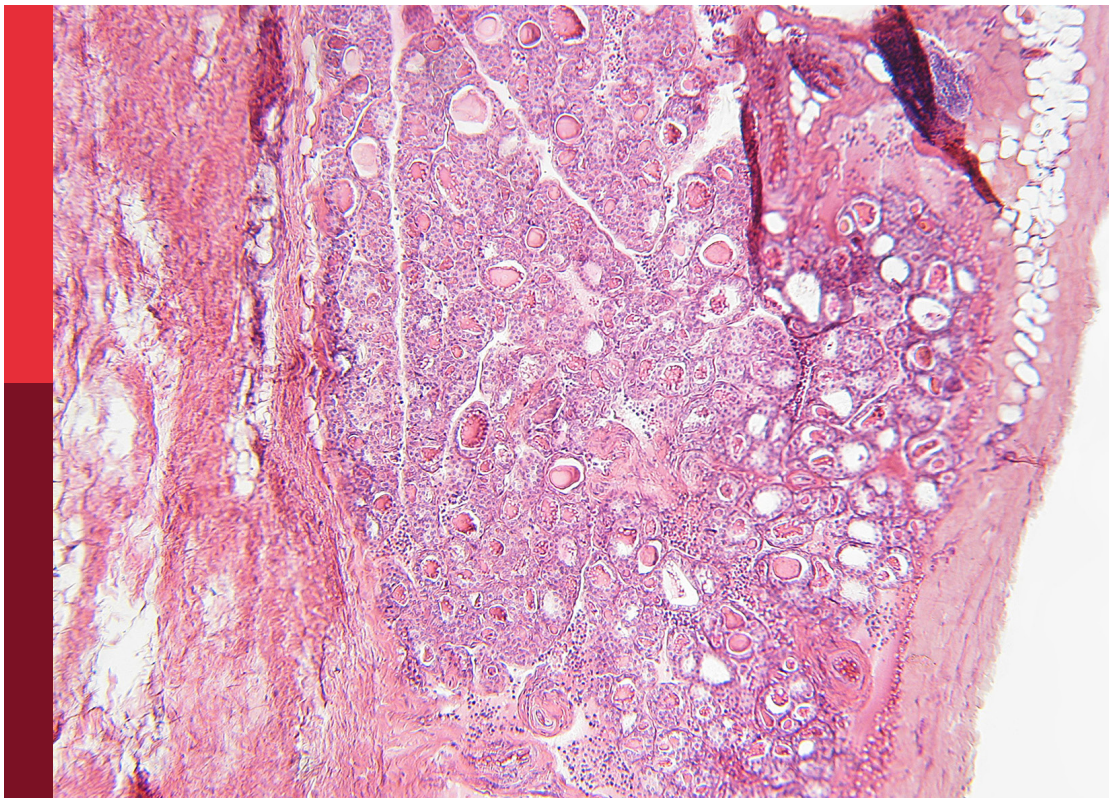

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Uterine factors associated with fertility impairment

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Editorial: Uterine factors associated with fertility impairment

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Editorial on the Research Topic
Uterine factors associated with fertility impairment

Infertilty is a prevalent medical condition affecting 9-18% of the global population with substantial impact on both the individual and societal level. While there have been noteworthy advancements in understanding the etiology, progression, and treatment of male and female factor infertilty, challenges remain for the medical community. Even at cutting-edge institutions, the success rate for treating infertility plateaus around 60%. We posit that augmented research efforts can help to address more complex cases and push the success rate further.

In particular, the uterine environment and endometrial health are essential contributors to fertility outcomes. The goal of this Special Reproduction Research Topic of Frontiers in Endocrinology is to address current challenges related to uterine and endometrial physiology and pathology and to highlight novel basic, translational, and clinical research in the field. We specifically seek to update readers on developments in endometriosis, adenomyosis, endometrial receptivity, recurrent implantation failure, management of uterine septum, pathophysiology of endometritis, endometrial microbiome, and endocrine disruptors' effect on endometrial function.

To begin, we want to draw readers' attention to a comprehesive review on endometriosis by Bonavina and Taylor. This article addresses everything you want to know about endometriosis, it covers pathophysiology, genetics and epigenetics, mechanisms of infertility, clinical presentation, and association with adenomyosis and other uterine issues. The authors then summarize the available treatment modalities including medical, surgical, and emerging therapies. This review provides a strong background for a nuanced translational study completed by Huang et al. addressing the potential use of human adipose-derived stem cell conditioned medium to improve endometriosis burden and subsequnt fertility in a murine model of endometriosis. The

success of this therapy is likely attributed to its anti-inflammatory and antiangiogenic properties. Readers should stay tuned for possible implementation of this strategy in humans using Good Tissue Practice and Good Manufacturing Practice regulations. Keeping in mind the multifaceted nature of endometriosis and its rare but potential transformation to malignancy, we want to highlight a study by Collins et al. The authors aimed to explore the molecular mechanisms behind the transformation of ovarian endometriosis (as a model for cystic lesions of deep endometriosis) to ovarian clear-cell carcinoma. Through deep transcriptomic and correlation analysis, they discovered dysregulation at the level of mRNA and miRNA, which was also confirmed during *in vitro* experiments. The authors eventually suggested miR-10a-5p as a molecule with oncogenic potential worth further evaluation.

Moving from endometriosis to adenomyosis, a similar but somewhat different condition, we would like to guide readers towards a paper by He et al. investigating a potential mechanism for impared endometrial receptivity in patients with adenomyosis. Elegantly using human endometrial cells *in vitro* and an adenomyosis mouse model *in vivo*, the authors showed that IL-33 is dysregulated in adenomyosis and linked to endometrial HOXA10 function. The reduced expression of IL-33 in the endometrium in adenomyosis impairs embryo implantation through modulation of HOXA10 expression through the STAT3 pathway. Tang et al. focused thei research efforts on understanding the mechanism of action of the dopamine receptor agonist bromocriptine as a novel medical management option for adenomyosis. Their publication reported that bromocriptine treatment exhibited an anti-proliferative effect within the endometrium of patients with adenomyosis *in vivo* and *in vitro*. This effect could be related to subsequent upregulation of several miRNAs and is consistent with symptomatic improvement in adenomyosis patients after bromocriptine therapy.

As the ultimate goal of infertility management is achieving a live birth, Wu et al. attempted to create a model to predict the chance of live birth after frozen embryo transfer (FET) in patients with adenomyosis, which was later validated in different sets of patients. The authors built a statistical nomogram incorporating age, uterine volume, developmental stage of the transferred embryo, FET protocol, and presence of singleton versus twin pregnancy after selecting from numerous variables. The results showed that younger age, smaller uterine volume, blastocyst transfer, and hormone replacement FET protocol with GnRH-agonist were associated with better live birth outcomes, while achieving a twin pregnancy was associated with a lower chance of live birth.

Switching gears to anatomic issues, we would like to update readers on the highly-debated management of a septate uterus. Chang et al. retrospectively analyzed a large database from a single institution where uterine septum resection was performed on all patients with infertility, miscarriage, or poor obsteric outcomes, regardless of prior pregnancy outcomes. The authors reported that live birth, clinical pregnancy, and early miscarriage rates were all significantly improved after surgery in patients with a septate uterus and maternal age ≥ 35 years. Primary infertility was associated with a lower chance of clinical pregnancy after septum resection. Although the results of this retrospective analysis contrast those of the only completed randomized controlled trial on this topic (1), certain methodological and clinical factors might have influenced the variation in outcomes.

Another controversial topic is recurrent implantation failure (RIF), a clinical phenomenon characterized by lack of implantation after at least 3 euploid blastocyst transfers (or the equivalent number of unscreened embryo transfers adjusted to the patient's age and corresponding euploidy rate). While true RIF arguably is extremely uncommon, occuring in less than 5% of couples with infertility (2), the nature of this reproductive impairment is not clearly established. One of the putative causes underlying RIF are alterations in endometrial receptivity. Several recent studies showed that "personalized" embryo transfer based on Endometrial Receptivity Assay (ERA) for an unselected group of patients (all comers) did not improve pregnancy outcomes compared to conventional transfer timing. In an effort to idenfity an appropriate test for the implantation window of patients with RIF, Chen et. al. conducted a study comparing an RNA-sequencing-based endometrial receptivity test (rsERT) with pinopode evaluation. After personalized embryo transfer, patients in rsERT group had significantly higher pregnancy rates and required fewer FET cycles, supporting the use of rsERT as a potential clinical tool. Other potential causes of RIF are immunological abnormalities. Guo et al. studied the alterations of cytokine profiles in patients with RIF, trying to shed new light on its pathomechanism. Several elevated proinflammatory cytokines, decreased anti-inflammatory cytokines, and an increased Th1/Th2 cytokine ratio were observed in the mid-luteal serum of RIF patients compared to control subjects. A constructed predictive model for RIF found that IL-10, G-CSF and IL-2/IL-10, in particular, had independent predictive values for occurrence of RIF. Furthermore, in combination with age, and endometrial thickness, IL-6 and IFN-γ/IL-10 demonstrated good diagnostic performance.

Various therapeutic options have been proposed to manage RIF without groundbreaking success. A study by Niu et al. aimed to explore the effects of low molecular-weight heparin (LMWH) treatment on the uterine inflammatory cytokine profile and pregnancy outcomes of patients with RIF but without trombophilia. The cytokine mRNA profile in endometrial samples obtained from 326 RIF patients undergoing FET with or without LMWH treatment demontrated different patterns potentially suggesting beneficial action of LMWH on implantation. Although the clinical and ongoing pregnancy rates did not significantly differ between the two groups, the cohort of patients treated with LMWH and who successfully concieved had significantly higher levels of IL-6, IL-15 and G-CSF. Another approach to improve success in patients with implantation failures was examined by Wang et. al. Several prior studies explored the effects of endometrial injury on clinical pregnancy outcomes with conflicting results. Wang et al. focused their prospective cohort study on the timing of endometrial scratch biopsy. In patients with a history of ≥ 2 failed transfers, the implantation and clinical pregnancy rates were significantly higher if endometrial injury took place in the luteal phase of the cycle preceding embryo transfer (7 days after ovulation), than in follicular phase of the embryo transfer cycle (cycle day 3-5). Interestingly, the endometrial injury did not improve the pregnancy outcomes in unselected patients.

Chronic endometritis (CE) is another condition potentially affecting endometrial receptivity and associated with poor *in vitro*

fertilization (IVF)/FET outcomes. The mechanisms underlying CE are not fully established, while unfavorable uterine microenvironment has been suggested as a potential cause. In the search of possible mechanisms, a study by Liu et al. investigated the hypoxic microenvironment and endometrial vascularization in the receptive phase endometrium from infertile women with CE. Their study found that upregulation of the hypoxia factor (H1F1α) and angiogenic factors, including VEGFA and VEGFR2, as well as excessive vascularization in the peri-implantation endometrium, seem to reduce endometrial receptivity in infertile patients with CE. Interestingly, the authors repeated the analyses after first-line antibiotic treatment, and suggested that antibiotic-mediated remodeling of hypoxic and angiogenic homeostasis in the endometrium might improve reproductive outcomes. Nevertheless, these initial results should be subject to further testing and confirmation in future studies.

A thought-provoking study by Molina et al. evaluated the influence of a Mediterranean diet on endometrial whole metabolome profile in the receptive phase. The study provided insight into the molecular background of "endometrial well-being" and, importantly, the possibilities for modulating endometrial environment and enhancing embryo implantation through nutrition.

In line with the uterine microenvironment, there is notable interest in detecting and identifying the endometrial core microbial composition, termed "microbiota," in physiology and pathophysiology. A study by Canha-Gouveia et al. assessed the female upper reproductive tract microbial composition in disease-free tissue and found that the endometrium and fallopian tubes share 70% of the bacteria, while the rest is organ-specific. They also showed that the endometrial sampling method may influence the results as well as the fact that each individual possesses their unique microbial profile. This knowledge adds to the appreciation of the vast complexity of the natural microenvironment of the female reproductive tract and further understanding with regard to implications on improving IVF/FET protocols.

Finally, we will shift our focus to the external environment. Increasing evidence suggests that environmental pollution has a direct impact on our health. Chemicals that can interfere with the endocrine system are termed endocrine disrupting chemicals (EDCs). Multiple studies have found associations between exposure to EDCs and reduced fertility in women (3). However, there is little knowledge of the effect of EDCs on the endometrium and its function. In a study by Lavogina et al. the effect of a wide selection of EDCs on primary human endometrial stromal cell (hESC) decidualization was evaluated *in vitro*. The authors showed that EDCs commonly present in the blood can reduce decidualization of hESCs. The effects of EDCs on endometrial dysfunction and risk of implantation failure are the next targets of study.

Although we have made significant advancements in understanding embryonic development and selection criteria, our comprehension of uterine and endometrial functions, as well as their associate anomalies, remains insufficient. We hope that this Special Reproduction Research Topic provides more insight into the uterine factors of infertility, stimulates readers' minds, and provokes new ideas for future research directions.

Author contributions

LA: Writing – original draft, Writing – review & editing. SA: Writing – original draft, Writing – review & editing. AS: Writing – original draft, Writing – review & editing.

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References

1. Rikken JFW, Kowalik CR, Emanuel MH, Bongers MY, Spinder T, Jansen FW, et al. Septum resection versus expectant management in women with a septate uterus: an international multicentre open-label randomized controlled trial. *Hum Reprod* (2021) 36:1260–7. doi: 10.1093/humrep/deab037

2. Pirtea P, Cedars MI, Devine K, Ata B, Franasiak J, Racowsky C, et al. Recurrent implantation failure: reality or a statistical mirage?: Consensus statement from the July 1, 2022 Lugano Workshop on recurrent implantation failure. *Fertil Steril* (2023) 120:45–59. doi: 10.1016/j.fertnstert.2023.02.014

3. Silva ABP, Carreiró F, Ramos F, Sanches-Silva A. The role of endocrine disruptors in female infertility. *Mol Biol Rep* (2023) 50:7069–88. doi: 10.1007/s11033-023-08583-2

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ORIGINAL RESEARCH
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A Validated Model for Individualized Prediction of Live Birth in Patients With Adenomyosis Undergoing Frozen–Thawed Embryo Transfer

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Purpose: This study aimed to develop a predictive tool for live birth in women with adenomyosis undergoing *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) treatment.

Methods: A total of 424 patients with adenomyosis who underwent frozen–thawed embryo transfer (FET) from January 2013 to December 2019 at a public university hospital were included. The patients were randomly divided into training (n = 265) and validation (n = 159) samples for the building and testing of the nomogram, respectively. Multivariate logistic regression (MLR) was developed on the basis of clinical covariates assessed for their association with live birth.

Results: In total, 183 (43.16%) patients became pregnant, and 114 (26.88%) had a live birth. The MLR showed that the probability of live birth was significantly correlated with age [odds ratio (OR), 3.465; 95% confidence interval (CI), 1.215–9.885, P = 0.020], uterine volume (OR, 8.141; 95% CI, 2.170–10.542; P = 0.002), blastocyst transfer (OR, 3.231; 95% CI, 1.065–8.819, P = 0.023), twin pregnancy (OR, 0.328; 95% CI, 0.104–0.344, P = 0.005), and protocol in FET (P < 0.001). The statistical nomogram was built based on age, uterine volume, twin pregnancy, stage of the transferred embryo, and protocol of FET, with an area under the curve (AUC) of 0.837 (95% CI: 0.741–0.910) for the training cohort. The AUC for the validation cohort was 0.737 (95% CI: 0.661–0.813), presenting a well-pleasing goodness-of-fit and stability in this model.

Conclusions: This visual and easily applied nomogram built on the risk factors of live birth in patients with adenomyosis provides useful and precise information for physicians on individualized decision-making during the IVF/ICSI procedure.

Keywords: adenomyosis, uterine size, live birth, GnRH-a, prediction model

BACKGROUND

Adenomyosis is a common gynecological disorder where endometrial glands and stroma surrounded by hyperplastic smooth muscle were found within the myometrium (1, 2). It affects up to 24.4% of infertile women, which represents a clinical issue associated with pelvic pain, excessive vaginal bleeding, enlarged uterus, and infertility (3, 4). Adenomyosis has been reported to adversely impact fertility *via* abnormal uterine contractility, including altered endometrial function and receptivity, and impaired implantation (5). Besides this, a patient with adenomyosis was also linked with poor obstetrical outcomes, including preeclampsia, placental malposition, preterm delivery, and preterm premature rupture of membrane (6–8).

Intracytoplasmic sperm injection (ICSI) and *in vitro* fertilization (IVF) are extensively being used for managing adenomyosis-related infertility. However, the results varied from one report to another, with some showing identical outcome as in patients without adenomyosis and others presenting increased miscarriage and lower clinical pregnancy rates (1). Several studies provided evidence that an enlarged uterus in adenomyosis had an adverse impact on pregnancy outcomes by the morphological and functional pathological changes (9, 10). In addition, results from some trials on adenomyosis showed that applying gonadotrophin-releasing hormone agonists (GnRH-a) before IVF/ICSI cycle exerts positive effects on increasing the clinical pregnancy rate in patients with adenomyosis (11–13). The success for a patient with adenomyosis-associated infertility in IVF/ICSI procedure to achieve pregnancy depends on a number of factors. Although several scoring systems have been published to evaluate the pregnancy rate after IVF/ICSI in infertile patients, the current guidelines are based on general rather than individual clinic data. Despite the availability of these models, few of them are applicable for patients with adenomyosis and cannot evaluate the chances of live birth for individual adenomyosis patient. In this aspect, setting up of a predictive calculation model in live birth with a combination of all the risk factors in patients with adenomyosis will be beneficial for the medical staff in the decision-making process and promoting adherence to medication through risk-informed counselling.

The aim of the current study was, therefore, to develop a nomogram model on retrospective data analysis to predict the probability of live birth for patients with adenomyosis.

PATIENTS AND METHODS

Data Sources

Women diagnosed with adenomyosis and undergoing frozen–thawed embryo transfer during 2013 and 2019 at Sun Yat-Sen Memorial Hospital, Guangzhou, China, were screened for this retrospective cohort study. The patients were included if they had received their first frozen–thawed embryo transfer (FET) cycle with autologous embryos. The exclusion criteria included congenital uterine malformation (unicornuate, bicornuate, and septate uterus), intrauterine adhesion, uterine malformation, and leiomyoma. Couples who received a preimplantation genetic screening or underwent preimplantation genetic diagnosis were excluded. The indications for IVF/ICSI included the tubal factor, male factor, and immunity factor. Demographic data on age, body mass index (BMI), infertility duration, basal sexual hormone levels (tested on days 2 to 3 of the menstrual cycle), uterine volume prior to embryo transfer (ET) (long diameter × width diameter ×anteroposterior diameter × $\pi/6$) (14), type of adenomyosis (diffusion or focal), endometrial thickness, and protocol of FET were obtained from the clinical database.

The diagnosis of adenomyosis was ascertained by a detailed chart review, including visit notes and ultrasound and operative reports as well as pathology reports. The diagnosis was defined with two or more transvaginal sonographic criteria that included heterogeneous myometrial area, globular asymmetric uterus, irregular cystic spaces, myometrial linear striations, poor definition of the endometrial myometrial junction, myometrial anterior posterior asymmetry, thickening of the anterior and posterior myometrial wall, and increased or decreased echogenicity (15, 16). All identified adenomyosis cases were confirmed by two experienced sonographers. Diffuse adenomyosis was defined as an outer myometrium extensive disease with the endometrial glands and stroma scattered throughout the uterine musculature, and focal adenomyosis included adenomyoma, defined as grossly circumscribed adenomyotic masses within the myometrium (9, 17).

Frozen–Thawed Embryo Transfer Procedure

FET was performed through a natural cycle (NC) or through hormone replacement therapy (HRT) cycles with endometrial preparation by exogenous estrogen and progesterone or through the cycle adding gonadotrophin-releasing hormone agonists (GnRH-a) before estradiol. Among the patients with GnRH agonist pre-treatment, long-acting GnRH-a was administrated with up to three injections of 3.75 mg of triptorelin acetate (Ipsen Pharma Biotech, France) (18). No more than two embryos were transferred. The luteal-supported phase was administered by the vaginal administration of micronized progesterone (400 mg/day). Pregnancies were diagnosed by an increasing concentration of serum β-hCG, which was tested 14 days after the embryo transfer (18). Clinical pregnancies were confirmed by the presence of the gestational sac on vaginal ultrasound examination during the fifth week. Twin pregnancy was confirmed by ultrasound examination during the 12th week. A live birth is defined as any live baby born after the 24th week of pregnancy.

Data Analysis

Statistics with Gaussian distribution were presented as mean ± SD, and categorical variables were described as absolute frequencies (**Table 1**). Youden Index was used to determine the optimal cutoff point of the uterine volume associated with

Abbreviations: BMI, body mass index; FSH, follicle-stimulating hormone; E2, estrogen; T, testosterone; HRT, hormone replacement therapy; FET, frozen–thawed embryo transfer; IVF/ICSI, *in vitro* fertilization/intracytoplasmic sperm injection; ART, assisted reproductive technology; GnRH-a, gonadotrophin-releasing hormone agonists; hCG, human chorionic gonadotropin; LBR, live birth rate; ROC, receiver operating characteristic curves; SD, standard deviation; ORs, odds ratios; CI, confidence interval.

TABLE 1 | Characteristics of the patients with and without live birth.

Characteristics[a]	Without live birth n = 310	With live birth n = 114	*P*
Age, years	34.94 ± 4.68	31.92 ± 3.95	<0.001*
Infertility duration, years	4.53 ± 3.79	4.20 ± 3.05	0.403
BMI, kg/m^2	21.58 ± 2.93	20.55 ± 2.20	0.001*
AMH, IU/L	3.22 ± 3.12	3.74 ± 2.88	0.337
FSH, IU/L	8.65 ± 3.77	7.98 ± 2.13	0.087
LH, IU/L	5.39 ± 2.48	5.60 ± 2.65	0.422
E2, pg/ml	50.29 ± 15.28	50.60 ± 36.60	0.968
T, ng/ml	1.24 ± 0.82	1.69 ± 4.90	0.150
Type of adenomyosis, *n* (%)			0.721
Diffuse	173 (65.29)	103 (64.78)	
Focal	92 (34.71)	59 (35.22)	
Uterine diameters prior to ET			
Width diameter, cm	5.51 ± 1.17	5.11 ± 0.99	0.001*
Anteroposterior diameter, cm	5.34 ± 1.17	4.97 ± 0.86	0.002*
Long diameter, cm	5.51 ± 1.07	5.51 ± 0.99	0.001*
Uterine volume, cm^3	91.13 ± 43.44	71.60 ± 26.53	0.000*
Stage of embryo transfer			0.165
Cleavage, *n* (%)	50 (16.13)	25 (21.93)	
Blastocyst, *n* (%)	260 (83.87)	89 (78.07)	
Protocol of FET			0.050
HRT	149 (48.06)	24 (21.05)	
GnRHa-HRT	127 (40.97)	53 (46.49)	
NC	34 (10.97)	37 (32.46)	
Endometrial thickness (mm)	10.04 ± 2.56	9.21 ± 3.00	0.128
Pregnancy type			
Singleton pregnancy, *n* (%)		83 (72.81)	
Twin pregnancy, *n* (%)		31 (27.19)	

BMI, body mass index; AMH, anti-Mullerian hormone; FSH, follicle-stimulating hormone; E2, estrogen; T, testosterone; ET, embryo transfer; HRT, hormone replacement therapy; NC, nature cycle.
[a]*Continuous variables are expressed as mean ± SD and categorical variables as absolute frequencies, n (%).*
**P < 0.05 was considered statistically significant.*

live birth. External validation was chosen in the study so that the patients enrolled were divided into a training set (n = 265) and a validation set (n = 159) by the sampling techniques of random numbers. Statistical analyses were performed using the STATA 14.0 MP software and Regression Modeling Strategies (R version 3.6.3). To build up the nomogram and measure the area under the curve (AUC), we used the "regplot", "pROC", and "rms" in R software (19). Differences between groups were compared using Student's *t*-test or chi-square test as appropriate.

Development and Validation of the Model

The training cohort of 265 patients was used to develop the nomogram for predicting the patient-specific probability of live birth in women with adenomyosis. The end-point of the study was live birth rate after FET cycles. Backward variable selection was performed to determine independent covariates. Logistic regression model was used for multivariate analysis, including the univariate analysis of significant variables (P < 0.05) (**Supplementary Table S1**). The coefficient for each independent covariate and the constant were generated in the equation by MLR analysis (20). The variables entered into the nomogram model were age, uterine volume, stage of the transferred embryo, twin pregnancy, and protocol of FET in the study. The values for each of the model covariates were mapped to points on a scale ranging from 0 to 100, and the total points obtained for each model corresponded to the probability of a live birth (19).

The model was applied to data from a sample of 159 patients (validation set) for external validation with a bootstrapping technique to obtain relatively unbiased estimates (1,000 repetitions). The bootstrapping method is based on resampling obtained by randomly drawing data and replacing them with samples from the original dataset (21). The predictive accuracy of the models was measured using the average optimism of the AUC. A precise prediction model would result in a plot where the observed and predicted probabilities fall along the diagonal (19).

RESULTS

Description of the Study Population

A total of 424 patients with adenomyosis who underwent frozen–thawed embryo transfer from January 2011 to December 2019 were identified as eligible and were analyzed in this study. In total, 183 (43.16%) patients became pregnant, and 114 (26.88%) had a live birth. The patients with live birth were younger (31.92 ± 3.95 vs. 34.94 ± 4.68, P < 0.001), slimmer (20.55 ± 2.20 vs. 21.58 ± 2.93, P = 0.001), and had a smaller uterine size (71.60 ± 26.53 vs. 91.13 ± 43.44, P < 0.001) compared with those patients without a live birth (**Table 1**). The blastocyst stage and GnRHa-HRT protocol for FET increased the live birth rate in patients with adenomyosis (P < 0.001) (**Table 1**). The patients were divided into a training set and a validation set by the sampling techniques of random numbers.

The model was built from a training cohort of 265 patients and was validated on an independent validation cohort of 159 patients. **Table 2** summarizes the epidemiological, clinical, biostatistical, and treatment strategies for the training and validation cohorts. No significant difference was observed in the patients' characteristics between the two cohorts. A total of 79 patients (29.81%) in the training cohort had a live birth, while 35 patients (22.01%) in the validation cohort had a live birth.

The logistic regression analysis revealed that blastocyst transfer, small uterine size, and GnRH-a pretreatment prior to FET improved the live birth, but twin pregnancy negatively impacted the live birth.

The optimal cutoff point of the uterine volume prior to ET related to live birth was 102.02 cm^3 (AUC = 0.603, P = 0.003) according to the Youden Index. **Supplementary Table S1** summarizes the univariable and multivariable analyses. The univariate logistic regression analysis showed that the live birth rate was significantly correlated with age (P = 0.018), uterine volume prior to ET <102.02 cm^3 (P < 0.001), twin pregnancy (P < 0.001), stage of the transferred embryo (P = 0.012), and protocol in FET (P < 0.001). In the MLR analysis of the training cohort, the probability of a live birth was significantly correlated with the age <37 years old [odds ratio (OR), 3.465; 95% CI, 1.215–9.885, P = 0.020], uterine volume prior to ET <102.02 cm^3 (OR, 8.141; 95% CI, 2.170–10.542; P = 0.002), blastocyst transfer (OR, 3.231; 95% CI, 1.065–8.819, P = 0.023), twin pregnancy (OR, 0.328; 95% CI, 0.104–0.344, P = 0.005), and protocol in FET (P < 0.001) (**Figure 1**). Blastocyst transfer, small uterine size, and GnRH-a pretreatment were associated with an increased probability of live birth, but twin pregnancy decreased the probability.

Development of the Models From the Training Cohort

Based on the univariable and multivariable logistic regression analyses that we performed, a nomogram incorporating the significant risk factors was established to predict the probability of a live birth (**Figure 2**). Total scores were calculated based on age, stage of the transferred embryo, uterine volume, twin pregnancy, and protocol of FET. The equation describing the probability of a live birth was as follows: $P = 1/[1 + \exp(-X)]$, where $X = 0.4755302 + 0.1091108 \times V1 + 0.0882141 \times V2 - 0.3309371 \times V3 + 0.1281561 \times V4 + 0.2339871$, where $V1$ was age (1 if <37 years old and 0 if ≥37 years old), $V2$ was blastocyst transfer (0 if no and 1 if yes), $V3$ was twin pregnancy (1 if no and 0 if yes), $V4$ was the protocol of FET (2 if GnRH-a HRT, 1 if NC, and 0 if HRT), and $V5$ was uterine volume (1 if <102.02 cm^3 and 0

TABLE 2 | Patient characteristics in the training and the validation cohorts.

Characteristics[a]	Training set n = 265	Validating set n = 159	*P*
Live birth, *n* (%)	79 (29.81)	35 (22.01)	0.080
Age, years	34.05 ± 4.79	34.26 ± 4.52	0.652
Infertility duration, Years	4.27 ± 3.41	4.57 ± 3.91	0.196
BMI, kg/m^2	21.41 ± 2.82	21.14 ± 2.74	0.331
AMH, IU/L	3.07 ± 2.83	3.76 ± 3.37	0.195
FSH, IU/L	8.65 ± 3.77	7.98 ± 2.13	0.087
LH, IU/L	5.27 ± 2.68	5.34 ± 2.87	0.822
E2, pg/ml	53.33 ± 15.78	46.60 ± 12.07	0.285
T, ng/ml	0.43 ± 0.24	0.46 ± 0.26	0.723
Type of adenomyosis, *n* (%)			0.721
Diffuse	173 (65.29)	103 (64.78)	
Focal	92 (34.71)	59 (35.22)	
Uterine diameters prior to ET			
Width diameter, cm	5.41 ± 1.14	5.38 ± 1.13	0.415
Anteroposterior diameter, cm	5.21 ± 1.11	5.30 ± 1.11	0.808
Long diameter, cm	5.56 ± 1.05	5.67 ± 1.09	0.300
Uterine volume, cm^3	84.81 ± 40.66	87.66 ± 40.35	0.485
Stage of embryo transfer			0.107
Cleavage, *n* (%)	53 (20.00)	25 (13.84)	
Blastocyst, *n* (%)	212 (80.00)	89 (86.16)	
Protocol of FET			0.219
HRT	115 (43.40)	58 (36.48)	
GnRHa-HRT	104 (39.25)	76 (47.80)	
NC	46 (17.35)	25 (15.72)	
Endometrial thickness (mm)	9.87 ± 2.72	9.91 ± 2.62	0.889
Pregnancy type			0.193
No pregnancy	142 (53.58)	99 (62.26)	
Singleton pregnancy, n (%)	71 (26.79)	37 (23.27)	
Twin pregnancy, n (%)	52 (19.62)	23 (14.47)	

BMI, body mass index; AMH, anti-Mullerian hormone; FSH, follicle-stimulating hormone; E2, estrogen; T, testosterone; ET, embryo transfer; HRT, hormone replacement therapy; NC, nature cycle.

[a]*Continuous variables are expressed as mean ± SD and categorical variables as absolute frequencies, n (%).*

**P < 0.05 was considered statistically significant.*

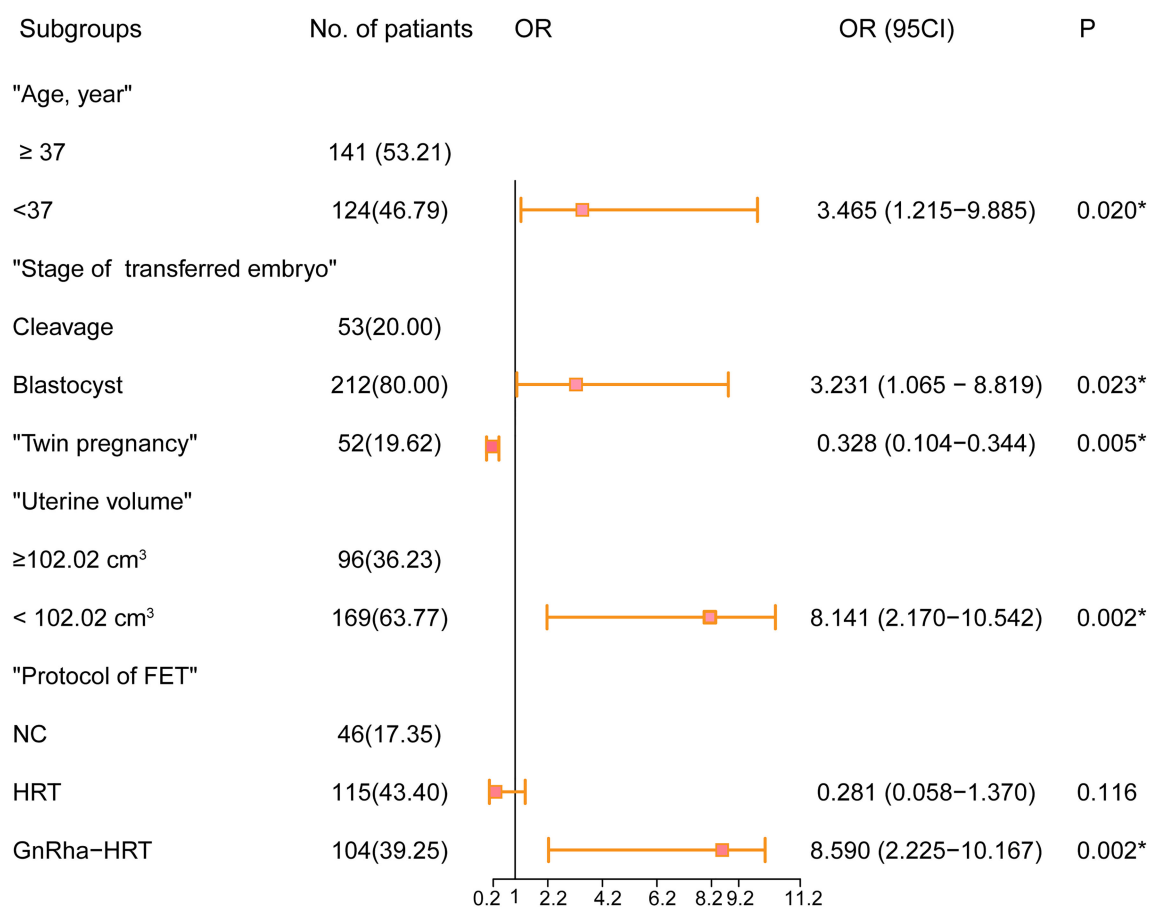

FIGURE 1 | Forest plot of the predictive factors of live birth in the multivariable analysis of the training cohort. OR and 95% CI are presented to show the risk of predictive factors. FET, frozen–thawed embryo transfer; OR, odds ratio; CI, confidence interval. *$P < 0.05$ was considered statistically significant.

if ≥102.02 cm^3). The nomogram derived from this equation is reported in **Figure 2**.

Validation of Predictive Accuracy

No significant difference was observed between the predicted probability obtained from the bootstrap correction and the actual probabilities of live birth ($P = 0.186$), which implied that the nomogram was well calibrated. The model demonstrated an AUC of 0.837 (95% confidence interval: 0.741–0.910) in the training cohort (**Figures 3A, B**), which denoted good performance. The AUC of the receiver operating characteristic (ROC) curve in the validation set was 0.737 (95% confidence interval: 0.661–0.813), which indicated fair performance.

DISCUSSION

On the basis of 424 infertile patients with adenomyosis who underwent FET, we have first created a predictive nomogram tailored to the individual patient and capable of reliably generating the numerical probabilities of live birth. The nomogram was developed in a training cohort including 265 patients and tested on an external independent validation cohort including 159 patients. Both calibration and discrimination were used to evaluate the performance. This graphical tool is a simple and straightforward calculator, integrating five predictive variables, that was easily accessible during the assisted reproductive technology (ART) treatment constituting of age, uterine volume, protocol of FET, type of pregnancy, and stage of the transferred embryo. Moreover, this model firstly integrated the potential risks in fetal loss of a patient with adenomyosis into one graphical calculator, which is of particular interest for clinicians to make an informed decision on the timing and protocol of FET, stage, and number of embryos to transfer.

An enlarged uterus in patients negatively impacted the live birth rate as revealed by MRL in our study. Endometrial tissues within the myometrium induce hyperplasia and hypertrophy of the adjacent smooth muscle, resulting in uterus enlargement which is considered an important feature of adenomyosis (10). The morphological and functional pathological changes caused by hyperplasia and hypertrophy of the adjacent smooth muscle weaken the scalability and coordination of the uterus, which adversely influence the patient's pregnancy and delivery

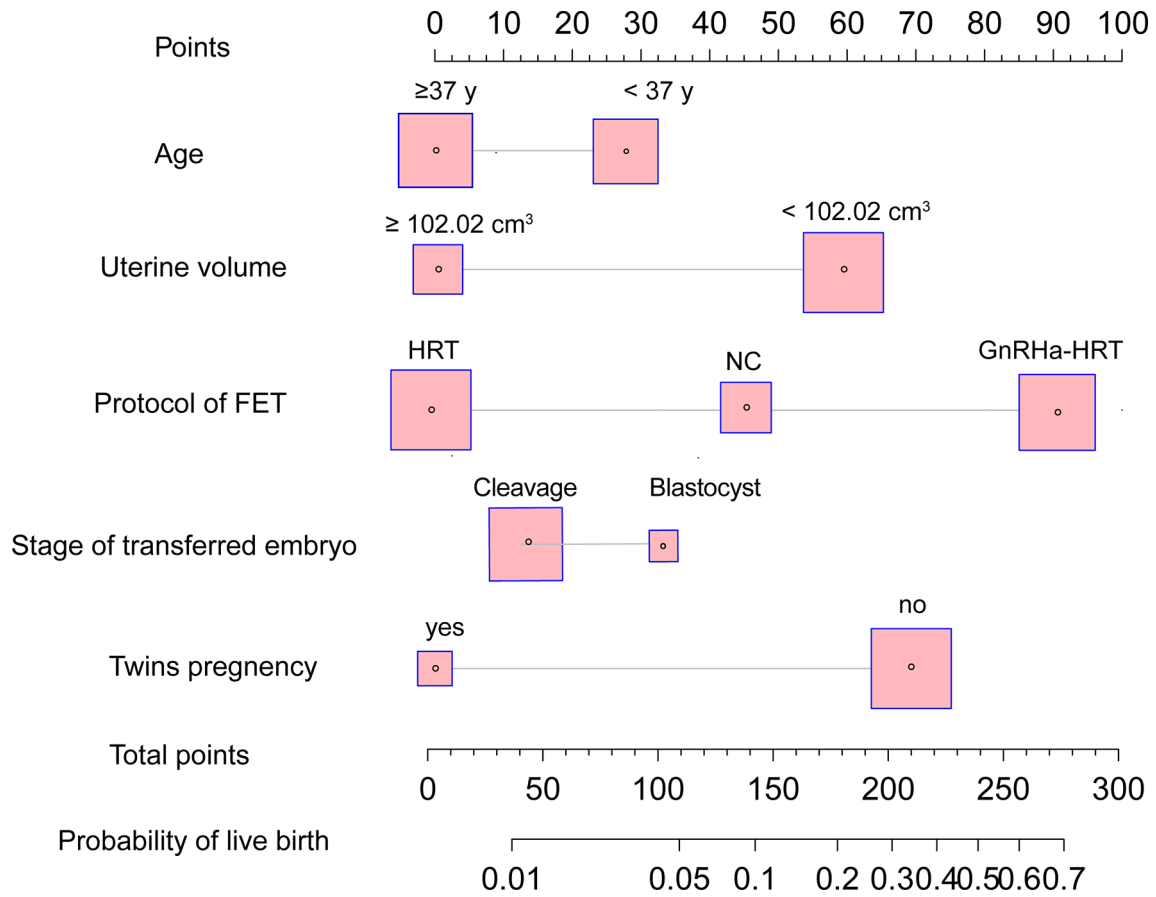

FIGURE 2 | Nomogram to predict the probability of live birth in adenomyosis-related infertility patients undergoing FET. The probability of a live birth is calculated by drawing a line to the point on the axis for each of the following variables: stage of transferred embryo, age, twin pregnancy, protocol of FET, and uterine volume prior to ET. The points for each variable are summed up and located on the total points line. Next, a vertical line is projected from the total points line to the predicted probability bottom scale to obtain the individual probability of a live birth. FET, frozen–thawed embryo transfer.

procedure (1, 10, 22). It was suggested that adenomyosis patients with an enlarged uterus suffer from a high rate of miscarriage and preterm delivery and have babies who were small for gestational age (10, 23). In addition, Kim et al. indicated that preterm delivery in pregnant patients with adenomyosis can be predicted through uterine wall thickness measurement in the second trimester (22). Recently, a retrospective study from Li et al. demonstrated that adenomyosis patients with a larger uterine volume suffered from lower live birth rate due to a higher incidence of miscarriage (9). A prospective study by Hawkins et al. also revealed that women with uterine lengths longer than 9 cm were more likely to experience spontaneous abortions (24). Consistently, a uterine volume larger than 102.02 cm^3 was associated with a lower live birth rate in our study. Therefore, the use of uterine volume as a significant determinant factor in pre-pregnancy examinations should never be ignored. Routine checks for uterine size during ART treatment are beneficial for detecting patients who were at an increased risk so that proper protocol for the subsequent FET can be chosen and preventive measures can be taken in early pregnancy.

GnRH-a pre-treatment in FET cycles significantly improved the live birth rate in our retrospective study. Consistently, several studies suggested that the administration of GnRH agonist increased the implantation rate, clinical pregnancy rate, and ongoing pregnancy rate of patients with adenomyosis in FET cycles (11, 25). The adenomyosis tissue, which contained estrogen, progesterone, and androgen receptors, develops in an estrogen-dependent manner (26). The administration of GnRH agonist can suppress the hypothalamic–pituitary axis, resulting in a hypoestrogenic status, and then suppress the proliferation of cells derived from the endometrium, reducing the size of pathologic lesions in patients with adenomyosis (27, 28). Moreover, the expression of aromatase cytochrome P450, a protein overexpressed in women with adenomyosis and that catalyzed the conversion of androgen to estrogen, can be decreased by GnRH agonist (29). Our results show that, after adjustment for confounding factors, GnRH agonist pre-treatment is associated with increased live births in patients with adenomyosis in FET cycles. With the increasing use of embryo freezing–thawing, pretreatment with GnRH agonist is recommended for adenomyosis patients in FET cycles.

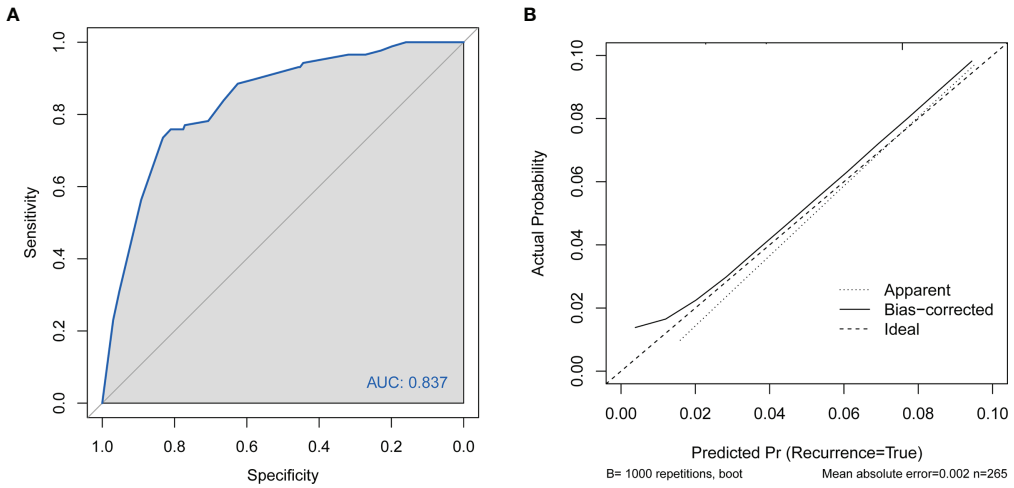

FIGURE 3 | **(A)** Discrimination for the training cohort. ROC curve of the model with an area under the curve of 0.837 (95% confidence interval: 0.741–0.910). **(B)** Calibration of the nomogram to predict live birth in patients with adenomyosis undergoing FET. FET, frozen–thawed embryo transfer; CI, cervical insufficiency; ROC, receiver operating characteristic curve.

A patient's age has been considered to be a significant prognostic factor in reproductive medicine and frequently involved in assessing the probability of a live birth or pregnancy (30). Uterine adenomyosis mostly occurs in women over the age of 35 years old, and the average age of patients included in our study was up to 34, which was associated with adverse pregnancy outcomes in this study.

Consistent with previously published studies (31, 32), our study showed that the increased live birth rate was significantly associated with blastocyst transfer than cleavage embryo transfer. Besides this, twin pregnancy—a well-understood risk factor of adverse obstetric outcomes (33)—was a strong collective factor in our model. Therefore, single blastocyst embryo transfer, which is highly recommended in ET cycles for its high live birth rate, is encouraged in patients with adenomyosis, especially those with an enlarged uterus.

However, some limitations of the current study have to be highlighted. First, we could not avoid the deviation of uterine diameter measurement by different clinicians. Second, the diagnosis for adenomyosis relied on ultrasound results, such that mild adenomyosis might have been misclassified. Third, the retrospective nature of the study cannot exclude all biases. Despite these limitations, our nomogram model for predicting live birth rates could be a useful tool to help doctors and adenomyosis patients undergoing IVF/ICSI procedure decide on embryo transfer option and to give special attention during prenatal visits.

CONCLUSION

In conclusion, an objective and accurate prediction nomogram model for live birth rate was drawn up and validated in infertile patients with adenomyosis. Relative risk assessment could be performed during infertility consultation, and appropriate measures could be carried out in advance to minimize the probability of fetal loss. In addition, our results support the concept that pretreatment of GnRH-a for reducing the lesion size before FET effectively increased the probability of a live birth.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors without undue reservation.

ETHICS STATEMENT

This study was approved by the ethical standards of the Ethics Committee of The Sun Yat-Sen Memorial Hospital of China (SYSEC-KY-KS-2020-127). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

QZ supervised the entire study, including the procedures, conception, design, and completion of the study data. HL and CC revised the article. RY and XJ were responsible for the collection of data. YW contributed to data collection and analysis and drafted the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.902083/full#supplementary-material

Supplementary Table 1 | Univariate and multivariate analyses of factors predicting the live birth in patients with adenomyosis undergoing frozen–thawed embryo transfer. BMI, body mass index; AMH, anti-Mullerian hormone; HRT, *in vitro* fertilization/intracytoplasmic sperm injection; NC, nature cycle, FET, frozen–thawed embryo transfer. *$P < 0.05$ was considered statistically significant.

REFERENCES

1. Younes G, Tulandi T. Effects of Adenomyosis on *In Vitro* Fertilization Treatment Outcomes: A Meta-Analysis. *Fertil Steril* (2017) 108:483–90 e3. doi: 10.1016/j.fertnstert.2017.06.025
2. Ferenczy A. Pathophysiology of Adenomyosis. *Hum Reprod Update* (1998) 4:312–22. doi: 10.1093/humupd/4.4.312
3. Chapron C, Vannuccini S, Santulli P, Abrao MS, Carmona F, Fraser IS, et al. Diagnosing Adenomyosis: An Integrated Clinical and Imaging Approach. *Hum Reprod Update* (2020) 26:392–411. doi: 10.1093/humupd/dmz049
4. Yu O, Schulze-Rath R, Grafton J, Hansen K, Scholes D, Reed SD. Adenomyosis Incidence, Prevalence and Treatment: United States Population-Based Study 2006-2015. *Am J Obstetr Gynecol* (2020) 223:94 e1– e10. doi: 10.1016/j.ajog.2020.01.016
5. Harada T, Khine YM, Kaponis A, Nikellis T, Decavalas G, Taniguchi F. The Impact of Adenomyosis on Women's Fertility. *Obstetr Gynecol Survey* (2016) 71:557–68. doi: 10.1097/OGX.0000000000000346
6. Juang CM, Chou P, Yen MS, Twu NF, Horng HC, Hsu WL. Adenomyosis and Risk of Preterm Delivery. *BJOG Int J Obstetr Gynaecol* (2007) 114:165–9. doi: 10.1111/j.1471-0528.2006.01186.x
7. Horton J, Sterrenburg M, Lane S, Maheshwari A, Li TC, Cheong Y. Reproductive, Obstetric, and Perinatal Outcomes of Women With Adenomyosis and Endometriosis: A Systematic Review and Meta-Analysis. *Hum Reprod Update* (2019) 25:592–632. doi: 10.1093/humupd/dmz012
8. Hashimoto A, Iriyama T, Sayama S, Nakayama T, Komatsu A, Miyauchi A, et al. Adenomyosis and Adverse Perinatal Outcomes: Increased Risk of Second Trimester Miscarriage, Preeclampsia, and Placental Malposition. *J Maternal-Fetal Neonatal Med* (2018) 31:364–9. doi: 10.1080/14767058.2017.1285895
9. Li X, Pan N, Zhang W, Wang Y, Ge Y, Wei H, et al. Association Between Uterine Volume and Pregnancy Outcomes in Adenomyosis Patients Undergoing Frozen-Thawed Embryo Transfer. *Reprod Biomed Online* (2021) 42:384–9. doi: 10.1016/j.rbmo.2020.10.002
10. Mochimaru A, Aoki S, Oba MS, Kurasawa K, Takahashi T, Hirahara F. Adverse Pregnancy Outcomes Associated With Adenomyosis With Uterine Enlargement. *J Obstetr Gynaecol Res* (2015) 41:529–33. doi: 10.1111/jog.12604
11. Chen M, Luo L, Wang Q, Gao J, Chen Y, Zhang Y, et al. Impact of Gonadotropin-Releasing Hormone Agonist Pre-Treatment on the Cumulative Live Birth Rate in Infertile Women With Adenomyosis Treated With Ivf/Icsi: A Retrospective Cohort Study. *Front Endocrinol* (2020) 11:318. doi: 10.3389/fendo.2020.00318
12. Gallagher JS, Missmer SA, Hornstein MD, Laufer MR, Gordon CM, DiVasta AD. Long-Term Effects of Gonadotropin-Releasing Hormone Agonists and Add-Back in Adolescent Endometriosis. *J Pediatr Adolesc Gynecol* (2018) 31:376–81. doi: 10.1016/j.jpag.2018.03.004
13. Park CW, Choi MH, Yang KM, Song IO. Pregnancy Rate in Women With Adenomyosis Undergoing Fresh or Frozen Embryo Transfer Cycles Following Gonadotropin-Releasing Hormone Agonist Treatment. *Clin Exp Reprod Med* (2016) 43:169–73. doi: 10.5653/cerm.2016.43.3.169
14. O'Donnell RL, Warner P, Lee RJ, Walker J, Bath LE, Kelnar CJ, et al. Physiological Sex Steroid Replacement in Premature Ovarian Failure: Randomized Crossover Trial of Effect on Uterine Volume, Endometrial Thickness and Blood Flow, Compared With a Standard Regimen. *Hum Reprod (Oxf Engl)* (2012) 27:1130–8. doi: 10.1093/humrep/des004
15. Meredith SM, Sanchez-Ramos L, Kaunitz AM. Diagnostic Accuracy of Transvaginal Sonography for the Diagnosis of Adenomyosis: Systematic Review and Metaanalysis. *Am J Obstetr Gynecol* (2009) 201:107.e1–6. doi: 10.1016/j.ajog.2009.03.021
16. Tellum T, Nygaard S, Lieng M. Noninvasive Diagnosis of Adenomyosis: A Structured Review and Meta-analysis of Diagnostic Accuracy in Imaging. *J Minimally Invasive Gynecol* (2020) 27:408–18.e3. doi: 10.1016/j.jmig.2019.11.001
17. Grimbizis GF, Mikos T, Tarlatzis B. Uterus-Sparing Operative Treatment for Adenomyosis. *Fertil Steril* (2014) 101:472–87. doi: 10.1016/j.fertnstert.2013.10.025
18. Li Y, Li R, Ouyang N, Dai K, Yuan P, Zheng L, et al. Investigating the Impact of Local Inflammation on Granulosa Cells and Follicular Development in Women With Ovarian Endometriosis. *Fertil Steril* (2019) 112:882–91.e1. doi: 10.1016/j.fertnstert.2019.07.007
19. Wu Y, Liang X, Cai M, Gao L, Lan J, Yang X. Development and Validation of a Model for Individualized Prediction of Cervical Insufficiency Risks in Patients Undergoing IVF/ICSI Treatment. *Reprod Biol Endocrinol: RB&E* (2021) 19:6. doi: 10.1186/s12958-020-00693-x
20. Gao L, Li M, Wang Y, Zeng Z, Xie Y, Liu G, et al. Overweight and High Serum Total Cholesterol Were Risk Factors for the Outcome of IVF/ICSI Cycles in PCOS Patients and a PCOS-specific Predictive Model of Live Birth Rate was Established. *J Endocrinol Invest* (2020) 43:1221–8. doi: 10.1007/s40618-020-01209-5
21. Ouldamer L, Bendifallah S, Naoura I, Body G, Uzan C, Morice P, et al. Nomogram to Predict Live Birth Rate After Fertility-Sparing Surgery for Borderline Ovarian Tumours. *Hum Reprod (Oxf Engl)* (2016) 31:1732–7. doi: 10.1093/humrep/dew137
22. Kim YM, Kim SH, Kim JH, Sung JH, Choi SJ, Oh SY, et al. Uterine Wall Thickness at the Second Trimester can Predict Subsequent Preterm Delivery in Pregnancies With Adenomyosis. *Taiwanese J Obstetr Gynecol* (2019) 58:598–603. doi: 10.1016/j.tjog.2019.07.004
23. Zhang XP, Zhang YF, Shi R, Zhang YJ, Zhang XL, Hu XM, et al. Pregnancy Outcomes of Infertile Women With Ultrasound-Diagnosed Adenomyosis for *In Vitro* Fertilization and Frozen-Thawed Embryo Transfer. *Arch Gynecol Obstetr* (2021) 304(4):1089–96. doi: 10.1007/s00404-021-06011-z
24. Hawkins LK, Correia KF, Srouji SS, Hornstein MD, Missmer SA. Uterine Length and Fertility Outcomes: A Cohort Study in the IVF Population. *Hum Reprod (Oxford England)* (2013) 28:3000–6. doi: 10.1093/humrep/det344
25. Niu Z, Chen Q, Sun Y, Feng Y. Long-Term Pituitary Downregulation Before Frozen Embryo Transfer Could Improve Pregnancy Outcomes in Women With Adenomyosis. *Gynecol Endocrinol* (2013) 29:1026–30. doi: 10.3109/09513590.2013.824960
26. Kitawaki J. Adenomyosis: The Pathophysiology of an Oestrogen-Dependent Disease. *Best Pract Res Clin Obstetr Gynaecol* (2006) 20:493–502. doi: 10.1016/j.bpobgyn.2006.01.010
27. Donnez O, Donnez J. Gonadotropin-Releasing Hormone Antagonist (Linzagolix): A New Therapy for Uterine Adenomyosis. *Fertil Steril* (2020) 114:640–5. doi: 10.1016/j.fertnstert.2020.04.017
28. Vannuccini S, Luisi S, Tosti C, Sorbi F, Petraglia F. Role of Medical Therapy in the Management of Uterine Adenomyosis. *Fertil Steril* (2018) 109:398–405. doi: 10.1016/j.fertnstert.2018.01.013

29. Ishihara H, Kitawaki J, Kado N, Koshiba H, Fushiki S, Honjo H. Gonadotropin-Releasing Hormone Agonist and Danazol Normalize Aromatase Cytochrome P450 Expression in Eutopic Endometrium From Women With Endometriosis, Adenomyosis, or Leiomyomas. *Fertil Steril* (2003) 79(Suppl 1):735–42. doi: 10.1016/S0015-0282(02)04813-6
30. Loy SL, Cheung YB, Fortier MV, Ong CL, Tan HH, Nadarajah S, et al. Age-Related Nomograms for Antral Follicle Count and anti-Mullerian Hormone for Subfertile Chinese Women in Singapore. *PloS One* (2017) 12:e0189830. doi: 10.1371/journal.pone.0189830
31. Holden EC, Kashani BN, Morelli SS, Alderson D, Jindal SK, Ohman-Strickland PA, et al. Improved Outcomes After Blastocyst-Stage Frozen-Thawed Embryo Transfers Compared With Cleavage Stage: A Society for Assisted Reproductive Technologies Clinical Outcomes Reporting System Study. *Fertil Steril* (2018) 110:89–94.e2. doi: 10.1016/j.fertnstert.2018.03.033
32. Glujovsky D, Farquhar C, Quinteiro Retamar AM, Alvarez Sedo CR, Blake D. Cleavage Stage Versus Blastocyst Stage Embryo Transfer in Assisted Reproductive Technology. *Cochrane Database Systematic Rev* (2016) (4): CD002118. doi: 10.1002/14651858.CD002118.pub5
33. Practice Committee of American Society for Reproductive M. Multiple Gestation Associated With Infertility Therapy: An American Society for Reproductive Medicine Practice Committee Opinion. *Fertil Steril* (2012) 97:825–34. doi: 10.1016/j.fertnstert.2011.11.048

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ORIGINAL RESEARCH
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Alterations of Cytokine Profiles in Patients With Recurrent Implantation Failure

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Serum cytokine profile and T helper (Th)1/Th2 cell balance are related to the success of embryo implantation, although not yet firmly linked to recurrent implantation failure (RIF), a repeated failure to achieve clinical pregnancy following multiple high-quality embryo transfer. In this prospective study, comprehensive bioinfomatic analysis and logistic regression analysis were used to compare the serum cytokine profiles of 41 RIF patients with those of 29 subjects with first-cycle successful pregnancy in the mid-luteal phase and to assess the alterations of cytokine profiles in patients with clinical pregnancy at five weeks post-transplantation. We found several elevated pro-inflammatory cytokines, decreased anti-inflammatory cytokines, and increased Th1/Th2 cytokine ratios in RIF patients compared to control subjects. Specifically, the receiver operating characteristic (ROC) curve generated using multiple indicators provides a high predictive value for diagnosing RIF (area under the curve [AUC] = 0.94, 95% confidence interval [CI] 0.87-1.00, $P < 0.0001$), with a sensitivity of 96.55% and a specificity of 87.50%. Meanwhile, at five weeks post-transplantation, patients in both groups diagnosed with clinical pregnancy exhibited increased levels of several cytokines compared with pre-pregnancy levels, and a gradual shift in Th1/Th2 balance toward Th2. These findings suggest that inflammatory serum cytokines and the predominance of Th1 cells likely contribute to RIF and possibly reflect the immune environment at the maternal-fetal interface, suggesting their value as outcome indicators in assisted reproductive therapy.

Keywords: ***in vitro*** **fertilization, recurrent implantation failure, cytokines, Th1/Th2, logistic regression analysis**

INTRODUCTION

In China, the incidence of infertility has reached as high as 15% in recent years, becoming the third most common medical problem affecting human health after tumors and cardiovascular diseases (1, 2). To address fertility-related issues, assisted reproductive technology (ART), especially *in vitro* fertilization-embryo transfer (IVF-ET) and derivative technologies, has developed rapidly and has

become the most widely used approach for the treatment of infertility patients. Currently, the cumulative live birth rate following IVF-ET is approximately 40% worldwide, and rates higher than 50% have been reported in some regions and reproductive centers (3). However, a large proportion of infertility patients still repeatedly fail to achieve a clinical pregnancy after multiple *in vitro* fertilization (IVF) cycles with high-quality embryos transferred. This outcome is termed recurrent implantation failure (RIF) and accounts for 5% to 10% of ART failures (4). Although the diagnostic criteria for RIF have not yet been unified, the currently accepted definition of RIF based on clinical research is "the failure to achieve a clinical pregnancy after transfer of at least four high-quality embryos in a minimum of three fresh or frozen cycles in a woman under the age of 40 years" (5). RIF thus poses one of the most urgent challenges facing the field of assisted reproduction, and it often results in severe physical and mental suffering for patients and their families. However, the pathogenesis of RIF remains unclear, and limited information is available regarding its indicators.

The etiology of RIF is complex and multifactorial, with contributions from maternal, embryonic, and external factors. Among them, maternal contributors include psychological factors, abnormalities in the reproductive system, aberrant endometrial development and function, and immunologic dysfunction at the maternal-fetal interface (6, 7). In particular, recent research efforts in reproductive immunology have focused on immune factors at the maternal-fetal interface, especially the role of peripheral blood immune cells in maternal-fetal communication and maternal remodeling. Despite these advances, the specific mechanisms governing this remodeling and its role in RIF remain unclear. Currently, the majority of studies on this topic have concluded that a variety of cytokines participate in the formation of maternal-fetal immune tolerance (8–10). Since the embryo acts as an allogeneic antigen, the cytokine profile in peripheral blood can significantly affect the stability of the immune system at the maternal-fetal interface during the implantation period, whether by direct or indirect regulatory roles. However, the expression patterns of the cytokine profile and its possible roles in the pathogenesis of RIF are poorly understood.

T lymphocytes, which account for approximately 60% of peripheral blood lymphocytes, originate from bone marrow lymphocyte stem cells, which differentiate and mature into immune cells capable of exerting immune activity in the thymus. Studies have found that the immune response mediated by T lymphocytes is an essential step in the process of embryo implantation (11, 12). In particular, CD4+ T and CD8+ T lymphocytes, defined by their characteristic expression of surface markers, are the predominant cell types found at the maternal-fetal interface. Among these cell types, helper T (Th) cells represent an independent subset of CD4+ T cells that have come to be recognized for their vital role in immune regulation at the maternal-fetal interface (13, 14). Th cells can be further categorized into Th1, Th2, Th17 and other subsets based on their expression of nuclear transcription factors, cytokines, and other functional characteristics (15). Notably, the dynamic balance of Th1/Th2 cells is a determining factor in immune system homeostasis at the maternal-fetal interface and consequently the pregnancy outcomes after IVF-ET, such as RIF (13, 14). Thus, this ratio could potentially serve as an effective indicator of maternal immune status during the implantation window period (10). However, relatively few studies have investigated the balance of Th1/Th2 cells in the peripheral blood of RIF patients, and as a result, the effects of these cells on IVF-ET remain controversial.

Therefore, to characterize the cytokine profiles of RIF patients, in this prospective study, we applied AimPlex multiplex immunoassays to detect serum levels of interleukin-2 (IL-2), IL-4, IL-6, IL-10, IL-17A, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), TNF-β, granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) in the middle luteal phase of 41 patients with RIF for comparison with those of 29 control subjects, with concurrent evaluation of differences in cytokines related to the ratio of Th1/Th2. We continued to follow up with the enrolled participants until five weeks after transplantation and compared the serum cytokine profiles of 10 patients with RIF and 14 controls who successfully achieved clinical pregnancy to identify pregnancy-associated changes in serum cytokine profiles. Thus, this study also aimed to identify cytokine-based biomarkers for RIF to facilitate the improvement of its clinical management through the development of more accurate diagnostics and targeted therapeutics.

MATERIALS AND METHODS

Participants

In this prospective study, a total of 41 patients with RIF and 29 control subjects with first-cycle successful pregnancy were enrolled from patients undergoing IVF/intracytoplasmic sperm injection (ICSI) cycles at the Center for Reproductive Medicine of Qilu Hospital of Shandong University from March, 2020 to December, 2020. The inclusion criteria for RIF patients were as follows: failure to achieve a clinical pregnancy after transfer of at least four high-quality embryos or underwent a minimum of three fresh or frozen cycles, age < 40 years old, basal follicle-stimulating hormone (FSH) < 10 mIU/ml, and antral follicle counts (AFCs) in both ovaries ≥ 5. The criteria for inclusion of controls were the same as those for RIF cases except that the included patients had no history of adverse maternal-fetal outcomes and achieved clinical pregnancy after the first IVF/ICSI-ET cycle. The exclusion criteria for all patients were chronic autoimmune disease, abnormal anatomy of the genital tract, chromosomal anomalies in either the female or male prospective parents, endometrial thickness < 7 mm on the day of embryo transfer, incomplete clinical data and other factors that may affect the inflammatory process.

IVF Procedure

All subjects included in this study adopted a standardized ovarian stimulation protocol, such as GnRH-agonist long-term protocol, ultra-long protocol, short-term protocol, micro-

stimulation protocol, and GnRH-antagonist protocol. Gonadotropin (Gn) was used individually based on evaluations of age, body mass index (BMI), and ovarian function. Follicular development was monitored by transvaginal ultrasound. Human chorionic gonadotropin (hCG) was injected to induce ovulation when there were at least two dominant follicles with an average diameter of ≥18 mm. Oocyte retrieval was performed at 34-36 hours (h) after hCG administration. Fertilization by IVF or ICSI was selected depending on sperm quality at 4-6 h after oocyte retrieval. Cleavage-stage embryos were evaluated into four grades by morphological criteria based on the regularity of blastomeres and the degree of fragmentation (16). In our center, embryos (grades I-III) are considered as transferable embryos. All patients enrolled in this study underwent frozen-thawed embryo transfer (FET). All transferable embryos were further cultured until the blastocyst stage with sequential media. All blastocysts were scored by Gardner morphological criteria (17) and cryopreserved by vitrification on day 5 or day 6 for later transfer. In our center, the embryos of grades over 4BC according to Gardner morphological criteria were defined as high-quality embryos. At the second menstrual cycle after oocyte retrieval, endometrial preparation was performed either with a natural cycle regimen or a programmed cycle regimen. After the frozen embryos were recovered, up to two high-quality embryos were transferred in each cycle during the implantation period. Biochemical pregnancy was determined by serum β-hCG >10 IU/L on days 10 to 14 after embryo transfer. Clinical pregnancy was defined by transvaginal ultrasound detection of the intrauterine gestational sac at five weeks after embryo transfer.

Sample Collection

In the mid-luteal phase prior to FET treatment, 5 ml fasting blood samples were collected from the anterior elbow vein of all subjects (including 41 RIF cases and 29 control cases). At five weeks after embryo transfer, blood samples were again drawn from patients diagnosed with clinical pregnancy. Among all subjects, as of the end of this study, 18 cases in the RIF group obtained clinical pregnancy, 10 of whom provided blood samples and complete follow-up data after pregnancy; blood samples were also obtained from 14 control cases after pregnancy. Blood samples were then centrifuged for 10 minutes at, 3000 X rpm. The serum was separated, and the supernatant was stored at −80° C for later analysis.

Cytokine Profiling

Individual serum samples were subjected to cytokine profile quantification by AimPlex multiplex immunoassay kits (Beijing Quantobio, China). The kit protocol for quantitative analysis included 10 total cytokines, including IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ, TNF-α, TNF-β, G-CSF, and GM-CSF. Briefly, 45 μL of capture bead working suspension was mixed with 45 μL of sample and shaken at 700 rpm for 60 min in the dark, followed by three washes with 100 μL 1 × wash buffer. Next, 25 μL of biotin-conjugated antibodies was added to each well, and the wells were sealed and shaken for 30 min in the dark. Then, after three washes with 1× wash buffer, 25 μL of streptavidin-PE was added to each well and shaken for 20 min in the dark. Finally, 100-200 μL of 1× reading buffer was added to each well, the fluorescence signals in each sample were acquired using a Navios flow cytometer (Beckman Coulter, USA), and the cytokine levels were analyzed with FCAP Array 3.0 software.

Statistical Analysis

Statistical analysis was performed using SPSS 26.0 for Windows (IBM, Armonk, NY, USA) and GraphPad Prism 8.3 (GraphPad Software, San Diego, CA, USA). Principal component analysis (PCA), heatmap and cluster analysis, and forest map were conducted using the website www.bioinformatics.com.cn. Normality of the data distributions was assessed by Kolmogorov–Smirnov tests. Data are presented as the means ± standard deviations (for normally distributed data) or medians with interquartile range (for nonnormally distributed data). Continuous variables with normal distributions were analyzed by independent *t test* and data with a non-normal distribution were compared by the Mann–Whitney U test. For paired comparisons, Wilcoxon paired test was used to compare the significant differences. Categorical variables are presented as counts (percentages) and were compared using either the chi-square or Fisher's exact test. Selected variables were entered into multivariable logistic regression analysis to assess predicting factors for RIF and compute odds ratios (ORs) with 95% confidence intervals (CIs) for each endpoint. Specificity and sensitivity were assessed using receiver operating characteristic (ROC) analysis. In all analyses, $P < 0.05$ was considered statistically significant.

RESULTS

Baseline Characteristics and Related Clinical Indicators

In total, 41 patients with RIF and 29 control patients who met the inclusion criteria were enrolled. Among the 41 patients in the RIF group, the average number of transfer failures was 3.12 ± 1.25, and the cumulative number of embryos transferred was 3.90 ± 1.87. As shown in **Table 1**, there were no significant differences between the RIF and control groups in the type of infertility, BMI, anti-Mullerian hormone (AMH), basal FSH, basal luteinizing hormone (LH), basal estradiol (E2), basal progesterone (P), basal prolactin (PRL), basal testosterone (T), free triiodothyronine (FT3), free thyroxin (FT4), thyroid-stimulating hormone (TSH), thyroglobulin antibody (TG-Ab), or thyroid peroxidase antibody (TPO-Ab) ($P > 0.05$); however, age was significantly higher in the RIF group, while endometrial thickness on the day of embryo transfer was lower in RIF patients than in the control group, which is in agreement with clinical assessments.

Controlled Ovarian Hyperstimulation and Laboratory Indicators

The numbers of participants in each group given different controlled ovarian hyperstimulation (COH) and fertilization procedures are compared in **Table 2**. There were no significant

TABLE 1 | Baseline characteristics and related clinical indicators of the participants.

Characteristics	RIF group (*N* = 41)	Control group (*N* = 29)	*P* values
No. of transfer failure	3.12 ± 1.25		
No. of embryo transferred	3.90 ± 1.87		
Age (years)	32.54 ± 3.81	29.07 ± 4.05	0.001*
Type of infertility (n, %)			0.225
Primary	18 (43.90%)	17 (58.62%)	
Secondary	23 (56.10%)	12 (41.38%)	
BMI (kg/m^2)	22.28 ± 2.41	22.12 ± 2.89	0.802
AMH (ng/ml)	3.36 (2.31-4.90)	3.73 (2.65-5.25)	0.183
Basal FSH (IU/L)	6.65 (5.61-7.74)	6.32 (5.41-7.89)	0.642
Basal E2 (pg/ml)	35.83 (26.65-47.16)	39.72 (32.83-51.62)	0.081
Basal P (ng/ml)	0.28 (0.17-0.37)	0.21 (0.10-0.38)	0.387
Basal PRL (ng/ml)	17.24 (14.17-23.24)	18.35 (15.21-24.04)	0.486
Basal LH (IU/L)	4.68 (3.62-6.66)	6.34 (4.60-7.20)	0.060
Basal T (ng/ml)	0.23 (0.20-0.30)	0.28 (0.21-0.43)	0.085
FT3 (pmol/L)	4.80 ± 0.62	4.67 ± 0.47	0.345
FT4 (pmol/L)	16.78 ± 2.95	17.11 ± 1.61	0.580
TSH (μIU/mL)	2.49 ± 1.05	2.59 ± 1.23	0.711
TG-Ab (IU/ml)	10.00 (10.00-10.00)	10.00 (10.00-13.41)	0.329
TPO-Ab (IU/ml)	7.99 (5.00-11.37)	9.71 (7.42-13.53)	0.056
Endometrial thickness (mm)	9.32 ± 1.67	10.32 ± 1.90	0.024*

Data are presented as the mean ± standard deviation, median (interquartile range) or n (%). P < 0.05 was considered statistically significant and indicated by an asterisk. BMI, body mass index; AMH, anti-Mullerian hormone; FSH, follicle-stimulating hormone; E2, estradiol; P, progestogen; PRL, prolactin; LH, luteinizing hormone; T, testosterone; FT3, free triiodothyronine; FT4, free thyroxin; TSH, thyroid-stimulating hormone; TG-Ab, thyroglobulin antibody; TPO-Ab, thyroid peroxidase antibody.

differences between the RIF and control groups in fertilization method, Gn duration, number of oocytes retrieved, number of metaphase II (MII) oocytes, number of normal fertilized oocytes, number of transferable embryos or number of high-quality embryos ($P > 0.05$).

TABLE 2 | Controlled ovarian hyperstimulation and laboratory indicators.

Characteristics	RIF group (*N* = 41)	Control group (*N* = 29)	*P* values
Fertilization (n, %)			0.588
IVF	32 (78.05)	21 (72.41)	
ICSI	9 (21.95)	8 (27.59)	
Gn duration (days)	9.00 (7.00-10.00)	8.00 (7.00-9.00)	0.127
No. of oocytes retrieved	12.23 ± 6.21	13.86 ± 7.26	0.323
No. of MII oocytes	11.49 ± 5.96	11.10 ± 5.26	0.784
No. of normal fertilized oocytes	8.36 ± 4.44	9.41 ± 4.21	0.326
No. of transferable embryos	5.00 ± 2.59	5.69 ± 3.08	0.320
No. of high-quality embryo	3.74 ± 2.45	4.72 ± 3.00	0.143

Data are presented as the mean ± standard deviation, median (interquartile range) or n (%). P < 0.05 was considered statistically significant. IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; Gn, gonadotropin; MII, metaphase II.

Serum Cytokine Profiles

In the mid-luteal phase prior to FET treatment, a panel of 10 total cytokines were measured for comparison of their levels between 41 women with RIF and 29 controls. The results indicated that several serum markers accumulated to different levels between the two groups and that a variety of cytokines contributed to first principal component (PC1), which may serve as the main reason for the variation (**Figure 1**). In particular, the levels of IL-6 were significantly higher in the RIF group, while IL-10 and G-CSF were both lower than those in the control group ($P < 0.05$). In addition, IL-2, IL-4, IL-17A, IFN-γ, and GM-CSF levels were all higherin the peripheral blood samples of the RIF group, whereas TNF-α and TNF-β showed lower, but nonsignificant ($P > 0.05$), compared with control group levels (**Table 3**, **Figure 2**).

To better understand how changes in cytokine levels before and after pregnancy may be related to the occurrence of RIF, we also collected peripheral blood samples from 10 patients in the RIF group and 14 patients in the control group at five weeks after embryo transfer who successfully achieved clinical pregnancy. The results showed that these patients in both the RIF and control groups displayed an overall increasing trend in cytokine levels from pre- to during-pregnancy. Among them, IL-10 and TNF-α levels in peripheral blood were significantly higher after clinical pregnancy than before pregnancy in the RIF group ($P < 0.05$) (**Figure S1A**). Meanwhile, in the control group, IL-6 concentrations were higher in peripheral blood samples after clinical pregnancy than that before pregnancy ($P < 0.05$) (**Figure S1B**).

Serum Th1/Th2 Ratios

This study also investigated differences in the Th1/Th2 ratio between the two groups by determining the ratio of Th1-/Th2-associated cytokines in the peripheral blood of patients. In particular, IL-2, IFN-γ, TNF-α and TNF-β are pro-inflammatory cytokines secreted by Th1 cells, while IL-4 and IL-10 are anti-inflammatory cytokines specifically secreted by Th2 cells. The results showed that the IL-2/IL-10 and IFN-γ/IL-10 ratios in the RIF group were higher than those in the control group ($P < 0.05$), which suggested a relationship between these proportions and the occurrence of RIF. However, we found no significant differences in other ratios, although the RIF group showed a trend of higher Th1/Th2 cytokine ratios than the control group (**Table 4**).

The cytokines were also quantified to calculate the Th1/Th2 ratios in pre- and during-pregnancy samples. The results showed that these patients in both the RIF and control groups displayed an overall decreasing trend in the Th1/Th2 ratio from pre- to during-pregnancy. Among them, the IL-2/IL-10 ratio was significantly lower after clinical pregnancy than before pregnancy in the RIF group ($P < 0.05$) (**Figure S1C**). Meanwhile, in the control group, the TNF-α/IL-4 ratio was significantly lower after clinical pregnancy than before pregnancy ($P < 0.05$) (**Figure S1D**).

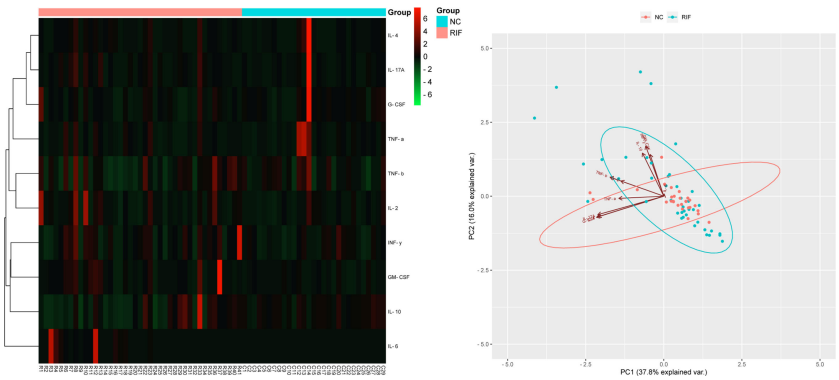

FIGURE 1 | Cytokine profile screening with heatmap and PCA. Heatmap and PCA of 10 cytokine profiles in serum. Each row in the heatmap represents an individual differentially expressed cytokine, and the column displays the sample number. Cytokines with lower levels are displayed in green, while cytokines with higher levels are displayed in red. The points in the PCA diagram represent the samples, red for the control group and blue for the RIF group. The arrows represent the contribution and correlation of the corresponding original variables to the principal component.

TABLE 3 | Serum cytokine profiles in the mid-luteal phase.

Cytokines (pg/mL)	RIF group (*N* = 41)	Control group (*N* = 29)	*P* values
IL-2	2.71 (1.69-4.55)	2.35 (2.04-3.14)	0.319
IL-4	1.01 (0.44-1.70)	0.86 (0.43-1.29)	0.604
IL-6	3.61 (1.97-16.48)	2.45 (1.88-3.33)	0.042*
IL-10	2.18 (1.02-4.17)	3.37 (2.70-3.61)	0.034*
IL-17A	0.94 (0.32-1.72)	0.78 (0.44-1.12)	0.445
IFN-γ	2.40 (1.30-3.76)	1.99 (1.40-2.98)	0.407
TNF-α	2.06 (1.00-3.11)	2.19 (1.26-3.41)	0.428
TNF-β	1.85 (0.72-2.90)	1.87 (1.38-2.93)	0.698
G-CSF	5.36 (3.24-7.69)	7.83 (5.83-9.06)	0.033*
GM-CSF	1.41 (1.09-2.01)	1.18 (1.00-1.48)	0.111

Data are presented as the median (interquartile range). P < 0.05 was considered statistically significant and indicated by an asterisk. IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor.

Logistic Regression Analysis and ROC Curve Evaluation

Logistic regression analysis was used to further explore the relationships between the serum cytokine profiles during the mid-luteal phase and the occurrence of RIF. We found that several cytokines and Th1/Th2 ratios were associated with RIF (**Figure 3A**). After adjusting for the influence of maternal age and endometrial thickness on the day of embryo transfer, IL-2, IL-2/IL-10 and IFN-γ/IL-10 were still found to have a high risk of developing RIF (**Figure 3B**). To generate a predictive model for RIF, we conducted multivariable logistic regression analysis of the associated indicators. We found that age, IL-10, G-CSF and IL-2/IL-10, in particular, had independent predictive values for the occurrence of RIF ($P < 0.05$) (**Table 5**). Then, the prediction

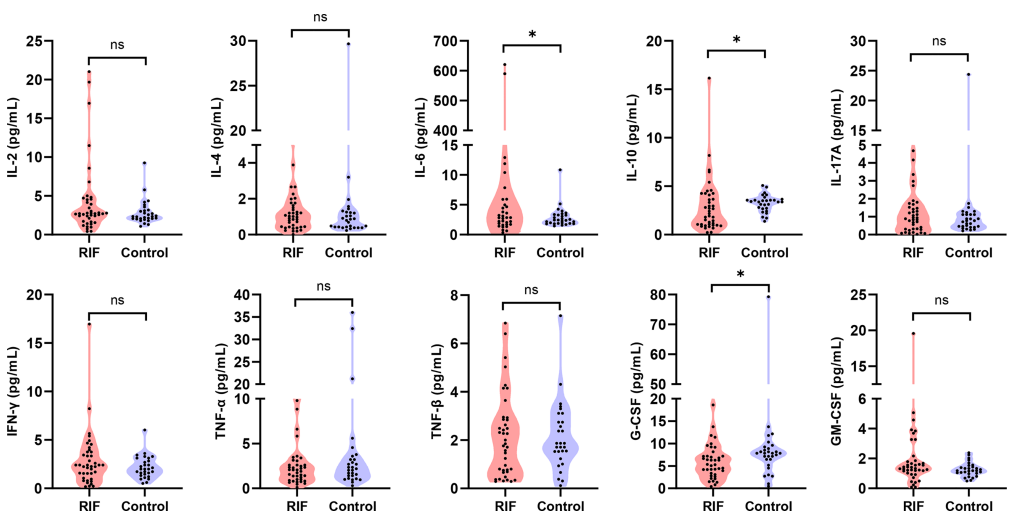

FIGURE 2 | Serum cytokine profiles in the mid-luteal phase between RIF patients and control women. Every different cytokine is shown as density (violin shap), and data are shown as the median (interquartile range) for the two groups. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; NS, not significant.

TABLE 4 | Serum Th1/Th2 ratios in the mid-luteal phase.

Characteristics	RIF group (N = 41)	Control group (N = 29)	*P* values
IL-2/IL-4	3.03 (1.89-5.71)	2.95 (2.02-5.54)	0.986
IL-2/IL-10	1.35 (0.69-2.57)	0.74 (0.60-1.07)	0.007*
IFN-γ/IL-4	2.00 (1.15-4.41)	2.19 (1.47-4.50)	0.825
IFN-γ/IL-10	0.95 (0.56-1.92)	0.58 (0.44-0.96)	0.012*
TNF-α/IL-4	2.03 (1.35-3.84)	2.94 (2.04-4.92)	0.075
TNF-α/IL-10	1.14 (0.53-1.47)	0.70 (0.51-0.97)	0.180
TNF-β/IL-4	1.78 (0.90-4.23)	1.74 (1.14-3.97)	0.761
TNF-β/IL-10	0.76 (0.48-1.18)	0.61 (0.36-0.88)	0.147

Data are presented as the median (interquartile range). P < 0.05 was considered statistically significant and indicated by an asterisk. IL, interleukin; IFN, interferon; TNF, tumor necrosis factor.

probability based on the binary logistic regression model was determined by ROC curve analysis. Notably, the predictive model constructed using age, endometrial thickness, IL-6, IL-10, G-CSF, IL-2/IL-10 and IFN-γ/IL-10 had a good diagnostic performance (AUC = 0.94, 95% CI 0.87-1.00, $P < 0.0001$), with a sensitivity of 96.55% and a specificity of 87.50% (**Figure 4**).

DISCUSSION

Successful embryo implantation requires synchronized and coordinated interactions between the embryo and the maternal endometrium (18, 19). Previous studies have proposed that the immune cells in the endometrium and peripheral blood and their various associated cytokines constitute a unique cytokine immunoregulatory network, especially during the implantation window (20) (which occurs in the mid-luteal phase). During this period, the endometrial morphology and transcriptional profile of cytokines change. This event leads to immune cell aggregation and subsequent secretion of a diverse array of cytokines to regulate embryo positioning and implantation (21, 22). Thus, the formation of immune tolerance at the maternal-fetal interface is essential for achieving normal pregnancy, while an imbalance in the immune microenvironment will lead to failure of embryo implantation or miscarriage (23, 24).

However, the precise regulatory mechanism controlling cytokine profiles and the immune response at the maternal-fetal interface in RIF patients remains unknown. In the current study, we characterized the cytokine profiles of RIF patients using IVF/ICSI-ET cases that were successful in the first cycle as a control group. Our results show that various pro-inflammatory cytokines are elevated, whereas anti-inflammatory cytokines are decreased, and Th1/Th2 ratios are increased during the mid-luteal phase in RIF patients compared with the control group, suggesting that alterations in the serum cytokine profile during the middle luteal phase in particular may play a role in the occurrence of RIF. Specifically, ROC curves generated using several indicators established here provide high predictive value for diagnosing RIF. Moreover, several cytokines were increased in both groups after pregnancy, but the Th1/Th2 balance gradually shifted toward Th2 cells, which may contribute to embryo implantation and the maintenance of normal pregnancy.

Studies have reported that the participation of cytokines in the immune response at the maternal-fetal interface can significantly impact pregnancy outcomes after IVF-ET (25). Among these cytokines, IL-2, IFN-γ, TNF-α, and TNF-β are pro-inflammatory cytokines mainly secreted by Th1 cells. In particular, IL-2 and IFN-γ were found to synergistically activate the cytotoxic effects of T lymphocytes and NK cells, which can destroy trophoblasts and kill embryos (26). Interestingly, TNF-α could induce positive regulatory effects on trophoblast invasion at low concentrations (27). In contrast, high TNF-α concentrations can inhibit endometrial decidualization and promote apoptosis in trophoblasts, leading to embryo implantation failure or pregnancy loss (28, 29). Distinct from Th1 cytokines, IL-4, IL-6 and IL-10 cytokines are primarily

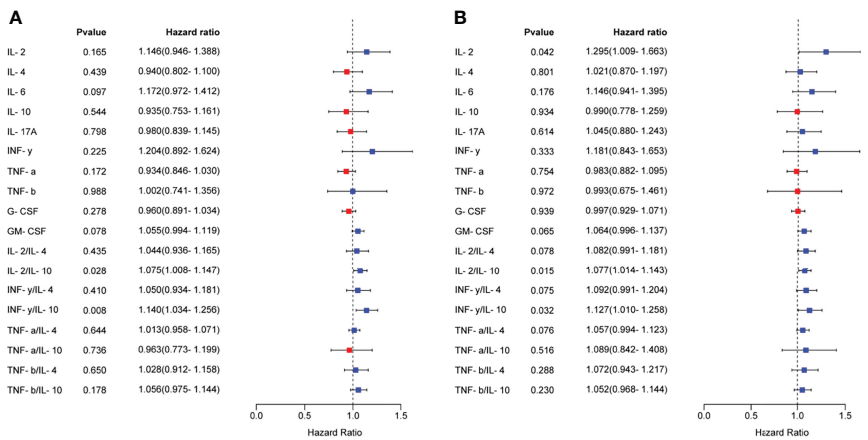

FIGURE 3 | Relationships between serum cytokine profiles and the occurrence of RIF. **(A)**, The relationship between the serum cytokine profiles in the mid-luteal phase and the occurrence of RIF was analyzed by univariate logistic regression analysis and visualized as a forest map. **(B)**, After correcting for maternal age and endometrial thickness on the day of embryo transfer, the relationship between the serum cytokine profiles and the occurrence of RIF was analyzed by logistic regression analysis. OR > 1 indicates a risk factor, and OR < 1 indicates a protective factor.

TABLE 5 | Logistic regression analysis of RIF risk prediction.

Characteristics	β	S.E.	Wald	*P* values	OR	95% CI
Age (years)	0.26	0.12	4.67	0.031*	1.30	(1.02,1.64)
Endometrial thickness (mm)	-0.24	0.22	1.14	0.286	0.79	(0.51,1.22)
IL-6 (pg/mL)	0.10	0.14	0.49	0.486	1.10	(0.84,1.44)
IL-10 (pg/mL)	0.67	0.24	7.69	0.006*	1.96	(1.22,3.14)
G-CSF (pg/mL)	-0.37	0.14	6.76	0.009*	0.69	(0.52,0.91)
IL-2/IL-10	2.25	0.91	6.06	0.014*	9.49	(1.58,56.97)
IFN-γ/IL-10	1.09	0.83	1.73	0.188	2.97	(0.59,15.05)
Constant	-9.19	4.52	4.13	0.042	0.00	

P < 0.05 was considered statistically significant and indicated by an asterisk. β, regression coefficient; S.E., standard error; OR, odds ratio; CI, confidence interval; IL, interleukin; G-CSF, granulocyte colony-stimulating factor; IFN, interferon.

secreted by Th2 cells. Among them, IL-4 and IL-10 can cooperate with Treg cells in the regulation of immune balance to ensure a conducive environment for embryo implantation and maintenance of pregnancy (30). Although IL-6 is secreted by Th2 cells, it belongs to the pro-inflammatory cytokine group and performs several diverse biological functions (31). Previous work has found that IL-6, at physiological concentrations, can promote trophoblast proliferation by activating matrix metalloproteinase (MMP), which facilitates embryo implantation. However, abnormally high expression of IL-6 is often associated with recurrent spontaneous abortion (RSA) and RIF (32–34). IL-17A is a pro-inflammatory cytokine secreted explicitly by Th17 cells, which can induce a robust inflammatory response and contributes to a variety of autoimmune diseases (35–37). G-CSF and GM-CSF are members of the colony-stimulating factor cytokine family, mainly secreted by activated monocytes and macrophages. These cytokines can positively regulate endometrial receptivity by promoting endometrial angiogenesis, which is crucial for embryo implantation (38, 39).

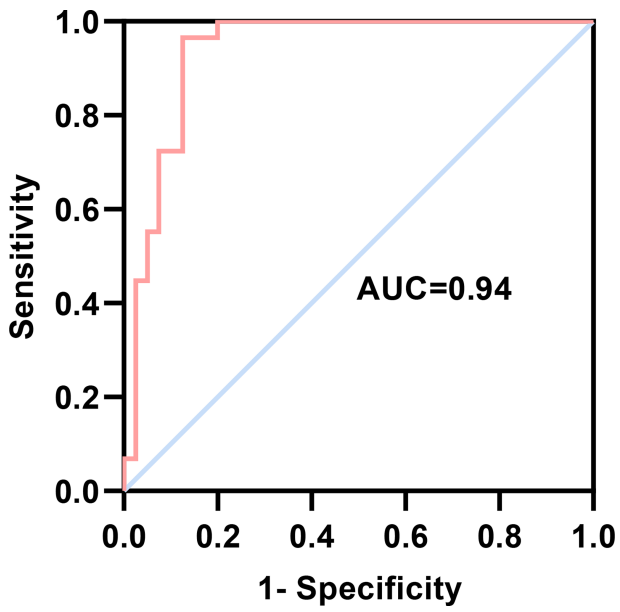

FIGURE 4 | ROC curve evaluation of the logistic regression model. The AUC of the predictive model was 0.94 (95% CI 0.87-1.00, *P* < 0.0001), with a sensitivity of 96.55% and a specificity of 87.50%. AUC, area under the curve; CI, confidence interval.

The results of our current study showed that IL-6 levels were significantly higher in the mid-luteal phase in RIF patients than in the control group, while IL-10 and G-CSF levels were significantly lower. Based on these findings, we tentatively conclude that aberrantly high serum IL-6 or insufficient levels of IL-10 and G-CSF during the peri-implantation period can adversely affect embryo implantation, suggesting that dysregulation of these cytokines is an immunological factor potentially leading to RIF. In addition, serum levels of IL-10 and TNF-α were significantly higher after pregnancy in the RIF group than before pregnancy, while during-pregnancy IL-6 levels were significantly higher than pre-pregnancy levels in the control group. It should be noted that we observed an overall increasing trend in the cytokine levels of both groups after pregnancy, which may suggest that a certain degree of inflammatory stimulation is required for successful embryo implantation.

Considerable research efforts have been dedicated to determining the role of the Th1/Th2 balance in maternal-fetal immune regulation. Previous reports have implicated Th1/Th2 imbalance in a variety of immune diseases, such as RSA (25), allergic bronchial asthma (40), and diabetes (41). However, the limited sample size, experimental design flaws, and inadequate follow-up of clinical trials have resulted in gaps in our understanding and persistent controversy surrounding the relationship between cytokine profiles and Th1/Th2 balance in the peripheral blood of RIF patients. Previous studies have concluded that the balance of Th1/Th2 shifts to Th2 cells during embryo implantation and pregnancy, resulting in a predominant Th2-type immune response, while the proportion of Th1 cells accordingly decreases (42). However, recent studies on this topic described a dynamic equilibrium in Th1/Th2 proportions. For example, a specific proportion of Th1 cells is necessary during embryo implantation to activate a slight inflammatory reaction at the maternal-fetal interface that is conducive to trophoblast invasion, tissue remodeling, and angiogenesis (25). The gradual shift toward predominant Th2 cell populations after embryo implantation likely inhibits the Th1-mediated immune response, thereby inducing immune tolerance that is beneficial to the maintenance of pregnancy (43).

Kwak-Kim (44) and Liang (45) found that Th1/Th2 cytokine ratios, such as IFN-γ/IL-4, IFN-γ/IL-10, TNF-α/IL-4, and TNF-α/IL-10, were significantly higher in the peripheral blood of RIF patients than in normal control subjects, suggesting that the immune response mediated by Th1 cells in peripheral blood may lead to RIF. Similarly, our results showed that the IL-2/IL-10 and IFN-γ/IL-10 cytokine ratios were markedly higher in the mid-luteal phase in the RIF group than in the control group and that after pregnancy, the IL-2/IL-10 ratio was lower in RIF patients, while TNF-α/IL-4 was lower in the control group than in pre-pregnancy values. We also noted an overall decreasing trend in Th1/Th2 cytokine ratios after pregnancy among patients in both groups. These collective findings strongly suggest that an obvious Th1/Th2 immune imbalance in the peripheral blood of IVF/ICSI-ET patients, particularly during the mid-luteal phase, could

lead to embryo implantation failure, while an increasing Th1/Th2 bias toward Th2 cells may be beneficial to implantation and normal pregnancy.

Although there are many studies on cytokines or the Th1/Th2 balance at the maternal-fetal interface, most studies have only described the different expression signatures. In our study, the predictive model constructed using multiple indicators showed good diagnostic performance for RIF. However, due to the particularity of RIF, this study is limited by a relatively small cohort size, and further analysis with a larger sample size is needed to validate our conclusions.

In conclusion, this study reveals alterations in the serum cytokine profiles of RIF patients, suggesting that dysregulation of these cytokines during the middle luteal phase in particular is an essential factor potentially leading to RIF. Moreover, we identify a cytokine-based predictive model for RIF that is informative in the prediction, diagnosis and treatment of this common disease that accounts for ART failures.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of Qilu Hospital of Shandong University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YL and CD conceived and designed the project. LG and AG performed the experiments and analyzed the data. FY, LL, JY and XD collected the clinical serum samples and information. LG and AG wrote the manuscript. JY, XD, CD, and YL critically revised the manuscript. All authors have been involved in interpreting the data and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.949123/full#supplementary-material

REFERENCES

1. Gurunath S, Pandian Z, Anderson RA, Bhattacharya S. Defining Infertility–a Systematic Review of Prevalence Studies. *Hum Reprod Update* (2011) 17:575–88. doi: 10.1093/humupd/dmr015
2. Montoya JM, Bernal A, Borrero C. Diagnostics in Assisted Human Reproduction. *Reprod Biomed Online* (2002) 5:198–210. doi: 10.1016/s1472-6483(10)61624-0
3. Polanski LT, Baumgarten MN, Quenby S, Brosens J, Campbell BK, Raine-Fenning NJ. What Exactly Do We Mean by 'Recurrent Implantation Failure'? A systematic review and opinion. *Reprod Biomed Online* (2014) 28:409–23. doi: 10.1016/j.rbmo.2013.12.006
4. Mak JSM, Chung CHS, Chung JPW, Kong GWS, Saravelos SH, Cheung LP, et al. The Effect of Endometrial Scratch on Natural-Cycle Cryopreserved Embryo Transfer Outcomes: A Randomized Controlled Study. *Reprod Biomed Online* (2017) 35:28–36. doi: 10.1016/j.rbmo.2017.04.004
5. Coughlan C, Ledger W, Wang Q, Liu F, Demirol A, Gurgan T, et al. Recurrent Implantation Failure: Definition and Management. *Reprod Biomed Online* (2014) 28:14–38. doi: 10.1016/j.rbmo.2013.08.011
6. Bashiri A, Halper KI, Orvieto R. Recurrent Implantation Failure-Update Overview on Etiology, Diagnosis, Treatment and Future Directions. *Reprod Biol Endocrinol* (2018) 16:121. doi: 10.1186/s12958-018-0414-2
7. Cakiroglu Y, Tiras B. Determining Diagnostic Criteria and Cause of Recurrent Implantation Failure. *Curr Opin Obstet Gynecol* (2020) 32:198–204. doi: 10.1097/GCO.0000000000000620
8. Chaichian S, Shoaee S, Saremi A, Pedar S, Firouzi F. Factors Influencing Success Rate of Leukocyte Immunization and Anti-Paternal Antibodies in Spontaneous Recurrent Miscarriage. *Am J Reprod Immunol* (2007) 57:169–76. doi: 10.1111/j.1600-0897.2006.00451.x
9. Tisoncik JR, Korth MJ, Simmons CP, Farrar J, Martin TR, Katze MG. Into the Eye of the Cytokine Storm. *Microbiol Mol Biol Rev* (2012) 76:16–32. doi: 10.1128/MMBR.05015-11
10. Lu HQ, Hu R. The Role of Immunity in the Pathogenesis and Development of Pre-Eclampsia. *Scand J Immunol* (2019) 90:e12756. doi: 10.1111/sji.12756
11. Gong Q, Zhu Y, Pang N, Ai H, Gong X, La X, et al. Increased Levels of CCR7 (lo)PD-1(hi) CXCR5(+) CD4(+) T Cells, and Associated Factors Bcl-6, CXCR5, IL-21 and IL-6 Contribute to Repeated Implantation Failure. *Exp Ther Med* (2017) 14:5931–41. doi: 10.3892/etm.2017.5334
12. Ghaebi M, Abdolmohammadi-Vahid S, Ahmadi M, Eghbal-Fard S, Dolati S, Nouri M, et al. T cell Subsets in Peripheral Blood of Women with Recurrent Implantation Failure. *J Reprod Immunol* (2019) 131:21–9. doi: 10.1016/j.jri.2018.11.002
13. Yang KM, Ntrivalas E, Cho HJ, Kim NY, Beaman K, Gilman-Sachs A, et al. Women With Multiple Implantation Failures and Recurrent Pregnancy Losses Have Increased Peripheral Blood T Cell Activation. *Am J Reprod Immunol* (2010) 63:370–8. doi: 10.1111/j.1600-0897.2010.00811.x
14. Zeng W, Liu Z, Liu X, Zhang S, Khanniche A, Zheng Y, et al. Distinct Transcriptional and Alternative Splicing Signatures of Decidual CD4(+) T Cells in Early Human Pregnancy. *Front Immunol* (2017) 8:682. doi: 10.3389/fimmu.2017.00682
15. Yuan J, Li J, Huang SY, Sun X. Characterization of the Subsets of Human NKT-Like Cells and the Expression of Th1/Th2 Cytokines in Patients With Unexplained Recurrent Spontaneous Abortion. *J Reprod Immunol* (2015) 110:81–8. doi: 10.1016/j.jri.2015.05.001

16. Puissant F, Van Rysselberge M, Barlow P, Deweze J, Leroy F. Embryo Scoring as a Prognostic Tool in IVF Treatment. *Hum Reprod* (1987) 2:705–8. doi: 10.1093/oxfordjournals.humrep.a136618
17. Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst Score Affects Implantation and Pregnancy Outcome: Towards a Single Blastocyst Transfer. *Fertil Steril* (2000) 73:1155–8. doi: 10.1016/s0015-0282(00)00518-5
18. Cha J, Sun X, Dey SK. Mechanisms of Implantation: Strategies for Successful Pregnancy. *Nat Med* (2012) 18:1754–67. doi: 10.1038/nm.3012
19. Tan Q, Shi S, Liang J, Zhang X, Cao D, Wang Z. MicroRNAs in Small Extracellular Vesicles Indicate Successful Embryo Implantation during Early Pregnancy. *Cells* (2020) 9. doi: 10.3390/cells9030645
20. Wang Y, Ma CH, Qiao J. [Differential Expression of microRNA in Eutopic Endometrium Tissue During Implantation Window for Patients With Endometriosis Related Infertility]. *Zhonghua Fu Chan Ke Za Zhi* (2016) 51:436–41. doi: 10.3760/cma.j.issn.0529-567X.2016.06.007
21. Gnainsky Y, Granot I, Aldo P, Barash A, Or Y, Mor G, et al. Biopsy-Induced Inflammatory Conditions Improve Endometrial Receptivity: The Mechanism of Action. *Reproduction* (2015) 149:75–85. doi: 10.1530/REP-14-0395
22. Ledee N, Petitbarat M, Chevrier L, Vitoux D, Vezmar K, Rahmati M, et al. The Uterine Immune Profile May Help Women With Repeated Unexplained Embryo Implantation Failure After In Vitro Fertilization. *Am J Reprod Immunol* (2016) 75:388–401. doi: 10.1111/aji.12483
23. Yang F, Zheng Q, Jin L. Dynamic Function and Composition Changes of Immune Cells During Normal and Pathological Pregnancy at the Maternal-Fetal Interface. *Front Immunol* (2019) 10:2317. doi: 10.3389/fimmu.2019.02317
24. Sheikhansari G, Pourmoghadam Z, Danaii S, Mehdizadeh A, Yousefi M. Etiology and Management of Recurrent Implantation Failure: A Focus on Intra-Uterine PBMC-Therapy for RIF. *J Reprod Immunol* (2020) 139:103121. doi: 10.1016/j.jri.2020.103121
25. Wang W, Sung N, Gilman-Sachs A, Kwak-Kim J. T Helper (Th) Cell Profiles in Pregnancy and Recurrent Pregnancy Losses: Th1/Th2/Th9/Th17/Th22/Tfh Cells. *Front Immunol* (2020) 11:2025. doi: 10.3389/fimmu.2020.02025
26. Raghupathy R, Kalinka J. Cytokine Imbalance in Pregnancy Complications and Its Modulation. *Front Biosci* (2008) 13:985–94. doi: 10.2741/2737
27. Todt JC, Yang Y, Lei J, Lauria MR, Sorokin Y, Cotton DB, et al. Effects of Tumor Necrosis Factor-Alpha on Human Trophoblast Cell Adhesion and Motility. *Am J Reprod Immunol* (1996) 36:65–71. doi: 10.1111/j.1600-0897.1996.tb00141.x
28. Haider S, Knofler M. Human tumour necrosis factor: physiological and pathological roles in placenta and endometrium. *Placenta* (2009) 30:111–23. doi: 10.1016/j.placenta.2008.10.012
29. Seki H, Matuoka K, Inooku H, Takeda S. TNF-Alpha From Monocyte of Patients With Pre-Eclampsia-Induced Apoptosis in Human Trophoblast Cell Line. *J Obstet Gynaecol Res* (2007) 33:408–16. doi: 10.1111/j.1447-0756.2007.00551.x
30. Zhang Y, Wang Y, Li MQ, Duan J, Fan DX, Jin LP, et al. IL-25 Promotes Th2 Bias by Upregulating IL-4 and IL-10 Expression of Decidual gammadeltaT Cells in Early Pregnancy. *Exp Ther Med* (2018) 15:1855–62. doi: 10.3892/etm.2017.5638
31. Prins JR, Gomez-Lopez N, Robertson SA. Interleukin-6 in Pregnancy and Gestational Disorders. *J Reprod Immunol* (2012) 95:1–14. doi: 10.1016/j.jri.2012.05.004
32. Hercor M, Anciaux M, Denanglaire S, Debuisson D, Leo O, Andris F. Antigen-Presenting Cell-Derived IL-6 Restricts the Expression of GATA3 and IL-4 by Follicular Helper T Cells. *J Leukoc Biol* (2017) 101:5–14. doi: 10.1189/jlb.1HI1115-511R
33. Sheikhansari G, Soltani-Zangbar MS, Pourmoghadam Z, Kamrani A, Azizi R, Aghebati-Maleki L, et al. Oxidative Stress, Inflammatory Settings, and microRNA Regulation in the Recurrent Implantation Failure Patients With Metabolic Syndrome. *Am J Reprod Immunol* (2019) 82:e13170. doi: 10.1111/aji.13170
34. Amjadi F, Zandieh Z, Mehdizadeh M, Aghajanpour S, Raoufi E, Aghamajidi A, et al. The Uterine Immunological Changes May Be Responsible for Repeated Implantation Failure. *J Reprod Immunol* (2020) 138:103080. doi: 10.1016/j.jri.2020.103080
35. Monin L, Gaffen SL. Interleukin 17 Family Cytokines: Signaling Mechanisms, Biological Activities, and Therapeutic Implications. *Cold Spring Harb Perspect Biol* (2018) 10. doi: 10.1101/cshperspect.a028522
36. Beringer A, Miossec P. Systemic Effects of IL-17 in Inflammatory Arthritis. *Nat Rev Rheumatol* (2019) 15:491–501. doi: 10.1038/s41584-019-0243-5
37. McGeachy MJ, Cua DJ, Gaffen SL. The IL-17 Family of Cytokines in Health and Disease. *Immunity* (2019) 50:892–906. doi: 10.1016/j.immuni.2019.03.021
38. Kamath MS, Chittawar PB, Kirubakaran R, Mascarenhas M. Use of Granulocyte-Colony Stimulating Factor in Assisted Reproductive Technology: A Systematic Review and Meta-Analysis. *Eur J Obstet Gynecol Reprod Biol* (2017) 214:16–24. doi: 10.1016/j.ejogrb.2017.04.022
39. Wurfel W. Treatment With Granulocyte Colony-Stimulating Factor in Patients With Repetitive Implantation Failures And/or Recurrent Spontaneous Abortions. *J Reprod Immunol* (2015) 108:123–35. doi: 10.1016/j.jri.2015.01.010
40. Asayama K, Kobayashi T, D'Alessandro-Gabazza CN, Toda M, Yasuma T, Fujimoto H, et al. Protein S Protects Against Allergic Bronchial Asthma by Modulating Th1/Th2 Balance. *Allergy* (2020) 75:2267–78. doi: 10.1111/all.14261
41. Arif S, Gomez-Tourino I, Kamra Y, Pujol-Autonell I, Hanton E, Tree T, et al. GAD-Alum Immunotherapy in Type 1 Diabetes Expands Bifunctional Th1/Th2 Autoreactive CD4 T Cells. *Diabetologia* (2020) 63:1186–98. doi: 10.1007/s00125-020-05130-7
42. Lin H, Mosmann TR, Guilbert L, Tuntipopipat S, Wegmann TG. Synthesis of T Helper 2-Type Cytokines at the Maternal-Fetal Interface. *J Immunol* (1993) 151:4562–73.
43. Saito S, Nakashima A, Shima T, Ito M. Th1/Th2/Th17 and Regulatory T-Cell Paradigm in Pregnancy. *Am J Reprod Immunol* (2010) 63:601–10. doi: 10.1111/j.1600-0897.2010.00852.x
44. Kwak-Kim JY, Chung-Bang HS, Ng SC, Ntrivalas EI, Mangubat CP, Beaman KD, et al. Increased T Helper 1 Cytokine Responses by Circulating T Cells Are Present in Women With Recurrent Pregnancy Losses and in Infertile Women With Multiple Implantation Failures After IVF. *Hum Reprod* (2003) 18:767–73. doi: 10.1093/humrep/deg156
45. Liang PY, Diao LH, Huang CY, Lian RC, Chen X, Li GG, et al. The Pro-Inflammatory and Anti-Inflammatory Cytokine Profile in Peripheral Blood of Women With Recurrent Implantation Failure. *Reprod Biomed Online* (2015) 31:823–6. doi: 10.1016/j.rbmo.2015.08.009

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Decreased intracellular IL-33 impairs endometrial receptivity in women with adenomyosis

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Adenomyosis is a common benign uterine lesion that is associated with female infertility, reduced clinical pregnancy rate and high miscarriage risk. While it has been known that the impaired endometrial receptivity is implicated in infertility in patients with adenomyosis, the underlying mechanism remains unclear. In the present study, we showed that intracellular protein level of IL-33 was downregulated in the endometrium of patients with adenomyosis, and IL-33 expression status was shown to be positively correlated with that of HOXA10, an endometrial receptivity marker. The subsequent analysis indicated IL-33 overexpression led to the increase of HOXA10 expression and enhancement of embryo implantation *in vitro*, which was accompanied with induction of STAT3 phosphorylation. Meanwhile, cryptotanshinone, a potent STAT3 inhibitor, was found to significantly suppress the increase of HOXA10 expression and embryo implantation caused by IL-33 overexpression *in vitro*, revealing the critical role of STAT3 activity. Consistently, the positive relationship between IL33 and HOXA10 expression in the endometrium was verified in the analysis of adenomyosis mouse model.

KEYWORDS

adenomyosis, endometrial receptivity, IL-33, STAT3, HOXA10

Introduction

Adenomyosis is a common benign uterine disorder that manifests as invasion of the endometrium in the muscular layer. According to epidemiological studies, many young women with pain, infertility, Abnormal Uterine bleeding (AUB) or even no symptoms are increasingly diagnosed with adenomyosis using imaging techniques such as transvaginal ultrasound or magnetic resonance (1). Adenomyosis is associated with a decreased clinical pregnancy rate, increased early miscarriage rate and infertility (2, 3). In our previous studies, we showed that impaired endometrial receptivity in patients with adenomyosis may be an important cause of infertility (4, 5). However, the specific mechanism of decreased endometrial receptivity is still unclear.

The research of endometrial receptivity was carried out about 80 years ago, followed with the technique of embryo transplantation (6, 7). Because not all high-quality embryo transfers achieve successful implantation, Navot et al. proposed a "window of implantation" to further describe endometrial receptivity, which is considered to be a rather short and important endometrial phase (7). Many studies have focused on the measurement of endometrial receptivity, including histologic criteria, electronic observation, and biochemical markers. The evaluation methods used tend to be highly microscopic and specific, and many biochemical markers have been discovered (8). The key transcription factor homeobox A10 (HOXA10) is considered to be a biomarker for the window of embryo implantation (9). HOXA10 has a conserved homeobox sequence that specifically recognizes the TTAT or TAAT sequence in the promoter region of the downstream gene. It can also activates or inhibits the binding of β3 integrin (ITGB3) (9), metalloproteinase MMP26 (10), and FK506 Protein 4 (FKBP4) (11, 12) as well as the expression of target genes such as the homeobox structural gene EMX2 (13), which has been demonstrated to regulate endometrial receptivity and embryo adhesion. The expression of HOXA10 in the endometrium changes with the menstrual cycle, and the highest expression level appears during the mid-secretory phase, which is the "implantation window period". Thus, the downregulation of HOXA10 in the endometrium is considered the main reason for impaired endometrial receptivity in some women, such as those with endometriosis (10), hydrosalpinx (11), and repeated implantation failure (12). Previous studies also found that HOXA10 expression was downregulated in the endometria of patients with adenomyosis, which led to impaired endometrial receptivity (13). However, the mechanism of abnormal HOXA10 expression is unclear.

Interleukin-33 (IL33), a novel member of the IL1 family, was discovered to be a nuclear factor in the high endothelial vein (NF-HEV) in 2003 (14). In 2005, IL33 was determined to be as a specific extracellular ligand for ST2, which belongs to the orphan IL-1 receptor family. Since its discovery, IL33 has been found to be expressed widely in tissues and is mainly located in the nucleus. Although some studies have suggested that IL33 acts as a transcriptional repressor, the function of intracellular IL33 needs to be further clarified (15). When facing trauma or infection, IL33 is released outside of cells as an endogenous danger signal or an alarm element. IL33 has been reported to play a major role in many diseases, including asthma, anaphylactic shock, cardiovascular disease, and rheumatoid arthritis. However, there has been little research on the role of IL33 in reproduction. A previous study found that the IL33/ST2 pathway may participate in the pathogenesis of endometriosis and that IL33 serum cytokine levels in women with adenomyosis were decreased compared to those in controls (16, 17). However, the function of IL33 in the endometrium is unknown.

Previous evidence underlies the important role of IL33/ST2 as a critical cytokine in pregnancy (18). Nevertheless, the functional roles of intracellular IL33 in the endometrium during the progression of embryo implantation have not been elucidated. Combined with previous evidence, we hypothesized that the intracellular IL33 in the eutopic endometrium of the infertile women with adenomyosis may cause failure of embryo implantation. The purpose of this study is to confirm the function of intracellular IL33 on embryo implantation and further clarify the mechanism of infertility in adenomyosis.

Materials and methods

Patient sample collection

20 infertile patients with adenomyosis and 20 fertile controls participated in this study. The endometrium samples were obtained during the 20-22 day of the menstrual cycle, which is the secretory phase. This study was approved by the Ethics Committee of Shanghai First Maternity and Infant Health Hospital. In this study, patients who experienced at least one normal pregnancy or delivery were included in the fertile control group. The diagnostic criteria for adenomyosis were described in our previous study (13). All participants had regular menstrual cycles of approximately 28-31 days, and none of the patients received hormone therapy within three months prior to sampling. Patients with PCOS, endometrial polyps, hydrosalpinx or uterine fibroids were excluded. Patient information is summarized in Table 1.

Cell culture, steroid hormones and inhibitors

Ishikawa cells and BeWo cells were cultured in DMEM supplemented with 10% (v/v) foetal bovine serum (Gibco BRL/Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (HyClone Laboratories, South Logan, UT, USA) at 37 °C in an atmosphere of 5% CO_2/95% air. Ishikawa cells were transfected with overexpression plasmids or siRNA. The administration of 17β-oestradiol (E, 10^{-8} M) and progesterone (P, 10^{-6} M) for different times was conducted in Ishikawa cells as described in the figure legends. STAT3-dependent signalling pathway inhibitor (Cryptotanshinone, 4.6 μM) (SigmaAldrich, St Louis, MO, USA) was added to the culture medium before the plasmids were transfected into Ishikawa cells.

RNA isolation and quantitative real-time PCR

Total RNA from Ishikawa cells, human endometrial tissues or mouse uteri was extracted with TRIzol reagent (Life Technologies, NY, USA). According to the manufacturer's

TABLE 1 Patients information.

	Nor (n=20)	Ade (n=20)	P
Age (years)	29.3±0.6	28.8±0.7	ns
Body mass index (kg/m^2)	21.4±0.5	21.8±0.4	ns
Smoker	6 (30.0)	4 (20.0)	ns
Drinker	7 (35.0)	8 (40.0)	ns
Menstrual cycle (days)	29.6±0.8	29.9±0.3	ns
Infertility duration (years)	3.3±2.3	3.3±1.4	ns
Basal sex hormone			
FSH (IU/L)	6.78±1.39	6.77±1.57	ns
LH (IU/L)	5.29±3.57	5.21±1.55	ns
E_2 (pg/ml)	40.92±9.77	44.51±9.64	ns
P (ng/ml)	0.53±0.15	0.58±0.21	ns
T (ng/ml)	0.41±0.19	0.42±0.19	ns

The data are presented as the mean ± SD unless otherwise indicated. Nor, Normal; Ade, Adenomyosis; ns, no significant. $P < 0.05$ was considered significant.

instructions, purified total RNA (1 μg) was reverse-transcribed into cDNA using the PrimeScript RT reagent kit (Bio–Rad, Hercules, CA, USA). Quantitative real-time PCR was performed using a MyiQ Single-Colour Real-Time PCR Detection System (BIO-RAD Laboratories, Hercules, CA, USA). The IL33, HOXA10 and ST2 mRNA expression levels were normalized to GAPDH with the $2^{-\triangle\triangle CT}$ method. The primer sequences for the indicated genes are shown in Table 2.

Western blot assay

Proteins are extracted from Ishikawa cells, human endometrial tissues or mouse uteri. Through the Bradford assay (Bio–Rad Laboratories), the protein content was measured, and equal amounts (20 μg) of protein were separated by SDS–PAGE. Immunoblotting was performed with primary antibodies against IL33 (Proteintech, USA, 1:1000), HOXA10 (Cell Signaling Technology, USA, 1:1000), ITGB3 (Cell Signaling Technology, USA, 1:1000), STAT3 (Cell Signaling Technology, USA, 1:1000), p-STAT3 (Cell Signaling Technology, USA, 1:1000) or GAPDH (Bioworld Technology, USA, 1:10000), followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody. An enhanced chemiluminescence kit (Amersham Biosciences Corp., USA) was used to detect the bands. Finally, the densitometric analysis of each band was performed with Quantity-one (Bio–Rad Laboratories) software.

Attachment assay of BeWo spheroids to Ishikawa cells

Ishikawa cells were digested and inoculated into a 24-well culture plate. When the cells reach 80%, the indicated plasmids were transfected into the cells: Mix the plasmids to be transfected with Lip2000 reagent (Invitrogen, China) in Opti-MEM® Reduced Serum Medium at a ratio of 1:2. 8h after transfection, the serum-free medium was replaced with normal medium containing 10% serum. After 48 h, 50 BeWo cell spheroids (at the same diameter of 150-200 μm) were transferred to a confluent monolayer of Ishikawa cells in each group. After incubation for 2 h at 37 °C, each well of the culture plate was washed with PBS 3 times, and the unattached spheroids were washed away. Spheroids were fixed in

TABLE 2 Primer Sequences for qPCR.

Genes		Sequence of primers 5' to 3'
IL33	Forward	GTGACGGTGTTGATGGTAAGAT
	Reverse	AGCTCCACAGAGTGTTCCTTG
ST2	Forward	ATGGGGTTTTGGATCTTAGCAAT
	Reverse	CACGGTGTAACTAGGTTTTCCTT
HOXA10	Forward	CTCGCCCATAGACCTGTGG
	Reverse	GTTCTGCGCGAAAGAGCAC
GAPDH	Forward	TGTGGGCATCAATGGATTTGG
	Reverse	ACACCATGTATTCCGGGTCAAT

4% paraformaldehyde at room temperature for 30 min, and the adhesion state of BeWo spheroids was observed and counted under a stereoscopic dissecting microscope, which was used to calculate the adhesion efficiency.

Transfection and luciferase assays

Ishikawa cells were digested and seeded into cell culture plates. Upon reaching 80% confluence, the cells were transfected with the indicated plasmids. After culturing for 48 h, 200 ng of the pGL3-ITGB3-Luc reporter gene plasmid was transiently transfected into Ishikawa cells using Lipofectamine 2000 transfection reagent. After incubating for 24 h, the cells were lysed with reporter gene lysis solution according to the method described in the Dual-Luciferase Reporter Assay Kit, and the supernatant was collected to measure the luciferase activity according to the Promega (Madison, WI, USA) Dual Luciferase Reporter Assay System.

Adenomyosis mouse model

Male and female ICR mice were mated in a 1:1 ratio. On the 2nd-5th day after birth, the newborn mice were separated from the mother mice every morning. After starvation for 5-6 hours, the adenomyosis group was drip-fed 5 μl/g (weight) of tamoxifen mixed in a peanut oil/lecithin/condensed milk mixture, and the control group was drip-fed only the peanut oil/lecithin/condensed milk mixture. After drip feeding, the mice were placed back in the same cage with the mother mice. All mice were exposed to light for 12 h every day and were fed by female mice from 1 to 21 days of age. From 22 days onwards, they were separated from the female mice. The mice were sacrificed at six months old. HE staining and immunohistochemistry were used to verify whether the model was successfully constructed.

Immunohistochemistry

Human endometrial tissue or mouse uterine sections (5 μm) were deparaffinized in xylene and ethanol. Incubation with 3% H_2O_2 removed endogenous peroxidase. Slides were blocked with 1.5% normal rabbit blocking serum at room temperature for 45 min. Then, the sections were incubated at 4 °C with primary anti-IL33 antibody (1:200; Affinity Biosciences, China) or anti-HOXA10 antibody (1:50; Abcam, USA). After washing with PBS, the sections were incubated with sufficient peroxidase label edpolymer secondary antibody for 30 min at room temperature. Finally, the sections were incubated with enough DAB for 2-5 min at room temperature until a brown colour developed. The slides were washed with PBS, and the staining was observed with a microscope. Nonspecific rabbit IgG and goat IgG was used as a negative control, and nonspecific staining was not detected in the controls.

HE staining

Mouse uterus section slides (5 μm) were deparaffinized in xylene and ethanol. The tissue samples were soaked and washed in PBS solution three times for 5 min each. Then, the slides were stained with haematoxylin solution. After staining, the excess haematoxylin staining solution was washed with distilled water. 1% hydrochloric acid ethanol was used for differentiation. After differentiation, the tissue sections were rinsed with double distilled water, and a weakly basic blue-stimulating solution was added to the tissue sections to stain the nuclei blue. After washing with double distilled water, tissue sample sections were stained with eosin for 3 minutes. After staining, the tissue sections were dehydrated in a gradient manner. The dehydrated tissue sample sections were soaked in xylene twice for 4 min each time, and then the tissue samples were dried and sealed with neutral gum. Finally, the sections were observed and imaged under a microscope.

Statistical analysis

Each result is shown as the mean ± SD of three independent experiments. Two-tailed Student's t-test was used to compare the mean expression values of two treatment groups; One-way ANOVA was performed for comparisons among more than two groups. Pearson's correlation analysis was used to assess the relationship between IL33 and HOXA10. P-values < 0.05 were considered statistically significant.

Results

IL33 is aberrantly expressed in the endometria of women with adenomyosis

A previous study found that serum levels of the cytokine IL33 were decreased in patients with adenomyosis (17), so we examined the levels of IL33 in the endometria of patients with adenomyosis. Patient information is shown in Table 1. We extracted total protein from endometrial tissues and detected IL33 protein expression *via* Western blotting. The results showed that the protein level of IL33 was significantly downregulated in the endometrial tissue of patients with adenomyosis (n=20) compared with the fertile controls (n=20) (Figures 1A, B). The protein level of HOXA10 was also significantly decreased (Figures 1A, C). In addition, the results indicated that the protein levels of IL33 and HOXA10 were positively related (Figure 1D). We further detected the expression and localization of IL33 in the endometrium by immunohistochemical staining (Figure 1E). We found that in the endometria of patients with adenomyosis, both IL33 and HOXA10 expression were lower. The results showed that IL33 was mainly localized in the nucleus of epithelial cells, similar to

the fertile controls. In addition, we quantified the expression of IL33 and HOXA10 in the endometrium using Image-Pro Plus System 6.0 image analysis software. The integrated optical densities (IOD) of IL33 for the normal controls (n=5) was 118,261 ± 33,491, while that for the patients with adenomyosis (n=5) was 54,415 ± 29,457 (p=0.0126); the IOD of HOXA10 for the normal controls (17,5024 ± 9,531) was significantly higher than that of the patients with adenomyosis (77,558 ± 56,198), p=0.0051 (Figure 1F). In addition, the mean expression of IL33 in normal controls (0.1783 ± 0.025) was higher than that in adenomyosis (0.1415 ± 0.0071), p=0.00182; the mean densities of HOXA10 in the fertile and adenomyosis individuals were 0.3003 ± 0.055 and 0.1878 ± 0.036, p=0.0050 (Figure 1G).

IL33 could induce HOXA10 expression

Previous studies demonstrated that the downregulation of HOXA10 in the endometrium was related to impaired endometrial receptivity in patients with adenomyosis (13). However, the molecular mechanism of HOXA10 downregulation in adenomyosis patients is unclear. In the present study, we utilized liposomes to transfect the high-expression plasmid Flag-IL33 into IK cells and verified the overexpression of IL33 by qRT–PCR and Western blot assays (Figures 2A, B). The results showed that overexpression of IL33 could significantly promote the mRNA and protein expression of HOXA10 (Figures 2B, C). When IL33 was silenced (Figure 2D), the HOXA10 mRNA and protein levels were reduced (Figures 2E, F).

IL33 participates in embryo implantation

Figures 3A–C shows that endogenous IL33 mRNA and protein levels were increased in Ishikawa cells treated with oestrogen and progesterone in a time-dependent manner. To determine whether IL33 participates in regulating embryo implantation, we found that IL33 expression was dramatically increased 4.5 days post-coitus (dpc) in pregnant mice (Figures 3D, E) ($p < 0.01$). In the luciferase reporter assay, we found that ITGB3 activity, which is transcriptionally regulated by HOXA10, was enhanced in IL33-overexpressing IK cells (Figure 3F). In addition, the BeWo spheroid attachment assay confirmed that IL33 expression promoted the adhesion of BeWo cell spheroids to Ishikawa cells (Figure 3G). Moreover, enhanced IL33 expression induced ITGB3 protein levels (Figure 3H).

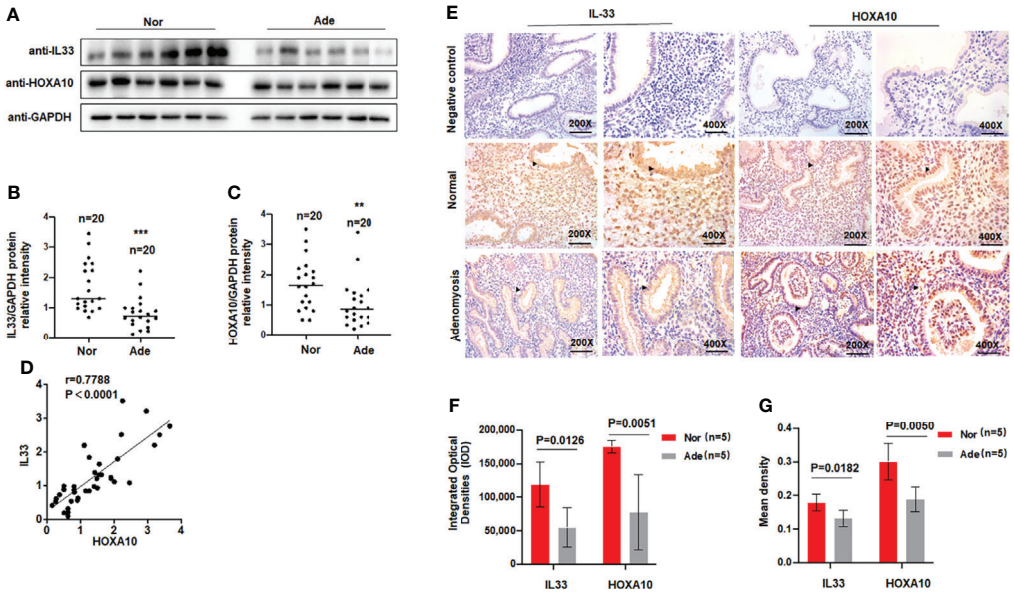

FIGURE 1
IL33 is aberrantly expressed in the endometria of women with adenomyosis. **(A)** IL33 and HOXA10 protein expression in normal (n = 20) and patients with adenomyosis (n = 20) was analyzed by Western blot. **(B)** The intensities of IL33 signals were quantified from the 20 samples and normalized. to GAPDH. ***p < 0.001. **(C)** The intensities of HOXA10 signals were quantified from the 20 samples and. normalized to GAPDH. **p < 0.01. **(D)** Correlation between IL33 and HOXA10 protein expression levels (r = 0.7788, p < 0.0001). **(E)** Timed mid-secretory endometrial biopsies from healthy control and infertile women with adenomyosis were analyzed using immunohistochemistry (IHC). Rabbit or Goat IgG was used as the negative control. The arrows show the decreased IL33 and HOXA10 conjugates in the endometrial epithelium cell. **(F)** The integrated optical densities (IOD) of the expression of IL33 and HOXA10 in the endometrium using Image-Pro Plus System 6.0 image analysis software. **(G)** The mean density of the expression of IL33 and HOXA10 in the endometrium. using Image-Pro Plus System 6.0 image analysis software.

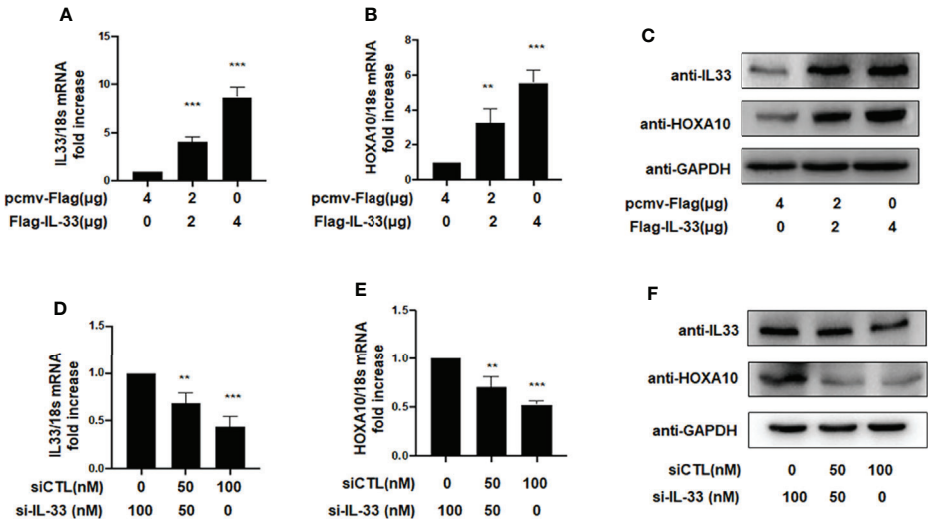

FIGURE 2
IL33 could induce HOXA10 expression. **(A)** Ishikawa cells were transfected with overexpression plasmid Flag-IL33 or pcmv-Flag negative control. IL33 mRNA levels were measured by qRT-PCR. ***p < 0.001. **(B)** Ishikawa cells were transfected with overexpression plasmid Flag-IL33 or pcmv-Flag negative control. HOXA10 mRNA levels were measured by qRT-PCR. **p < 0.01, ***p < 0.001. **(C)** Ishikawa cells were transfected with overexpression plasmid Flag-IL33 or pcmv-Flag negative control. IL33 and HOXA10 protein levels were measured by Western blot. **(D)** Ishikawa cells were transfected with si-IL33 or negative control. IL33 mRNA levels. were measured by qRT-PCR. **p < 0.01, ***p < 0.001. **(E)** Ishikawa cells were transfected with si-IL33 or negative control. HOXA10 mRNA levels were measured by qRT-PCR. **p < 0.01, ***p < 0.001. **(F)** Ishikawa cells were transfected with si-IL33 or negative control. IL33 and HOXA10 protein levels were measured by Western blot.

ST2 silence dose not affect the IL33-promoted HOXA10 expression and embryo implantation *in vitro*

When IL33 acts as a cytokine, it binds to the ligand ST2 on the surface of the cell membrane, thereby causing a series of signal changes (14). Meanwhile, some studies have also shown that intracellular IL33 may play a role in regulating the expression of transcription factors (14). To further study the underlying mechanism, we silenced ST2 when IL33 was overexpressed and found that the promotive effects of IL33 on HOXA10 was not affected (Figures 4A, B). In the luciferase reporter assay, we found that the downregulation of ST2 expression does not affect the IL33-enhanced transcriptional activity of HOXA10 and the IL33-improved BeWo spheroids adhesion (Figures 4C, D). These results suggested that intracellular IL33 may play a role independently of ST2.

IL-33 enhances embryo implantation *via* phosphorylation of STAT3

We further explored the molecular mechanism by which IL33 promoted embryo adhesion, and we found that phosphorylation of STAT3 was enhanced after IL33 was overexpressed in IK cells (Figure 5A). When IL33 expression was silenced, STAT3 phosphorylation was subsequently attenuated (Figure 5B). After treatment with cryptotanshinone (4.6 μM), an inhibitor of p-STAT3, the promotion of HOXA10 by IL33 was weakened (Figure 5C). Furthermore, when the phosphorylation of STAT3 was blocked by cryptotanshinone, the activity of ITGB3 and the adhesion of BeWo cell spheroids were also decreased (Figures 5D, E). These results demonstrated that IL33 increased endometrial receptivity *via* phosphorylation of STAT3.

IL33 expression was decreased in a mouse model of adenomyosis

We utilized HE staining to ensure that we successfully constructed a mouse model of adenomyosis. HE staining showed that endometrial glands appeared in the myometrium of the mouse uterus (Figure 6A). Figure 6B showed that IL33 mainly localized in the epithelial cells of the mouse uterus and decreased in the adenomyosis mouse model. We further found that IL33 protein expression, consistent with the HOXA10 level, was dramatically downregulated compared with that in the control group (Figures 6C–E). In addition, IL33 and HOXA10 protein expression was positively related (Figure 6F).

Discussion

Adenomyosis has a negative impact on fertility, accounting for 25% of infertile women. Among women who underwent IVF

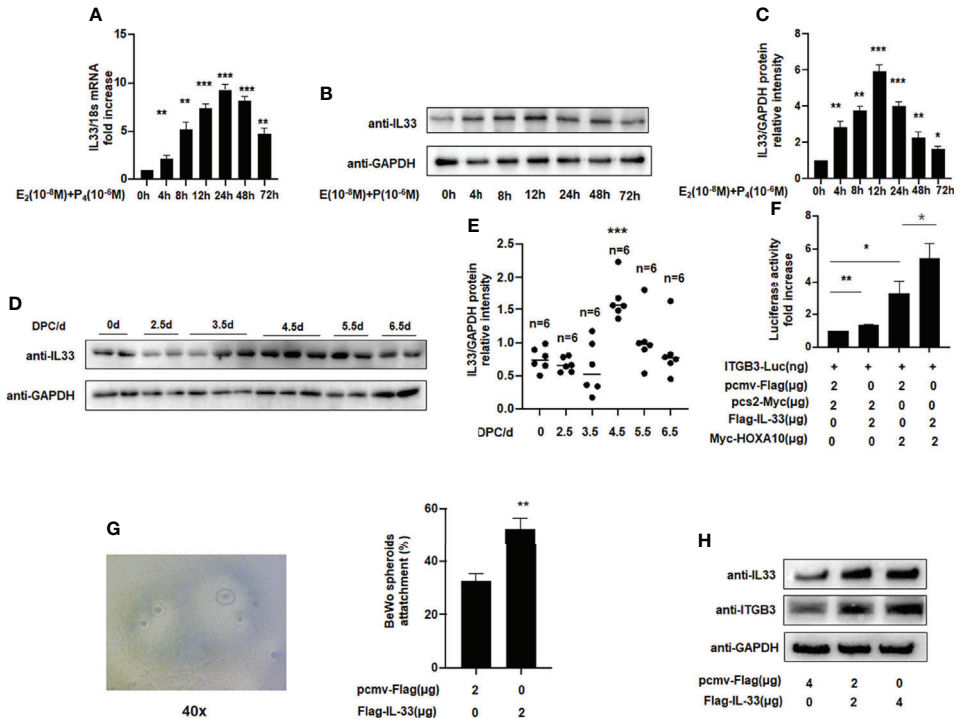

FIGURE 3
IL33 participates in embryo implantation. **(A)** Ishikawa cells were administered estrogen (10^{-8} M) and progesterone (10^{-6} M) at different times, as indicated. IL33 mRNA levels were measured by qRT-PCR. **p < 0.01, ***p < 0.001. **(B)** Ishikawa cells were administered estrogen (10^{-8} M) and progesterone (10^{-6} M) at different times, as indicated. Whole-cell lysates were analyzed by western blot analysis with the indicated antibodies. **(C)** Protein levels were normalized to GAPDH protein expression level in **(B)** (**p< 0.01, ***p < 0.001. vs control). **(D)** Western blot analysis of uterine IL33 during the peri-implantation period in mice, with the strongest decreased signal detected at 4.5 dpc. **(E)** Protein levels were normalized to GAPDH protein expression level in **(D)** (**p< 0.01, ***p < 0.001. vs control, n is shown in each group). **(F)** Ishikawa cells were transfected with ITGB3-Luc, Myc-HOXA10 (2 μg), and Flag-IL33 (2 μg), as indicated. After 48 h, the luciferase activities were measured and are presented as fold induction. Values represent the mean ± SEM (n = 3), *p < 0.05, **p < 0.01. **(G)** BeWo spheroids (150–200 μm diameter) were attached to Ishikawa cells after 2 h of co-culture. Adhesion experiments with BeWo spheroids attached to the Ishikawa cell monolayer. The data are the average of three independent experiments. An ANOVA test was used to compare the percentage of the attached spheroids with each treatment in comparison to the control. The error bars indicate SD of three independent experiments. Values represent the mean ± SEM (n = 3), **p < 0.01. **(H)** Ishikawa cells were transfected with Flag-IL33 (2 μg) as indicated. Whole-cell lysates were analyzed by western blot analysis with the indicated antibodies.

therapy, adenomyosis deceased the clinical outcome (19–21).However, the underlying mechanism which adenomyosis cause implantation failure remains unclear. This study first demonstrated that the reduced IL33 expression in the endometrium impaired embryo implantation through modulation of HOXA10 expression.

In 1840, pathologists Carl von Rokitansky first described adenomyosis (22). Adenomyosis is believed to be a benign gynaecological disease in which endometrial glands and stroma are present in the myometrium. With the continuous development of diagnostic criteria, the incidence of adenomyosis is increasing, especially in reproductive women (23).Understanding how adenomyosis leads to infertility is complex; some studies have shown that, physically speaking, adenomyosis affects uterine motility and hinders sperm transport (24), while others have suggested that adenomyosis impairs decidualization and embryo implantation by impacting molecular expression (4, 25). The endometrial receptivity marker HOXA10 and its downstream target molecule ITGB3 were both decreased in the adenomyosis mouse model as well as during the secretory phase in patients with adenomyosis (26–28). However, the underlying mechanism remains unclear.

Changes in immune cells and the imbalance between proinflammatory and anti-inflammatory cytokines are involved in the subfertility of women affected by adenomyosis (29). Some studies have focused on macrophages and T cells, which were found to be increased in the endometrium from adenomyosis-affected uteri compared to disease-free uteri (30, 31). These changes ultimately led to the abnormal secretion of cytokines and other immune factors. Proinflammatory factors such as IL1β, IL6, TNFα and NF-kB and anti-inflammatory factors such as IL10, TGF-β1, HGF, and EGF were also found to be dysregulated (29). This dysregulation modifies the immune environment and causes the abnormal expression of some

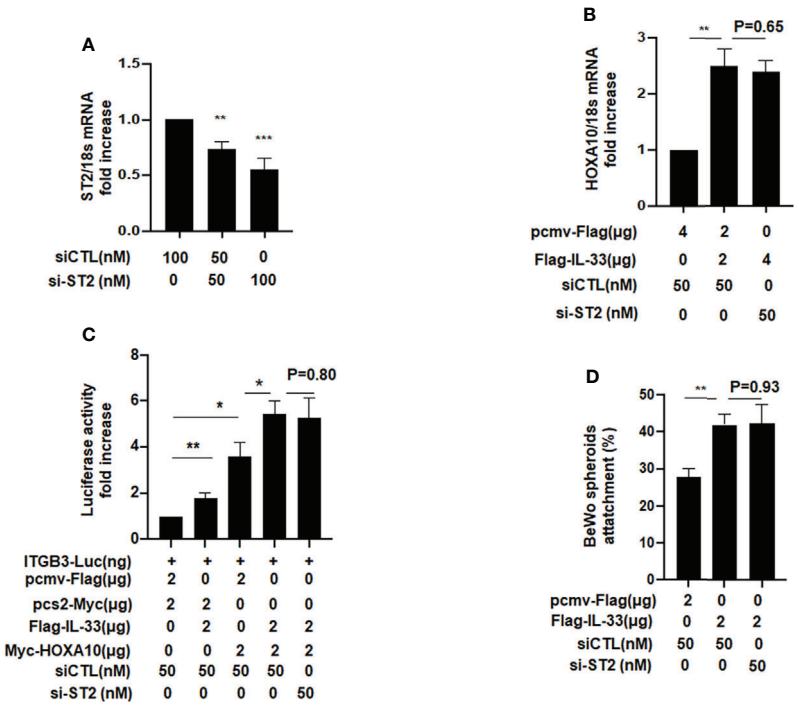

FIGURE 4
ST2 silence dose not affect the IL33-promoted HOXA10 expression and embryo implantation *in vitro*. **(A)** Ishikawa cells were transfected with si-ST2 or negative control. ST2 mRNA levels were measured by qRT-PCR ** $p < 0.01$, *** $p < 0.001$. **(B)** Ishikawa cells were transfected with si-ST2 or negative control. After 24h, IL33. was overexpression *via* the transfection of Flag-IL33. HOXA10 mRNA levels were measured by qRT-PCR. ** $p < 0.01$ **(C)** Ishikawa cells were transfected with si-ST2 or negative control. After 24h, ITGB3-Luc, Myc-HOXA10 (2 μg), and Flag-IL33 (2 μg) were transfected into the cells, as indicated. After 48 h, the luciferase activities were measured and are presented as fold induction. Values represent the mean ± SEM (n = 3), *$p < 0.05$, **$p < 0.01$. **(D)** BeWo spheroids (150–200 μm diameter) were attached to Ishikawa cells after 2 h. of co-culture. Adhesion experiments with BeWo spheroids attached to the Ishikawa cell monolayer. The data are the average of three independent experiments. An ANOVA test was used to compare the percentage of the attached spheroids with each treatment in comparison to the control. The error bars indicate SD of three independent experiments. Values represent the mean ± SEM (n = 3), **$p < 0.01$.

proteins related to embryo implantation. Few studies have focused on the effect of IL33 on adenomyosis. Only one study investigated the serum IL33 levels in adenomyosis-affected women (17). In the study, the researcher analysed the serum cytokine profiles in different types of adenomyosis: diffuse adenomyosis (DIF-ADE), focal adenomyosis (FOC-ADE), and combination of focal and diffuse lesions (FOC/DIF-ADE) (17). They demonstrated that the serum IL33 levels were reduced in the adenomyosis, especially in the type of FOC/DIF-ADE. In our study, we illustrated that in both the endometrium of infertile women and the mouse model with adenomyosis, IL33 expression was downregulated. However, we did not undergo further studies on the different types of adenomyosis. Previous studies have proven the lower embryo implantation rate and higher miscarriage rate in adenomyosis-affected women undergoing IVF (32). Some researchers proposed that the Uterine Junctional Zone (JZ) thickness evaluated by MRI is the best predictive factor of implantation failure (33). When the average JZ thickness was more than 7 mm, the implantation failure was found to be higher (34). According to the classification of adenomyosis *via* MRI examination, the JZmax in DIF-ADE should be more than 12mm, which would be a major factor affecting embryo implantation (35, 36). In our study, we found endometrial IL33 expression was positively correlated to HOXA10 expression and may involve embryo implantation in patients with adenomyosis. Regarding to the embryo implantation, we supposed that the expression of IL33 in the endometrium should be more affected in the type of DIF-ADE. This proposal requires further research, and studies on deeper molecular mechanism should be conducted in the future.

IL33 is widely expressed in many tissues and predominantly located in tissue cell types, including endothelial cells, epithelial cells and fibroblasts (37, 38). Human IL-33 genes are located on chromosome 9p24.1 and are composed of 270 amino acids, including N-terminal and C-terminal domains. Nuclear localization sequences and chromosome binding regions are located in the N-terminal structure (AA1-65); the IL1-like cytokine domain is included in the C-terminal domain (AA112-270) (39). Because of these properties of the protein structure, IL-33 has dual functions, acting both as a cytokine and

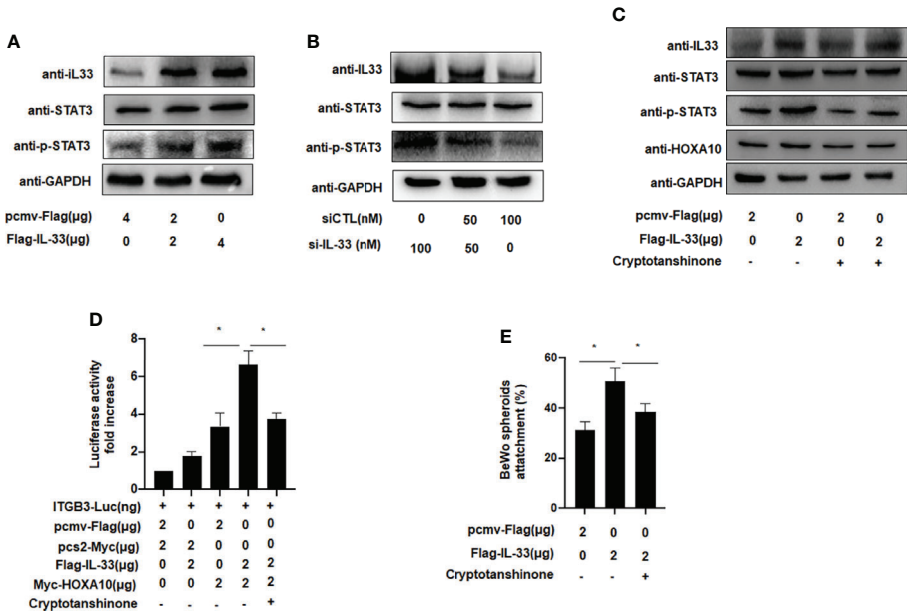

FIGURE 5
IL-33 enhances embryo implantation *via* phosphorylation of STAT3. **(A)** Ishikawa cells were transfected with Flag-IL33 (2 μg). IL33, STAT3 and p-STAT3 protein levels were analyzed by western blot analysis. **(B)** Ishikawa cells were transfected with siIL33 (50 or 100 nM), Whole-cell lysates. were analyzed by western blot analysis with the indicated antibodies. **(C)** Ishikawa cells were administrated with cryptotanshinone (4.6 μM) for 8h before. the transfection of Flag-IL33 (2 μg), Whole-cell lysates were analyzed by western blot analysis with the indicated antibodies. **(D)** Ishikawa cells were administrated with cryptotanshinone (4.6 μM) for 8h before. the transfection of ITGB3-Luc, Myc-HOXA10 (2 μg), and Flag-IL33 (2 μg), as indicated. The luciferase activities were measured and are presented as fold induction. Values represent the mean ± SEM (n = 3), *p < 0.05. **(E)** Ishikawa cells were administrated with cryptotanshinone (4.6 μM) for 8h before the. transfection of Flag-IL33 (2 μg). BeWo spheroids (150–200 μm diameter) were attached to Ishikawa cells after 2 h of co-culture. Adhesion experiments with BeWo spheroids attached to the Ishikawa cell monolayer. The data are the average of three independent experiments. An ANOVA test was used to compare the percentage of the attached spheroids with each treatment in comparison to the control. The error bars indicate SD of three independent experiments. Values represent the mean ± SEM (n = 3), *p < 0.05.

as a transcriptional regulator. When facing danger or stress, IL33 is released from cells and binds to the ligand ST2 to exert an immune effect. IL33/ST2 promotes the phosphorylation and activation of PI3K/AKT, p38, ERK1/2, and JNK signalling, participating in both normal physiology and various diseases (40–42). In addition, IL33 in the nucleus may play a role in some physiological activity independently of ST2. However, recent studies have pointed out that nuclear IL33 has no function in gene transcription in endothelial or epithelial cells (43, 44). Initial studies illustrated that IL-33 acted as a transcriptional regulator to inhibit NF-KB transcriptional activity (45). Nuclear IL-33 in epidermal keratinocytes is involved in the pathogenesis of atopic dermatitis and serves as a cofactor of STAT3, inhibiting keratinocyte differentiation and skin barrier function *via* phosphorylation of STAT3 and STAT6 (46). In intervertebral disc degeneration, IL33 regulates ECM degradation independent of ST2 (47). Previous studies suggested that disordered IL33/ST2 signalling is involved in endometrial decidualization and embryo implantation, but the specific mechanism has not been elucidated. In the present study, we use Ishikawa cells, which express estrogen and progesterone receptors, as an endometrial epithelium model. We found the endogenous IL33 mRNA and protein levels were increased in Ishikawa cells treated with estrogen and progesterone in a time-dependent manner. In the mouse model, IL33 expression was strengthened at Dpc.4.5, which was the period of mouse embryo implantation. Correspondingly, the expression of IL33 in secretory phase endometrium is higher than that in proliferative phase in previous study (18). These studies indicated that intracellular IL33 may be regulated by estrogen and progesterone and involved in embryo implantation *in vivo and in vitro*. Besides, our results were consistent with previous findings that nuclear IL33 expression is upregulated during the secretory phase (the implantation window), suggesting the important role of nuclear IL33 in this process. In addition, when ST2 was silenced, the promoting effect of IL33 was not weakened, indicating that IL33 in the nucleus could still exert its corresponding function. There were some limitations to our study, which should be mentioned. Although we identified one possible mechanism of IL33, that is, the regulation of STAT3 phosphorylation, we did not further explore the transcriptional regulation of IL33 in the nucleus. This requires additional research in the future.

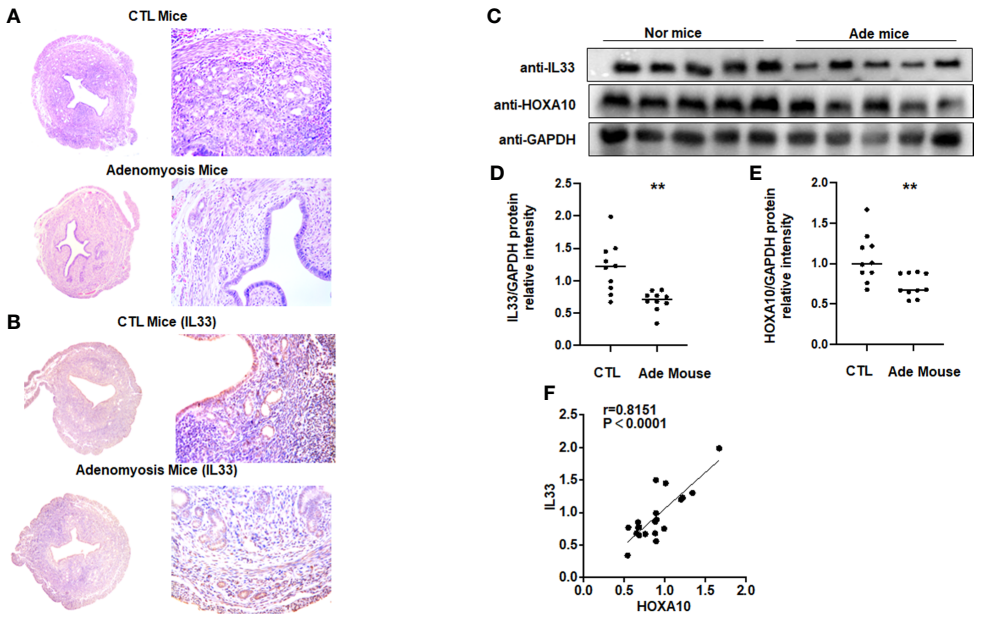

FIGURE 6
IL33 expression is decreased in a mouse model of adenomyosis. **(A)** HE staining confirmed that the mouse model of adenomyosis was successfully constructed. **(B)** Uterus from control mouse and adenomyosis mouse model were analyzed using immunohistochemistry (IHC). Rabbit IgG was used as the negative control. The arrows show the decreased IL33 conjugates in the endometrial cell epithelium. **(C)** IL33 and HOXA10 protein expression in control (n =10) and mouse with adenomyosis (n=10) was analyzed by Western blot. **(D)** The intensities of IL33 signals were quantified from the 10 samples and normalized to GAPDH. **p < 0.01. **(E)** The intensities of HOXA10 signals were quantified from the 10 samples and normalized to GAPDH. **p < 0.01. **(F)** Correlation between IL33 and HOXA10 protein expression levels (r = 0.8151, p < 0.0001).

Conclusion

In conclusion, the present study first demonstrated that nuclear IL33 plays an important role in epithelial cells by inducing HOXA10 expression *via* the phosphorylation of STAT3, which is critical for mouse and human pregnancy establishment and maintenance. This study provides a new pathological mechanism study for infertility in adenomyosis and may bring new insights for the treatment of infertility in adenomyosis. The specific molecular mechanism still needs to be further studied.

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics Statement

The studies involving human participants were reviewed and approved by Ethics Committee of Shanghai First Maternity and Infant Health Hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Ethics Committee of Shanghai First Maternity and Infant Health Hospital.

Author contributions

K-ML and QY contributed to the conception and design of the work. MZ, Z-QC contributed to the acquisition. BH, FH and X-MT drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Chapron C, Vannuccini S, Santulli P, Abrão MS, Carmona F, Fraser IS, et al. Diagnosing Adenomyosis: An Integrated Clinical and Imaging Approach. *Hum Reprod Update* (2020) 26(3):392–411. doi: 10.1093/humupd/dmz049

2. Szubert M, Koziróg E, Olszak O, Krygier-Kurz K, Kazmierczak J, Wilczynski J. Adenomyosis and infertility-review of medical and surgical approaches. *Int J Environ Res Public Health* (2021) 18(3):1235. doi: 10.3390/ijerph18031235

3. Barbanti C, Centini G, Lazzeri L, Habib N, Labanca L, Zupi E, et al. Adenomyosis and infertility: the role of the junctional zone. *Gynecological Endocrinol Off J Int Soc Gynecological Endocrinol* (2021) 37(7):577–83. doi: 10.1080/09513590.2021.1878131

4. Yan Q, Yan G, Zhang C, Wang Z, Huang C, Wang J, et al. Mir-21 reverses impaired decidualization through modulation of Klf12 and nr4a1 expression in human endometrial stromal cells†. *Biol Reprod* (2019) 100(5):1395–405. doi: 10.1093/biolre/ioz026

5. Wang PH. Endometrial receptivity and adenomyosis. *Taiwanese J obstetrics gynecol* (2018) 57(5):625–6. doi: 10.1016/j.tjog.2018.08.002

6. Rock J, Bartlett MK. Biopsy studies of human endometrium: criteria of dating and information about amenorrhea, menorrhagia, and time of ovulation. *J Am Med Assoc* (1937) 108(24):2022–8. doi: 10.1001/jama.1937.02780240014006

7. Craciunas L, Gallos I, Chu J, Bourne T, Quenby S, Brosens JJ, et al. Conventional and modern markers of endometrial receptivity: a systematic review and meta-analysis. *Hum Reprod Update* (2019) 25(2):202–23. doi: 10.1093/humupd/dmy044

8. Hernández-Vargas P, Muñoz M, Domínguez F. Identifying biomarkers for predicting successful embryo implantation: applying single to multi-omics to improve reproductive outcomes. *Hum Reprod Update* (2020) 26(2):264–301. doi: 10.1093/humupd/dmz042

9. Bi Y, Huang W, Yuan L, Chen S, Liao S, Fu X, et al. Hoxa10 improves endometrial receptivity by upregulating e-cadherin. *Biol Reprod* (2022) 106 (5):992–9. doi: 10.1093/biolre/ioac007

10. Zanatta A, Rocha AM, Carvalho FM, Pereira RM, Taylor HS, Motta EL, et al. The role of the Hoxa10/Hoxa10 gene in the etiology of endometriosis and its related infertility: A review. *J Assist Reprod Genet* (2010) 27(12):701–10. doi: 10.1007/s10815-010-9471-y

11. Kong C, Sun L, Zhang M, Ding L, Zhang Q, Cheng X, et al. Mir-133b reverses the hydrosalpinx-induced impairment of embryo attachment through down-regulation of Sgk1. *J Clin Endocrinol Metab* (2016) 101(4):1478–89. doi: 10.1210/jc.2015-1588

12. Nazarenko TA, Kalinina EA, Knyazeva EA, Kiselev VI, Smolnikova VY, Sukhikh GT. The role of abnormal hypermethylation of the Hoxa10 and Hoxa11 promoters in implantation failures in ivf programs. *Gynecol Endocrinol* (2019) 35 (sup1):31–4. doi: 10.1080/09513590.2019.1632087

13. Wang J, Huang C, Jiang R, Du Y, Zhou J, Jiang Y, et al. Decreased endometrial il-10 impairs endometrial receptivity by downregulating Hoxa10 expression in women with adenomyosis. *BioMed Res Int* (2018) 2018:2549789. doi: 10.1155/2018/2549789

14. Baekkevold ES, Roussigne M, Yamanaka T, Johansen FE, Jahnsen FL, Amalric F, et al. Molecular characterization of nf-hev, a nuclear factor preferentially expressed in human high endothelial venules. *Am J Pathol* (2003) 163(1):69–79. doi: 10.1016/s0002-9440(10)63631-0

15. Zhao W, Hu Z. The enigmatic processing and secretion of interleukin-33. *Cell Mol Immunol* (2010) 7(4):260–2. doi: 10.1038/cmi.2010.3

16. Lin TC, Wang KH, Chuang KH, Kao AP, Kuo TC. Interleukin-33 promotes invasiveness of human ovarian endometriotic stromal cells through the St2/Mapk/Mmp-9 pathway activated by 17β-estradiol. *Taiwan J Obstet Gynecol* (2021) 60 (4):658–64. doi: 10.1016/j.tjog.2021.05.013

17. Bourdon M, Santulli P, Chouzenoux S, Maignien C, Bailly K, Andrieu M, et al. The disease phenotype of adenomyosis-affected women correlates with specific serum cytokine profiles. *Reprod Sci (Thousand Oaks Calif)* (2019) 26 (2):198–206. doi: 10.1177/1933719118816852

18. Salker MS, Nautiyal J, Steel JH, Webster Z, Sućurović S, Nicou M, et al. Disordered il-33/St2 activation in decidualizing stromal cells prolongs uterine receptivity in women with recurrent pregnancy loss. *PloS One* (2012) 7(12):e52252. doi: 10.1371/journal.pone.0052252

19. Puente JM, Fabris A, Patel J, Patel A, Cerrillo M, Requena A, et al. Adenomyosis in infertile women: Prevalence and the role of 3d ultrasound as a marker of severity of the disease. *Reprod Biol Endocrinol RB&E* (2016) 14(1):60. doi: 10.1186/s12958-016-0185-6

20. Abu Hashim H, Elaraby S, Fouda AA, Rakhawy ME. The prevalence of adenomyosis in an infertile population: A cross-sectional study. *Reprod Biomed Online* (2020) 40(6):842–50. doi: 10.1016/j.rbmo.2020.02.011

21. Zhang XP, Zhang YF, Shi R, Zhang YJ, Zhang XL, Hu XM, et al. Pregnancy outcomes of infertile women with ultrasound-diagnosed adenomyosis for in vitro fertilization and frozen-thawed embryo transfer. *Arch gynecol obstetrics* (2021) 304 (4):1089–96. doi: 10.1007/s00404-021-06011-z

22. Munro MG. Classification and reporting systems for adenomyosis. *J Minimally Invasive Gynecol* (2020) 27(2):296–308. doi: 10.1016/j.jmig.2019.11.013

23. Upson K, Missmer SA. Epidemiology of adenomyosis. *Semin Reprod Med* (2020) 38(2-03):89–107. doi: 10.1055/s-0040-1718920

24. Munro MG. Uterine polyps, adenomyosis, leiomyomas, and endometrial receptivity. *Fertil Steril* (2019) 111(4):629–40. doi: 10.1016/j.fertnstert.2019.02.008

25. Peng Y, Liu X, Jin Z, Liu H, Xu C. Scribble downregulation in adenomyosis compromises endometrial stromal decidualization by decreasing Foxo1 expression. *Hum Reprod (Oxford England)* (2021) 37(1):93–108. doi: 10.1093/humrep/deab234

26. Guo S, Li Z, Yan L, Sun Y, Feng Y. Gnrh agonist improves pregnancy outcome in mice with induced adenomyosis by restoring endometrial receptivity. *Drug Design Dev Ther* (2018) 12:1621–31. doi: 10.2147/dddt.s162541

27. Fischer CP, Kayisili U, Taylor HS. Hoxa10 expression is decreased in endometrium of women with adenomyosis. *Fertil steril* (2011) 95(3):1133–6. doi: 10.1016/j.fertnstert.2010.09.060

28. Xiao Y, Li T, Xia E, Yang X, Sun X, Zhou Y. Expression of integrin B3 and osteopontin in the eutopic endometrium of adenomyosis during the implantation window. *Eur J Obstetrics Gynecol Reprod Biol* (2013) 170(2):419–22. doi: 10.1016/j.ejogrb.2013.05.007

29. Bourdon M, Santulli P, Jeljeli M, Vannuccini S, Marcellin L, Doridot L, et al. Immunological changes associated with adenomyosis: A systematic review. *Hum Reprod Update* (2021) 27(1):108–29. doi: 10.1093/humupd/dmaa038

30. An M, Li D, Yuan M, Li Q, Zhang L, Wang G. Different macrophages equally induce emt in endometria of adenomyosis and normal. *Reproduction* (2017) 154(1):79–92. doi: 10.1530/rep-17-0174

31. Bonilla FA, Oettgen HC. Adaptive immunity. *J Allergy Clin Immunol* (2010) 125(2 Suppl 2):S33–40. doi: 10.1016/j.jaci.2009.09.017

32. Harada T, Khine YM, Kaponis A, Nikellis T, Decavalas G, Taniguchi F. The impact of adenomyosis on women's fertility. *Obstetric Gynecol Survey* (2016) 71 (9):557–68. doi: 10.1097/ogx.0000000000000346

33. Piver P. [Uterine factors limiting art coverage]. *J Gynecol Obstetrique Biol la Reprod* (2005) 34(7 Pt 2):5S30–5S3. doi: JGYN-11-2005-34-7-C2-0368-2315-101019-200509169

34. Maubon A, Faury A, Kapella M, Pouquet M, Piver P. Uterine junctional zone at magnetic resonance imaging: A predictor of in vitro fertilization implantation failure. *J Obstetrics Gynaecol Res* (2010) 36(3):611–8. doi: 10.1111/j.1447-0756.2010.01189.x

35. Chapron C, Tosti C, Marcellin L, Bourdon M, Lafay-Pillet MC, Millischer AE, et al. Relationship between the magnetic resonance imaging appearance of adenomyosis and endometriosis phenotypes. *Hum Reprod (Oxford England)* (2017) 32(7):1393–401. doi: 10.1093/humrep/dex088

36. Bazot M, Cortez A, Darai E, Rouger J, Chopier J, Antoine JM, et al. Ultrasonography compared with magnetic resonance imaging for the diagnosis of adenomyosis: Correlation with histopathology. *Hum Reprod (Oxford England)* (2001) 16(11):2427–33. doi: 10.1093/humrep/16.11.2427

37. Moussion C, Ortega N, Girard JP. The il-1-Like cytokine il-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: A novel 'Alarmin'? *PloS One* (2008) 3(10):e3331. doi: 10.1371/journal.pone.0003331

38. Pichery M, Mirey E, Mercier P, Lefrancais E, Dujardin A, Ortega N, et al. Endogenous il-33 is highly expressed in mouse epithelial barrier tissues, lymphoid organs, brain, embryos, and inflamed tissues: *in situ* analysis using a novel il-33-

Lacz gene trap reporter strain. *J Immunol* (2012) 188(7):3488–95. doi: 10.4049/jimmunol.1101977

39. Cayrol C, Girard JP. Interleukin-33 (Il-33): A nuclear cytokine from the il-1 family. *Immunol Rev* (2018) 281(1):154–68. doi: 10.1111/imr.12619

40. Huang SJ, Yan JQ, Luo H, Zhou LY, Luo JG. Il-33/St2 signaling contributes to radicular pain by modulating mapk and nf-Kb activation and inflammatory mediator expression in the spinal cord in rat models of noncompressive lumber disk herniation. *J Neuroinflamm* (2018) 15(1):12. doi: 10.1186/s12974-017-1021-4

41. Han P, Liu S, Zhang M, Zhao J, Wang Y, Wu G, et al. Inhibition of spinal interlukin-33/St2 signaling and downstream erk and jnk pathways in electroacupuncture analgesia in formalin mice. *PloS One* (2015) 10(6):e0129576. doi: 10.1371/journal.pone.0129576

42. Lin L, Li Y, Liu M, Li Q, Liu Q, Li R. The interleukin-33/St2 axis promotes glioma mesenchymal transition, stemness and tmz resistance *Via* jnk activation. *Aging (Albany NY)* (2020) 12(2):1685–703. doi: 10.18632/aging.102707

43. Travers J, Rochman M, Miracle CE, Habel JE, Brusilovsky M, Caldwell JM, et al. Chromatin regulates il-33 release and extracellular cytokine activity. *Nat Commun* (2018) 9(1):3244. doi: 10.1038/s41467-018-05485-x

44. He Z, Chen L, Furtado GC, Lira SA. Interleukin 33 regulates gene expression in intestinal epithelial cells independently of its nuclear localization. *Cytokine* (2018) 111:146–53. doi: 10.1016/j.cyto.2018.08.009

45. Choi YS, Park JA, Kim J, Rho SS, Park H, Kim YM, et al. Nuclear il-33 is a transcriptional regulator of nf-Kb P65 and induces endothelial cell activation. *Biochem Biophys Res Commun* (2012) 421(2):305–11. doi: 10.1016/j.bbrc.2012.04.005

46. Dai X, Shiraishi K, Muto J, Utsunomiya R, Mori H, Murakami M, et al. Nuclear il-33 plays an important role in il-31—Mediated downregulation of flg, keratin 1, and keratin 10 by regulating signal transducer and activator of transcription 3 activation in human keratinocytes. *J Invest Dermatol* (2022) 142 (1):136–44.e3. doi: 10.1016/j.jid.2021.05.033

47. Hu J, Yan Q, Jiang H, Xu C, Chen Y, Yuan W. A decrease in il-33 regulates matrix degradation and apoptosis in intervertebral disc degeneration *Via* hif-1alpha. *Am J Transl Res* (2021) 13(11):12724–33.

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Endocrine disrupting chemicals interfere with decidualization of human primary endometrial stromal cells *in vitro*

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Multiple studies have shown associations between exposure to endocrine disrupting chemicals (EDCs) and reduced fertility in women. However, little is known about the target organs of chemical disruption of female fertility. Here, we focus on the hormone-sensitive uterine lining, the endometrium, as a potential target. Decidualization is the morphological and functional change that endometrial stromal cells undergo to support endometrial receptivity, which is crucial for successful implantation, placentation, and pregnancy. We investigated the effect of nine selected EDCs on primary human endometrial stromal cell decidualization *in vitro*. The cells were exposed to a decidualization-inducing mixture in the presence or absence of 1 µM of nine different EDCs for nine days. Extent of decidualization was assessed by measuring the activity of cAMP dependent protein kinase, Rho-associated coiled-coil containing protein kinase, and protein kinase B in lysates using photoluminescent probes, and secretion of prolactin into the media by using ELISA. Decidualization-inducing mixture upregulated activity of protein kinases and prolactin secretion in cells derived from all women. Of the tested chemicals, dichlorodiphenyldichloroethylene (p,p'-DDE), hexachlorobenzene (HCB) and perfluorooctanesulfonic acid (PFOS) significantly reduced decidualization as judged by the kinase markers and prolactin secretion. In addition, bisphenol A (BPA) reduced prolactin secretion but did not significantly affect activity of the kinases. None of the EDCs was cytotoxic, based on the assessment of total protein content or activity of the viability marker casein kinase 2 in lysates. These results indicate that EDCs commonly present in the blood circulation of reproductive-aged women can reduce decidualization of human endometrial stromal cells *in vitro*. Future studies should focus on

detailed hazard assessment to define possible risks of EDC exposure to endometrial dysfunction and implantation failure in women.

KEYWORDS

endocrine disrupting chemical, phthalate, persistent organic pollutants (POPs), human endometrium, decidualization, per- and polyfluorinated alkyl substances (PFASs), primary stromal cells, infertility

Introduction

The environment is polluted by a wide range of human-made industrial chemicals, which are deliberately or accidently released by humans (1). Some of them can interfere with the endocrine system leading to adverse health effects in humans and wildlife, and can be termed as endocrine disrupting chemicals (EDCs) according to commonly accepted definitions (2). The mechanisms of endocrine disruption have diversified from the classical EATS modalities (Estrogen, Androgen, Thyroid and Steroidogenesis) to encompass *e.g.* signal transduction, epigenetics and cell fate in hormone-producing or hormone-sensitive cells (3). EDCs can be found in various natural environments including water, soil, and air (4). Many consumer products such as food packaging, kitchen utensils, plastics, personal care products, home electronics, textiles, and furniture contain EDCs (5, 6). Therefore, humans and wildlife are continuously exposed to mixtures of possibly hazardous contaminants.

Despite extensive and continuous exposure documented in multiple humanbiomonitoring studies (*e.g.* National Biomonitoring Program by the Centers for Disease Control and Prevention in the US, and the HBM4UE in the European Union), hazard identification and characterization of most chemicals on the market remains limited. For example, out of the 100 000 chemicals on the European market, only 500 have been thoroughly characterized for their hazards and exposures (7). In addition, typical guideline assays for reproductive toxicity are not well suited for detecting fertility disrupting effects of chemicals in women, which has led to the European Commission flagging female reproductive toxicity as a priority research area (8). More research is needed to better understand the effects of EDCs on reproductive health in women, and to develop novel predictive and human-relevant assays that can be used to characterize reproductive hazards of chemicals.

The female reproductive system has multiple essential functions that enable fertility. Ovaries produce competent oocytes that can be fertilized by sperm in the fallopian tubes. The fertilized embryos then travel to the uterus where they implant into the endometrium for further development. Embryo implantation is only possible during the short period of endometrial receptivity, referred as the window of implantation when the epithelial cells have obtained an adhesive phenotype and stromal cells have decidualized. Decidualization is a morphological and functional change of endometrial stromal fibroblasts in the early secretory phase to epithelial-like decidual stromal cells in the mid-secretory phase. Rising levels of the steroid hormones estradiol and progesterone from ovaries together with increased intracellular cyclic adenosine monophosphate (cAMP) levels activate a crosstalk of different downstream gene transcription and protein kinases such as cAMP-dependent protein kinase (PKA) - among other outcomes, resulting in secretion of prolactin (PRL) and insulin growth factor-binding protein 1 (IGFBP-1), classical markers of decidualization (9, 10). Subsequent embryo implantation relies on tight and timely communication between the embryo and the endometrium, which paves the way to controlled trophoblast invasion into the stromal tissue. Decidualized stromal cells are more supportive for trophoblast expansion than undifferentiated stromal cells (11) and decidualization is required to distinguish poor and good quality embryos, favoring the continuation of the pregnancy resulting from the latter (12).

Approximately 15% of known pregnancies end in miscarriage in humans, and this number is likely higher because the very early miscarriages often go unnoticed (13). Miscarriages contribute to longer time-to-pregnancy (14) and are associated with increased risk for future miscarriages or recurrent pregnancy loss (15) and infertility - the inability to achieve pregnancy after 12 months of regular unprotected sexual intercourse (16). Many epidemiological studies have found significant associations between exposure to EDCs, miscarriage and pregnancy complications such as preeclampsia, preterm birth, and fetal growth restriction (17–20). As decidualization is a strictly hormone-dependent, key process in establishment of a pregnancy, and disrupted implantation and placentation can lead to complications mentioned, it is essential to investigate the effects of EDCs on the human endometrium. For example, EDCs affect steroid hormone receptors, affecting endometrial stromal cell signalling pathways and proliferation *in vivo* in rodents (21, 22). Importantly, there is a need to study EDC effects on

decidualization in human-relevant settings because there are major differences in endometrial biology between experimental animals like mice and humans (23, 24). Effects of EDCs on human endometrial stromal cells can be studied in controlled settings for example using *in vitro* models where decidualization is triggered with hormones in combination with the cell-permeable analogues of cAMP.

Up to date, only a few studies have investigated the effects of EDCs on human *in vitro* decidualization (Table 1) (25–33). Six out of nine published studies have focused on the plastic additive BPA and found that BPA exposure has significant effects on markers of decidualization such as PRL, although results were inconsistent. Some studies found that BPA reduced PRL at higher (87.6 μM) (32) and others at lower (1 μM) (31) concentrations, while some found no effects (28), and others suggested that BPA might even increase PRL secretion (25). This lack of consistency may depend on different decidualization protocols, exposure times and concentrations of EDCs used, and source of cells. Cells for *in vitro* decidualization are often derived from hysterectomy material or endometrial biopsies at different stages of the menstrual cycle, but even commercially available cell lines (THESC) have been used. Patient-derived primary stromal cells typically show high inter-individual variation while commercial cell lines are more homogeneous. However, immortalized commercial cell lines might not reflect true endometrial biology. All these factors complicate the comparison of results between different studies (Table 1). In addition to BPA, PFOS, alkylphenols, and triclosan have been found to affect decidualization markers *in vitro* (Table 1). Collectively, these nine studies suggest that *in vitro* decidualization is sensitive to EDC exposures.

Here, our ambition was to study the impact of a wide selection of EDCs on human endometrial cell decidualization using our recently established *in vitro* model for decidualization (34). This model is based on primary endometrial stromal cells from hysterectomies (or endometrial biopsies) that are simulated to decidualize with a mixture containing progesterone and estradiol; the process is monitored using a suite of kinases reflecting activation of intracellular signaling, and secretion of PRL as the final outcome (34). In this way, instead of focusing on one marker, we assessed a characteristic fingerprint involving several previously identified markers of decidualization, which is a complex physiological process that cannot be explained by modulation of a single pathway. Apart from assessing PRL secretion and activity of PKA, we explored activity of Rho-dependent protein kinase (ROCK), protein kinase B (Akt/PKB), casein kinase 2 (CK2) and total protein concentration in lysates. The nine EDCs were chosen for the following reasons: BPA and its replacement chemical bisphenol F (BPF) because they have been studied in similar models before (Table 1) and BPA is a known human endocrine disrupter (European Chemicals Agency); phthalate MEHP (metabolite of DEHP) because it is also a known human endocrine disrupter (European Chemicals Agency) and associated to miscarriage in humans (18, 20); organochlorine pesticides p,p'-DDE (metabolite of DDT) and HCB, and the polychlorinated biphenyls PCB180 and PCB170, because they associate to longer time-to-pregnancy in our cohort studies (35, 36); HCB is also associated with lower odds for clinical pregnancy and live birth (36); and PFOS and perfluorooctanoic acid (PFOA) because they are associated to preterm birth, fetal growth restriction and pre-eclampsia in humans (19). In addition, the chosen EDCs have different physiochemical properties. BPA, BPF and MEHP are quickly metabolized and excreted from the body; PFOS and PFOA are resistant to degradation (*i.e.*, are persistent), amphiphilic and predominantly bind to plasma proteins and accumulate in liver, while p,p'-DDE, HCB, and PCBs are persistent, lipophilic and accumulate in adipose tissue. A concentration of 1 μM was chosen for all EDCs to allow identification of hazards at a non-toxic exposure level. First, the nine different EDCs were tested in cells obtained from two women. Then, those chemicals showing effects (BPA, p,p'-DDE, HCB and PFOS) were studied further in cells from two additional women. Altogether, our results suggest that certain EDCs pose a hazard to endometrial biology. Follow-up studies should focus on detailed characterization of the hazard to allow for risk assessment in the population. It will be essential to study the sensitivity of this endpoint in more detail and develop test systems that are suitable for screening of large numbers of chemicals.

Materials and methods

Women, endometrial samples and isolation of stromal cells

Endometrial tissue was collected from the uterus immediately after hysterectomy from four women: endometrial stromal cells (eSCs) coded as 4, 5, 8 and 12, at the gynecological surgical unit at Uppsala University hospital (Table 2). All women were in the proliferative phase of the menstrual cycle, had no endometriosis, and were generally healthy (Table 2). The tissue was transported to the laboratory on ice in isolation medium: DMEM/F-12 with HEPES without phenol red (Gibco, Thermofisher, USA) containing 1% (v/v) penicillin/streptomycin (Thermofisher, USA). The eSCs 5 and 8 were isolated on the day of surgery. Due to late surgery, eSCs 4 and 12 were kept at 4°C overnight and cells were isolated the next morning.

The studies were approved by Uppsala Regional ethical review board, Sweden, now the Swedish ethical review authority (reference number 2011/430). Informed written consent was given by all participants in accordance with the declaration of Helsinki. Clinical information was retrieved from patient journals. All samples and data were pseudonymized.

TABLE 1 Summary of literature on the effects of EDCs on human endometrial stromal cell decidualization *in vitro*.

Origin of cells	EDC exposure (concentrations, time)	Decidualization protocol	Key results	Reference
Endometrial biopsies from gynecological surgery, proliferative state (n=8)	BPA and TCL 100 µM, 100 nM, 100 pM, 24 h, 48 h and 72 h	10 µM P4 for 10 days	TCL and BPA 100 nM and 100 pM arrested cell cycle. Increased expression of decidualization markers *PRL* and *IGFBP1* after 48 h and 72 h. Effects on cell migration genes at 100 µM and 100 nM. Both affected cell migration and PRL and IGFBP-1 secretion at 100 nM.	(25)
First trimester (6–8 weeks) decidual tissue from induced abortion (n=3-5)	PFOS first range from 0.0001 to 1 µM, then 0.01 µM was used 24 h or 48 h	0.5 mM 8-Br-cAMP and 1 µM MPA for 24 h or 48 h	PFOS decreased decidualization markers in a concentration-dependent-way. PFOS attenuates cortisol-induced *PRL* and *IGFBP1* increase. PFOS attenuates cortisone-induced anti-inflammatory response.	(26)
Telomerase-immortalized human endometrial stromal cells (THESC, ATCC CRL-4003) (n=3)	OP and NP 5, 10, 15, 20, 25, or 30 µM. Then 5 µM and 10 µM OP, and 5 µM NP were used 12 days	0.5 mM db-cAMP and 1 µM MPA for 12 days	OP (from 20 µM) and NP (from 15 µM) reduced cell viability, but not for lower concentrations. Decrease in *LEFTY2* and *FOXO1* by OP and NP. PRL and IGFBP-1 expression decreased at day 1 of decidualization with 10 µM OP and 5 µM NP. Effects on secreted decidualization markers at later days.	(27)
Endometrial biopsies from women receiving infertility treatments (n=15)	1 nM to 10 µM BPA, BPF, and BPS 9 days	0.5 mM 8-Br-cAMP, 10 nM E2 and 1 µM P4 for 9 days	No effect on PRL and IGFBP-1 expression. 10 µM BPA or 10 nM and 10 µM BPF, not BPS, increased spheroid invasion area in indirect co-culture. 10 µM BPA or BPF, but not BPS increased spheroid outgrow in direct co-culture. 10 µM BPA increased *LIF* expression and 10 µM BPA and BPF affected anti-invasion molecules.	(28)
Endometrial biopsies from hysterectomies early state cervical cancer, proliferative state and HESC (cell line) (n=6)	BPA 10 pM, 100 pM, 1 nM, 10 nM, 100 nM, 1 µM, 10 µM. 10 µM to study ER 48 h or 6 days	1 µM MPA, and 0.5 mM cAMP for 6 days	Morphological change impaired by 10 nM and 10 µM BPA. Dose-dependent decrease in *PRL*, *IGFBP1* and *HOXA10* mRNA expression. BPA affects methyltransferases dose dependently. BPA decreases histone methylation of *HOXA10, PRL* and *IGFBP-1* promoter region. BPA down regulates MLL1 protein expression and increased EZH2 protein expression through ER.	(29)
Endometrial biopsies from hysterectomy, benign reason (n=8)	BPA 5, 25, 50 and 100 µM 48 h	0.5 mM 8-Br-cAMP 96 h	5 µM and 100 µM BPA decreased proliferation. High doses affect mRNA and protein expression of IGFBP-1 and mRNA of steroid enzymes.	(30)
Endometrial biopsies from hysterectomy or hysteroscopy, proliferative state (n=6)	BPA (1 pM - 1 µM) 24 h	3 days E2 100 nM, then E2+P4 100 nM and 10 µM for 12 days	No effect on proliferation. Reduction of PRL secretion at 1 µM. No effect on IGFBP-1 secretion. Secretion of MIF stimulated.	(31)

(Continued)

TABLE 1 Continued

Origin of cells	EDC exposure (concentrations, time)	Decidualization protocol	Key results	Reference
			Effect on mRNA/protein levels of different hormone receptors.	
Decidua parietalis of the placental membrane (n=4)	BPA 1 ng/mL to 20 µg/mL 8 days	E2 36 nM, MPA 1 µM and db-cAMP 0.5 mM for 8 days	20 µg reduces *PRL* mRNA expression. 20 µg reduces cell proliferation. 20 µg reduces *ESR1* and *PGR* expression. 20 µg reduces cell cycle gene *CCND2* expression.	(32)
Endometrial biopsies from patients receiving IVF treatment (n=4)	Mancozeb 3 µg/mL 9 days	0.5 mM cAMP, 10 nM 17β-E2 and 1 µM P4 for 9 days	No effect on *PRL* and *IGFBP1* gene expression. Effect on morphology change.	(33)

BPA, Bisphenol A; BPF, Bisphenol F; BPS, Bisphenol S; cAMP, Cyclic adenosine monophosphate; CCND2, Cyclin D2; E2, Estrogen; ER/ESR1, Estrogen receptor alpha; EZH2, Enhancer of Zeste Homolog 2; FOXO1, Forkhead Box Protein O1; HOXA10, Homeobox A10; IGFBP-1/IGFBP1, Insulin-like Growth Factor Binding Protein 1; IVF, In Vitro Fertilisation; LEFTY2, Left-Right Determination Factor 2; LIF, Leukemia Inhibitor Factor; MIF, Macrophage Migration Inhibitory Factor; MLL1, Mixed-Lineage Leukemia 1; MPA, Medroxyprogesterone acetate; NP, Nonylphenol; OP, Octylphenol; P4, Progesterone; PFOS, Perfluorooctane sulfonic acid; PGR, Progesterone Receptor; PRL/PRL, prolactin; TCL, Triclosan.

Data processing was done in agreement with relevant guidelines (the Swedish data protection law PUL and the General Data Protection Regulation GDPR).

For eSC isolation, the endometrial tissue was placed in a 50 mL tube with isolation medium and isolated as previously described with some adjustments (37–39). Tissue was washed three times in isolation media and cut in smaller pieces using a scalpel. Tissue digestion was done by a mixture of 5 mg/mL collagenase type IA (Sigma Aldrich, Germany) and 0.1 mg/mL DNase I (Roche, Sigma Aldrich, Germany) in isolation media for 1 h at 37°C on a rocking table. After enzymatic tissue digestion, the tube was left to stand for 10 min at room temperature (RT) for sedimentation. The supernatant with eSCs was filtered through a 40 µm cell strainer into a 50 mL collection tube. Sedimentation and filtering were repeated three times. Thereafter, cells were pelleted by centrifugation at 200 × *g* RT.

The cells were then resuspended in 1 mL of DMEM/F12 with phenol red (Gibco, Thermofisher, USA) containing 10% fetal bovine serum (FBS; Thermofisher, USA), 4% Amniomax C100 (Gibco, Thermofisher, USA), 0.2% glutamine (Gibco, Thermofisher, USA) and 0.2% penicillin-streptomycin (growth medium). Erythrocytes were lysed using 90% ACK Lysing Buffer (Gibco, Thermofisher, USA) with 10% growth medium. The eSCs were pelleted by centrifugation (200 x *g* for 6 minutes RT) and cultured in growth medium in a humidified incubator at 37°C with 5% CO_2 in air.

After reaching ca 80% confluency, cells were detached with trypsin-EDTA (Gibco, Thermofisher, USA) pelleted and resuspended in recovery cell culture freezing medium (Thermofisher, USA) to freeze the cells. MR Frosty (VWR, Avantor, USA) was used with 2-propanol stored at 4°C to achieve a -1°C/min rate of cooling. Cells were slowly frozen down to -80°C overnight and then stored in liquid nitrogen until used.

Characterization of eSCs

Purity of the eSC suspensions was characterized by immunostaining for stromal and epithelial cell markers using the UltraVision One Detection System HRP Polymer kit (Fisher Scientific TL-060-HLJ) for a chromogenic reaction. The primary antibody anti-vimentin clone V9 (DAKO, Agilent, USA) at 1:250 dilution was used to stain stromal cells, while the primary antibody anti-cytokeratin-8/18 clone Zym5.2 (Thermofisher, USA) at 1:100 dilution was used to stain epithelial cells. Mouse IgG1 (Dako, Agilent, USA) at 1:100 dilution served as a negative control. 100 000 cells/well were plated in an 8-well chamber slide and incubated overnight. The cells were fixed with 4% buffered formaldehyde solution and permeabilized with cold ethanol at -20°C. Hydrogen peroxide block was performed with 0.3% H_2O_2 in methanol.

TABLE 2 Characteristics of tissue donors.

Donor	Age	Fertility	Indication	BMI (kg/m^2)	Hormone treatment	Smoker	Gravidity	Parity
eSC4	28	unknown	Pelvic pain, especially during menstruation	18	No	No	0	0
eSC5	41	yes	Pelvic pain, especially around and at menstruation	24	No	No	4	3
eSC8	26	yes	Pelvic pain, especially around and at menstruation	26	No	No	2	2
eSC12	41	yes	Menorrhagia and menstrual pain	29	No	No	4	1

Immunostaining was done according to manufacturer's protocol and the results were recorded with light microscopy.

Preparation of EDC stocks

HCB, p,p'-DDE, and PCB180 (Sigma-Aldrich, Cat Nos. 45522, 35487, 35495) were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Cat No. D8418) to 1-5 mM concentrations by heating in a Thermoblock at 65°C for 15-60 min with intermittent vortexing. Thereafter, the chemicals were sonicated in a water bath for 15-60 min. PCB170 (Sigma-Aldrich, Cat No. 34107), PFOS (Sigma-Aldrich, Cat No. EHERC15987120), PFOA (Sigma-Aldrich, Cat No. 171468) and BPA (Sigma-Aldrich, Cat No. 239658) were dissolved in DMSO to the 1-5 mM concentrations by vortexing. Prof. Christian Lindh (Division of Occupational and Environmental Medicine, Lund University, Sweden) provided 1 M solutions of BPF and MEHP in DMSO. All stock solutions were diluted to 1 mM nominal concentration using DMSO prior to use in cell culture experiments. The stocks were stored in borosilicate glass vials. The persistent chemical stocks (HCB, p,p'-DDE, PCB180, PCB170, PFOS and PFOA) were stored at 4°C and other chemicals (BPA, BPF and MEHP) at -20°C. For mass-spectrometric validation of the concentrations, the DMSO stocks were diluted in starvation medium to achieve 2 µM nominal concentrations. Concentrations of HCB, p,p'-DDE, PCB170 and PCB180 in the DMSO stocks were validated using gas chromatography – tandem mass spectrometry (GC-MS/MS) by following a previously published method (40). MEHP, BPF, BPA, PFOS and PFOA concentrations were directly analysed from the DMSO stocks without any pretreatment by previously published liquid chromatography tandem mass spectrometry (LC-MS/MS) methods (40–42). Data of these validation analyses are shown in Supplement (Table S1).

Treatment and lysis of eSCs

Prior to the *in vitro* decidualization studies, isolated eSCs were cultured for 2 passages in assay medium: Dulbecco's Modified Eagle's medium (DMEM)/Ham's F12 medium without phenol red (Sigma-Aldrich, Steinheim, Germany) supplemented with 10% charcoal-purified fetal bovine serum (ccFBS), 2 mM L-glutamine (both from Sigma-Aldrich; Steinheim, Germany) and a mixture of penicillin (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (0.25 µg/mL; from Capricorn, Ebsdorfergrund, Germany). After reaching confluency, the cells were seeded onto 6-well plates with the density of 100 000 – 170 000 cells per well; after 1-3 days, the assay medium was replaced with starvation medium (containing the same components as the assay medium, but 2% ccFBS), and after additional 24 h, treatment was started. Decidualization mixture (DC-mix) hormones and chemicals were added to the starvation medium to obtain the following final concentrations: 10 nM β-estradiol (E2); 100 nM progesterone (P4); 200 µM 8-bromoadenosine 3′,5′-cyclic monophosphate sodium salt (8-Br-cAMP); and 100 µM 3-isobutyl-1-methylxanthine (IBMX). All DC-mix components were from Sigma-Aldrich (Steinheim, Germany); the corresponding solutions were prepared in cell culture grade DMSO (AppliChem; Darmstadt, Germany) as 1000-fold concentrated stocks (1 000x), and stored at -20°C. The EDCs were added at 1 µM final concentration. By volume, the final concentration of the vehicle (DMSO) in the treatment mixtures was as follows: 0.1% in case of E2 control, 0.4% in case of DC-mixture, and 0.5% in case of DC-mixture + any EDC. The *in vitro* decidualization protocol lasted for a total of 9 days; the treatment mixtures were replaced every 72 h (full medium change).

Spent culture media were collected and stored at -80°C. At the end of the 9-day treatment, the cells were rinsed with phosphate-buffered saline (PBS; from Sigma-Aldrich, Steinheim, Germany) and lysed as previously reported (34). The lysis buffer components were from the following sources: HEPES and NaCl – Calbiochem (Darmstadt, Germany); EDTA – Scharlau (Barcelona, Spain); Triton X-100 – Ferak (Berlin, Germany); phenylmethylsulfonyl fluoride (PMSF) – AppliChem (Darmstadt, Germany); cOmplete™ protease inhibitor cocktail – Roche (Basel, Switzerland). After 1 h lysis on ice, the membranes were pelleted by centrifugation, and the supernatants were collected.

Assessment of viability

Cell viability and proliferation were estimated by quantifying total protein concentration in lysates and by measuring the activity of the viability marker CK2 at the end of the 9-day decidualization assay. The total protein concentration in cell lysates was measured using Bradford assay utilizing Pierce™ Coomassie Plus (Bradford) Assay Reagent (Thermo Fischer Scientific; Rockford, IL, USA) according to the manufacturer's instructions. The bovine serum albumin (BSA) dilutions (2 - 128 µg/mL) used for calibration and PBS (supplemented with Ca^{2+}, Mg^{2+}) used as a negative control for the Bradford assay were from Sigma-Aldrich (Steinheim, Germany). The absorbance was measured with PHERAstar multi-mode reader (BMG Labtech; Ortenberg, Germany) with the following parameters: filter block 608A (590 nm), 20 flashes per well, focal height 10.5 mm, optical pathway correction for volume of 150 µL.

The total protein concentration in each sample was then normalized to the same level between the samples lysed on the same day using the kinase assay buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 0.005% Triton X-100, 0.5 mg/mL BSA, 5 mM dithiothreitol). For measuring activity of CK2, the previously described protocol was used (34). Briefly, ARC-1530 probe (final

total concentration of 2 nM) was added to the lysate dilutions (total protein concentration of 200-400 µg/mL). ARC-1530 was synthesized as reported previously (43). Following 60 min incubation, the photoluminescence of the probe was measured with PHERAstar multi-mode reader (BMG Labtech; Ortenberg, Germany) using the following parameters: time-delayed photoluminescence, excitation 337 (300–360) nm, emission 590 (50) nm, 200 flashes per well, integration start 60 µs, integration time 400 µs. Next, the selective competitive CX-4945 (from Synkinase, Shanghai, China) was added (final total concentration of 2-4 µM); after 30-60 min incubation to achieve equilibrium, photoluminescence was measured again.

Measurement of decidualization markers

Decidualization was measured by quantification of PRL in spent culture media samples (from the last 72 h of the *in vitro* treatment) and assessment of kinase activities in cell lysates following the 9-day *in vitro* decidualization as described (34). In media samples, the total protein concentration was measured as described above. Then, PRL concentration was measured using human PRL ELISA kit (Cayman Chemical; Ann Arbor, MI, USA); the absorbance readings at 450 nm were measured using Cytation 5 multi-mode reader (Biotek; Winooski, VT, USA). Finally, the PRL concentration calculated from the calibration curve was normalized to the total protein concentration in the same media aliquot.

For spent culture media samples collected in experiments with p,p'-DDE and HCB-containing mixtures, levels of the IGFBP-1 were also assessed for the non-decidualized control cells, decidualized cells (9-day treatment with DC-mix), and cells treated for 9 days with DC-mix supplemented with 1 µM p,p'-DDE or HCB. IGFBP-1 concentration was measured using human IGFBP1 ELISA kit (Invitrogen; Waltham, MA, USA); the absorbance readings at 450 nm were measured using Cytation 5 multi-mode reader (Biotek; Winooski, VT, USA). Again, the IGFBP-1 concentration calculated from the calibration curve was normalized to the total protein concentration in the same media aliquot.

The activities of protein kinases PKAc, ROCK, and Akt/PKB were assessed using a competitive photoluminescent displacement assay based on kinase-responsive probes as described (34). After the lysis of cells and normalization of the total protein concentration (measured by Bradford as described above) to the same level between the samples lysed on the same day, ARC-1139 probe (final total concentration of 2 nM) was added to the lysate dilutions (total protein concentration of 200-400 µg/mL); ARC-1139 was synthesized as reported previously (44). Following 60 min incubation, the photoluminescence of the probe was measured with PHERAstar multi-mode reader (BMG Labtech; Ortenberg, Germany) using the following parameters: time-delayed photoluminescence, excitation 337 (300–360) nm, emission 675 (50) nm, 100 flashes per well, integration start 60 µs, and integration time 400 µs. Next, the selective competitive kinase inhibitors H89 (PKAc), Y-27632 (ROCK) or GSK690693 (Akt/PKB) were added (final total concentrations of 2-4 µM, 2-4 µM and 0.2-0.4 µM, respectively); after 30-60 min incubation to achieve equilibrium, photoluminescence was measured again. Protein kinase inhibitors were obtained from the following sources: H89 – Biaffin (Kassel, Germany); Y-27632 – Cayman Chemical (Ann Arbor, MI, USA); GSK690693 – Tocris (Bristol, UK).

Effects of EDCs and control compounds on kinases in cell-free conditions

In order to address whether the EDCs might directly bind the protein kinases thereby affecting their activities, cell-free binding assays were carried out with recombinant PKAcα (human full-length protein; Biaffin), ROCK2 (human His_6-tagged protein, amino acids 11-552; Millipore), Akt3/PKBγ (human His_6-tagged S472D mutant protein, amino acids 117-C-terminus; Millipore), and CK2α (human protein, amino acids 1-335; a kind gift from Prof. Karsten Niefind, University of Cologne) according to the previously established protocols (43–45). Briefly, solutions of EDCs or control inhibitors in kinase assay buffer were prepared and a complex consisting of a kinase and a fluorescent or photoluminescent probe was added; the signals were measured following 30-60 min incubation (Table S2). In case of PKAcα, two different probes were used to ensure the sufficient dynamic range of assay. The final total concentrations of EDC in the reaction mixtures were 1 µM.

The selectivity of the chosen inhibitors within the set of tested kinases was confirmed using a similar format, yet the final concentrations of the probes (2 nM) were chosen to mimic the conditions used in the lysate assays. The final total concentrations of active kinases in the assay mixture were equal to 3 nM. For assessment of the dose-response profile, the dilution series of displacing compounds (3-fold dilutions starting from 20 µM final concentration) were tested (Figure S1). Two independent experiments were carried out.

Effect of EDCs on ELISA and Bradford assay components

To assess whether EDCs alone have impact on the measurement of PRL, elevated concentrations of EDCs (5 µM in the final assay mixture) were individually spiked into the 100 ng/mL PRL standard included in the assay kit, and the ELISA was further carried out according to the manufacturer's instructions. As a control, 100 ng/mL PRL standard spiked with 0.5% DMSO was used. The experiment was performed once in duplicates.

To assess whether EDCs alone have impact on the measurement of the total protein content, elevated concentrations of EDCs (5 μM in the final assay mixture) were individually spiked into the PBS buffer, and two-fold dilution series of a standard calibration protein BSA into the spiked buffers were made. As a control, analogous dilution of BSA was carried out in the non-spiked PBS. The Bradford assay was subsequently carried out according to the manufacturer's instructions. The experiment was performed once in duplicates.

Statistical analysis

For general data analysis, GraphPad Prism 9.2.0 (San Diego, CA, USA) and Excel 2016 (Microsoft Office 365; Redmond, WA, USA) were used.

In case of PRL ELISA, the normalized PRL content (in ng/mg total protein) was pooled for samples treated in the same conditions in each independent experiment (please note that several vials of cells were available for samples from each patient, so more than one independent experiment could be made using eSCs from one patient: N = 5 for eSC4; N = 5 for eSC5; N = 7 for eSC8; N = 4 for eSC12; all measurements performed in duplicates). For assessment of effect of EDCs on decidualization, additional normalization was carried out by setting the normalized PRL content in the DC-mix to 100% in each independent experiment, prior to pooling of data. The statistical significance of differences between the DC-mix *versus* other groups was established for pooled data by one-way ANOVA with Dunnett's test for multiple comparisons. For assessment of EDC effect on the ELISA kit components, the apparent concentrations of spiked standards were calculated using the calibration curve. The statistical significance of difference between the 0.5% DMSO control *versus* other groups was established by one-way ANOVA with Dunnett's test for multiple comparisons.

In case of IGFBP-1 ELISA, the experiment was carried out using samples from a total of four independent experiments: a single experiment with eSC4, a single experiment with eSC5, and two experiments with eSC8. Normalization of data was carried out as in case of PRL ELISA. The statistical significance of differences between the DC-mix *versus* other groups was established for pooled data by one-way ANOVA with Dunnett's test for multiple comparisons.

In case of protein kinase activity assay in lysates, for each target kinase the difference of probe signal prior to and after addition of competing inhibitor was calculated. For assessment of effect of EDCs on decidualization, the values calculated for each target kinase in different lysates within a single independent experiment were normalized to the value calculated for lysate from eSCs treated with the DC-mix (activity set to 100%). The results of all measurements were then pooled (N = 5 for eSC4; N = 5 for eSC5; N = 6 for eSC8; N = 4 for eSC12; all measurements performed in duplicates). The statistical significance of differences between the DC-mix *versus* other groups was established for pooled data by one-way ANOVA with Dunnett's test for multiple comparisons.

In case of recombinant protein kinase activity assay, the probe signal measured for the inhibitor-containing mixtures was normalized to the signal of kinase-probe complex (activity = 100%) and free probe (activity = 0%). The results of all measurements were then pooled (N = 2 for each kinase; all measurements performed in duplicates). The statistical significance of difference between the kinase-probe complex *versus* inhibitor-containing mixtures was established for pooled data by one-way ANOVA with Dunnett's test for multiple comparisons. Data is presented as mean with standard error of mean (SEM). P-values <0.05 were considered significant and are marked in the figures as follows: *P<0.05, **P<0.01, ***P<0.001.

Results

Study design

Primary uterine eSCs were used to study the effects of EDCs on decidualization *in vitro* using kinase activities and PRL secretion as endpoints (Figure 1). We selected nine EDCs based on their ubiquitous presence in biomonitoring samples from reproductive age women (46), as well as their significant associations to lower fertility of possible endometrial origin (longer time-to-pregnancy, miscarriage, pre-eclampsia) (17–19, 35). Some of the chosen EDCs were non-persistent (BPA, BPF, MEHP) and others persistent with lipophilic (p,p'-DDE, HCB, PCB170, PCB180) or amphiphilic (PFOS, PFOA) properties. EDC stocks were prepared in DMSO, and the concentrations were validated by mass spectrometry (Table S1). All stocks were confirmed to have concentrations close to expected (80 – 130%) except for the two PCBs that had markedly lower concentrations than anticipated: PCB170 was 20% and PCB180 was 45% of the expected concentration (Table S1). PCBs are notoriously difficult to dissolve, even by heating, vortexing and sonication. Here, nominal concentrations will be used when results are presented.

Endometrial cells were isolated from hysterectomized uteri in proliferative stage from non-smoking women aged 26 to 41 years not using hormonal contraception. They underwent hysterectomies due to pelvic pain and menorrhagia (Table 2). The purity of the endometrial stromal cells was studied by immunostaining and found to be satisfactory (Figure S2). The decidualization protocol was optimized in our previous study and consisted of a nine-day exposure of the cells to the DC-mix composed of 8-Br-cAMP, P4, E2 and IBMX (34). 8-Br-cAMP has been used in multiple *in vitro* decidualization protocols because it activates the cAMP/PKA pathway that is physiologically presumably initiated by prostaglandin E2

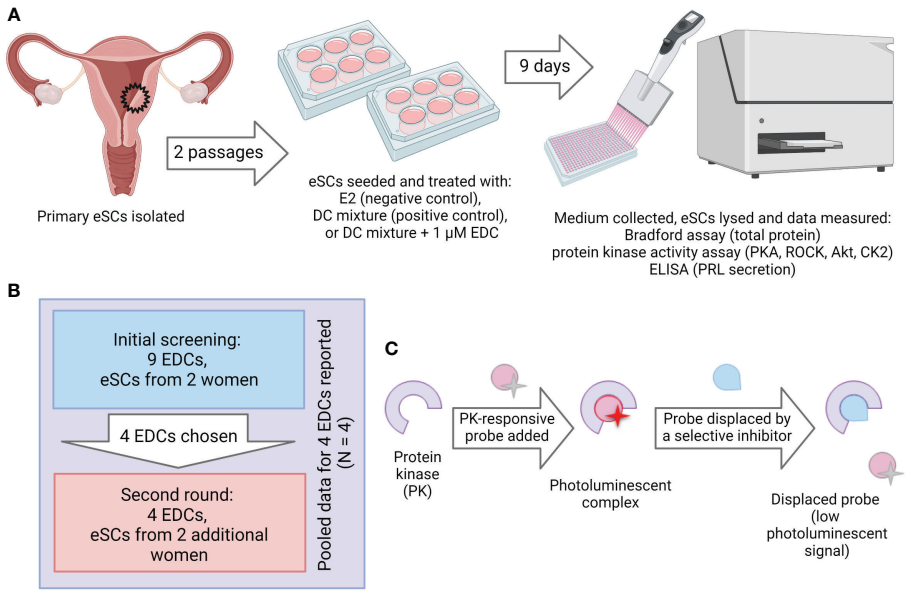

FIGURE 1
Experimental setup. **(A)** Workflow of the decidualization disruption assay. Human endometrial tissue samples were digested to single-cell suspension and endometrial stromal cells (eSCs) were isolated. The eSCs were cultured for two passages before they were cryopreserved for later use in experiments. To induce decidualization, the cells were exposed to decidualization mixture (DC-mix) composed of estradiol (E2), progesterone (P4), 8-Br-cAMP and IBMX for nine days. To test the effect of EDCs on decidualization, the cells were co-exposed to DC-mix and 1 µM of EDCs. Decidualization was assessed by measuring prolactin (PRL) secretion in culture media and activation of protein kinases PKA, ROCK and Akt in lysates of treated cells. Effects on cell proliferation and viability were estimated by the total protein concentration and activity of the viability marker CK2 in lysates of treated cells. **(B)** Initial screening was performed with nine EDCs on eSCs from two different women. From these nine chemicals, four were chosen for the exposure studies with ESCs from two additional women. **(C)** The principle of the protein kinase activity assay in lysates. Binding of a generic kinase probe to the mixture of target kinases present in lysate samples produces long-lifetime photoluminescent signal. Activity of each protein kinase of interest is subsequently established by displacing the probe with a competing selective inhibitor (H89 in case of PKA, Y-27632 in case of ROCK, GSK-690693 in case of Akt, and CX-4945 in case of CK2).

(PGE2) (47) or relaxin receptor-mediated signaling (48). However, we have shown that 8-Br-cAMP alone is not sufficient but requires the presence of P4 to trigger the whole spectrum of changes occurring during the transition from the proliferative to the secretory endometrium (34). Additionally, we added IBMX to prevent the PDE-catalyzed hydrolysis of 8-Br-cAMP, and E2 to reflect the constant presence of the hormone in women. The duration of the decidualization protocol was optimized in our previous studies where we demonstrated that shorter treatment (4 days) only resulted in a partial development of the decidualization-characteristic fingerprint of markers, whereas the prolonged 9-day protocol enabled us to achieve higher measurement windows for all markers (34).

EDCs are not cytotoxic

To verify that the selected concentrations are not cytotoxic to the cells, cell proliferation and viability were estimated by measuring protein concentration and activity of the cell proliferation kinase CK2 in cell lysates after the nine-day culture. CK2 is a well-characterized pro-survival kinase that sustains viability of cells by phosphorylating the major chaperone machinery represented by the Cdc37-Hsp90 complex (49); the reduced activity of CK2 thus mirrors reduction in number of viable cells in the sample. Compared to DC-mix, none of the EDCs significantly reduced protein content or CK2 activity, suggesting that the exposures were not cytotoxic (Figure S3). Only MEHP increased protein content, which may indicate increased proliferation.

EDCs disrupt markers of decidualization *in vitro*

In the first step, the nine EDCs were screened for effects using eSCs from two women. The cells were treated with DC-mix for 9 days with or without EDCs present at 1 µM concentrations. Media were changed every three days, and

effects on kinases and PRL were measured in lysates or in media aliquots collected on the last day of culture. Expectedly, the DC-mix significantly increased PRL secretion and activity of all kinases compared to E2 treatment alone (Figure 2). In all experiments, DC-mix was thus used as positive control and set to 100%, and 10 nM E2 alone was used as negative control. Our previous experiments have shown that E2 and DMSO are comparable negative controls in this system (34) and we chose to use E2 here to reflect the constant presence of this hormone in the female reproductive system during fertile years.

When we compared the signal from the positive control (DC-mix) to the experimental groups (DC-mix+EDC) we found that some EDCs significantly reduced markers of decidualization. BPA, p,p'-DDE, HCB and PFOA significantly reduced the secretion of the classical decidualization marker PRL in culture medium to 70 ± 8%, 59 ± 4%, 72 ± 2% and 84 ± 6% (Figure 2). These effects were associated with significantly reduced kinase activities although not consistently: BPA caused reduced Akt activity (86 ± 3%); p,p'-DDE caused reduced PKAc (66 ± 6%), ROCK (71 ± 4%) and Akt (75 ± 4%) activities; HCB caused reduced PKA (81 ± 1%) and ROCK (83 ± 3%) activities; and PFOA did not significantly affect activity of any kinases. Interestingly, PFOS and PCB170 also caused reduced kinase activities although these EDCs did not have statistically significant effects on PRL secretion. However, it can be observed that PFOS exposure also reduced PRL secretion, but the effect did not reach significance at $p<0.05$ ($p=0.052$). Finally, BPF and PCB180 did not have any significant effects on the kinase activities or PRL secretion. Collectively, EDCs representing different physicochemical properties significantly reduced hormone-stimulated decidualization process *in vitro*. Reduced PRL secretion, a hallmark of decidualization residing downstream of the cAMP/PKA signaling (50), was accompanied with reduced kinase activities although certain variation was observed, suggesting that multiple upstream pathways can lead to disrupted PRL secretion.

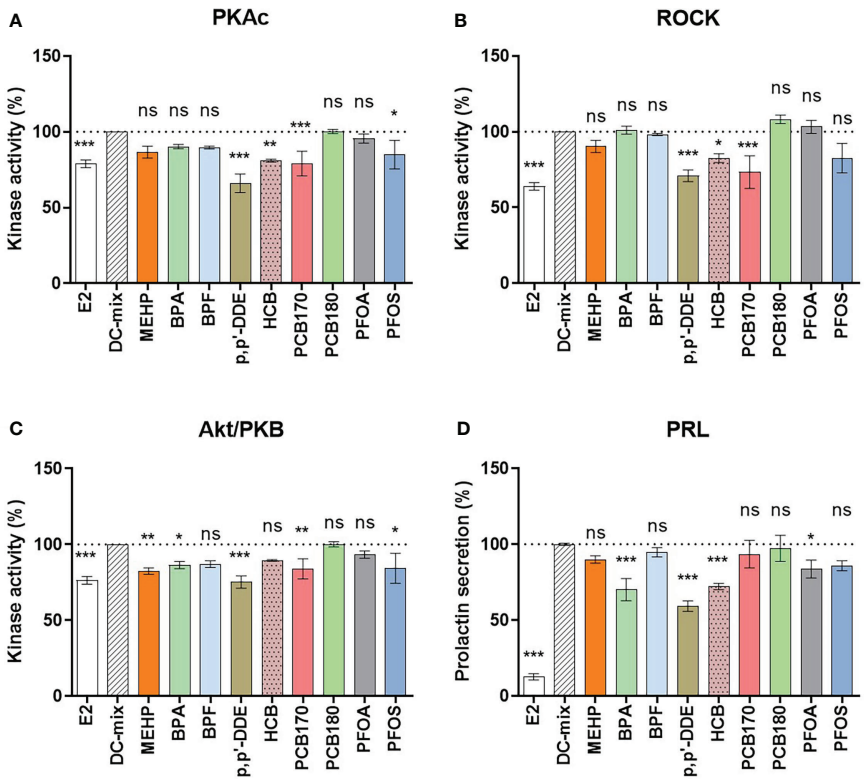

FIGURE 2
Screening of nine EDCs for effects on decidualization. The eSCs of two women (N=2) were exposed to the decidualization-inducing mixture (DC-mix) in the absence or presence of 9 different EDCs (1 µM) for 9 days, and 10 nM E2 was used as a negative control. Altogether 10 independent assays were carried out (n=10). Protein kinase activity was assessed in lysates (normalized to protein content) using a competitive photoluminescent probe displacement assay, and calculated as percentage of DC-mix (100%) for **(A)** PKAc, **(B)** ROCK and **(C)** Akt/PKB. **(D)** Secreted PRL was measured in spent media representing the last 72 h of the in vitro treatment using a human PRL ELISA kit. Values are expressed as mean percentage of DC-mix alone ± SEM (N = 2, n = 10). Significance is calculated by one-way ANOVA with Dunnett test for multiple comparisons (95% CI) relative to DC-mix and indicated as *P<0.05, **P<0.01 and ***P<0.001 and ns, not significant. The dotted line corresponds to 100%.

To confirm the observed trends, we additionally assessed whether secretion of yet another decidualization biomarker, IGFBP-1, was affected by the presence of p,p'-DDE or HCB in the treatment mixture (Figure S4). These analyses confirmed a statistically significant reduction of IGFBP-1 secretion in the presence of EDCs. Normalized secretion of 81 ± 5% ($p<0.01$ relative to DC-mix) and 84 ± 8% ($p<0.05$ relative to DC-mix) was measured for the cells treated with mixtures containing p,p'-DDE and HCB, respectively.

EDCs do not directly inhibit kinases

We also wanted to study whether the EDCs at 1 µM concentration might directly interfere with the activity of the kinases. For this, cell-free kinase binding assay was performed using recombinant protein kinases. As controls, selective inhibitors of the corresponding protein kinases were used (H-89 in case of PKACα, Y27632 in case of ROCK2, GSK690693 in case of Akt3, and CX-4945 in case of CK2α; the selectivity of the corresponding inhibitors was validated by the dose-response studies of each compound with all four protein kinases of interest, see Figure S1). Expectedly, the selective inhibitors for all four kinases strongly reduced kinase residual activity (Figure 3). None of the EDCs affected activity of recombinant PKACα (Figure 3A). Interestingly, activities of the other kinases were slightly upregulated by the EDCs. Compared to the control set to 100%, the activity of recombinant ROCK2 was increased by several EDCs (BPF 107 ± 2%; p,p'-DDE 108 ± 1%; HCB 108 ± 0%; PCB170 109 ± 1%; PFOA 108 ± 1%; PFOS 111 ± 2%), activity of recombinant Akt3 was increased by MEHP (118 ± 2%), p,p'-DDE (119 ± 4%), HCB (118 ± 4%), PCB170 (124 ± 5%) and PFOS (124 ± 3%), and that of recombinant CK2α increased by MEHP (110 ± 2%), HCB (110 ± 3%), PCB180 (109 ± 3%) and PFOS (112 ± 1%). This could be explained by reduction of non-specific binding of the kinase or the photoluminescent probe in the presence of EDCs containing hydrophobic moieties. BPA did not affect any of the recombinant protein kinases. Overall, all observed differences showed increased activity, not decreased, which suggests that the effects of EDC on reduced kinase

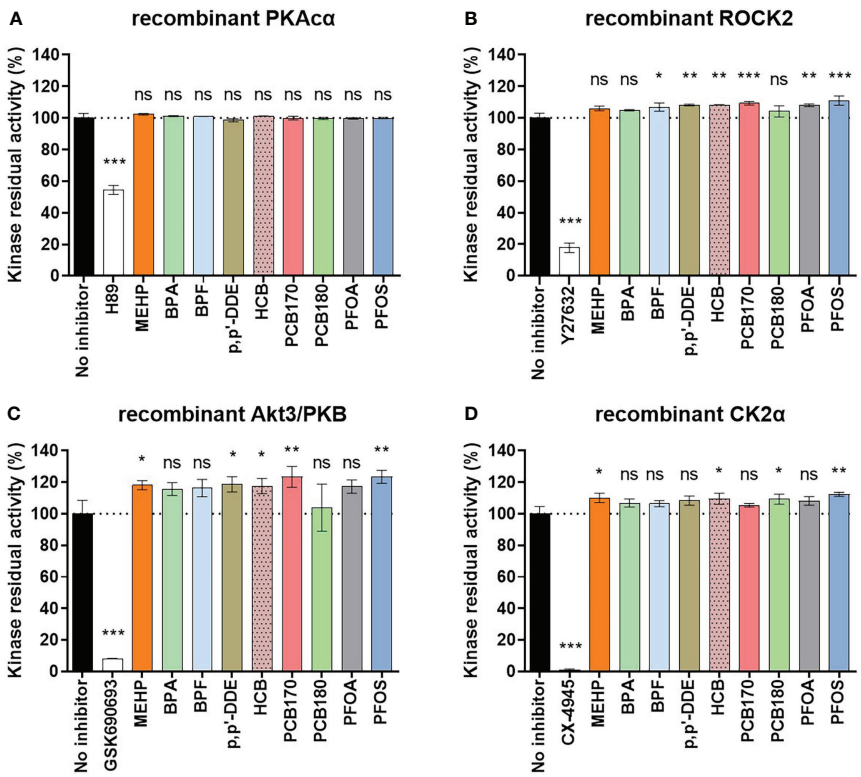

FIGURE 3
Effect of EDCs (1 µM) and selective control inhibitors (1 µM) on activities of recombinant protein kinases to assess whether EDCs have a direct effect on the protein kinases. Recombinant protein kinases **(A)** full-length PKACα; **(B)** His_6-ROCK2 (amino acids 11-552); **(C)** His_6-Akt3 (S472D mutant, amino acids 117-end); **(D)** CK2α (amino acids 1-335) were used. The graph shows the averaged data of the three measurements as mean ± SEM (n = 2 for each kinase, performed in duplicates). Significance is calculated by one-way ANOVA with Dunnett test for multiple comparisons (95% CI) relative to no inhibitor and indicated as *P<0.05; **P<0.01 ***P<0.001 and ns, not significant. The dotted line corresponds to 100%.

activities in eSC lysates do not originate from direct inhibition of the kinases but rather from overall impact on hormone-triggered signal transduction leading to decidualization. The decidualization marker kinases explored here (PKAc, ROCK and Akt) all require upstream activation (by cAMP, Rho and PI3K/PDK1, respectively) which adds further levels of complexity into the model of decidualization disruption by EDCs. The exact molecular targets of EDCs need to be explored in future studies, for instance by utilizing large-scale profiling techniques such as proteomics, transcriptomics, or metabolomics. Knowledge of precise mechanisms could help to develop novel assays for decidualization that are not dependent on primary patient materials.

Validation of BPA, p,p'-DDE, HCB and PFOS effects

Additional replicate experiments were carried out with BPA, p,p'-DDE, HCB and PFOS using cells from two more women to validate the results. The pooled results of the experiments with samples from four women suggest that these chemicals significantly disrupt hormone-induced decidualization *in vitro* (Figure 4). Even though there was variation in responses between the samples (Figure S5), statistical analyses showed that all four EDCs significantly reduced secretion of PRL into culture medium (p,p'-DDE 75 ± 6%, HCB 76 ± 3%, BPA 80 ± 5%, PFOS 90 ± 3% compared to DC-mix set to 100%). Considering markers of cell viability and proliferation, there was a significant difference between the negative control E2 and positive control DC-mix for both CK2 activity and the total protein content (Figure 4), which was consistent with our previously reported observations regarding the reduced proliferation rate of eSCs upon treatment with DC-mix (34). Furthermore, consistent with the screening of the nine different EDCs (Figure S3), viability of the cells was not significantly affected by EDC exposure (Figure 4). The variability between samples from different women in response to hormone-stimulated decidualization *in vitro* was also noted in our previous studies utilizing primary cells (34, 51) and this, at least in part, reflects varying sensitivity of individuals to hormones (components of the DC-mix) and EDCs. The reduced PRL secretion in cell culture media was accompanied by varying degrees of reduction in kinase activities. BPA had the least effects on kinases (none significantly affected in the pooled data), p,p'-DDE caused reduced PKA activity (85 ± 6%), PFOS caused reduced PKA (87 ± 5%) and Akt (87 ± 5%) activities, and HCB affected all markers (82 ± 3% PKA; 85 ± 4% Akt; 85 ± 4% ROCK).

To exclude that the measurements would be affected by EDCs interfering with PRL or protein concentrations measurements in general, additional control experiments were carried out by spiking PRL assay standard and BSA (Bradford assay standard) with 5 µM EDCs. A slight reduction of approximately 10% in PRL by 5 µM DDE and HCB was observed (Figure S6). This can be considered as a minor effect compared to the 25% decrease in PRL secretion measured in the cell assays at the 5-fold lower concentrations of the EDCs (Figure 4). There were no effects on the Bradford assay. In conclusion, the impact of the tested EDCs on markers of decidualization of human eSCs *in vitro* is likely not due to technical reasons, but reflects disruption of hormone-stimulated cellular signal transduction underlying decidualization process. The shift in the decidualization marker 'fingerprint' pattern of the DC-mix caused by addition of 1 µM p,p'-DDE, HCB, BPA, or PFOS is summarized in Figure 5.

Discussion

There is a lack of relevant model systems for screening and characterization of chemicals that disrupt fertility in women (2, 8, 52). It is clear that elevated EDC serum levels correlate with worse female fertility in cohort studies (17–20, 35), but since correlation does not mean causation, experimental studies are needed to provide the proof for causality, to identify most sensitive target organs, and also to understand mechanisms behind their effect. Human primary eSCs can be obtained from hysterectomies, isolated, and studied in culture. Such cells are hormone-sensitive and recapitulate many aspects of endometrial biology, and have been used for example to study contraceptive effects on embryo attachment *in vitro* (37, 53–55), or even applied to EDC research (Table 1). To the best of our knowledge, our study is the first one testing a large set of EDCs, at relatively low levels, in primary cells. Although clinical primary samples are prone to substantial inter-individual variation, like observed in our studies (Figure S5), we found significant disruption of decidualization by EDCs using cells from four women. The EDC concentrations tested were higher than typically found in women or reproductive age, but so was the concentrations of steroid hormones in the DC-mix supraphysiological too. As such, the results from our model show that the process of decidualization is sensitive to EDCs of various physico-chemical properties. Future studies should test whether immortalized cell lines could be used to detect same effects as this would allow wider exploitation of the decidualization model in a broader range of chemicals. On the other hand, based on our previous experience with immortalized cell lines *vs* primary cells, the profile of markers related to the *in vitro* decidualization is substantially different, posing questions regarding the adequacy of applicability of the immortalized cell lines for such studies.

Among the markers assessed here, PKAc is a canonical player in decidualization, being activated by the increasing intracellular levels of cAMP and triggering changes in gene expression *via* CREB and other transcription factors (9, 50).

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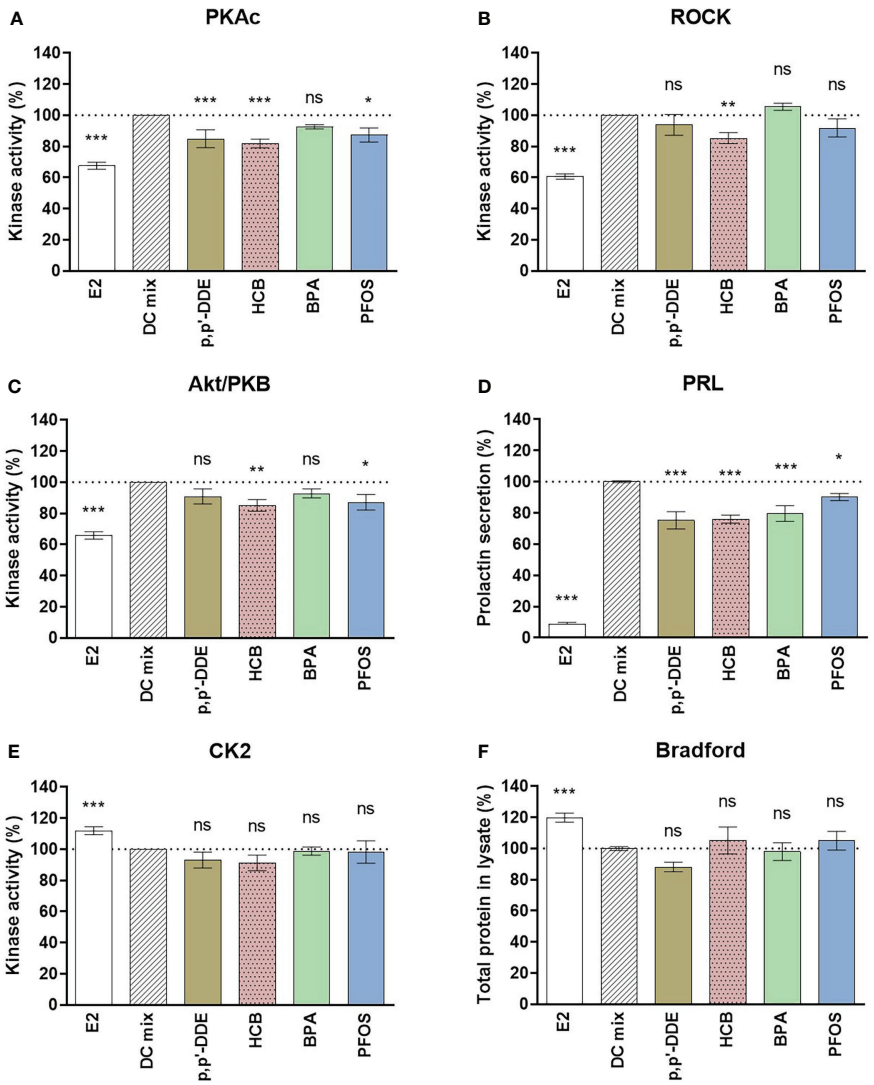

FIGURE 4
Effect of DDE, HCB, BPA and PFOS on decidualization and viability markers. The eSCs of two additional women were exposed to the decidualization-inducing mixture (DC-mix) in the absence or presence of 1 µM DDE, HCB, BPA, PFOS for 9 days. The effects of EDCs were compared to the positive control DC-mix, and 10 nM E2 was used as negative control. Total protein concentration for different treatments was normalized after lysis of the eSCs. Protein kinase activity for **(A)** PKAc, **(B)** ROCK2 and **(C)** Akt/PKB was assessed in lysates using a competitive photoluminescent probe displacement assay and calculated as percentage of DC-mix (100%). **(D)** Secreted PRL was measured in media aliquots (collected following the last 72 h of the in vitro treatment) using a human PRL ELISA kit. The impact of the exposures on viability of the endometrial stromal cells in culture during decidualization assays was assessed by the activity of the viability marker kinase CK2 in lysates **(E)** as well as evaluating the total protein concentrations in lysates at the end of the assay **(F)**. Values are expressed as mean percentage of DC-mix alone ± SEM based on cells derived from 4 women (N=4) tested in 4 to 6 independent experiments (n=4-6). Significance is calculated by one-way ANOVA with Dunnett test for multiple comparisons (95% CI) relative to DC-mix and indicated as *P<0.05, **P<0.01 and ***P<0.001 and ns, not significant. The dotted line corresponds to 100%.

The reports on the status of Akt signaling in decidualization are somewhat conflicting, as several groups have previously reported reduction in phosphorylation levels and thus activity of Akt during *in vitro* decidualization (56); our previous studies have, however, shown increase in Akt activity (34). We presume that Akt might be involved in metabolic reprogramming characteristic for the transition from proliferative to secretory endometrium; furthermore, other studies (57, 58) have also highlighted the importance of anti-apoptotic effects of Akt for the successful decidualization, embryo implantation and pregnancy onset. ROCK was identified in our previous study (34) as a novel downstream target of progesterone; in the context

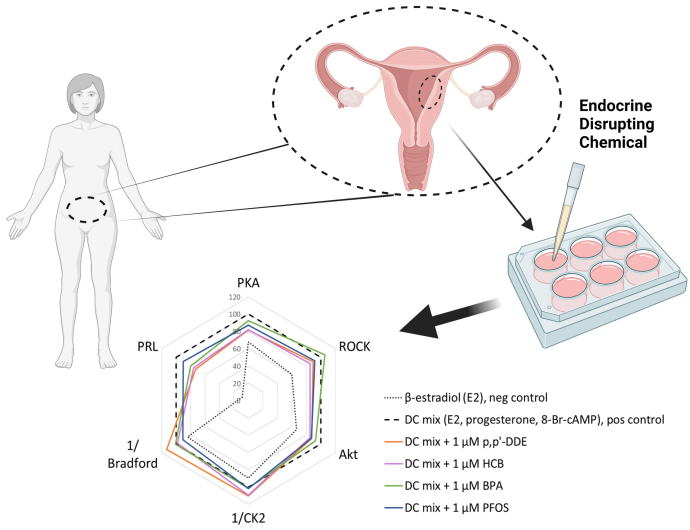

FIGURE 5
Summary of the 1 µM EDC impact on decidualization markers following in vitro treatment of ESCs. The radar plot depicts a characteristic 'fingerprint' of various decidualization- and viability-related markers measured in this study and the alterations caused by the presence of EDCs (as seen by shift of pattern related to that of the DC-mix). The y-axis shows normalized activities (%) of PKA, ROCK and Akt, normalized secretion (%) of prolactin (PRL), normalized reciprocal of CK2 activity (%) and normalized reciprocal of total protein content (indicated as Bradford) (%). In each case, 100% corresponds to the value measured for the decidualization mixture; the reciprocal values for CK2 activity and total protein content reflect the fact that these markers are reduced during decidualization. The graph summarizes data from all independent experiments performed within the study (N = 4 and n = 21 for each EDC); for better clarity, error bars are not shown.

of decidualization, ROCK is responsible for the stabilization of cytoskeleton and changes in the migratory properties of eSCs associated with the mesenchymal-epithelial transition (59).

The exact decidualization-relevant targets affected by the EDCs are beyond the scope of this study. The cell-free kinase activity measurements showed that there was no direct inhibitory effect of EDCs on the protein kinases tested. Presumably, modulation of the kinase activities by EDCs can occur on the level of upstream activation – *e.g.*, by binding of EDCs to the membrane G-protein couple estrogen receptor that mediates cAMP and Ca^{2+} signalling (60, 61). Moreover, the reduction in decidualization-related kinase markers but not PRL secretion observed in this study (Figure 2) for PFOS and PCB170 suggests that PRL expression can be regulated not only *via* PKAc, ROCK or Akt pathways, or that the kinase activity must be reduced below a certain threshold value before reduction of downstream gene expression is triggered. The complexity of the EDC-mediated effects on the level of gene expression is further augmented by the documented interactions of EDCs with the steroid receptors.

eSCs are central to embryo attachment and implantation, and disruption of their function could lead to implantation and/or placentation failure contributing to pregnancy complications and loss of pregnancy (62, 63). Our aim was to study whether decidualization can be disrupted by chemicals commonly found in reproductive age women. The concentration of EDCs chosen (1 µM) is relatively high compared to the population exposure levels (46, 64), yet lower than in several previous studies (Table 1). If safety factors are to be considered, effects of EDCs at 1 µM concentration would convert to a 1000-fold lower safe exposure levels (1 nM), which are relevant from the typical human exposure point of view. In reality, solubility issues led to a situation where the levels of PCBs in the experiment were lower than anticipated (Table S1); however, they were likely higher than the typical pM levels measured in serum of women. At these conditions, half of the studied EDCs disrupted at least one marker of decidualization *in vitro* (Figure 5), which is perhaps not surprising, as we chose them for their known associations to infertility, pregnancy loss and longer time-to-pregnancy in cohort studies (18–20, 35, 36).

A limitation of our study is that we used eSCs from four different women only. However, the most significant trends could be reliably pinpointed already at this statistical level. Regarding the decidualization protocol used here, two limitations can be emphasized. First, the measurements were only performed after 9 days of decidualization, yet we have confirmed before that for the set of monitored markers, such duration of the experiment is optimal (34). Second, we used E2 instead of DMSO as a negative control, yet it was previously shown that either DMSO or E2 are suitable treatments representing the non-decidualized cells in case of the measured set of markers (34); furthermore, we considered application of E2 important in this study, as the EDCs would have to compete with physiological levels of E2 also within the

female organism. Yet another limitation is represented by the use of protein kinase inhibitors for dissection of individual kinases (PKA, ROCK, Akt, and CK2), as the selectivity of compounds is not absolute. Still, at least from the aspect of the cross-selectivity within the set of the chosen markers (Figure S1), the selectivity of the displacing compounds is sufficient to draw the outlined conclusions.

Altogether, the results suggest that EDCs may pose a hazard to decidualization, and endometrial cells should be considered as a target of endocrine disruption. Notably, endometrium normally renews every month (65), so accumulation of more persistent EDCs in this tissue is unlikely; thus the exposure *via* the blood circulation seems more relevant. We focused on the effects of single EDC exposures. However, women are never exposed to one chemical only, but to complex mixtures of contaminants where combination effects have been shown to occur leading to adverse health consequences (66). Together with the many cohort studies (18–20, 35, 36), our *in vitro* screen suggests that EDC exposure may have causal link to miscarriage and infertility in women. Follow-up studies need to address concentration-response relationship to enable evaluation of risks. The role of EDCs in endometrial biology, implantation failure and miscarriage in women could be studied in more detail with the help of women attending infertility clinics, for example. Finally, by determining how EDCs affect fertility in endometrial samples, adjusted treatments for these women could be implemented leading to more successful pregnancies and live births in the future.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Uppsala Regional Ethical review board. The patients/participants provided their written informed consent to participate in this study.

Author contributions

DL: protein kinase assay, PRL assay, writing and checking the manuscript. NV: stromal cell isolation, literature analysis, writing and checking the manuscript. KS: cell culturing, checking the manuscript. ED: stromal cell isolation and validation, checking the manuscript. RB: Preparation of chemical stocks, checking the manuscript.. JH: Preparation of chemical stocks, checking the manuscript. JK: Chemical validation by mass-spectrometry, checking the manuscript. PR: Chemical validation by mass-spectrometry, checking the manuscript. HK: Chemical validation by mass-spectrometry, checking the manuscript. AR: PI, acquisition of funding, checking the manuscript. MO: PI, study conceptualization, acquisition of funding and checking manuscript. AS: PI, study conceptualization, acquisition of funding and checking manuscript. PD: main PI, study conceptualization, acquisition of funding, writing and checking the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary materials

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.903505/full#supplementary-material.

References

1. Persson L, Carney Almroth BM, Collins CD, Cornell S, de Wit CA, Diamond ML, et al. Outside the safe operating space of the planetary boundary for novel entities. *Environ Sci Technol* (2022) 56(3):1510–21. doi: 10.1021/acs.est.1c04158

2. Gore AC, Chappell VA, Fenton SE, Flaws JA, Nadal A, Prins GS, et al. EDC-2: The endocrine society's second scientific statement on endocrine-disrupting chemicals. *Endocrine Rev Endocrine Soc* (2015) 36:1–150. doi: 10.1210/er.2015-1010

3. La Merrill MA, Vandenberg LN, Smith MT, Goodson W, Browne P, Patisaul HB, et al. Consensus on the key characteristics of endocrine-disrupting chemicals as a basis for hazard identification. *Nat Rev Endocrinol* (2020) 16(1):45. doi: 10.1038/s41574-019-0273-8

4. Bergman Å, Heindel JJ, Jobling S, Kidd KA, Thomas Zoeller R. *State of the science of endocrine disrupting chemicals 2012 summary for decision-makers*. INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS. (Geneva, Switzerland; United Nations Environment Programme and the World Health Organization) (2013). Available at:https://apps.who.int/iris/bitstream/handle/10665/78102/WHO_HSE_PHE_IHE_2013.1_eng.pdf

5. Muncke J. Exposure to endocrine disrupting compounds *via* the food chain: Is packaging a relevant source? *Sci Total Environ* (2009) 407:4549–59. doi: 10.1016/j.scitotenv.2009.05.006

6. Parlett LE, Calafat AM, Swan SH. Women's exposure to phthalates in relation to use of personal care products. *J Expo Sci Environ Epidemiol* (2013) 23(2):197–206. doi: 10.1038/jes.2012.105

7. European Commission. *Chemicals strategy for sustainability towards a toxic-free environment*. Brussels (2020): https://ec.europa.eu/environment/pdf/chemicals/2020/10/Strategy.pdf

8. Directorate-General for Environment (European Commission) R& PAL (RPA). *Setting priorities for further development and validation of test methods and testing approaches for evaluating endocrine disruptors - publications office of the EU* (2018). Available at: https://op.europa.eu/en/publication-detail/-/publication/6b464845-4833-11e8-be1d-01aa75ed71a1/language-en.

9. Fowler DJ, Nicolaides KH, Miell JP. Insulin-like growth factor binding protein-1 (IGFBP-1): a multifunctional role in the human female reproductive tract. *Hum Reprod Update* (2000) 6(5):495–504. doi: 10.1093/humupd/6.5.495

10. Gellersen B, Brosens J. Cyclic AMP and progesterone receptor cross-talk in human endometrium: A decidualizing affair. *J Endocrinol* (2003) 178:357–72. doi: 10.1677/joe.0.1780357

11. Gonzalez M, Neufeld J, Reimann K, Wittmann S, Samalecos A, Wolf A, et al. Expansion of human trophoblastic spheroids is promoted by decidualized endometrial stromal cells and enhanced by heparin-binding epidermal growth factor-like growth factor and interleukin-1β. *Mol Hum Reprod* (2011) 17(7):421–33. doi: 10.1093/molehr/gar015

12. Teklenburg G, Salker M, Molokhia M, Lavery S, Trew G, Aojanepong T, et al. Natural selection of human embryos: Decidualizing endometrial stromal cells serve as sensors of embryo quality upon implantation. *PLoS One* (2010) 5(4): e10258. doi: 10.1371/journal.pone.0010258

13. Quenby S, Gallos ID, Dhillon-Smith RK, Podesek M, Stephenson MD, Fisher J, et al. Miscarriage matters: the epidemiological, physical, psychological, and economic costs of early pregnancy loss. *Lancet* (2021) 397(10285):1658–67. doi: 10.1016/S0140-6736(21)00682-6

14. Sapra KJ, McLain AC, Maisog JM, Sundaram R, Buck Louis GM. Successive time to pregnancy among women experiencing pregnancy loss. *Hum Reprod* (2014) 29(11):2553–9. doi: 10.1093/humrep/deu216

15. Devall AJ, Coomarasamy A. Sporadic pregnancy loss and recurrent miscarriage. *Best Pract Res Clin Obstet Gynaecol* (2020) 69:30–9. doi: 10.1016/j.bpobgyn.2020.09.002

16. Zegers-Hochschild F, Adamson GD, de Mouzon J, Ishihara O, Mansour R, Nygren K, et al. International committee for monitoring assisted reproductive technology (ICMART) and the world health organization (WHO) revised glossary of ART terminology, 2009∗. *Fertil Steril* (2009) 92(5):1520–4. doi: 10.1016/j.fertnstert.2009.09.009

17. Chiang C, Mahalingam S, Flaws JA. Environmental contaminants affecting fertility and somatic health. *Semin Reprod Med* (2017) 35(3):241–9. doi: 10.1055/s-0037-1603569

18. Zhang H, Gao F, Ben Y, Su Y. Association between phthalate exposure and risk of spontaneous pregnancy loss: A systematic review and meta-analysis. *Environ pollut* (2020) 267:115446. doi: 10.1016/j.envpol.2020.115446

19. Gao X, Ni W, Zhu S, Wu Y, Cui Y, Ma J, et al. Per- and polyfluoroalkyl substances exposure during pregnancy and adverse pregnancy and birth outcomes: A systematic review and meta-analysis. *Environ Res* (2021) 201:111632. doi: 10.1016/j.envres.2021.111632

20. Chang WH, Chou WC, Waits A, Liao KW, Kuo PL, Huang PC. Cumulative risk assessment of phthalates exposure for recurrent pregnancy loss in reproductive-aged women population using multiple hazard indices approaches. *Environ Int* (2021) 154:106657. doi: 10.1016/j.envint.2021.106657

21. Li Q, Davila J, Bagchi MK, Bagchi IC. Chronic exposure to bisphenol a impairs progesterone receptor-mediated signaling in the uterus during early pregnancy. *Recept Clin Investig* (2016) 3(3):e1369. doi: 10.14800/rci.1369

22. Kim J, Cha S, Lee MY, Hwang YJ, Yang E, Choi D, et al. Chronic and low dose exposure to nonlyphenol or Di(2-ethylhexyl) phthalate alters cell proliferation and the localization of steroid hormone receptors in uterine endometria in mice. *Dev Reprod* (2019) 23(3):263–75. doi: 10.12717/DR.2019.23.3.263

23. Ramathal CY, Bagchi IC, Taylor RN, Bagchi MK. Endometrial decidualization: Of mice and men. *Semin Reprod Med* (2010) 28:17–26. doi: 10.1055/s-0029-1242989

24. Gellersen B, Brosens JJ. Cyclic decidualization of the human endometrium in reproductive health and failure. *Endocrine Rev* (2014) 35:851–905. doi: 10.1210/er.2014-1045

25. Forte M, Mita L, Cobellis L, Merafina V, Specchio R, Rossi S, et al. Triclosan and bisphenol a affect decidualization of human endometrial stromal cells. *Mol Cell Endocrinol* (2016) 422:74–83. doi: 10.1016/j.mce.2015.11.017

26. Yang Q, Wang W, Liu C, Wang Y, Sun K. Effect of PFOS on glucocorticoid-induced changes in human decidual stromal cells in the first trimester of pregnancy. *Reprod Toxicol* (2016) 63:142–50. doi: 10.1016/j.reprotox.2016.06.003

27. Ko DS, Lee DR, Song H, Kim JH, Lim CK. Octylphenol and nonylphenol affect decidualization of human endometrial stromal cells. *Reprod Toxicol* (2019) 89:13–20. doi: 10.1016/j.reprotox.2019.06.003

28. Fan H, Jiang L, Lee YL, Wong CKC, Ng EHY, Yeung WSB, et al. Bisphenol compounds regulate decidualized stromal cells in modulating trophoblastic spheroid outgrowth and invasion *in vitro*. *Biol Reprod* (2020) 102(3):693–704. doi: 10.1093/biolre/ioz212

29. Xiong Y, Wen X, Liu H, Zhang M, Zhang Y. Bisphenol a affects endometrial stromal cells decidualization, involvement of epigenetic regulation. *J Steroid Biochem Mol Biol* (2020) 200:105640. doi: 10.1016/j.jsbmb.2020.105640

30. Aghajanova L, Giudice LC. Effect of bisphenol a on human endometrial stromal fibroblasts *in vitro*. *Reprod BioMed Online* (2011) 22(3):249–56. doi: 10.1016/j.rbmo.2010.12.007

31. Mannelli C, Szóstek AZ, Lukasik K, Carotenuto C, Ietta F, Romagnoli R, et al. Bisphenol a modulates receptivity and secretory function of human decidual cells: An in vitro study. *Reproduction* (2015) 150(2):115–25. doi: 10.1530/REP-14-0601

32. Olson MR, Su R, Flaws JA, Fazleabas AT. Bisphenol a impairs decidualization of human uterine stromal fibroblasts. *Reprod Toxicol* (2017) 73:339–44. doi: 10.1016/j.reprotox.2017.07.008

33. Akthar I, Wang Z, Wijayagunawardane MPB, Ratnayake CJ, Siriweera EH, Lee KF, et al. In vitro and in vivo impairment of embryo implantation by commonly used fungicide mancozeb. *Biochem Biophys Res Commun* (2020) 527 (1):42–8. doi: 10.1016/j.bbrc.2020.04.051

34. Lavogina D, Stepanjuk A, Peters M, Samuel K, Kasvandik S, Khatun M, et al. Progesterone triggers rho kinase-cofilin axis during in vitro and *in vivo* endometrial decidualization. *Hum Reprod* (2021) 36(8):2230–48. doi: 10.1093/humrep/deab161

35. Björvang RD, Gennings C, Lin PI, Hussein G, Kiviranta H, Rantakokko P, et al. Persistent organic pollutants, pre-pregnancy use of combined oral contraceptives, age, and time-to-pregnancy in the SELMA cohort. *Environ Heal A Glob Access Sci Source* (2020) 19(1):1–14. doi: 10.1186/s12940-020-00608-8

36. Björvang RD, Hallberg I, Pikki A, Berglund L, Pedrelli M, Kiviranta H, et al. Follicular fluid and blood levels of persistent organic pollutants and reproductive outcomes among women undergoing assisted reproductive technologies. *Environ Res* (2021) 208:112626. doi: 10.1016/j.envres.2021.112626

37. Lalitkumar PGL, Lalitkumar S, Meng CX, Stavreus-Evers A, Hambiliki F, Bentin-Ley U, et al. Mifepristone, but not levonorgestrel, inhibits human blastocyst attachment to an in vitro endometrial three-dimensional cell culture model. *Hum Reprod* (2007) 22(11):3031–7. doi: 10.1093/humrep/dem297

38. Kasvandik S, Samuel K, Peters M, Eimre M, Peet N, Roost AM, et al. Deep quantitative proteomics reveals extensive metabolic reprogramming and cancer-like changes of ectopic endometriotic stromal cells. *J Proteome Res* (2016) 15 (2):572–84. doi: 10.1021/acs.jproteome.5b00965

39. Krjutškov K, Katayama S, Saare M, Vera-Rodriguez M, Lubenets D, Samuel K, et al. Single-cell transcriptome analysis of endometrial tissue. *Hum Reprod* (2016) 31(4):844–53. doi: 10.1093/humrep/dew008

40. Koponen J, Rantakokko P, Airaksinen R, Kiviranta H. Determination of selected perfluorinated alkyl acids and persistent organic pollutants from a small volume human serum sample relevant for epidemiological studies. *J Chromatogr A*. (2013) 1309:48–55. doi: 10.1016/j.chroma.2013.07.064

41. Rajasärkkä J, Koponen J, Airaksinen R, Kiviranta H, Virta M. Monitoring bisphenol a and estrogenic chemicals in thermal paper with yeast-based bioreporter assay. *Anal Bioanal Chem* (2014) 406(23):5695–702. doi: 10.1007/s00216-014-7812-x

42. Porras SP, Koponen J, Hartonen M, Kiviranta H, Santonen T. Non-occupational exposure to phthalates in Finland. *Toxicol Lett* (2020) 332:107–17. doi: 10.1016/j.toxlet.2020.06.021

43. Vahter J, Viht K, Uri A, Manoharan GB, Enkvist E. Thiazole- and selenazole-comprising high-affinity inhibitors possess bright microsecond-scale photoluminescence in complex with protein kinase CK2. *Bioorg Med Chem* (2018) 26(18):5062–8. doi: 10.1016/j.bmc.2018.09.003

44. Enkvist E, Vaasa A, Kasari M, Kriisa M, Ivan T, Ligi K, et al. Protein-induced long lifetime luminescence of nonmetal probes. *ACS Chem Biol* (2011) 6(10):1052–62. doi: 10.1021/cb200120v

45. Vaasa A, Viil I, Enkvist E, Viht K, Raidaru G, Lavogina D, et al. High-affinity bisubstrate probe for fluorescence anisotropy binding/displacement assays with protein kinases PKA and ROCK. *Anal Biochem* (2009) 385(1):85–93. doi: 10.1016/j.ab.2008.10.030

46. Woodruff TJ, Zota AR, Schwartz JM. Environmental chemicals in pregnant women in the united states: NHANES 2003–2004. *Environ Health Perspect* (2011) 119(6):878. doi: 10.1289/ehp.1002727

47. Yee GM, Kennedy TG. Prostaglandin E2, cAMP and cAMP-dependent protein kinase isozymes during decidualization of rat endometrial stromal cells *in vitro*. *Prostaglandins* (1993) 46(2):117–38. doi: 10.1016/0090-6980(93)90038-9

48. Kern A, Bryant-Greenwood GD. Characterization of relaxin receptor (RXFP1) desensitization and internalization in primary human decidual cells and RXFP1-transfected HEK293 cells. *Endocrinology* (2009) 150(5):2419. doi: 10.1210/en.2008-1385

49. Miyata Y. Protein kinase CK2 in health and disease: CK2: the kinase controlling the Hsp90 chaperone machinery. *Cell Mol Life Sci* (2009) 66(11–12):1840–9. doi: 10.1007/s00018-009-9152-0

50. Yoshie M, Kusama K, Tamura K. Molecular mechanisms of human endometrial decidualization activated by cyclic adenosine monophosphate signaling pathways. *J Mamm Ova Res* (2015) 32(3):95–102. doi: 10.1274/jmor.32.95

51. Khatun M, Meltsov A, Lavogina D, Loid M, Kask K, Arffman RK, et al. Decidualized endometrial stromal cells present with altered androgen response in PCOS. *Sci Rep* (2021) 11(1):16287. doi: 10.1038/s41598-021-95705-0

52. World Health Organisation. *Identification of risks from exposure to endocrine-disrupting chemicals at the country level* (2014). Available at: https://www.euro.who.int/en/publications/abstracts/identification-of-risks-from-exposure-to-endocrine-disrupting-chemicals-at-the-country-level.

53. Carver J, Martin K, Spyropoulou I, Barlow D, Sargent I, Mardon H. An in-vitro model for stromal invasion during implantation of the human blastocyst. *Hum Reprod* (2003) 18(2):283–90. doi: 10.1093/humrep/deg072

54. Berkhout RP, Lambalk CB, Huirne J, Mijatovic V, Repping S, Hamer G, et al. High-quality human preimplantation embryos actively influence endometrial stromal cell migration. *J Assist Reprod Genet* (2018) 35(4):659. doi: 10.1007/s10815-017-1107-z

55. Ojosnegros S, Seriola A, Godeau AL, Veiga A. Embryo implantation in the laboratory: an update on current techniques. *Hum Reprod Update* (2021) 27 (3):501–30. doi: 10.1093/humupd/dmaa054

56. Fabi F, Grenier K, Parent S, Adam P, Tardif L, Leblanc V, et al. Regulation of the PI3K/Akt pathway during decidualization of endometrial stromal cells. *PloS One* (2017) 12(5):e0177387. doi: 10.1371/journal.pone.0177387

57. Fabi F, Asselin E. Expression, activation, and role of AKT isoforms in the uterus. *Reproduction* (2014) 148(5):R85–95. doi: 10.1530/REP-14-0270

58. Tessier C, Prigent-Tessier A, Ferguson-Gottschall S, Gu Y, Gibori G. PRL antiapoptotic effect in the rat decidua involves the PI3K/Protein kinase b-mediated inhibition of caspase-3 activity. *Endocrinology* (2001) 142(9):4086–94. doi: 10.1210/endo.142.9.8381

59. Zhang XH, Liang X, Liang XH, Wang TS, Qi QR, Deng WB, et al. The mesenchymal-epithelial transition during in vitro decidualization. *Reprod Sci* (2013) 20:354–60. doi: 10.1177/1933719112472738

60. Belcher SM, Chen Y, Yan S, Wang HS. Rapid estrogen receptor-mediated mechanisms determine the sexually dimorphic sensitivity of ventricular myocytes to 17β-estradiol and the environmental endocrine disruptor bisphenol a. *Endocrinology* (2012) 153(2):712–20. doi: 10.1210/en.2011-1772

61. Bouskine A, Nebout M, Brücker-Davis F, Banahmed M, Fenichel P. Low doses of bisphenol a promote human seminoma cell proliferation by activating PKA and PKG via a membrane G-protein-coupled estrogen receptor. *Environ Health Perspect* (2009) 117(7):1053–8. doi: 10.1289/ehp.0800367

62. Brosens JJ, Gellersen B. Death or survival – progesterone-dependent cell fate decisions in the human endometrial stroma. *J Mol Endocrinol* (2006) 36(3):389–98. doi: 10.1677/jme.1.02060

63. Garrido-Gómez T, Castillo-Marco N, Cordero T, Simón C. Decidualization resistance in the origin of preeclampsia. *Am J Obstet Gynecol* (2020) 226:S886-S894. doi: 10.1016/j.ajog.2020.09.039

64. CDC. *Fourth national report on human exposure to environmental chemicals update* (2021). Available at: https://www.cdc.gov/nchs/nhanes/.

65. Critchley HOD, Maybin JA, Armstrong GM, Williams ARW. Physiology of the endometrium and regulation of menstruation. *Physiol Rev* (2020) 100:1149–79. doi: 10.1152/physrev.00031.2019

66. Caporale N, Leemans M, Birgersson L, Germain PL, Cheroni C, Borbély G, et al. From cohorts to molecules: Adverse impacts of endocrine disrupting mixtures. *Science* (2022) 375(6582):eabe8244. doi: 10.1126/science.abe8244

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Comparing the effects of endometrial injury in the luteal phase and follicular phase on *in vitro* fertilization treatment outcomes

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Background: Several studies have shown that endometrial injury improves clinical pregnancy outcomes in patients undergoing *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) treatment with a history of implantation failure. However, endometrial injury can be performed in the follicular phase (FP) followed by embryo transfer in the same menstrual cycle or in the luteal phase (LP) before the embryo transfer cycle.

Method: This prospective cohort study was conducted from January 2015 to September 2021, and a total of 487 patients were included. All included patients had a history of a failed implantation cycle. They were divided into two groups: the FP group (N = 330), in which endometrial injury was performed on menstrual day 3-5, and the LP group (N = 157), in which endometrial injury was performed in the cycle preceding embryo transfer 7 days after ovulation.

Results: First, in unselected patients, the implantation rate and clinical pregnancy rate were comparable between the LP and FP groups. However, in patients with a history of ≥ 2 failed transfer cycles, the implantation rate was significantly higher in the LP group than in FP group (43.09% versus 33.33%, P = 0.03). Moreover, the clinical pregnancy rate was also significantly higher in patients in the LP group than in patients in the FP group (60.17% versus 46.15%, P=0.02). In addition, logistic regression analysis showed that endometrial injury in the LP group was an independent factor affecting clinical pregnancy outcome in patients with a history of ≥ 2 failed transfer cycles (aOR = 2.05, 90% CI:1.22-3.47, P=0.01).

Conclusion: Endometrial injury improves pregnancy outcomes when performed in the luteal phase compared with the follicular phase in patients with a history of ≥ 2 failed transfer cycles but not in unselected patients.

KEYWORDS

endometrial injury, luteal phase, follicular phase, *in vitro* fertilization, pregnancy outcome

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Introduction

In the past few years, assisted reproductive techniques (ART), which mainly includes *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI), has evolved rapidly. However, embryo implantation failure is still a substantial problem that places considerable pressure on both patients and clinicians. For some young patients, even if high-quality embryos are transferred, implantation is still difficult. Since embryo quality and endometrial receptivity are two main factors for a successful pregnancy during IVF/ICSI, a possible explanation for this phenomenon is that these failed cycles have poor endometrial receptivity.

Endometrial injury, which is also known as endometrial scratching, is usually defined as intentional trauma to the endometrium by a biopsy catheter (1, 2). Recently, several studies have reported that endometrial injury improves clinical pregnancy outcomes in patients undergoing IVF/ICSI treatment with a history of implantation failure (3–5). Other clinical studies, as well as several meta analyses, did not show any difference in regard to pregnancy outcomes between women who underwent endometrial injury and those who did not (6, 7). In our center, it has been more than 10 years since the first endometrial injury was performed in patients with implantation failure. Our data confirm that endometrial injury improves pregnancy outcomes for women undergoing repeated IVF cycles.

In previous studies, endometrial injury was performed in either the follicular phase (FP) followed by embryo transfer in the same menstrual cycle or in the luteal phase (LP) before the embryo transfer cycle. A recent study showed that the timing of endometrial injury can make a major difference for the final pregnancy outcome (8). However, only few RCTs with a small sample size compared the difference in pregnancy outcomes in patients with endometrial injury in the FP and LP (9).

Thus, the current prospective observational study aimed to explore the impact of endometrial injury (FP vs. LP) on pregnancy outcomes in women undergoing frozen thawed embryo transfer (FET) cycles, especially in patients with different histories of implantation failure.

Materials and methods

A total of 487 women with a history of failed implantation cycles were included into this study. This study was approved by the Medical Ethics Committee Board of First Affiliated Hospital of Zhengzhou University. All patients included were fully informed and signed written informed consent to allow us using their data for publication. Exclusion criteria were: Uterine anomaly; uterine adhesion, oocyte donation cycles; Pre-implantation Genetic Testing (PGT) cycles.

Treatment protocol

According to our Standard Operating Procedure, preparation of endometrium in frozen thawed embryo transfer was mainly divided into natural cycles (NCs) and artificial (estrogen-progesterone [EP]) cycles. Briefly, for NCs, patients were advised to perform ultrasonic evaluation starting on day 7-9 of the menstrual cycle. Blood samples were obtained for progesterone and LH levels once leading follicle reached 14 mm. After ovulation, embryos were transferred 3 or 5 days later, based on the stage of embryos cryopreserved. For EP cycles, low dose of oral estradiol (1 mg [progynova]; Bayer, Germany) was begun twice a day on cycle day 3 for the first 4 days. This dose was adjusted based on endometrial thickness every 4 days. Around 12-14 days later, progesterone in oil was added. Embryo transfer was performed 3 or 5 days later. Endometrial thickness was measured on the day of progesterone admission. All patients included were transferred with 2 cleavage embryos or 1 blastocyst according to Standard Operating Procedure in our center. To assess IVF outcome, serum human chorionic gonadotropin (hCG) was measured 14 days after embryo transfer. Clinical pregnancy was confirmed by ultrasound 5 weeks after embryo transfer.

Endometrial injury

For patients in FP group, endometrial injury was performed on menstrual day 3-5. Embryos were transferred approximately 2 weeks after injury in the same cycle. Patients in the LP group underwent endometrial injury in the cycle preceding embryo transfer. Luteal phase was defined as 7 days after ovulation. Endometrial injury was performed by the same physician with Pipelle (Disposable Endometrium Suction Tube, S-3.2; Nuode Medical, Jiangxi, China). The standard procedure was as follows: the patient was placed in the bladder lithotomy position under the guidance of abdominal ultrasound, and the Pipelle was gently probed into the uterine cavity. Endometrial tissue was taken from the Pipelle with little negative pressure.

First, patient basic parameters and pregnancy outcomes were compared between patients in the FP and LP groups. In addition, these parameters were compared in a special group of patients with a history of ≥ 2 failed transfer cycles. Finally, adjusted logistic regression analysis was used to explore potential factors affecting clinical pregnancy rate in unselected patients and patients with ≥ 2 failed transfer cycles.

Several definitions in the current study were as follows: Implantation rate was the number of sacs detected on ultrasound divided by the number of embryos transferred. Clinical pregnancy was defined as the presence of at least one gestational sac on ultrasound at 5 weeks after embryo transfer.

Ectopic pregnancy was defined as in previous study (10). The early miscarriage rate was defined as the number of miscarriages before 20 weeks divided by the number of women with a positive pregnancy test.

Data were analyzed using SPSS (Statistical Package for Social Science, SPSS Inc, Chicago, IL, USA) 17.0 software. The continuous data were shown as Mean ± Standard Deviation, and Student T-test was used for comparison between groups. The countable data were compared using Chi-square test. In adjusted logistic regression analysis, confounding factors included were female age, endometrial thickness on day of progesterone administration, stage of embryos transferred, endometrial preparation protocol, and time of endometrial injury (LP versus FP). $P < 0.05$ was considered statistically significant.

Results

From January 2015 to September 2021, a total of 487 patients (157 luteal phase and 330 follicular phase) were included into this study. As shown in Table 1, there were no differences between these two groups with regard to female age, body mass index, infertility duration, infertility diagnosis, stage of embryos transferred, or endometrial preparation protocol. However, the endometrial thickness on the progesterone administration day was significantly thicker in the LP group (11.36 mm versus 10.23 mm; $P = 0.01$). The clinical pregnancy outcomes in patients undergoing endometrial injury in the two groups are also shown in Table 1. No significant differences regarding the implantation rate and clinical pregnancy rate were detected in these two groups, even though both parameters were higher in the LP group. Moreover, both ectopic pregnancy and early miscarriage rates were lower in the LP group, and the difference was not statistically significant.

In patients with a history of ≥ 2 failed transfer cycles, patients' basic parameters were also comparable in the LP and FP groups (Table 2). Both the implantation rate (43.09% versus 33.33%, P=0.03) and clinical pregnancy rate (60.17% versus 46.15%, P=0.02) were significantly higher in the LP group than in the FP group. Interestingly, endometrial thickness was also higher in the LP group (11.27 mm vs 9.89 mm; $P = 0.00$). Similar to that from unselected patients, the ectopic pregnancy rate and early miscarriage rate were also comparable between the LP and FP groups.

As shown in Table 3, multivariate logistic regression analysis was performed to explore factors associated with clinical pregnancy in unselected patients and patients with ≥ 2 failed transfer cycles after adjusting for confounding factors. In the unselected group, only female age was an independent factor (aOR=0.82, 90% CI: 0.78-0.86, P=0.00). However, in patients with ≥ 2 failed transfer cycles, apart from female age,

TABLE 1 Basic parameters and pregnancy outcomes in patients undergoing endometrial injury in two groups.

	Luteal phase N=157	Follicular phase N=330	*P* value
Age (year)	31.71 ± 4.63	31.54 ± 5.01	0.34
Infertility duration (year)	4.36 ± 3.28	4.66 ± 3.67	0.59
BMI (kg/m^2)	22.43 ± 2.67	22.74 ± 3.43	0.46
Infertility diagnosis			
Tubal factor	44.59% (70/157)	40.91% (135/330)	0.37
PCOS	6.37% (10/157)	16.67% (55/330)	
Male infertility	20.38% (32/157)	14.55% (48/330)	
Others	28.66% (45/157)	27.88% (92/330)	
Endometrium preparation protocol			
Natural cycle	49.04% (77/157)	50.60% (167/330)	0.77
EP cycle	50.96% (80/157)	49.40% (163/330)	
Stage of embryo transferred			
Cleavage	57.32% (90/157)	60.61% (200/330)	0.15
Blastocyst	42.68% (67/157)	39.39% (130/330)	
No. of embryos transferred	1.57 ± 0.50	1.61 ± 0.49	0.49
Endometrial thickness (mm)	11.36 ± 1.61	10.23 ± 1.71	0.01
Implantation rate	43.48% (100/230)	38.10% (208/546)	0.17
Clinical pregnancy rate	60.51% (95/157)	51.21% (169/330)	0.06
Ectopic pregnancy rate	2.11% (2/95)	2.37% (4/169)	0.84
Early Miscarriage rate	12.63% (12/95)	15.38% (26/169)	0.59

BMI, body mass index; PCOS, polycystic ovarian syndrome; EP, estrogen-progesterone.

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TABLE 2 Basic parameters and clinical pregnancy outcomes in patients with ≥ 2 failed transfer cycles.

	Luteal phase N=118	Follicular phase N=234	*P* value
Age (year)	32.40 ± 4.88	32.31 ± 5.75	0.82
Infertility duration (year)	4.52 ± 3.43	4.85 ± 3.45	0.63
BMI (kg/m^2)	23.12 ± 3.22	23.41 ± 3.63	0.34
Infertility diagnosis			
Tubal factor	25.42% (30/118)	17.09% (40/234)	0.26
PCOS	5.93% (7/118)	19.23% (45/234)	
Male infertility	19.49% (23/118)	10.68% (25/234)	
Others	49.15% (58/118)	52.99% (124/234)	
Endometrium preparation protocol			
Natural cycle	46.61% (55/118)	49.57% (116/234)	0.81
EP cycle	53.39% (63/118)	50.43% (118/234)	
Stage of embryo transferred			
Cleavage	69.49% (82/118)	73.08% (171/234)	0.24
Blastocyst	30.51% (36/118)	26.92% (63/234)	
No. of embryos transferred	1.69 ± 0.54	1.73 ± 0.51	0.52
Endometrial thickness (mm)	11.27 ± 1.91	9.89 ± 1.71	0.00
Implantation rate	43.09% (78/181)	33.33% (131/393)	0.03
Clinical pregnancy rate	60.17% (71/118)	46.15% (108/234)	0.02
Ectopic pregnancy rate	1.41% (1/71)	1.85% (2/108)	0.67
Early Miscarriage rate	9.86% (7/71)	19.4% (21/108)	0.10

BMI, body mass index; PCOS, polycystic ovarian syndrome; EP, estrogen-progesterone.

endometrial injury in the LP group was significantly associated with the clinical pregnancy rate (aOR=2.05, 90% CI:1.22-3.47, *P*=0.01).

Discussion

Currently, IVF/ICSI-ET is an effective technology to treat infertility, but the embryo implantation rate is still approximately 30%-40%. Improving pregnancy outcomes of IVF/ICSI is a substantial challenge for reproductive clinicians.

Embryo implantation, which is the first step for a successful pregnancy, includes embryo localization, adhesion, and then invasion of the endometrium. Many scholars have advocated that endometrial injury can stimulate the inflammatory response, adjust gene expression, and thus improve endometrial receptivity. As early as 2003, Barash et al. reported that local endometrial injury before embryo transfer could significantly

TABLE 3 Factors associated with clinical pregnancy rate in unselected patients and patients with ≥ 2 failed transfer cycles.

	Unselected patients		Patients with ≥ 2 failed cycles	
	aOR (95% CI)	P	aOR (95% CI)	P
Age (year)	0.82 (0.78-0.86)	0.00	0.85 (0.80-0.90)	0.00
Endometrial thickness (mm)	1.03 (0.92-1.07)	0.44	1.04 (0.84-1.04)	0.51
Protocols				
Natural vs. EP	0.96 (0.88-1.06)	0.46	0.97 (0.91-1.05)	0.48
Stage of embryos				
Blastocyst vs. cleavage	1.05 (0.85-1.07)	0.28	1.01 (0.92-1.03)	0.67
Time of endometrial injury				
Luteal phase vs. follicular phase	1.25 (0.95-1.65)	0.07	2.05 (1.22-3.47)	0.01

EP, estrogen-progesterone; aOR, adjusted odds ratio; CI, confidence interval.

improve the clinical pregnancy rate and live birth rate (11). In 2007, Raziel et al. reported that in patients with high-order implantation failure (≥ 4 IVF trials and ≥ 12 transferred embryos), local injury to the endometrium prior to controlled ovarian stimulation improved implantation rates and pregnancy outcomes (12). In addition, a meta-analysis including 4 randomized studies and 3 observational studies showed that in patients with repeated implantation failure, endometrial injury also significantly improved pregnancy outcomes (13). More recently, another meta-analysis including 10 studies with 1468 participants showed that both a higher live birth rate (RR 1.38, 95% CI 1.05-1.80) and clinical pregnancy rate (RR 1.34, 95% CI 1.07-1.67) were observed in patients with endometrial injury than in controls (14).

However, unlike the previous studies mentioned above, in which all participants had failed implantation cycles, other studies have also investigated the impact of endometrial injury on pregnancy outcomes in unelected patients. In 2014, a randomized controlled trial including 300 unselected subfertile women showed that endometrial injury performed in the mid-luteal phase did not change pregnancy outcomes (6). Moreover, another RCT in 2017 that included an unselected group of patients also showed that there was no significant difference in the implantation rate or live birth rate between patients with or without local endometrial injury (9). In 2019, a multicenter RCT published in The New England Journal of Medicine including 1364 women showed that endometrial injury did not result in a higher live birth rate than no intervention among women undergoing IVF (15).

Several reasons may explain the inconsistency among these studies. First, it is obvious that the type of patients (unselected patients or patients with repeated implantation failure) included is important (16). Our results also showed that the pregnancy outcomes in unselected patients were comparable between the LP and FP groups at first. However, in the subgroup (patients with a history of ≥ 2 failed transfer cycles) analysis, it was interesting to find that endometrial injury in the LP significantly improved pregnancy outcomes in this group of patients. Then, we also noticed that the timing of endometrial injury may also have an impact on pregnancy outcomes. In 2020, data showed that there was insufficient evidence to support the use of endometrial injury in the follicular phase in intrauterine insemination treatment cycles (17). Another RCT demonstrated that endometrial injury in the luteal phase before ovarian stimulation significantly enhanced the clinical pregnancy rate in women with three or more prior implantation failures (18).

Then, when is the proper time to perform endometrial injury? Recently, a randomized study directly compared the effect of endometrial injury between the proliferative vs. luteal phase on unselected women. They found that there was no significant difference in the clinical outcomes between endometrial injury in the proliferative phase (38 patients) and injury in the luteal phase (32 patients) (9). However, our data were in contrast with those from this small sample-sized study. In our study, a total of 487 patients were included, which makes the conclusion more convincing.

Several possible mechanisms by which local endometrial injury improves pregnancy outcome have also been discussed. First, the part of the endometrium damaged due to local injury recruits immune cells, which can be further differentiated into macrophages or dendritic cells and may promote implantation (19). Second, implantation-related genes were observed to be highly expressed in women after endometrial injury when compared with controls. Moreover, after endometrial injury, a series of cytokines and growth factors are secreted to induce wound healing, such as leukemia inhibitory factor (LIF), and endothelial growth factor (VEGF), which can facilitate embryo implantation. Compared with the follicular phase, the luteal phase is characterized by a large content of growth factors, cytokines and immune cells in the endometrium (20). Thus, it is more reasonable that endometrial injury performed in the luteal phase can activate more cytokines and eventually result in better pregnancy outcomes eventually. In addition, we noticed that endometrial thickness was significantly improved in both unselected patients and patients with ≥ 2 failed transfer cycles. It is known that measuring endometrial thickness is a noninvasive method to predict endometrial receptivity in IVF cycles. In addition, data from our center and a large-sample meta-analysis also confirmed that endometrial thickness was positively associated with pregnancy outcomes in patients with embryo transfer (21–23). Thus, in our next work, we plan to collect endometrial tissue after endometrial injury in both the LP and FP and to detect endometrial receptivity-associated genes or cytokines to confirm this hypothesis.

Even though our study was one of the very few studies comparing the different effects of endometrial injury in the LP and FP with a larger sample size, there were indeed several limitations. First, the first endometrial injury was performed in our center in 2009, and it has been a routine practice for patients with repeated IVF cycles as we observed an improvement in pregnancy outcomes after endometrial injury. However, it would be better to include a sham group. In addition, this was a prospective cohort study. We tried to control for other confounding factors, such as stage of embryo transferred and the endometrial preparation protocol. Moreover, there were only 118 patients with ≥ 2 failed transfer cycles in the luteal phase group. Thus, well-designed RCTs with more patients are needed in the future.

In summary, our study with a larger sample showed that endometrial injury, a slightly traumatic intervention procedure that causes little harm to patients, significantly improves pregnancy outcomes when performed in the luteal phase

compared with the follicular phase in a particular group of patients with a history of ≥ 2 failed transfer cycles but not in unselected patients.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Medical Ethics Committee Board of First Affiliated Hospital of Zhengzhou University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

YW and ZB contributed to the conception, design, acquisition and interpretation of data, and drafting of the manuscript. LH supervised the study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Tk A, Singhal H, Premkumar S P, Acharya M, Kamath S M, George K. Local endometrial injury in women with failed IVF undergoing a repeat cycle: A randomized controlled trial. *Eur J Obstet Gynecol Reprod Biol* (2017) 214:109–14. doi: 10.1016/j.ejogrb.2017.05.005

2. Nastri CO, Lensen SF, Gibreel A, Raine-Fenning N, Ferriani RA, Bhattacharya S, et al. Endometrial injury in women undergoing assisted reproductive techniques. *Cochrane Database Syst Rev* (2015) 3):CD009517. doi: 10.1002/14651858.CD009517.pub3

3. Levin D, Hasson J, Cohen A, Or Y, Ata B, Barzilay L, et al. The effect of endometrial injury on implantation and clinical pregnancy rates. *Gynecol Endocrinol* (2017) 33(10):779–82. doi: 10.1080/09513590.2017.1318369

4. Reljic M, Knez J, Kovac V, Kovacic B. Endometrial injury, the quality of embryos, and blastocyst transfer are the most important prognostic factors for *in vitro* fertilization success after previous repeated unsuccessful attempts. *J Assist Reprod Genet* (2017) 34(6):775–9. doi: 10.1007/s10815-017-0916-4

5. Siristatidis C, Kreatsa M, Koutlaki N, Galazios G, Pergialiotis V, Papantoniou N. Endometrial injury for RIF patients undergoing IVF/ICSI: A prospective nonrandomized controlled trial. *Gynecol Endocrinol* (2017) 33(4):297–300. doi: 10.1080/09513590.2016.1255325

6. Yeung TW, Chai J, Li RH, Lee VC, Ho PC, Ng EH. The effect of endometrial injury on ongoing pregnancy rate in unselected subfertile women undergoing *in vitro* fertilization: A randomized controlled trial. *Hum Reprod* (2014) 29(11):2474–81. doi: 10.1093/humrep/deu213

7. Dunne C, Taylor B. Does endometrial injury improve implantation of frozen-thawed embryos? *Arch Gynecol Obstet* (2014) 290(3):575–9. doi: 10.1007/s00404-014-3258-9

8. Acet F, Sahin G, Goker E, Tavmergen E. The effect of hysteroscopy and conventional curretage versus no hysteroscopy on live birth rates in recurrent *in vitro* fertilisation failure: A retrospective cohort study from a single referral centre experience. *J Obstet Gynaecol* (2022) p:1–5. doi: 10.1080/01443615.2022.2033963

9. Liu W, Tal R, Chao H, Liu M, Liu Y. Effect of local endometrial injury in proliferative vs. luteal phase on IVF outcomes in unselected subfertile women undergoing *in vitro* fertilization. *Reprod Biol Endocrinol* (2017) 15(1):75. doi: 10.1186/s12958-017-0296-8

10. Bu Z, Xiong Y, Wang K, Sun Y. Risk factors for ectopic pregnancy in assisted reproductive technology: A 6-year, single-center study. *Fertil Steril* (2016) 106 (1):90–4. doi: 10.1016/j.fertnstert.2016.02.035

11. Barash A, Dekel N, Fieldust S, Segal I, Schechtman E, Granot I. Local injury to the endometrium doubles the incidence of successful pregnancies in patients undergoing *in vitro* fertilization. *Fertil Steril* (2003) 79(6):1317–22. doi: 10.1016/S0015-0282(03)00345-5

12. Raziel A, Schachter M, Strassburger D, Bern O, Ron-El R, Friedler S. Favorable influence of local injury to the endometrium in intracytoplasmic sperm injection patients with high-order implantation failure. *Fertil Steril* (2007) 87(1):198–201. doi: 10.1016/j.fertnstert.2006.05.062

13. Potdar N, Gelbaya T, Nardo LG. Endometrial injury to overcome recurrent embryo implantation failure: A systematic review and meta-analysis. *Reprod BioMed Online* (2012) 25(6):561–71. doi: 10.1016/j.rbmo.2012.08.005

14. Vitagliano A, Di Spiezio SA, Saccone G, Valenti G, Sapia F, Kamath MS, et al. Endometrial scratch injury for women with one or more previous failed embryo transfers: A systematic review and meta-analysis of randomized controlled trials. *Fertil Steril* (2018) 110(4):687–702.e2. doi: 10.1016/j.fertnstert.2018.04.040

15. Lensen S, Osavlyuk D, Armstrong S, Stadelmann C, Hennes A, Napier E, et al. A randomized trial of endometrial scratching before *In vitro* fertilization. *N Engl J Med* (2019) 380(4):325–34. doi: 10.1056/NEJMoa1808737

16. Nahshon C, Sagi-Dain L, Dirnfeld M. The impact of endometrial injury on reproductive outcomes: results of an updated meta-analysis. *Reprod Med Biol* (2020) 19(4):334–49. doi: 10.1002/rmb2.12348

17. Ghuman NK, Raikar S, Singh P, Gothwal M, Yadav G. Improving reproductive outcomes of intrauterine insemination: Does endometrial scratch injury help? *A randomised Controlled trial. Eur J Obstet Gynecol Reprod Biol* (2020) 253:225–31. doi: 10.1016/j.ejogrb.2020.08.010

18. Olesen MS, Hauge B, Ohrt L, Olesen TN, Roskaer J, Baek V, et al. Therapeutic endometrial scratching and implantation after *in vitro* fertilization: A multicenter randomized controlled trial. *Fertil Steril* (2019) 112(6):1015–21. doi: 10.1016/j.fertnstert.2019.08.010

19. Tu YA, Chou CH, Yang PK, Shun CT, Wen WF, Tsao PN, et al. Intentional endometrial injury enhances angiogenesis through increased production and activation of MMP-9 by TNF-alpha and MMP-3 in a mouse model. *Mol Hum Reprod* (2021) 27(10):1–11. doi: 10.1093/molehr/gaab055

20. Gnainsky Y, Granot I, Aldo PB, Barash A, Or Y, Schechtman E, et al. Local injury of the endometrium induces an inflammatory response that promotes successful implantation. *Fertil Steril* (2010) 94(6):2030–6. doi: 10.1016/j.fertnstert.2010.02.022

21. Bu Z, Wang K, Dai W, Sun Y. Endometrial thickness significantly affects clinical pregnancy and live birth rates in frozen-thawed embryo transfer cycles. *Gynecol Endocrinol* (2016) 32(7):524–8. doi: 10.3109/09513590.2015.1136616

22. Kasius A, Smit JG, Torrance HL, Eijkemans MJ, Mol BW, Opmeer BC, et al. Endometrial thickness and pregnancy rates after IVF: A systematic review and meta-analysis. *Hum Reprod Update* (2014) 20(4):530–41. doi: 10.1093/humupd/dmu011

23. Liao Z, Liu C, Cai L, Shen L, Sui C, Zhang H, et al. The effect of endometrial thickness on pregnancy, maternal, and perinatal outcomes of women in fresh cycles after IVF/ICSI: A systematic review and meta-analysis. *Front Endocrinol (Lausanne)* (2021) 12:814648. doi: 10.3389/fendo.2021.814648

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The RNA-seq based endometrial receptivity test (rsERT) compared to pinopode: A better diagnostic tool for endometrial receptivity for patients with recurrent implantation failure in Chinese population

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Displaced window of implantation (WOI) is one of the endometrial origins that accounts for implantation failure, especially for patients with recurrent implantation failure (RIF), yet no standard diagnostic tool has been recognized. The study consists of two parts, aiming to compare the concordance and efficacy of the diagnostic tools, the newly developed RNA-seq based endometrial receptivity test (rsERT) to the conventional pinopode, in diagnosing WOI and guiding personalized embryo transfer (pET). With the same group of RIF patients, the rsERT diagnosed 32 patients (65.31%) with normal WOIs, and most of the displacements were advancements (30.61%). While according to pinopode, only 14 patients (28.57%) were found with normal WOIs, and most patients (63.27%) presented delayed growth patterns. After conducting pET, patients in the rsERT group had higher successful pregnancy rates while requiring fewer ET cycles (50.00% vs. 16.67%, p=0.001). The study proved poor consistency between the diagnostic tools of endometrial receptivity based on cellular structure and gene profiling, and it supported rsERT as a reliable tool with potential clinical value.

KEYWORDS

endometrial receptivity, window of implantation, pinopode, gene profiling, transcriptomic (RNA-Seq), rsERT, personalized embryo transfer

Background

Successful embryo implantation is a crucial event that relies upon elaborate communication between the capable embryo and the receptive endometrium (1). Although there have been significant advances in embryo culture technology, endometrial receptivity remains a hurdle in today's ART (2). In humans, the endometrium undergoes dynamic and complex changes during the menstrual cycle and becomes receptive to blastocysts in a brief period during the mid-luteal phase, which is known as the window of implantation (WOI), usually around days 19-23 of the menstrual cycle (3). However, WOIs could be variant among patients, and implantation failure may occur despite a viable embryo due to embryo-endometrium asynchrony (4, 5). It has been suggested that the implantation failure of endometrial origin is not a result of pathology but a failure to synchronize the developing embryo with a receptive endometrium (6), thus making diagnosing WOI a critical task, especially for patients with repeated implantation failure(RIF).

Pinopode has been considered one of the standard indicators that have been proposed and extensively studied with WOI and fertility. Pinopodes are smooth mushroom or balloon-shaped projections that arise from the apical surface of the luminal epithelium of the endometrium in mice, rats, and humans (7). Its development coincides with the implantation window, as well as other receptivity changes including loss of progesterone receptors (8), peak expression of integrins (9), osteopontin (10), LIF (11), etc., indicating a possible function in embryo implantation. However, strong disagreement still exists in the literature as to the function and clinical value of pinopode, and the necessity of pinopodes in the implantation process has yet to be firmly established (12). Using pinopode as an indicator of endometrial receptivity was considered a promising method. Poor IVF outcomes were related to altered pinopode shape and poor pinopode development (13–17), and personalized embryo transfer(pET) based on pinopode scoring has been proved feasible (15, 18). However, other investigators held uncertain attitudes. Some found pinopode presented throughout the luteal phase rather than a specific period during WOI, and women experiencing infertility do not exhibit a significant difference in pinopode coverage or morphology (19–21).

Recent developments in omics have heightened the need for personalized medicine, and earlier studies proved the feasibility of distinguishing different phases of the menstrual cycle or different diseases based on transcriptomic characteristics (22–24). The disrupted gene expression pattern was found in RIF patients, and 12.0% ~59.2% of RIF patients were found with displaced WOIs, significantly higher rates compared to the controls (25–30). Personalized embryo transfer based on transcriptomic tools has been proven feasible and valuable, especially for RIF patients. Significantly higher pregnancy rates were reported in the pET group instructed by the commercial Endometrial Receptivity Array (ERA) test, and rescue of non-receptive patients by pET in a displaced WOI resulted in similar clinical outcomes to controls (25, 31–34). Other investigators, however, considered the value was rather limited when applied ERA in ICSI cycles, first embryo transfer cycles, and in good prognosis patients considering the current level of development (26, 30, 35–38). Based on RNA-seq technology while aiming to improve the diagnostic tool, especially for Chinese population, we constructed an RNA-seq based endometrial receptivity test (rsERT) in a previous study and validated its clinical value in RIF patients (29). Prospective clinical trials, including multicenter trials, are being conducted for further validation.

Previous studies have demonstrated poor concordance between the ERA tool and traditional histologic dating while supporting the superiority of the transcriptomic tool in diagnosing WOI-displacement (39, 40). However, a comparison between transcriptomic tools and pinopode evaluation is yet to be reported. In this study, we aim to compare the concordance and efficacy of the tools, the pinopode and rsERT, in diagnosing WOI and instructing personalized embryo transfer.

Materials and methods

Study design and patients

This study was conducted at the Department of Reproductive Medicine of Xiangya Hospital, Changsha, Hunan, China from November 2017 to July 2019 and consists of two separate parts (Figure 1). Study Part 1 aims to compare the concordance of the diagnostic tools of endometrial receptivity, the rsERT and pinopode, in a paired biopsies population, in which endometrial biopsies obtained from the same patient were divided and sent for both tests. The study was approved by the ethics committee of the department of reproductive medicine, Xiangya Hospital, Central South University (reference number 2017002) and registered in the Chinese Clinical Trial Registry (http://www.chictr.org.cn/ .Registration No. ChiCTR-DDD-17013375). Part 2 aims to compare the accuracy and efficacy of the tools in instructing pET. The pregnancy outcomes of RIF patients with rsERT- or pinopode-instructed pET cycles were compared retrospectively. The study was approved by the same ethics committee (reference number 2019016). Patients were informed and consented to the use of their anonymized data for research purposes. Informed consents were obtained from all the patients.

Inclusion and exclusion criteria

The inclusion criteria included age between 20 and 38, a body mass index (BMI) of 18-25 kg/m^2, and a history of RIF, which was defined as failure to achieve a clinical pregnancy after receiving at least 4 morphologically high-quality cleavage embryos or 2 high-

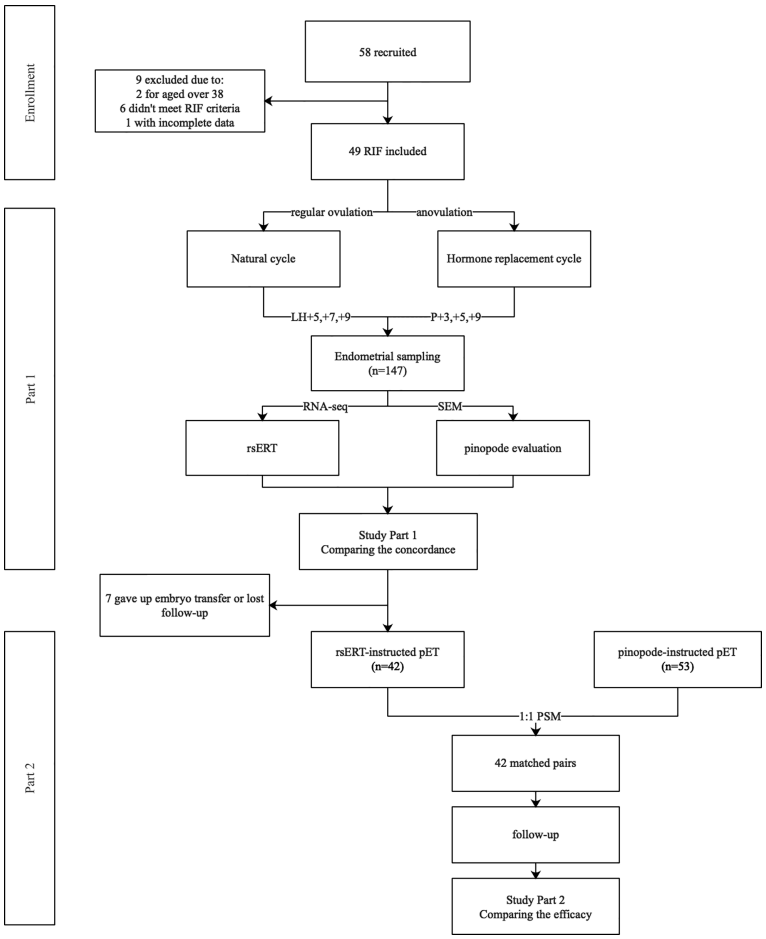

FIGURE 1
Flow chart of the study. A total of 58 RIF patients were recruited in Part 1 of the study, 9 were removed due to variant of reasons. Three consecutive endometrial biopsies from a same menstrual cycle were obtained from each patient, being sent for both rsERT test and pinopode evaluation, and the concordance was evaluated. In Part 2, 42 patients that received rsERT instructed pET were matched with 42 patients who once received pinopode-instructed pET in our center by using PSM in a 1:1 ratio. Clinical outcomes were followed up to compare the efficacy of the diagnostic tools.

quality blastocysts in a minimum of 2 fresh or frozen transfer cycles. Patients were excluded for the following reasons: endometrial lesions (such as intrauterine adhesions, endometrial tuberculosis, endometritis, endometrial hyperplasia, and a thin endometrium, etc.); severe hydrosalpinx; severe endometriosis (stage III-IV); uterine malformations; other medical complications (including hypertension, diabetes, etc.). The criteria for embryos of high quality were as follows: (i) cleavage-stage embryo: 20% fragmentation or less on day 3 and absence of multinucleated blastomeres (41); (ii) Blastocysts: ≥3BB on day 5 and 6 (42).

Endometrial preparing and sampling

In Part 1 of the study, three endometrial biopsies were taken consecutively during the same menstrual cycle, while avoiding repeated sampling from the same uterine wall. 49 RIF patients consented to participate in the study and 147 endometrial biopsies were collected. All procedures were performed following the applicable rules and guidelines. All patients were informed and written consents were obtained.

For endometrial preparation, patients with or without regular ovulation used ovulation monitoring (natural cycle) and hormone replacement therapy (HRT), respectively. In brief, for ovulatory patients, ultrasound monitoring was initiated from Day 10 of the menstrual cycle and plasma LH was dynamically measured when the diameter of the dominant follicle was ≥14mm. The LH peak day was recorded as LH+0 and endometrial biopsies were obtained on 5, 7 and 9 days thereafter (LH+5/+7/+9) using an endometrial sampler (AiMu Medical Science & Technology Co.; Liaoning; China). For anovulatory patients, hormone replacement treatment (HRT) was applied.

Estradiol administration was started on Day 3 of the menstrual cycle, and progesterone was added after at least 12 days if the endometrium was >7 mm. The first day with progesterone supplementation was seen as P+0, and endometrial tissues were obtained after 3, 5 and 7 days (P+3/+5/+7).

Specimens were first rinsed in saline and then divided evenly. One was stored in RNA-later buffer (AM7020; Thermo Fisher Scientific, Waltham, MA, USA) for RNA sequencing and rsERT testing, while the other was fixed in 2.5% glutaraldehyde solution for more than 48 hours, rinsed twice with PBS buffer, and dehydrated in series of ethanol concentrations (50%, 70%, 80%, 95%, 100%). The samples were then dried in a critical point drier using carbon dioxide and coated with palladium gold before being sent for scanning electron microscopy (SEM) and pinopode evaluation.

WOI delineation

In humans, pinopode morphology changes during the luteal phase. Three different pinopode development stages have been identified, known as developing, fully developed, and regressing, with each phase lasting approximately 24 hours (43, 44). In brief, developing pinopodes are smooth and slender membrane projections that arise from the entire cell apex; fully developed pinopodes are maximally folded, smooth and devoid of microvilli; and regressing pinopodes are slightly wrinkled and microvilli tips reappear. In the study, 10 fields at a magnification of 2,000 were randomly chosen under the SEM, and two independent observers were responsible for evaluating pinopode. By observing endometrial biopsies, a consecutive growth pattern of pinopode of a patient was obtained, and the most receptive day was determined to be the day on which the most fully developed pinopodes appeared, the day after developing pinopodes appeared, or the day before regressing pinopodes appeared.

For rsERT testing, the tool comprises 175 biomarkers genes and demonstrated an average accuracy of 98.4% *via* tenfold cross-validation. It can distinguish precisely between pre-receptive, receptive, and post-receptive endometrium while remaining unaffected by different endometrium preparing methods (HRT or natural) (29).

Personalized embryo transfer and propensity score matching

Part 2 of the study aims to compare the efficacy of the tools in instructing personalized embryo transfer (pET). rsERT group comprises 42 patients from Part 1, who consented to receive rsERT-instructed embryo transfer. Additional 53 patients who had previously received pinopode-instructed personalized embryo transfer were retrospectively included and compared. Propensity score matching (PSM) was used considering the potential bias introduced by the retrospective design. Groups were matched for age, BMI, endometrium preparation method and number of transferred high-quality embryos in a 1:1 ratio. After matching, 42 pairs of patients were selected for statistical comparisons out of a total of 95.

For both examination and transfer cycles, the same endometrial preparing method was used. One or two embryos were transferred, aiming to synchronize blastocysts with the predicted optimal WOI. Day 3 cleavage-stage embryos were transferred 2 days earlier accordingly.

The primary outcome was intrauterine pregnancy rate (IPR), which was defined as the presence of a gestational sac containing a fetal heart as evaluated by ultrasound. Secondary outcomes were the live birth rate (LBR) and implantation rate (IR). LBR refers to the number of pET cycles that result in deliveries. IR refers to the number of gestational sacs observed divided by the number of transferred embryos.

Statistical analysis

SPSS Statistics 25.0 was used for statistical analysis. Chi-square test, independent sample t-test, Mann Whitney-U test and Cohen's Kappa index were used for statistical description and comparison. P-value <0.05 was considered statistically significant.

Results

In the first part of the study, 147 endometrial biopsies were collected from 49 RIF patients and sent for both tests. The demographic characteristics of patients are detailed in Supplementary Table S1. According to rsERT, the majority of patients (32 of 49, 65.31%) had normal-WOIs (on P+5 or LH +7), while two of them were found with prolonged-WOIs, that their endometrium remained receptive for more than 72 hours (Figure 2). The remaining 17 patients were diagnosed with displaced-WOIs, of which 15 were advanced (30.61%) and 2 were delayed (4.08%). In contrast, by evaluating pinopode, only 14 patients (28.57%) were considered to have normal-WOIs. Most patients (31 of 49, 63.27%) presented delayed pinopode growth patterns and four patients (8.16%) were diagnosed with advanced WOIs. Test results for only 15 patients (30.61%) were congruent, indicating poor consistency.

42 of the 49 participants consented to receive personalized frozen embryo transfer following the instruction of the rsERT. There is no intervention for the 2 patients who were found to have prolonged-WOIs. 23 patients (54.76%) conceived successfully after transferring one or two embryos to match the predicted optimal WOI. Their basic information and WOI distributions were presented in Table 1 and Supplementary Table S2. While WOIs were confirmed by pregnancy, only 8

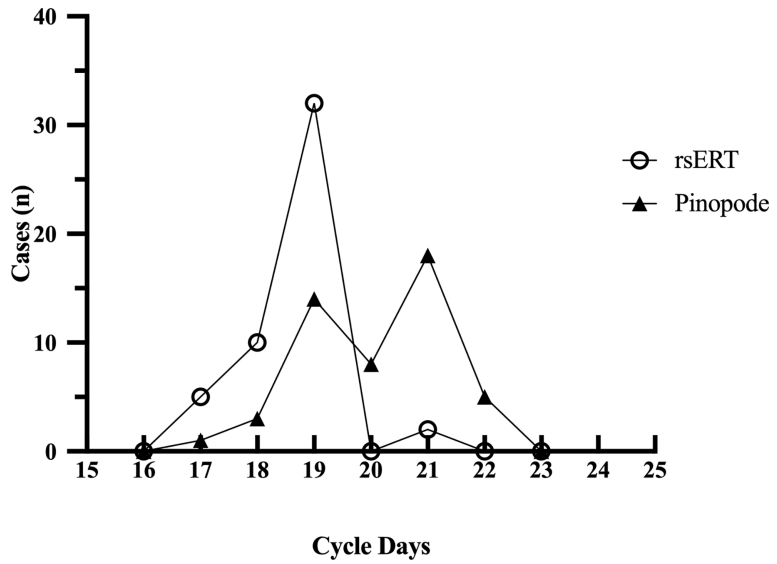

FIGURE 2
Optimal WOIs predicted by rsERT and pinopode of a same RIF group (n=49). The rsERT diagnosed 65.31% of the patients (n=32) with normal WOIs (including 2 patients with prolonged-WOIs), and most of the displacements were advancements (15/49, 30.61%). While according to pinopode, 28.57% (n=14) of the same group were considered with normal-WOIs and 63.27% (n=31) presented delayed pinopode growth pattern.

patients (34.78%) exhibited concordant pinopode patterns, with a Cohen's Kappa index of 0.090 (P=0.112), demonstrating poor concordance.

Notably, there are 7 patients who once received pinopode-instructed pET in our center but failed to conceive after several attempts. They were recruited for the rsERT test, and 6 of them received a different result. Five patients conceived on the first attempt after pET (Supplementary Table S3).

To evaluate the accuracy and efficacy of the tools, patients with rsERT- or pinopode-instructed pET histories were included retrospectively, and their clinical outcomes during the first pET cycle were compared as shown in Table 2. 42 pairs of patients were matched for age, BMI, endometrial preparation method, and number of transferred high-quality embryos with PSM in a 1:1 ratio. Age, BMI, infertility type, baseline FSH, LH, AMH and number of previous transferred cycles were comparable between groups; however, but infertility duration of patients in rsERT group was significantly longer. Particularly, a higher proportion of patients were diagnosed with displaced-WOI in the pinopode group.

All the patients received pET instructed either by rsERT or pinopode. No statistically significant differences were observed between the groups in terms of the endometrial preparation method, endometrial thickness, or endometrial pattern. Although more high-quality embryos were transferred in the pinopode group, the intrauterine pregnancy rate, implantation rate and live birth rate were significantly higher in the rsERT group (50.00 vs. 16.67%, P=0.001; 33.33% vs. 10.96%, P=0.001; 42.86% vs. 14.29, P=0.004). Three patients in the rsERT group and one patient in the pinopode group had miscarriages due to embryonic chromosomal abnormalities and fetal malformation. One patient experienced an ectopic pregnancy.

Patients in the rsERT group have a higher successful pregnancy rate while requiring fewer ET cycles, as shown in Figure 3. 21 of 42 patients conceived successfully in the first ET cycle compared to 7 of 42 of the pinopode group (50.00% vs. 16.67%, P=0.001). 14 and 29 patients, respectively, remained persistent infertile after three attempted treatment cycles (33.33% vs. 69.05%, P=0.001).

Discussion

Endometrial receptivity is a crucial event that its understanding has been one of the centers of dispute for researchers studying human reproduction (45). While histological and morphological markers being gold standards for almost half of a century, their accuracy and consistency are contested (46, 47). Rapid developments took place in omics technology in the past few decades from theoretical research to clinical applications, providing us with novel insights and a deeper understanding of endometrial receptivity (28). However, clinical evidence is required before widespread use.

Previous studies found poor consistency between the transcriptomic tool and histological dating while supporting the superiority of the former (38–40), here we bridge the gap

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TABLE 1 Basic information and predicted optimal WOIs of the 23 pregnant patients.

No.	Age (y)	Infertility Type[1]	Previous Failed Cycles(n)	No. of Previous Transferred good-quality embryos(n)	rsERT[2]	Pinopode	pET Cycles (n)
1	31	1	3	4	N	D	1
2	27	1	3	3	N	D	1
3	32	2	3	5	N	N	1
4	31	1	4	8	A	D	1
5	32	2	7	10	N	D	1
6	30	1	4	8	A	D	1
7	29	1	2	1	D	N	1
8	34	1	2	3	N*	N	1
9	33	2	3	4	D	D	1
10	31	2	2	3	N	D	1
11	36	4	2	3	N	N	1
12	27	1	2	4	N	D	1
13	28	1	2	3	A	D	1
14	34	1	4	6	N	N	1
15	34	2	2	4	N	D	1
16	33	1	4	5	A	D	1
17	28	1	2	3	A	D	1
18	35	1	6	3	N	N	1
19	32	2	2	2	N	D	2
20	27	1	4	6	N	N	2
21	32	1	2	2	A	A	2
22	31	1	3	3	N	D	2
23	37	1	3	4	N	D	3

Basic information and predicted optimal WOIs of the 23 patients who conceived successfully after rsERT-instructed pET. [1]Infertility type:1= primary infertility, 2 = secondary infertility; [2]Predicted optimal WOI by rsERT or pinopode evaluation: A = Advanced WOI (P+3/+4 or LH+5/+6), N = Normal WOI (P+5 or LH+7), D = Delayed WOI (P+6/+7 or LH+8/+9). *Patient No.8 was diagnosed with prolonged-WOI by rsERT, from P+5 to P+7.

by presenting the first evidence comparing rsERT to pinopode evaluation in predicting WOI and instructing personalized embryo transfer, where similar poor concordance was found. According to rsERT, most RIF patients had normal WOIs, and the majority of displaced WOIs were advanced, which is consistent with earlier research (30, 31, 38, 40). However, most of the same group present delayed pinopode growth patterns.

Pinopode has been suggested as a controversial marker of endometrial receptivity. It displays cycle-dependent changes in morphology and is most prominent during the putative WOI (48). Several studies established pinopode as a reliable indicator of WOI, and pinopode-instructed pET showed promising clinical outcomes. However, its indicator role in humans remains obscure and controversial (12). Prior studies found no significant difference in pinopode morphology and coverage between the recurrent pregnancy loss group and the typical fertile group (20) or between infertile patients with and without endometriosis (49). According to our previous work, patients presenting with few or no mature pinopode nevertheless have a chance of becoming pregnant (15). Possible explanations include the randomness of sampling, the limited fields being counted, and the inevitable subjectivity and variability between observers. Endometrium luminal surface is highly heterogeneous. It is found that most of the endometrial samples obtained from the luteal phase showed only 5% to 20% coverage of pinopodes (19). While increasing the number of counting fields may help reduce sample error, as recommended 60 fields in each specimen for calculating pinopode coverage (13), it is impractical for clinical use compared to other diagnostic tools such as ultrasound, histological analyze, ERA or rsERT, etc. Another suspicion was raised by Usadi et al., who found pinopodes persisted for the entire duration of the secretory phase (48), accordant with Quinn et al., who reported pinopodes present throughout the luteal phase of the menstrual cycle, even up to the eleventh week of pregnancy (19), contradicting the perceptive role of an indicator.

The transcriptomic tools demonstrate natural advantages in terms of objectivity and precision. According to rsERT, the transcriptomic signatures of endometrium were identified and classified into pre-receptive, receptive, and post-receptive stages, hence establishing a precise individualized WOI and aiding clinical decisions. According to our previous study, pET based on pinopode could assist 33.82% of RIF patients to achieve clinical pregnancy, whereas only 8.11% of patients in the

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TABLE 2 Baseline information and clinical outcomes of patients that received pET (after PSM).

Parameter	rsERT	Pinopode	P-value
Patients, n	42	42	–
Age, y	31.93 ± 3.07	31.56 ± 3.30	0.636
BMI, kg/m^2	21.10 ± 2.23	21.30 ± 2.49	0.719
Infertility duration, y	5.74 ± 3.61	4.05 ± 2.61	**0.019**
Infertility type			
Primary infertility	28	22	0.182
Secondary infertility	14	20	
Etiology of infertility			
Tubal	33	39	0.176
Endometriosis	1	1	
DOR	5	2	
Other	3	0	
Previous transferred cycles, n	3 (2-4)	3 (2-3)	0.134
Baseline FSH, mIU/ml	6.82 (5.85-8.15)	6.10 (5.10-7.15)	0.074
Baseline LH, mIU/ml	4.96 (4.19-6.47)	4.90 (4.01-6.83)	0.687
AMH, ng/ml	3.454 ± 3.576	2.594 ± 1.512	0.431
Displaced-WOI, n (%)	12 (28.57)	34(80.95)	**0.000**
Endometrial preparing method, n			
HRT	19	27	0.079
NC	23	15	
Endometrial thickness, mm	9.10 (7.90-10.90)	9.10 (8.00-10.70)	0.707
Endometrial pattern*, n			0.602
A	8	7	
B	31	34	
C	3	1	
P levels on the day of progesterone administration	0.11(0.08-0.30)	0.30(0.24-0.40)	0.056
No. of transferred embryos, n	75	73	0.590
Stage of embryo development, n			
D3	36	41	0.320
D5	39	32	
No. of good-quality embryos, n	52	64	**0.007**
Intrauterine pregnancy rate, n (%)	21(50.00)	7(16.67)	**0.001**
Implantation rate, n (%)	25(33.33)	8(10.96)	**0.001**
Miscarriage rate, n (%)	3(14.29)	1(14.29)	0.708
Ectopic pregnancy rate, n (%)	1(2.38)	0(0.00)	0.500
Live birth rate, n (%)	18(42.86)	6(14.29)	**0.004**

Values presented as mean ± SD or median (IQR). Endometrial thickness and endometrial pattern were measured on the day of progesterone administration. *Endometrial pattern was classified as A (a triple-line pattern), B (an intermediate isoechogenic pattern) and pettern C (homogenous hyperechogenic pattern). Bold value represents statistically significant.

conventional FET group conceived (15). Here by conducting rsERT-instructed embryo transfer, both the intrauterine pregnancy rate and the implantation rate were further elevated (50.00% vs. 16.67%, 33.33% vs. 10.96%), and patients in the rsERT group require fewer ET cycles for a successful pregnancy. Similar promising results were observed by applying pET based on ERA, the cumulative pregnancy rate was significantly higher in the pET group compared with the regular ET group (93.6% vs. 79.9%) (33), and a comparable pregnancy rate was obtained in the receptive patients and nonreceptive patients (58.8% vs. 50.0%) (31). All of which demonstrated the feasibility and potential value of the tools.

In this study, we found 65.31% of RIF patients with normal WOIs, however, for patients that received pET, 33.33% of them remained persistent failure after multiple attempts. That, apart from a displaced-WOI, other factors coexisted still hampering successful pregnancy. Disrupted WOI might be one of the possible explanations. By analyzing endometrial transcriptomics of RIF patients, Koot et al. (28) considered RIF to be a result combining displacement and disrupted WOI, whereas Leon et al.

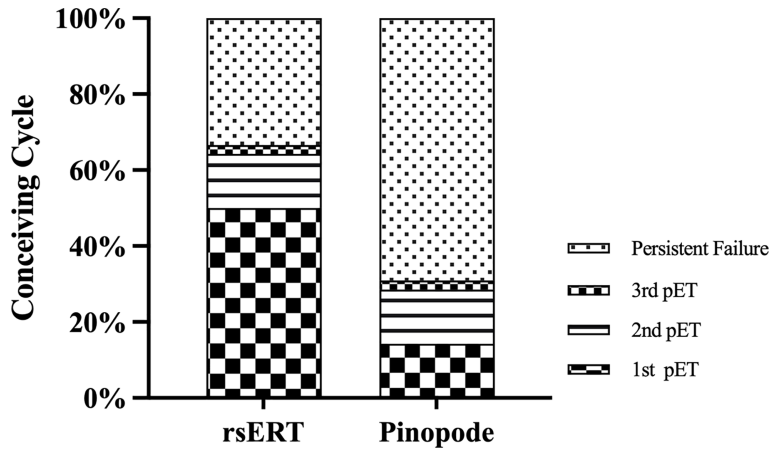

FIGURE 3
Histogram with the distribution of conceiving cycle between the groups. Patients in the rsERT group have a higher successful pregnancy rate and require fewer ET cycles.

(50) suggested that those factors either alone or together lead to repeated implantation failure. Other factors, including hyperplasia, submucosal myomas, endometrial polyps, endometritis, etc., are common reasons hampering embryo implantation (51). However, despite all the pathologies being corrected, insufficient endometrial receptivity might persist. While modified embryo transfers could rescue asynchrony, pathology remains a tough problem.

Another notable issue is the between-cycle consistency of the tools. Considering its invasive nature and the expected indicating role, most of the tests are conducted before the transfer cycle, making cycle variability an inescapable question. As for pinopode, a study conducted by Ordi et al. shows that, when taking one biopsy per cycle for three consecutive spontaneous cycles, most of the patients showed poor intra-patient consistency in pinopode scores that indicated low predictive value (52). While considering transcriptomic tools, a previous study recruited seven women for a second ERA test on the same day, approximately one month later, where consistent results were found (39). However, Arianne et al. (40) found poor reproducibility of the ERA test, as only 5 of 14 patients initially diagnosed as "non-receptive" with ERA obtained the same diagnosis. Further studies with larger populations are needed before a safe conclusion.

While transcriptomics revolutionized the field, its expense and invasive nature continue to be obstacles. Displaced-WOIs were identified in 24% to 84.9% of RIF patients and 12% of the controls (25, 26, 30), and rescue of "non-receptive" was found a promising therapy (25, 29, 31). However, it has been shown that ERA does not improve pregnancy outcomes compared to standard ET strategy in the first embryo transfer cycles, or in populations including RIF or patients with good prognosis (26, 36, 38). Considering the present state of research, transcriptomic tools were not recommended as a routine test for unselected patient populations (26). As it has been reported, half of the couples quit before any fertility treatment was started, and one-third stopped after at least one IVF cycle (53). Psychological stress and lost hope for success are the primary considerations. Therefore, while diagnosing recurrent implantation failure calls for consideration and prudence, for patients who have experienced two or more failed cycles, positive actions, including endometrial receptivity assessments, are recommended.

Lastly, our study has points of limitations. Firstly, we took three endometrial samples from the same patient at 48-hour intervals to observe a growth pattern of pinopode. Although we attempted to avoid repeated sampling from the same uterine wall, it is difficult to preclude the possible effect of repeat sampling. Secondly, due to the retrospective design of Part 2 of the study, the sample size is limited, and the study is lacking randomization. A prospective randomized controlled trial would allow for more accurate comparison and clarify its clinical value. Our study was conducted quite early between 2017 and 2019, and we have recently revised the rsERT model. It can now predict a precise time window based on a single endometrial biopsy and shows potential clinical value. Clinical trials including multi-center studies are being conducted for validation. Thirdly, a further comparison is required between transcriptomic tools, such as rsERT and ERA.

Conclusion

In conclusion, our study demonstrated that the novel transcriptomic tool rsERT is superior to the standard marker pinopode in diagnosing WOI and instructing personalized embryo transfer. However, considering the limited sample size,

prospective clinical trials and basic research are needed for further validation and clinical application.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee of the department of reproductive medicine, Xiangya Hospital, Central South University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

JC contributed to data analysis and drafted the article. AH and QZ participated in data acquisition. JZ and JF participated in sample collection. HL and YL are the corresponding author, they contributed to the design of the work. All the authors agreed with their contributions and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1009161/full#supplementary-material

References

1. Aghajanova L, Hamilton AE, Giudice LC. Uterine receptivity to human embryonic implantation: histology, biomarkers, and transcriptomics. *Semin Cell Dev Biol* (2008) 19(2):204–11. doi: 10.1016/j.semcdb.2007.10.008

2. Messaoudi S, El Kasmi I, Bourdiec A, Crespo K, Bissonnette L, Le Saint C, et al. 15 years of transcriptomic analysis on endometrial receptivity: what have we learnt? *Fertil Res Pract* (2019) 5:9. doi: 10.1186/s40738-019-0059-7

3. Nikas G, Aghajanova L. Endometrial pinopodes: some more understanding on human implantation? *Reprod BioMed Online* (2002) 4 Suppl 3:18–23. doi: 10.1016/s1472-6483(12)60111-4

4. Franasiak JM, Ruiz-Alonso M, Scott RT, Simón C. Both slowly developing embryos and a variable pace of luteal endometrial progression may conspire to prevent normal birth in spite of a capable embryo. *Fertil Steril* (2016) 105(4):861–6. doi: 10.1016/j.fertnstert.2016.02.030

5. Wilcox AJ, Baird DD, Weinberg CR. Time of implantation of the conceptus and loss of pregnancy. *N Engl J Med* (1999) 340(23):1796–9. doi: 10.1056/NEJM199906103402304

6. Valdes CT, Schutt A, Simon C. Implantation failure of endometrial origin: it is not pathology, but our failure to synchronize the developing embryo with a receptive endometrium. *Fertil Steril* (2017) 108(1):15–8. doi: 10.1016/j.fertnstert.2017.05.033

7. Nikas G. Cell-surface morphological events relevant to human implantation. *Hum Reprod* (1999) 14 Suppl 2:37–44. doi: 10.1093/humrep/14.suppl_2.37

8. Stavreus-Evers A, Nikas G, Sahlin L, Eriksson H, Landgren BM. Formation of pinopodes in human endometrium is associated with the concentrations of progesterone and progesterone receptors. *Fertil Steril* (2001) 76(4):782–91. doi: 10.1016/s0015-0282(01)01993-8

9. Nardo LG, Nikas G, Makrigiannakis A, Sinatra F, Nardo F. Synchronous expression of pinopodes and alpha v beta 3 and alpha 4 beta 1 integrins in the endometrial surface epithelium of normally menstruating women during the implantation window. *J Reprod Med* (2003) 48(5):355–61.

10. Lessey BA, Appa Rao KB, Bagnell RC. Osteopontin normally localized to the apical surface of receptive endometrium, is lacking in women with endometriosis with aberrant αvβ3 integrin expression. *Fertil Steril* (2001) 76(Supplement 1):S60.

11. Kabir-Salmani M, Nikzad H, Shiokawa S, Akimoto Y, Iwashita M. Secretory role for human uterodomes (pinopods): secretion of LIF. *Mol Hum Reprod* (2005) 11(8):553–9. doi: 10.1093/molehr/gah218

12. Quinn CE, Casper RF. Pinopodes: a questionable role in endometrial receptivity. *Hum Reprod Update* (2009) 15(2):229–36. doi: 10.1093/humupd/dmn052

13. Jin XY, Zhao LJ, Luo DH, Liu L, Dai YD, Hu XX, et al. Pinopode score around the time of implantation is predictive of successful implantation following frozen embryo transfer in hormone replacement cycles. *Hum Reprod* (2017) 32(12):2394–403. doi: 10.1093/humrep/dex312

14. Bahar L, Kahraman S, Eras N, Pirkevi C. Comparison of endometrial biopsies of fertile women and women with repeated implantation failure at the ultrastructural level. *Turk J Med Sci* (2015) 45(3):706–13. doi: 10.3906/sag-1402-153

15. Qiong Z, Jie H, Yonggang W, Bin X, Jing Z, Yanping L. Clinical validation of pinopode as a marker of endometrial receptivity: a randomized controlled trial. *Fertil Steril* (2017) 108(3):513–517.e2. doi: 10.1016/j.fertnstert.2017.07.006

16. D'Ippolito S, Di Nicuolo F, Papi M, Castellani R, Palmieri V, Masciullo V, et al. Expression of pinopodes in the endometrium from recurrent pregnancy loss women. *Role Thrombomodulin Ezrin J Clin Med* (2020) 9(8):2634. doi: 10.3390/jcm9082634

17. Aunapuu M, Kibur P, Järveots T, Arend A. Changes in morphology and presence of pinopodes in endometrial cells during the luteal phase in women with infertility problems: A pilot study. *Medicina (Kaunas)* (2018) 54(5):69. doi: 10.3390/medicina54050069

18. Sudoma I, Goncharova Y, Zukin V. Optimization of cryocycles by using pinopode detection in patients with multiple implantation failure: preliminary report. *Reprod BioMed Online* (2011) 22(6):590–6. doi: 10.1016/j.rbmo.2011.02.004

19. Quinn C, Ryan E, Claessens EA, Greenblatt E, Hawrylyshyn P, Cruickshank B, et al. The presence of pinopodes in the human endometrium does not delineate the implantation window. *Fertil Steril* (2007) 87(5):1015–21. doi: 10.1016/j.fertnstert.2006.08.101

20. Xu B, Sun X, Li L, Wu L, Zhang A, Feng Y. Pinopodes, leukemia inhibitory factor, integrin-β3, and mucin-1 expression in the peri-implantation endometrium of women with unexplained recurrent pregnancy loss. *Fertil Steril* (2012) 98 (2):389–95. doi: 10.1016/j.fertnstert.2012.04.032

21. Quinn CE, Detmar J, Casper RF. Pinopodes are present in lif null and Hoxa10 null mice. *Fertil Steril* (2007) 88(4 Suppl):1021–8. doi: 10.1016/j.fertnstert.2006.11.157

22. Rekker K, Altmäe S, Suhorutshenko M, Peters M, Martinez-Blanch JF, Codoñer FM, et al. A two-cohort RNA-seq study reveals changes in endometrial and blood miRNome in fertile and infertile women. *Genes (Basel).* (2018) 9 (12):574. doi: 10.3390/genes9120574

23. Borthwick JM, Charnock-Jones DS, Tom BD, Hull ML, Teirney R, Phillips SC, et al. Determination of the transcript profile of human endometrium. *Mol Hum Reprod* (2003) 9(1):19–33. doi: 10.1093/molehr/gag004

24. Ponnampalam AP, Weston GC, Trajstman AC, Susil B, Rogers PA. Molecular classification of human endometrial cycle stages by transcriptional profiling. *Mol Hum Reprod* (2004) 10(12):879–93. doi: 10.1093/molehr/gah121

25. Ruiz-Alonso M, Blesa D, Díaz-Gimeno P, Gómez E, Fernández-Sánchez M, Carranza F, et al. The endometrial receptivity array for diagnosis and personalized embryo transfer as a treatment for patients with repeated implantation failure. *Fertil Steril* (2013) 100(3):818–24. doi: 10.1016/j.fertnstert.2013.05.004

26. Riestenberg C, Kroener L, Quinn M, Ching K, Ambartsumyan G. Routine endometrial receptivity array in first embryo transfer cycles does not improve live birth rate. *Fertil Steril* (2021) 115(4):1001–6. doi: 10.1016/j.fertnstert.2020.09.140

27. Tapia A, Gangi LM, Zegers-Hochschild F, Balmaceda J, Pommer R, Trejo L, et al. Differences in the endometrial transcript profile during the receptive period between women who were refractory to implantation and those who achieved pregnancy. *Hum Reprod* (2008) 23(2):340–51. doi: 10.1093/humrep/dem319

28. Koot YE, van Hooff SR, Boomsma CM, Van LD, Groot KM, Goddijn M, et al. An endometrial gene expression signature accurately predicts recurrent implantation failure after IVF. *Sci Rep* (2016) 6:19411. doi: 10.1038/srep19411

29. He A, Zou Y, Wan C, Zhao J, Zhang Q, Yao ZY, et al. The role of transcriptomic biomarkers of endometrial receptivity in personalized embryo transfer for patients with repeated implantation failure. *J Transl Med* (2021) 19 (1):176. doi: 10.1186/s12967-021-02837-y

30. Jia Y, Dong YJ, Sha YL, Cai SC, Diao LH, Qiu Z, et al. Effectiveness comparison between endometrial receptivity array, immune profiling and the combination in treating patients with multiple implantation failure. *Am J Reprod Immunol* (2022) 87(3):e13513. doi: 10.1111/aji.13513

31. Hashimoto T, Koizumi M, Doshida M, Toya M, Sagara E, Oka N, et al. Efficacy of the endometrial receptivity array for repeated implantation failure in Japan: A retrospective, two-centers study. *Reprod Med Biol* (2017) 16(3):290–6. doi: 10.1002/rmb2.12041

32. Mahajan N. Endometrial receptivity array: Clinical application. *J Hum Reprod Sci* (2015) 8(3):121–9. doi: 10.4103/0974-1208.165153

33. Simón C, Gómez C, Cabanillas S, Vladimirov L, Castillón G, Giles J, et al. A 5-year multicentre randomized controlled trial comparing personalized, frozen and fresh blastocyst transfer in IVF. *Reprod BioMed Online* (2020) 41(3):402–15. doi: 10.1016/j.rbmo.2020.06.002

34. Tan J, Kan A, Hitkari J, Taylor B, Tallon N, Warraich G, et al. The role of the endometrial receptivity array (ERA) in patients who have failed euploid embryo transfers. *J Assist Reprod Genet* (2018) 35(4):683–92. doi: 10.1007/s10815-017-1112-2

35. Saxtorph MH, Hallager T, Persson G, Petersen KB, Eriksen JO, Larsen LG, et al. Assessing endometrial receptivity after recurrent implantation failure: a prospective controlled cohort study. *Reprod BioMed Online* (2020) 41(6):998–1006. doi: 10.1016/j.rbmo.2020.08.015

36. Cozzolino M, Diaz-Gimeno P, Pellicer A, Garrido N. Evaluation of the endometrial receptivity assay and the preimplantation genetic test for aneuploidy in overcoming recurrent implantation failure. *J Assist Reprod Genet* (2020) 37 (12):2989–97. doi: 10.1007/s10815-020-01948-7

37. Fodina V, Dudorova A, Erenpreiss J. Evaluation of embryo aneuploidy (PGT-a) and endometrial receptivity (ERA) testing in patients with recurrent implantation failure in ICSI cycles. *Gynecol Endocrinol* (2021) 37(sup1):17–20. doi: 10.1080/09513590.2021.2006466

38. Bassil R, Casper R, Samara N, Hsieh TB, Barzilay E, Orvieto R, et al. Does the endometrial receptivity array really provide personalized embryo transfer? *J Assist Reprod Genet* (2018) 35(7):1301–5. doi: 10.1007/s10815-018-1190-9

39. Díaz-Gimeno P, Ruiz-Alonso M, Blesa D, Bosch N, Martinez-Conejero JA, Alama P, et al. The accuracy and reproducibility of the endometrial receptivity array is superior to histology as a diagnostic method for endometrial receptivity. *Fertil Steril* (2013) 99(2):508–17. doi: 10.1016/j.fertnstert.2012.09.046

40. Cohen AM, Ye XY, Colgan TJ, Greenblatt EM, Chan C. Comparing endometrial receptivity array to histologic dating of the endometrium in women with a history of implantation failure. *Syst Biol Reprod Med* (2020) 66(6):347–54. doi: 10.1080/19396368.2020.1824032

41. Van Royen E, Mangelschots K, De Neubourg D, Valkenburg M, Van de Meerssche M, Ryckaert G, et al. Characterization of a top quality embryo, a step towards single-embryo transfer. *Hum Reprod* (1999) 14(9):2345–9. doi: 10.1093/humrep/14.9.2345

42. Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertil Steril* (2000) 73(6):1155–8. doi: 10.1016/s0015-0282(00)00518-5

43. Nikas G. Pinopodes as markers of endometrial receptivity in clinical practice. *Hum Reprod* (1999) 14 Suppl 2:99–106. doi: 10.1093/humrep/14.suppl_2.99

44. Nikas G, Psychoyos A. Uterine pinopodes in peri-implantation human endometrium. *Clin relevance. Ann N Y Acad Sci* (1997) 816:129–42. doi: 10.1111/j.1749-6632.1997.tb52136.x

45. Horcajadas JA, Pellicer A, Simón C. Wide genomic analysis of human endometrial receptivity: new times, new opportunities. *Hum Reprod Update* (2007) 13(1):77–86. doi: 10.1093/humupd/dml046

46. Quinn KE, Matson BC, Wetendorf M, Caron KM. Pinopodes: Recent advancements, current perspectives, and future directions. *Mol Cell Endocrinol* (2020) 501:110644. doi: 10.1016/j.mce.2019.110644

47. Murray MJ, Meyer WR, Zaino RJ, Lessey BA, Novotny DB, Ireland K, et al. A critical analysis of the accuracy, reproducibility, and clinical utility of histologic endometrial dating in fertile women. *Fertil Steril* (2004) 81(5):1333–43. doi: 10.1016/j.fertnstert.2003.11.030

48. Usadi RS, Murray MJ, Bagnell RC, Fritz MA, Kowalik AI, Meyer WR, et al. Temporal and morphologic characteristics of pinopod expression across the secretory phase of the endometrial cycle in normally cycling women with proven fertility. *Fertil Steril* (2003) 79(4):970–4. doi: 10.1016/s0015-0282(02)04929-4

49. Da Broi MG, Rocha CVJr, Carvalho FM, Martins WP, Ferriani RA, Navarro PA. Ultrastructural evaluation of eutopic endometrium of infertile women with and without endometriosis during the window of implantation: A pilot study. *Reprod Sci* (2017) 24(10):1469–75. doi: 10.1177/1933719117691142

50. 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(4):626–35. doi: 10.1093/humrep/dey023

51. Bellver J, Simón C. Implantation failure of endometrial origin: what is new? *Curr Opin Obstet Gynecol* (2018) 30(4):229–36. doi: 10.1097/GCO.0000000000000468

52. Ordi J, Creus M, Quintó L, Casamitjana R, Cardesa A, Balasch J. Within-subject between-cycle variability of histological dating, alpha v beta 3 integrin expression, and pinopod formation in the human endometrium. *J Clin Endocrinol Metab* (2003) 88(5):2119–25. doi: 10.1210/jc.2002-021659

53. Brandes M, van der Steen JO, Bokdam SB, Hamilton CJCM, de Bruin JP, Nelen WLDM, et al. When and why do subfertile couples discontinue their fertility care? a longitudinal cohort study in a secondary care subfertility population. *Hum Reprod* (2009) 24(12):3127–35. doi: 10.1093/humrep/dep340

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Endometriosis-associated infertility: From pathophysiology to tailored treatment

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Despite the clinically recognized association between endometriosis and infertility, the mechanisms implicated in endometriosis-associated infertility are not fully understood. Endometriosis is a multifactorial and systemic disease that has pleiotropic direct and indirect effects on reproduction. A complex interaction between endometriosis subtype, pain, inflammation, altered pelvic anatomy, adhesions, disrupted ovarian reserve/function, and compromised endometrial receptivity as well as systemic effects of the disease define endometriosis-associated infertility. The population of infertile women with endometriosis is heterogeneous, and diverse patients' phenotypes can be observed in the clinical setting, thus making difficult to establish a precise diagnosis and a single mechanism of endometriosis related infertility. Moreover, clinical management of infertility associated with endometriosis can be challenging due to this heterogeneity. Innovative non-invasive diagnostic tools are on the horizon that may allow us to target the specific dysfunctional alteration in the reproduction process. Currently the treatment should be individualized according to the clinical situation and to the suspected level of impairment. Here we review the etiology of endometriosis related infertility as well as current treatment options, including the roles of surgery and assisted reproductive technologies.

KEYWORDS

endometriosis, infertility, pathogenesis, ovarian reserve, endometrial receptivity, *in-vitro* fertilization (IVF), stem cell

Background

Endometriosis is a complex and systemic clinical syndrome that can negatively impact on women's reproductive health and quality of life (1). Chronic inflammation and hormonal dependance are the main underlying pathophysiologic mechanisms that drive endometriosis, and the association of these two key biological features make the natural history of this disease distinct.

A possible relationship between endometriosis and infertility was first suggested in the *Corpus Hippocraticum*, as women suffering from dysmenorrhea were urged to conceive as quickly as possible to increase the chance of become pregnant (2). Today, nearly 10% of women in their reproductive age suffer from endometriosis and about one third of them experience infertility, almost twice the rate observed among women without the disease (3). Up to 50% of infertile women are found to suffer from endometriosis (4).

Despite the clinically recognized association between endometriosis and infertility, the mechanisms implicated in endometriosis-associated infertility are unclear and this condition is currently considered multifactorial. In addition, the diagnosis of endometriosis is currently underestimated due to the almost exclusive reliance on surgical findings, which delays diagnosis until symptoms require surgical intervention. The ability to identify endometriosis also critically depend on surgeon's expertise and may preclude early recognition and treatment. The average time to diagnostics ranges from 4 to 11 years is reported in these patients, and this delay has a significant impact on health-care utilization and costs (5, 6). Indeed, the absence of macroscopic lesions or clinical features does not exclude the diagnosis of endometriosis, as infertility is often the only health concern. Furthermore, only one-half of women with endometriosis-associated infertility show typical lesions (7). In women with infertility, an early diagnosis of endometriosis is crucial from the perspective of fertility because the burden of the disease could be even more deleterious when compounded by the effect of increasing age on ovarian reserve.

The focus of this review is to provide an update of pathophysiology of endometriosis-associated infertility. We will also discuss current medical and surgical strategies, and the role of fertility preservation and of assisted reproductive technologies (ART) in patients with endometriosis.

Pathogenesis of endometriosis

Understanding the pathogenesis of endometriosis is crucial as it may have meaningful clinical and therapeutical implications. To date, none of the proposed theories have been able to comprehensively explain the natural history of the disease and its associated diverse clinical presentations. The common thread to all theories is a complex dysregulated hormonal signaling, enhanced proinflammatory microenvironment that has the potential to drive the initiation, maintenance, and progression of the disease (Figure 1).

Retrograde menstruation theory

The most widely accepted pathogenetic hypothesis is based on retrograde menstruation as proposed by Sampson in 1927 (8–10). Viable endometrial tissue moves into the pelvic cavity through the fallopian tubes at the time of menses, adheres to the peritoneal mesothelial cells, proliferates, and finally invades pelvic structures. Retrograde menstruation is a physiological event that occurs in approximately 90% of women (11–13). Viable endometrial tissue has been identified in the shed menstrual endometrium (13). However, the differences in its morpho-histological, hormonal, and biological composition compared with the eutopic endometrium of healthy women still remain matter of investigation. Endometrial reflux seems to be enhanced in women with endometriosis and possibly driven by the action of prostaglandins that may cause disorganized myometrial contraction (14–16). Moreover, the incidence of endometrial reflux is much higher in women with congenital anomalies causing menstrual outflow obstruction (17). This theory has been well supported by animal models of endometriosis. Normal endometrial tissue placed into the peritoneal cavity recapitulates the disease, including the effects on eutopic endometrium, suggesting that an abnormal endometrium is not a prerequisite for initiation and development of endometriosis (18–21).

Early age at menarche, long duration and heavy menstrual flow are all well-recognized epidemiological risk factors for the development of endometriosis. The anatomical predominance of endometriosis in the right side of both hemipelvis and diaphragm, further supports this theory (22). This asymmetry has been attributed to both a physiological process (the clockwise intraperitoneal current) and an anatomical factor (the presence of the sigmoid colon and falciform ligament). However, the retrograde menstruation hypothesis is not sufficient to explain the development of rare forms of the disease.

Coelomic metaplasia and mullerian remnants hypotheses

The coelomic metaplasia and mullerian remnants hypothesis are both based on the concept that endometriotic lesions originate *in-situ* from embryological remnants or by metaplasia. Based on the mullerian remnants hypothesis ("mullerianosis") (23), endometriosis is a consequence of the aberrant migration and differentiation of embryonic cell rests originating from the Mullerian ducts during organogenesis. This hypothesis can explain the presence of endometriosis in in adolescents before or shortly after menarche and in fetuses (24–26). Embryological studies (24) support the presence of Mullerian remnants in the cul-de-sac area, uterosacral ligaments, and medial broad ligaments. Alternatively, both germinal ovarian epithelium and peritoneum may undergo a Mullerian metaplasia and differentiate into endometrium (27). This latter theory would explain the presence of endometriosis in ovary, sigmoid colon, appendix, or more distal sites such as the diaphragm and pleura (28), although direct infiltration through

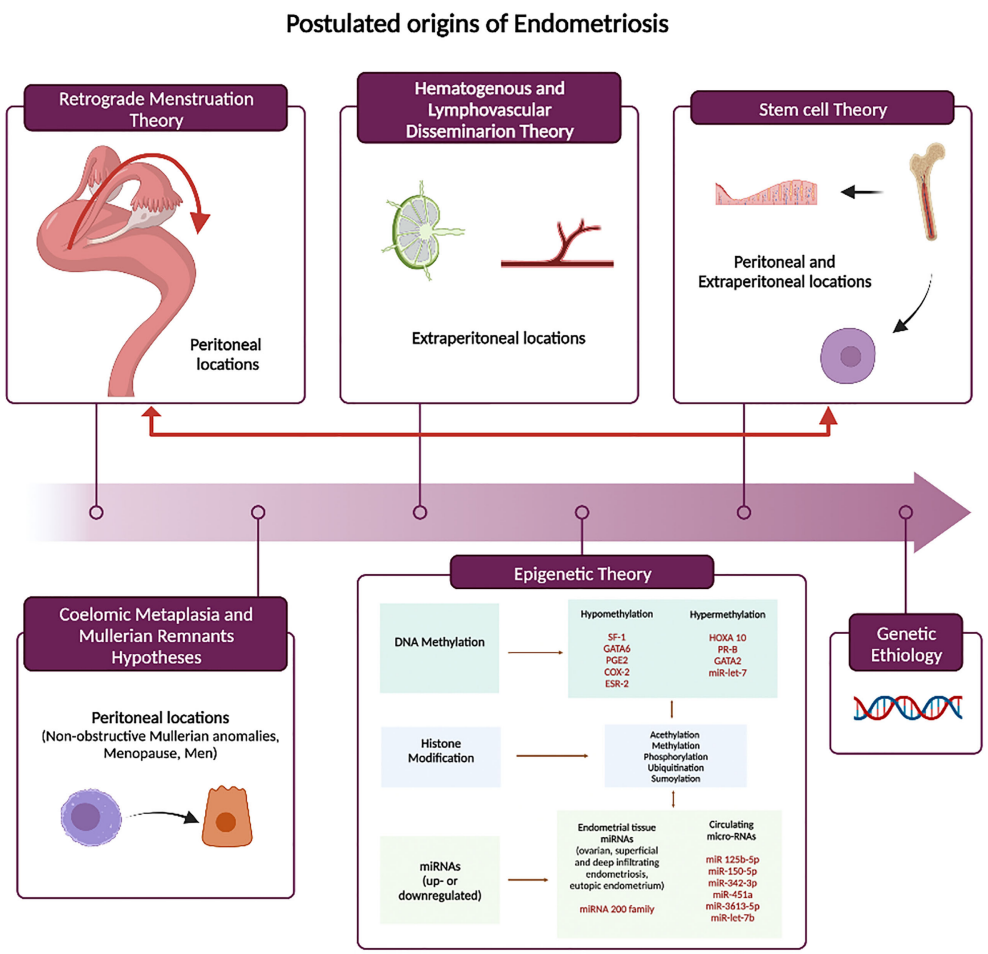

FIGURE 1
Theories of Endometriosis Pathogenesis. SF-1, steroidogenic factor 1;GATA6, GATA binding protein 6;PGE2, Prostaglandin E_2;COX2, cyclooxygenase-2;ESR-2, estrogen receptor alpha; HOXA10, homebox protein A10;PR-B, progesterone receptor isoform B; GATA2, GATA binding protein 2.

diaphragmatic fenestrations is possible. Additionally, both hypotheses may explain rare cases of endometriosis in women with Mayer-Rokitansky-Kuster-Hauser syndrome and other non-obstructive Mullerian anomalies (29–31), in the absence of menstruation (menopause) (32) and in men (33–36).

Hematogenous and lymphovascular dissemination

Sampson recognized that retrograde menstruation does not explain uncommon extraperitoneal locations and diverse clinical presentations with symptoms remote form the pelvis (37). He first suggested hematogenous or lymphatic dissemination of endometrial like-tissue as an alternative theory. This hypothesis implies that endometrial cells enter the uterine vasculature or lymphatic system at menstruation, and they spread to ectopic sites (38). In murine models of surgically induced endometriosis, endometriosis-derived cells are capable of migration and micrometastasis to different extra-pelvic organs including lung, spleen, liver and brain (39). Clinically, this theory has been supported by the presence of endometrial tissue in the uterine vasculature (37) and by evidence of emboli in sentinel lymph nodes (40).

Stem cell theory

In the last few years, it has become clear that altered stem cell trafficking contribute to the etiology and pathophysiology of endometriosis. The first evidence on the contribution of bone marrow derived stem cells (BMDCs) in the regeneration of the endometrium was reported in 2004 (41); subsequent studies have confirmed the bone marrow contribution to endometrium

(42, 43). Both progenitor cells within the endometrium and multipotent cells from bone marrow contribute to endometrial homeostasis. The BMDSCs travel through the circulatory system and contribute to the composition of eutopic endometrium (41). After travelling to endometrium these BMDSCs can become restricted to an endometrial cell lineage, contributing to the pool of both stromal and epithelial endometrial progenitor cells. Some become located in the basal layer of the eutopic endometrium and regenerate on a monthly basis under the influence of estrogens. Furthermore, women with endometriosis have a higher number of these pluripotential cells compared with healthy women during menses (44).

During menstruation, women with endometriosis shed more basalis cells, including progenitor cells, than healthy individuals, these cells can more easily generate endometrium in ectopic locations than differentiated cells and further expand on Sampson's theory of retrograde menstruation (45).

BMDSCs can directly differentiate into endometrium without first being localized in the uterus. Ectopic differentiation of circulating stem cells has been proposed as a pathogenetic mechanism of endometriosis. Mesenchymal extrauterine stem cells derived from bone marrow and other sources may also be involved in the pathogenesis of the disease both in the peritoneal cavity as well as distant sites. Their inappropriate differentiation to endometrial cells at ectopic locations is likely the principal source of extraperitoneal endometriosis (46, 47).

The ability of BMDSCs to contribute to endometriotic lesion and differentiate into endometrial phenotypes help to explain how ectopic tissue can occur in locations outside the peritoneal cavity and in non-peritoneal-derived cells, such as lungs (48–50), central nervous system (51) and in men (33–36). Furthermore, BMDSC are attracted by eutopic endometrium under injury and inflammatory conditions (52). Endometriotic lesions, through the production and release of pro-inflammatory cytokines and chemokines (47) and under estrogenic influence (53) recruit more stem cells to further promote lesion growth. Additionally circulating endothelial progenitor cells contribute to the vascularization of endometriotic lesions. Stem cells are also capable of trafficking between endometriotic lesions and the eutopic endometrium, and therefore likely contribute to the impaired uterine receptivity in these women (54). These cells, derived from endometriosis, migrate as mesenchymal stem cells (MSC), engraft the uterine stroma, however activation epithelial Wnt signaling that likely distorts the epithelial-stromal dialog needed for optimal endometrial development and receptivity.

Genetic etiology

Familiar clusters of endometriosis have been found in humans (55) and nonhuman primates (56, 57). However, no distinct inheritance pattern has been established and the notion that multiple genes contribute to endometriosis is widely accepted. Studies on monozygotic twins show that endometriosis has an estimated total heritability of approximately 51% (58–60). Daughters of mothers with surgically confirmed endometriosis have more than double risk of developing the disease (61). Moreover, familial inherited of endometriosis tends to be more severe with an earlier onset of symptoms compared with sporadic cases (55, 62). Meta-analyses of genome-wide association studies of diverse populations have identified a robust association of endometriosis with certain risk loci involved in sex steroid hormone pathways, indicating a possible role in the development of advanced stages of endometriosis (63–68). However, none are common and in total these genetic variants account for only a small fraction disease risk. In general, a multitude of genetic variants with only weak individual effects care likely responsible for the increased hereditary risk of endometriosis (69).

In the context of infertility associated with endometriosis, a recent cross-sectional study including 213 infertile women with endometriosis who underwent IVF procedures, found that single nucleotide variants of FSHB and FSHR separately interfered with the hormonal profile (both FSH and LH levels) and ultimately with the number of oocytes retrieved in these patients at any stage of the disease (70).

The epigenetic theory

There is a growing body of evidence that epigenetics has a key role in the pathogenesis of endometriosis. Epigenetic modifications involve dynamic and reversible changes in the chromatin structure influencing gene expression in a heritable fashion. Epigenetic phenomena are likely to have implications for diagnosis, prognosis and for the possibility of developing targeted therapeutic strategies. The hallmarks of epigenetic gene regulation are DNA methylation (hypo and hypermethylation), histone modifications, and microRNA production, which lead to expression or suppression of specific proteins. Comparative studies of both ectopic lesions and eutopic endometrium stromal cells have provided data on the role of epigenetic factors in the etiopathogenesis of endometriosis and its related infertility (19, 71–73).

DNA methylation is one of the most common epigenetic modifications and active in endometrium. Numerous studies have revealed a direct correlation with the expression of genes influencing the implantation process in eutopic endometrium of women with endometriosis. Homebox protein-A10 (HOXA10) is a gene that has a well characterized and essential role in generating a receptive endometrium. Hypermethylation of the HOXA gene promoter has been demonstrated both in animals and in the eutopic endometrium of women with endometriosis compared to healthy controls (19, 74, 75). As promoter

hypermethylation is generally associated with gene silencing, the reduced HOXA10 gene expression in the endometrium of women with endometriosis is, at least in part, responsible for the impaired uterine receptivity. Conversely, one recent study (76) found hypomethylation of the HOXA10 gene in the endometrium of women with a previous history of endometriosis and under hormonal treatment at the time of surgery, opening the possibility that long-term therapy may reverse epigenetic signatures classically seen in the disease. Aberrant DNA methylation patterns have also been found in endometriotic tissue compared with eutopic endometrium. The promoter of progesterone receptor (PR) isoform B gene is hypermethylated in endometriosis, with subsequent reduced PR-B expression (77–79) contributing to the relatively persistent progesterone resistance. Similarly, the different level of expression and methylation (hypo or hyper) of certain transcriptional factors (GATA6, GATA 2 and steroidogenic factor 1(SF1)) may account for estrogen dependency and progesterone resistance by changing the expression of both estrogen receptor-beta and progesterone receptor (80–82). Lastly, the invasive proprieties of endometriotic cells have also found to be regulated by hypermethylation in endometriosis (83–85). Let-7 microRNA is hypermethylated in endometriosis leading to decreased Let-7 expression and disinhibition of KRAS and other genes that drive endometriosis growth and invasion (86).

Little is known about the role of histone modifications in the pathogenesis of endometriosis, and results are often conflicting. A marked histone hypoacetylation has been shown in endometriotic stromal cells of both eutopic and ectopic tissue of affected women compared to healthy endometrium (87) and HDAC enzymes seems to play a key role in this process (88–91). Also, acetylation levels of H3 and H4 histones are lower in ectopic lesions and eutopic endometrium of women with endometriosis compared with healthy women (92, 93).

MicroRNAs (MiRNAs) are small RNA molecules of approximately 22 bases. They interact with mRNA and change gene expression by inhibiting translation or inducing mRNA degradation. Their increased expression causes repression of translation from the mRNA while decreased MiRNA expression can lead to upregulation of protein production from mRNA. They also target and regulate both methylation and acetylation processes, thereby modifying the epigenome. Unlike other epigenetic mechanisms, miRNAs regulate gene expression at a post-transcriptional level, and they are found both intra- and extracellularly (94). They target genes involved in hormone metabolism, cell cycle proliferation, migration, and invasion, immune- inflammatory response, epithelial-mesenchymal transition (EMT), apoptosis and angiogenesis (95). Differential expression of more than 100 miRNAs has been found in paired endometriotic lesions and eutopic endometrium of women with and without endometriosis (96, 97). In addition, different expression profiles were detected and reported as characteristic to each lesion subtype (ovarian, superficial, and deep infiltrating endometriosis) (98). Moreover, miRNA signatures in endometrium are likely to change with the respect of the different phases of the menstrual cycle (99, 100). The most frequently detected miRNA both in endometriomas and endometriotic lesions, found to be downregulated in six studies was miR-200 family, known to play a crucial role in the EMT, a relevant process in the establishment of endometriotic lesions (101, 102). Other miRNA reported to be differentially regulated (up- or downregulated) in endometriotic lesions in more than two studies were miR-1, -29c, -34c, -100, -141, -145, -183, -196b, -200a, -200b, -200c, -202, -365, and -375 (103). Several of these are also known to be involved in EMT, as well as, cell proliferation, cell adhesion, invasion and angiogenesis and demonstrating binding to target mRNAs is an important step to validate their role in the pathogenesis of the disease. Extracellular miRNAs are found in all body fluids, including the circulation (both serum and plasma) (104). Circulating microRNAs can potentially impact endometriotic lesion development by mediating intercellular communication between eutopic endometrium and ectopic implants (105).

Pathophysiology of endometriosis associated infertility

Translational animal models of endometriosis associated infertility

Considering the limited knowledge of endometriosis pathophysiology, research has long focused on finding animal models to study suspected pathogenic mechanisms and to find novel targets for therapy. As with human endometriosis, animal models of endometriosis reveal an impact on fecundity in terms of impaired folliculogenesis, ovulation, fertilization, implantation or embryonic developement (106). Non-human primates have been extensively used as experimental models for endometriosis because of their phylogenetic proximity to humans. They menstruate cyclically and therefore they can develop endometriosis spontaneously, resembling the human disease based on retrograde menstruation. To date, 11 species of menstruating non-human primates have been reported (107). Ectopic lesions are laparoscopically and histologically identical and at a similar pelvic sites (108). However, spontaneous endometriosis develops slowly, at a lower rate compared to humans and might be multifactorial. Therefore, alternative methods of artificially induced endometriosis have been introduced in these species: cervical repositioning (109), cervical occlusion (110) or surgical induction (18, 21). The use of the non-human primate model of endometriosis, either inducible or spontaneous, seems to provide an excellent tool to investigate not only the pathogenesis of disease but also its

associated infertility and impaired endometrial function. These models offer the opportunity to investigate the effect of endometriosis on the eutopic endometrium because of their similar reproductive physiology and endometrial pattern compared to humans. However, high costs, restricted facilities and ethical challenges are limiting their use for experimental purposes. Conversely, rodents do not menstruate and therefore they do not develop endometriosis spontaneously. Only homologuos models of surgically induced endometriosis have been used so far in this setting. Heterologous mouse models consisting of immunodeficient mouses do not seem offer obvious advantages in the study of endometriosis associated infertility. Ectopic transplanted tissue grows and behaves in a hormone-dependent manner, and they exhibit similar histological patterns compared with human endometriotic lesions (106). Despite of these limitations, the rodent model offers a low cost option and the opportunity to perform studies on large homogeneous groups of genetically similar animals. Transgenerational and long-term studies can also be performed because there is no rejection of the transplanted ectopic tissue. For an accurate model of the human condition, an intact hypothalamic-pituitary-ovarian axis in the animal recipient is essential for the evaluation of endometriosis and its related infertility. Another challenge is the difficulty in developing models that recapitulate all subtypes of endometriotic disease and therefore individualize and target. For example, there is a lack of specific *in vivo* models which resemble characteristics of ovarian endometriosis and its related infertility. To date, few animal models of ovarian endometriosis have been successfully implemented (108, 111, 112). Spontaneous ovarian endometriosis in non-human primates is also not as common as in humans (108). Lastly, a major limitation of these models is the concomitant establishment of other subtypes of the disease within the peritoneal cavity causing potential confounding effects. To capture the full extent of human disease it is possible to transplant human endometriosis into an immunodeficient mouse. This model may best recapitulate human disease (113).

Role of pain

For a successful natural conception, the feasibility of sexual intercourse is an important prerequisite, and one that is often neglected, however this is a potentially relevant mechanism involved in endometriosis-associated infertility. Pain may be a factor involved in endometriosis-related infertility when superficial dyspareunia (pain occurring in or around the vaginal introitus) makes intercourse difficult to achieve or deep dyspareunia makes intercourse difficult to sustain, leading to avoidance to sexual activity. However, only a few studies have focused on the relationship between superficial dyspareunia and endometriosis and is often concomitant with deep dyspareunia (114, 115). One cross-sectional study conducted on 300 women with histologically confirmed endometriosis reported that the severity of superficial dyspareunia was associated with increased odds of infertility concerns (116). Endometriosis is associated with a 9-fold increased risk of deep dyspareunia mostly due to the infiltrative form and severe stages of the disease affecting the posterior vaginal fornix, the pouch of Douglas, the uterosacral ligaments, and the rectum (117–119). Although relatively frequent, dyspareunia, is not the exclusive sexual complaint in women with endometriosis. Systematic reviews have highlighted that about two thirds of women with endometriosis have some form of sexual dysfunction not limited to deep dyspareunia (120–122). Chronic, nonmenstrual pelvic pain associated with the disease might influence sexual life by reducing desire, frequency of sexual intercourse, arousal, or orgasm. This will have a significant negative impact on intimate relationships, emotional well-being, and quality of life in general. In this regard, a holistic approach, rather than just a mechanistic approach, is mandatory given the complex nature of human sexuality.

Mechanical factors

Pelvic adhesions and anatomical distortion potentially affect the conception process in endometriosis. Inflammation, fibrosis, adhesions, and surgical sequela are the main pathophysiologic processes involved. Anatomical distortion and mechanical factors may impair oocyte release from the ovary, inhibit tubal ovum pick up or ovum transport, and/or block sperm transfer into the fallopian tube. Interestingly, no term pregnancies occurred in a non-human primate animal model of induced endometriosis when adnexal adhesions were noted on the same side as the ovulation occured (123).

Ovarian reserve

The ovary is the most common location of endometriosis. Ovarian reserve is one of the main prognostic factors regarding fertility and is in large part related to a woman's age. Ovarian reserve is defined as the supply of non-growing, unrecruited primordial follicles (124); currently, a clinical tool that accurately predicts ovarian reserve does not exist. Despite concerns over their role and its specificity in clinical practice, antral follicle count (AFC) and serum anti-Mullerian hormone levels (AMH) are currently the most widely used indices of ovarian function (125). AMH is best used in identifying women who may be poor responders to gonadotropin stimulation in the setting of assisted reproductive technologies (ART) (126). AMH concentrations are not greatly affected by the menstrual cycle or oral contraceptives, making measurement possible at any time.

At present, the pathophysiologic mechanism of diminished ovarian reserve in endometriosis remains unclear. Nevertheless, there is a growing molecular, histological, and morphological evidence that endometriomas have a detrimental effect on

ovarian function. Whether the endometrioma reduces the amount of functional tissue available by space-occupying effect (mechanical stretching damage) or by a direct toxic effect remains unknown.

An endometrioma is a peculiar benign cyst without a real capsule; therefore, there is exchange of cysts contents with the adjacent healthy ovarian cortex. Unlikely other benign cysts, the fluid of endometriotic cyst is able to induce oxidative stress in viable cells and potentially cause damage to healthy tissue. Molecular comparative analysis of the cystic fluid revealed high concentrations of free iron which is able to mediate the production of reactive oxygen species (ROS) that are highly diffusible through cellular compartments. An increase in the iron concentration in the follicular fluid from follicles in contact with the endometrioma was found in comparison with the contralateral healthy ovary (127). Moreover, proteolytic enzymes, inflammatory and adhesion molecules were also found in the endometriotic cyst fluid microenviroment (128). Thus, the release of toxic cysts contents in the adjacent ovarian parenchyma may lead to oxidative stress, fibrosis, loss of cortical stroma, smooth muscle cell metaplasia, impaired vascularization, and, at later stage, reduced follicular maturation and atresia in early follicles (128). Notably, the demonstration of increased oxidative stress affecting the normal ovarian cortex surrounding an endometrioma strongly suggest a ROS-induced fibrogenic response, leading to inhibition of angiogenesis and to follicular damage (129).

Maneschi et al. (130) first found a reduced follicular number and activity prior to surgery compared in histopathological specimens of endometriomas compared to other benign cysts; these findings were later confirmed in other similar studies evaluating follicular density (131–133). Another histopathological study also found increased fibrotic tissue surrounding endometrioma in comparison with that of other benign cysts (134). Interestingly, focal inflammation in the ovarian cortex of affected ovaries was suggested to cause enhanced follicular recruitment and atresia as a result of fibrosis and loss of cortex-specific stroma that maintains the follicular niche (135). Hence, excessive primordial follicle activation was proposed as a mechanism of "burn-out" of the follicular reservoir in ovarian endometriosis (136). Primordial follicle activation is an irreversible process and results in follicular depletion. The PI3K/PTEN/Akt/FOXO3 and PI3K/Akt/mTOR signaling pathways are the best-characterized regulators of primordial follicle activation during the initial recruitment. Takeuchi et al. (136) demonstrated that the number of primordial follicles was diminished, whereas primary, secondary, antral and growing follicle numbers increased in human ovaries with endometrioma, and this effect was mediated by the PI3K-PTEN-Akt-Foxo3 pathway. Similarly luteinized granulosa cells of women with endometriosis are characterized by increased apoptosis (137), however the specific putative mechanism leading to cell loss has not been yet identified. One recent study (138) found that proteins involved in apoptotic pathways were significantly increased in cortical tissue surrounding small endometriotic cysts (<3cm) but not in those surrounding other benign cysts.

Clinical findings confirm this trend; one recent, large, prospective cohort study including 106,633 premenopausal, laparoscopically confirmed endometriosis patients found higher risk for early natural menopause compared to those without endometriosis, especially in nulliparous women and in those who never used oral contraceptives (139). In two recent meta-analysis both serum AMH and AFC were found to be reduced in patients with unoperated endometriomas compared to patients with other benign ovarian cysts without endometriosis (140, 141). Moreover, in a prospective longitudinal study, a time-dependent effect was recently where serum AMH decline in women with untreated endometriomas faster than in age-matched healthy controls (142). Five meta-analyses (143–146) evaluating reproductive outcomes of women with endometrioma who had not undergone previous adnexal surgery found a reduced responsiveness to ovarian stimulation as measured by higher cycle cancellation rate, lower number of oocytes retrieved and lower number of formed embryos despite similar pregnancy and live birth rates. Besides their overall effect, important questions have been raised concerning endometrioma and the effect of size and bilaterality. Indeed, several comparative studies (147–150) in patients with unilateral endometriomas undergoing IVF showed that the affected and the healthy ovary produce a similar number of codominant follicles and oocytes perhaps indicating more than a local effect in the affected ovary; a single visible endometrioma maybe a marker of bilateral disease or alternatively there may be a systemic effect of the single endometrioma on both ovaries. Women with bilateral endometriomas demonstrate an even lower response to stimulation, however clinical pregnancy rate are not affected (151–153), likely overcome by the availability of multiple eggs and embryos

Another key concern is whether surgery has a negative impact on residual ovarian function. Despite the many studies that have been performed to evaluate the impact of surgical treatment of ovarian endometrioma on ovarian reserve, the data are still inconclusive. The potential detrimental impact of adnexal surgery on ovarian reserve has been elucidated in several histological studies confirming that cystectomy is generally associated with inadvertent removal of healthy ovarian tissue and primordial follicles adjacent to the cyst's pseudocapsule (154, 155); this effect increases proportionally with cyst diameter (156), and ultimately is poorly correlated with the level of expertise in reproductive surgery (157, 158). Unlike other benign cysts, in which a well-defined capsule is present, endometrioma is not surrounded by a capsule (154) and technical difficulties may arise due to the absence of a clear cleavage plane. However, the damage inflicted by surgery may also be due to the related local inflammation or vascular

compromise secondary to excessive manipulation of the cortex with subsequent tearing, bleeding, and the need for electrosurgical coagulation (159). Five meta-analyses showed a significant reduction in serum AMH concentrations after surgical treatment of endometriomas (160–162) and this effect is persistent post-operatively up to 18 months (162) and more pronounced in case of bilateral adnexal surgery (163–165). In contrast, two meta-analyses showed that ovarian reserve evaluated by AFC is not decreased after surgical treatment of endometriomas (162, 166). Concerning reproductive outcomes following IVF treatment, two recent meta-analyses (167, 168) showed a lower number of oocytes retrieved in women who had surgical treatment for endometrioma compared to women with expectant management; this finding was previously confirmed separately in case of unilateral treatment, compared with the contralateral normal ovary without endometrioma in the same patient (144). However, two meta-analyses (144, 169) concluded that women who had surgical treatment before IVF/ICSI had a similar live birth rate, clinical pregnancy rate, miscarriage rate, number of oocytes retrieved, and cancellation rate per cycle compared with those with untreated endometrioma. Lastly, according to a recent cohort study (170), the SAFE (surgery and ART for endometriomas) trial, about 50% of women with endometrioma were able to conceive spontaneously within 6 to 12 months after surgery. On the other hand, higher FSH and LH levels between the 2nd and the 5th day of the cycle prior to IVF required higher doses of gonadotropins for ovarian stimulation, and lower number of oocytes were retrieved after surgery for endometrioma in the remaining cohort of patients addressed for IVF, compared with women with unexplained infertility.

Despite all these efforts, further clinical analysis implementing standardization of endometrioma size, bilaterality, surgical technique, post-operative time-interval evaluations and clinical measurements are needed to help in elucidating both contributions and the magnitude of the effect.

Oocyte quality, embryo transport, sperm function and motility, sperm-oocyte interaction

The possible effect of ovarian endometriosis on oocyte quality is still under debate. Deeper understanding of the impact of the disease on oocyte quality is crucial as fertility preservation techniques are gaining attention in the counseling and treatment of this patients. Only few studies have investigated the impact of endometriosis on embryological competence. A recent meta-analysis including 22 studies indicate that endometriosis does not affect embryo morphology: Women with endometriosis have comparable high-quality embryo rate, cleavage rate, and embryo formation rate, regardless the stage of the disease (171). Results from several meta-analyses analyzing IVF outcomes, are controversial due to the high heterogeneity of the included studies (172–175). One recent large cohort study including 3818 embryos in cleavage stage found similar fertilization rate and embryo quality, despite a reduction in viable pregnancy rate. Conversely, one recent retrospective analysis using time-lapse technology observed altered relative kinetics in embryos from patients with endometriosis, supporting poorer embryo quality (176). Lastly, from the oocyte donation perspective, reduced pregnancy and implantation rates are observed when oocytes come from donors with endometriosis (177–179), supporting an effect of endometriosis on embryo quality. In contrast no difference was seen in recipients of donated oocytes based on the presence or absence of endometriosis. However, the cases used in these studies do not reflect the general population of women with endometriosis. The recipients in oocyte donation programs are relatively older compared with the majority of women with endometriosis seeking for pregnancy. With diminishing ovarian reserve and menopause endometriosis typically resolves. A history of endometriosis in a recipient of donor oocytes may not reflect current disease status. Therefore, use of results from oocyte donation does not provide a valid model to evaluate implantation and pregnancy rates in young women with infertility related to endometriosis.

Dysregulation of steroidogenesis and alterations of intrafollicular microenvironment are the main pathophysiological processes investigated in the context of endometriosis. E_2 is crucial for follicular maturation and oocyte competence; follicular fluid also plays an important role in the reproductive performance of oocytes. Alterations in the normal physiology of the granulosa cells such as increased apoptosis and dysregulations of molecular pathways involved in its development and have been intensively studied. Granulosa cells of women with endometriosis are characterized by a decreased expression of P540 aromatase, a key enzyme in estrogen production. Similarly, some evidence also indicates an altered progesterone secretion from granulosa cells that might affect normal oocyte maturation (180, 181). Symmetrical lower E_2 levels and higher progesterone levels were found in the follicular fluid of patients with endometriosis compared to controls (182). Moreover, follicular fluid has been shown to be subject to an important oxidative stress (183–188). An imbalance in ROS and antioxidant systems in the oocyte microenvironment could promote abnormal oocyte development, causing DNA damage, which would result in lower oocyte quality. In another study, cryopreserved human oocytes exposed to endometriotic fluid from patients with advanced stages of the disease had excess cellular fragmentation of derived embryos that may lead to impaired embryo development by inducing apoptosis in surrounding blastomeres or by altering blastomere division (189).

An altered systemic and peritoneal immune and inflammatory profile that characterize women with endometriosis has also been proposed to directly influence the follicular fluid composition. Altered levels of pro-inflammatory

cytokines and growth factors (IL1B, TNFa, IL2, IL8, IL12, IL6, RANTES) have been reported in the follicular fluid of women with endometriosis compared to controls (190–192). Follicular fluid is released into the peritoneal cavity at each ovulation. Three studies have shown spindle and chromosome damage after incubating murine (193, 194) and bovine (185, 195) oocytes in metaphase II with both peritoneal fluid and follicular fluid derived from infertile women with endometriosis. A reduced implantation rate in normal rabbits was observed when the peritoneal fluid from rabbits with surgically induced endometriosis was transferred (196). On the other hand, intraperitoneal injection of peritoneal fluid from women with endometriosis significantly reduced implantation rates in rabbits as well as in hamsters (197, 198).

Gamete transport is also affected by the inflammatory microenvironment, anatomical distortions and uterotubal dysperistalsis associated with endometriosis (15). The endometriotic pro-inflammatory peritoneal fluid microenvironment may also affect sperm function (199–201) by inducing sperm DNA fragmentation (201), disrupt sperm membrane permeability or integrity (202), reduced sperm mobility (203), impaired sperm-oocyte interaction (204) and abnormal sperm acrosome reaction (205).

Impaired ovulation

Clinical data concerning spontaneous ovulation rate in these women is poor and controversial (206, 207). Prolactin levels are significantly higher in women with endometriosis when compared to those of women without endometriosis. Hyperprolactinemia prevents luteinizing hormone pulsatility and interferes with hypothalamic function by blocking estrogen receptors, thus producing anovulation. Another potential cause of ovulation failure in women with endometriosis is the luteinized unruptured follicle syndrome (208), a condition challenging to estimate in clinical settings in which the dominant follicle undergoes luteinization but fails to rupture at or to release the oocyte. Altered patterns of estrogen and progesterone secretion leading to an abnormal luteal phase may also compromise ovulation in these women (209). An association between endometriosis, luteinized unruptured follicle syndrome, and impaired fertility was observed in non-human primates animal models of endometriosis (123, 210) as well as in a mouse model of surgically-induced endometriosis (211, 212).

Endometrial receptivity

The implantation rate is clearly diminished in women with endometriosis during both natural cycles and ART treatments, even in patients with minimal disease (213–215). However, data from clinical studies suggesting that endometriosis leads to implantation defects implicating the endometrium is still conflicting (216, 217). Two recent reports showed similar outcomes in terms of implantation rates through ART cycles when compared to healthy controls (218, 219).

Defective implantation could be due to a reduced endometrial receptivity or decidualization capacity in these women.

The eutopic endometrium of women with endometriosis displays several molecular and functional, abnormalities compared to healthy women's endometrium (220–223). Gradual and profound alterations have also been detected in the endometrium of endometriosis-induced baboons (224, 225). However, the mechanism and specific signal that leads to alterations in the endometrial microenvironment of women with endometriosis is not fully characterized and is still unknown whether changes in the endometrial pattern are the cause for the infertility and for presence of ectopic lesions or vice versa.

Endometrial receptivity and decidualization is dependent upon hormonally regulated molecular processes. Estradiol (E_2) and progesterone (P_4) responsive signaling pathways are regulated in an epithelial and stromal compartment-specific manner in the endometrium. Progesterone is the main hormone responsible for the transient receptive endometrial phenotype, essential for embryo implantation. The endometrial response to P_4 is characterized by inhibition of estrogen-dependent proliferation of epithelial cells, secretory maturation of the glands, and transformation of stromal cells into specialized decidual cells. Functional dysregulation of steroid hormone signaling in endometriosis, such as upregulation of E_2-induced cell proliferation, inflammation and progesterone resistance, seems to play an important role in impairing endometrial receptivity in these patients (226, 227). The shift toward estrogen dominance promotes inflammation, angiogenesis, cell proliferation, and immunosuppression. Both total endometrial PR expression and PR-A/PR-B isoforms ratios are dysregulated in the endometrium of women with endometriosis (222, 228–230) and in mice with induced endometriosis (19). Moreover, progesterone receptor expression levels are lower in women with endometriosis associated-infertility (231), whereas estrogen receptor 1 (ESR-1) levels are increased in the mid- secretory phase endometrium of these women compared to controls (232, 233).

From a histological perspective, Noyes et al. in 1950 have been proposed eight morphological criteria to evaluate endometrial receptivity, and for many decades they were adopted as the main diagnostic tool for detection of endometrial receptivity defects. However, these criteria have been questioned in recent years and several randomized control trials (RCT) (234, 235) have invalidated their use based on data demonstrating that histological dating of the endometrium does not discriminate between fertile and infertile women. Similarly, the negative predictive value of the endometrial thickness and the endometrial pattern as ultrasonographic parameters (236–239) in predicting endometrial receptivity are insufficient (240).

A transition from an anatomical and histological to a molecular perspective led to the genome-wide screening of all transcribed genes. Transcriptomic analysis of both eutopic and ectopic endometrium from women with or without endometriosis found dysregulations of selected genes that are implicated in the implantation process (241). Interestingly, HOXA10 is a progesterone target in the endometrium. The homebox gene family is critical in the development of the female reproductive tract during embryonic stages as well as in the regulation of endometrial receptivity during adulthood in response to steroid hormones (242). Decreased HOXA10 and HOXA 11 expression has shown to be involved in impaired endometrial receptivity, resulting in decreased implantation rates (75, 243). Patients with endometriosis do not show the normal physiologic rise in HOXA10 and HOXA11 during the mid-luteal phase of the menstrual cycle (75, 243, 244). HOX gene expression is also subjected to epigenetic modifications that leads to long-lasting alterations in endometrial receptivity (245). HOXA10 hypermethylation is an important mechanism responsible for its diminished expression (74). Both murine and baboon endometriosis models showed hypermethylation of the promoter region of HOXA10 and decreased expression of HOXA10 genes in the eutopic endometrium (19, 246). In humans, hypermethylation of HOXA10 was also identified in the endometrium of women with endometriosis (71, 74). Lastly, under normal conditions, high expression of the *HOXA10* gene suppresses the transcription of the EMX2 gene, which is also essential in regulating endometrial receptivity and implantation. With the diminished expression of HOXA10 in endometriosis, the increased level of endometrial EMX2 directly affects endometrial cell proliferation and function during the peri-implantation period, resulting in aberrant implantation (247).

Integrins are cell adhesion molecules expressed in the endometrium during the receptive window and therefore involved in successful implantation. Interestingly, B3-integrin subunit is a direct downstream target gene of both HOXA10 and ESR-1 (248) and its aberrant expression have been described in the endometrium of women with endometriosis. Moreover, in the clinical setting, ART is less effective in patients with lower expression level of B3-integrin in the eutopic endometrium (249). Other transcriptional factors involved in regulation and mediation of progesterone signaling (IGFBP1, GATA2, FOXO1, ARID1A, NOTCH1and WNT4), required for successful implantation are also reduced in endometrium of women with endometriosis. In human endometrial stromal cells, silencing of GATA2, diminishes markers of decidualization (82) and interestingly the expression level is significantly reduced in the endometrium of women with endometriosis (80). Defects in decidual response have been also investigated in the eutopic endometrium of women with endometriosis. Compromised decidualization of cultured stromal cells was found in fresh shed endometrium as well as in the eutopic endometrium of women with endometriosis compared with matched healthy controls (250, 251). Several pathways have found to be aberrant in endometriosis (251–256) contributing to the unfavorable environment and promoting aberrant effects on the maternal/embryo interface. Increased activation of PI3K/AKT (252) pathway and decreased NOTCH signaling (256) contributes to inactivates FOXO1, an important mediator of decidualization involved in the progesterone signaling. Moreover, AKT pathway has been shown to downregulate and upregulate ESR-2 and ESR-1, respectively, with the net effect of promoting estrogen dominance (257, 258). Lastly, IGFBP1, a downstream target gene of HOXA 10 and a marker of decidualization, is reduced in the endometrium of women with endometriosis (251), and it is also downregulated in the eutopic endometrium of mice with induced endometriosis (19).

It is still poorly understood how the immune system contributes to and influences the endometrial microenvironment and the implantation window. Data is conflicting on the immune cell population of both ectopic and eutopic endometrium of women with endometriosis and controls, especially regarding absolute numbers, markers, activation states and cycle dependence due to heterogeneity of studies (259). Eutopic endometrium microenvironment of women with endometriosis seems to be more pro-inflammatory than controls and aberrant functions of certain immune population may lead to an inhospitable environment for embryo implantation. Interestingly, type I classically activated macrophages, that secretes proinflammatory factors, are the main population in eutopic endometrium of women with endometriosis, across all cycle phases, compared with normal controls (260–262). This proinflammatory predominance may decrease embryo nidation. The relative less cytotoxicity of natural killers together with their higher immaturity in the eutopic endometrium of women with endometriosis was significantly correlated with the infertility status in the same group (263). Concerning the role of B cells, anti-endometrial antibodies may also play a role in impaired implantation by affecting directly endometrial function for embryo receptivity (264). Finally, circulating and endometrial/decidual regulatory T cells (Tregs) have shown to be reduced in women with recurrent pregnancy loss, recurrent implantation failure and endometriosis (265).

Adenomyosis and other uterine factors

Endometriosis and adenomyosis often co-exist, especially in infertile women (266, 267). Additionally, the concomitant presence of both conditions seems to worsen fertility outcomes (267). In baboons, endometriosis was found to be statistically significantly associated to adenomyosis and the latter was found to be strongly associated with primary infertility (268). Several pathogenetic hypotheses have been postulated regarding adenomyosis and its associated infertility, including junctional zone thickness and subsequent perturbed uterine peristalsis that may alter utero-tubal transport, as well as biochemical, functional

and epigenetic alterations in both eutopic and ectopic endometrium (269). The eutopic endometrial microenviroment in adenomyosis differs from the endometrium of unaffected women (270, 271). However, it remains conflicting whether these changes are of clinical significance and, in particular, in the setting of assisted reproductive technologies. According to the most recent meta-analysis in the field (213, 272) the presence of adenomyosis was associated with lower clinical pregnancy rate, higher risk of miscarriage following ART, and (independently of the mode of conception) with adverse pregnancy and neonatal outcomes. However, because of the limited number of comparative studies and their heterogeneous design, the real influence of adenomyosis alone on fertility is still controversial and poorly understood. One recent retrospective cohort study including more than 2000 subjects who underwent ART and by excluding those with decreased ovarian reserve and coexistence of endometriosis and fibroids, found that adenomyosis has a negative effect on IVF outcomes including an increased risk of miscarriage and a reduced live birth rate (273). There are several major limitations in investigating the impact of adenomyosis on infertility. First, there are major diagnostic limitations related with coexistence of endometriosis and adenomyosis, making the interpretation of the available literature difficult. Second, with the advent of 3D ultrasound and the use of magnetic resonance imaging the diagnosis of adenomyosis can now be relatively reliable without the need of histological examination of the surgical specimen (274). However, there is no consensus regarding diagnostic features of adenomyosis using imaging making the interpretation of observational studies challenging; different imaging criteria to define adenomyosis are commonly used. Lastly, adenomyosis frequently coexists with other gynecological disorders and potential confounders, such as uterine leyomiomas. As with adenomyosis, uterine fibroids, in particular submucous leyomiomas, has been associated with lower implantation rates and increased risk for early pregnancy loss (275, 276). The main pathophysiological processes implicated in endometriosis associated infertility are summarized in Figure 2.

Management of endometriosis associated infertility

Clinical management of infertility associated with endometriosis is challenging due to lack of high-quality scientific evidence and conflicting available guidelines (277). The complexity in therapeutic decision-making is mainly related to the heterogeneous population of infertile women with endometriosis which includes diverse patient's phenotypes. This often requires innovative diagnostic and therapeutic tools to target the specific dysfunctional step of the reproduction process. Therefore, care of women with endometriosis-associated infertility is best undertaken in referral centers where a multidisciplinary approach can be offered and where both surgery and IVF services are present.

From the patient perspective, a shared and informed decision is mandatory because different treatment options may involve both clinical and personal aspects. The treatment must be individualized according to the clinical situation and to the suspected level of impairment. Factors such as woman's age, ovarian reserve, duration of infertility, additional infertility factors (male, tubal), ASRM stage, previous surgical treatment for endometriosis, concomitant pain, and indications for IVF-ET must be considered because they will influence the choice of treatment and may also have socio-economic implications.

In American and European guidelines (278, 279), the management of endometriosis is still based on the disease stage defined according to the revised American Society of Reproductive Medicine (rASRM) classification. Despite the high consensus and multiple revisions, the currently used classifications system has several limitations, including failure in predicting fertility outcomes and in accounting for the different types of endometriosis. For this reason, Adamson and Pasta developed a validated and predictive endometriosis staging system, the Endometriosis Fertility Index (EFI), to estimate the non-ART pregnancy rate (natural intercourse or IUI) in women with surgically documented endometriosis (280). This scoring system, which takes into account patient-related factors (age, length of infertility, history of previous pregnancy) and surgical factors (rASRM total score, endometriosis lesions and "least function score" from the tubes and ovaries), is highly accurate (281) and reproducible (282) and represents an important clinical decision tool to counsel patients on their reproductive options after surgery.

While ART can correct many defects that prevent conception, implantation failure is not easily identified, and in most cases, there are no available treatments. The endometrial status is rarely investigated during the standard work-up of infertile women performed in infertility clinics worldwide, even prior to ART (283). Thus, in the era of precision medicine and tailored therapy, a reliable endometrial receptivity assay would be of huge clinical and economical benefit for the patient's selection process. Starting from functional analysis of the endometrium, Kliman et al. (284) introduced an innovative endometrial functional diagnostic tool (endometrial function test EFT®) based on the use of antibodies two cyclins, as expression patterns of this type of mitotic cycle regulators have been associated with implantation success or failure. Moreover, revolutionary diagnostic tests based on transcriptomic and bioinformatic technologies, that can inform clinicians about the status of endometrial receptivity, have been proposed for diagnostic and prognostic purposes. The endometrial receptivity array (ERA) (285) is a and reproducible (286) microarray-based machine-learning predictive model for assessing endometrial status in the work-up for infertile patients

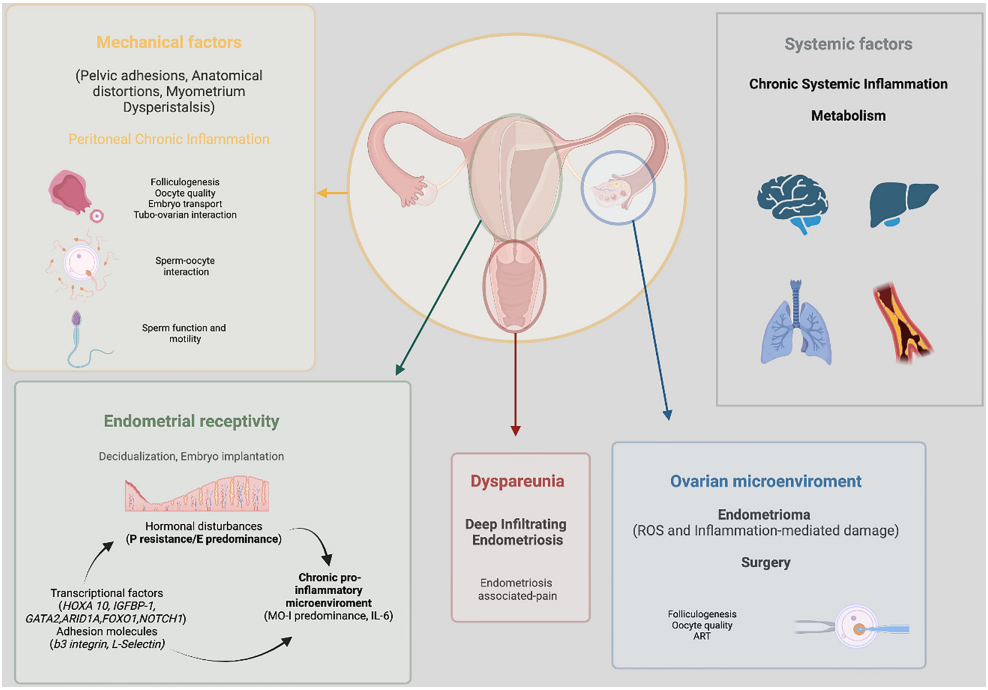

FIGURE 2
Summary of the main pathophysiological processes implicated in endometriosis associated infertility.

based on the specific signature of 238 differentially expressed genes in the receptive endometrium. The ReceptivaDX™ test a new screening and diagnostic test has been proposed (287) based on the findings that B-cell-lymphoma 6 (BCL6) overexpression in the secretory endometrium of these women contribute to the progesterone resistance (288) and could potentially serve as a surrogate inflammatory marker for a dysfunctional endometrium in endometriosis associated with infertility (289, 290).

Given the important diagnostic delay, strenuous research has been made to identify potential non-invasive diagnostic tool in endometriosis and to date, remains one of the major research priorities in this disease. Several potential biomarkers have been evaluated; however, none has demonstrated sufficient sensitivity and specificity for clinical use.

Cancer antigen-125 (CA-125), a high-molecular-weight glycoprotein antigen expressed in some derivatives of the celomic epithelium, has been previously reported to be elevated in serum of women with advanced forms of the disease (291, 292); however, its overall sensivity is reported to be extremely low (53%) (293).

Based on their pivotal role in the etiopathogenesis of endometriosis, a substantial body of work has shed light on circulating/exosomal miRNAs as potential leading biomarkers for early diagnosis, prognosis, and surveillance for endometriosis. They are they are found both intra- and extracellularly, contained and released *via* exosomes (294).

MiRNAs are attractive due to their simple structure and their stability at the post-translational level and in extracellular biofluids. Conversely, their highly variable content and their low abundance in extracellular biofluids, make detection very demanding. Real time PCR (qRT-PCR) remains the gold standard for miRNA quantification (295). Next-generation sequencing platforms are also used for miRNA sequencing, and they showed high sensivity and excellent reproducibility (296), nevertheless a great performance variation exists among the different platforms. MicroRNA have several targets in cells and each single mRNA transcript may be subjected to regulation by various miRNAs, Thus, the same pathway maybe regulated by a panel of miRNA.

Many studies have investigated the role of circulating miRNA in endometriosis (104, 297–312). To date, more than 60 miRNAs were found to be differentially expressed in the circulation (plasma and/or serum) of women with endometriosis (313). Very few (20%) miRNAs have been replicated in more than one study (314). While generally miRNAs are not highly evolutionarily conserved, serum let-7 family miRNA showed similar dysregulation in a murine model of endometriosis (315).

In general, serum-derived miRNAs seems to yield higher sensitivity and specificity (92 and 95,5% respectively) compared

with plasma-derived miRNAs and the highest biomarkers potential was found to be represented by a panel of serum-derived miRNAs comprised of miR-125-5p, miR-150-5P, mir-342-3p, miR-451a, miR-3613-5P, and let-7b (area under the curve (AUC) of 0.94) (313). Cosar et al. (302) reported a logistic regression model combining miR-125-5p, miR-451a, and miR-3613-3p. Later, the same group (300) confirmed a significant diagnostic value of a combination of six miRNAs, with a final AUC>0.9 across two independent clinical data sets.

The reason for the limited consistency of results across studies is related with the dynamic nature/behavior of miRNA expression which is influenced by lack of standardization in the study protocols, such as sample collection (menstrual phase, circadian rhythm), miRNA analysis method, case-control matching and subject's background (age, ethnicity, health status), stage (minimal-mild vs. moderate/severe) and type of endometriosis (ovarian, peritoneal, deep infiltrating). In addition, different cutoff points were considered to define a meaningful change in expression. An important variable is whether their expression is influenced by the menstrual cycle phase.

Results need to be replicated on large series of well-phenotyped patients and under stringent conditions of sampling. The availability of a reliable non-invasive test for endometriosis will allow more accurate and accessible diagnosis as well as the potential for identification and treatment of endometriosis related infertility. Current and emerging treatment options for endometriosis associated infertility are summarized in Figure 3.

Reproductive surgery

Surgical indications should be guided by the presence or absence of pain, patient's age, history of previous surgery for endometriosis, presence of other infertility factors, ovarian reserve, and estimated EFI. In general, it is clear that multiple surgeries should not be attempted to improve fecundity. Current guidelines suggest fertility counseling before surgery which should include AMH measurement (278, 279).

r-ASRM stages I and II

Stages I and II are not visible during the clinical and ultrasound examination and they are diagnosed mainly during diagnostic laparoscopy. A recent meta-analysis (315) of moderate quality evidence, including three RCTs on rASRM stage I/II endometriosis, concluded that operative laparoscopy increases natural viable intrauterine pregnancy rates compared to diagnostic laparoscopy only (OR 1.89, 95% CI 1.25-2.86; I^2 = 0%). Similar findings were later confirmed in a network-metanalysis by Hodgson et al. (316) comparing operative laparoscopy with placebo (OR 1.63; 95% CI 1.13-2.35). Only one meta-analysis (317) analyzed the live birth rate outcome and concluded that laparoscopic surgery in this disease stages has an overall advantage in improving the chances of live birth (RR 1.52, 95% CI 1.26-1.84). Based on this evidence, operative laparoscopy is currently an option for

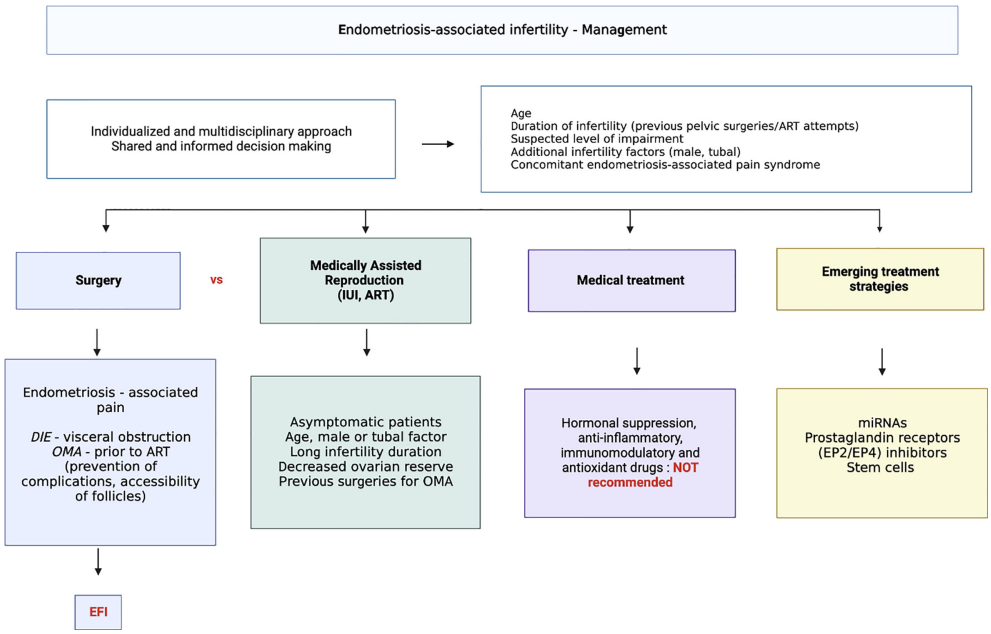

FIGURE 3
Current and emerging treatment options for endometriosis related infertility.

endometriosis-associated infertility in rASRM stage I/II (278, 279) when is performed for other indications such as pain. The absolute benefit is modest with a number of women needed to be treated of 12 to achieve one additional pregnancy.

r-ASRM stages III and IV

The situation is more complex for advanced disease where high quality evidence regarding the role of surgery for infertile patients is lacking and the risk of major complications due to the surgery itself must be considered. There are no RCT to determine whether clinical pregnancy rates are improved after surgery in patients with stage III-IV of the disease. Women with advanced disease are normally counseled toward surgery in case of significant pain symptoms, large endometriomas, or ureter and bowel clinical involvement.

Apart from the deep infiltrating endometriosis (DIE)-induced alteration of pelvic anatomy and adhesions, evidence supporting a direct link between DIE and infertility is weak and the lack of high-quality data preclude firm conclusions on the effect of surgery. Moreover, DIE alone is found in only 6% of endometriosis patients (318) and surgery-related major complications in this context must be taken into account. According to three independent systematic reviews, pregnancy rate after surgery for rectovaginal endometriosis varies from 24% to 44% (319–321). An additional systematic review (322) of heterogeneous prospective and retrospective studies reported postoperative spontaneous pregnancy rates in women with DIE with and without bowel involvement of approximately 50% and 20%, respectively. Lastly, a recent meta-analysis including observational studies on both rectovaginal and rectosigmoid DIE patients showed a statistically significant benefit of surgery before IVF (323) in terms of live birth and pregnancy rates per patient and per cycle. Nonetheless results need to be interpreted with caution before attributing this rate of success entirely to surgery (324). Interestingly it has been shown in a recent study that extensive surgery in women with deep and intraperitoneal endometriosis, when compared with intraperitoneal surgery only, does not modify the fertility outcome (325). At present, operative laparoscopy for DIE represent a well-established indication in endometriosis associated-pain and in case of visceral obstruction, and is a treatment option in symptomatic patients wishing to conceive (278, 279).

Potential benefits and harms of adnexal surgery for endometrioma must be considered in this context because of its direct effect on ovarian reserve and the risk of recurrence. There is still no consensus regarding the optimal indication for surgery depending on cyst diameter due to the lack of comparative studies. According to guidlines (278, 279), key indications for surgical management of the "asymptomatic" ovarian endometrioma in patients with infertility is to improve the accessibility of follicles and to prevent potential complications (endometrioma rupture, contamination) prior to ART.

A recent meta-analysis (326) conducted by Alborzi et al. of eight prospective studies comparing pregnancy rate from four different approaches of OMA (surgery + ART, surgery + spontaneous pregnancy, aspiration with or without sclerotherapy + ART, and ART alone) found no significant difference between the four study groups. Another meta-analysis (167) analyzing surgical versus expectant management of endometriomas reported similar live birth rates per cycle after IVF in both groups. Surgery is generally not recommended for the sole purpose of enhancing fertility in an otherwise asymptomatic patient.

Medically assisted reproduction (IUI, ART)

The utility of Intrauterine Insemination (IUI) with or without ovarian stimulation in patients with endometriosis is supported by only one single RCT (327) including patients undergoing ovarian stimulation with gonadotrophins and IUI versus expectant management. The study found a 5-6 times higher live birth rate per cycle in the treatment group. IUI in combination with controlled ovarian stimulation (clomiphene citrate) is currently recommended only in infertile women with ASRM stage I/II (328).

Currently, up to 25% of *in vitro* fertilization (IVF)-embryo transfer procedures are performed in patients with endometriosis (329). Despite its high implementation, both the influence of endometriosis on pregnancy rates after ART, and the effectiveness of ART treatments in women with endometriosis are still a matter of debate. Main indications for ART remain tubal impairment, male factor, low EFI, and failure of other treatments. There are currently no RCTs evaluating the efficacy of this treatment option versus no intervention in women with endometriosis, and only indirect evidence is available from studies comparing ART outcomes in women with endometriosis to women without the disease. Several meta-analyses (172–175) have investigated ART outcomes of women with and without endometriosis, but results appear conflicting due to the low quality and the high heterogeneity of the selected studies. According to a recent meta-analysis (213), endometriosis consistently leads to reduced oocyte yield and a reduced fertilization rate. Milder forms of endometriosis are most likely to affect fertilization and implantation processes as discussed earlier, whereas advanced stages of the disease may influence all stages of reproduction.

Reasons for the suspected suboptimal performance of ART in endometriosis may include the affected ovarian responsiveness during the ART cycles (low

ovarian reserve), impaired endometrial receptivity, and altered folliculogenesis.

Finally, a specific protocol of controlled ovarian stimulation for ART in women with endometriosis is not currently recommended as both antagonist and agonist protocols are still widely used and no difference in pregnancy or live birth rates has already been demonstrated (330).

Medical approach

Based on a presumed altered steroidogenesis in endometriosis associated infertility, the use hormonal suppression has been investigated. Based on current recommendations (278, 279) ovarian suppression (danazol, GnRH agonists, progestogens, OCP) should not be offered alone or in combination with surgery in endometriosis-related infertility because there is no evidence of its benefit on pregnancy outcomes (331). A recent Cochrane review (332), comparing the effectiveness of different timing of hormonal suppression in the setting of surgery, concluded that postsurgical medical therapy compared with no treatment or placebo may increase pregnancy rates and reduce disease recurrence, and that it should be recommended in women who cannot, or decide not to conceive immediately after surgery.

The role of downregulation with GnRH agonists prior to ART has been extensively investigated and several meta-analysis have been performed; however results are still contradictory. It has been proposed that medical treatment with gonadotropins prior to IVF may result in improved fertility outcomes in terms of both oocyte quality and endometrial receptivity.

An updated Cochrane review by Georgiu et al. (333) that included 8 RCT concluded that the effect of long-term GnRH agonist pre-treatment for at least 3 months versus no pre-treatment is uncertain in terms of live birth rate (primary outcome), clinical pregnancy rate, multiple pregnancy rate, miscarriage rate, mean number of oocytes and mean number of embryos.

Another more recent meta-analysis (334) investigated the effectiveness of three different down-regulating protocols based on the use of GnRH-agonist (ultra-long, long and short protocol) in infertile women with endometriosis prior to ART. The authors concluded that the ultra-long protocol may improve the clinical pregnancy rate especially in patients with stages III-IV endometriosis based on data from two RCTs. Conversely, more recently, two RCTs (335, 336) failed to demonstrate a beneficial effect of the ultra-long protocol in terms of live birth rate, clinical pregnancy rate, or embryo quality; instead, it was associated with a longer duration of ovarian stimulation, a higher consumption of gonadotropins, and a lower ovarian estradiol production (335). No studies have been conducted to evaluate the efficacy of GnRH antagonists for the treatment of endometriosis-related infertility. A large multicenter RCT (337) is ongoing in the US with the aim to investigate for the first time the pre-IVF treatment with a GnRH antagonist (Elagolix) in women with endometriosis (PREGnant). Compared with GnRH-agonists, the rapid reversibility and recovery of the hormone secretion once the treatment is concluded using GnRH antagonist may allow for better outcomes at the time of ART.

Data addressing pretreatment with continuous oral contraceptives are very limited and do not allow firm conclusion (338, 339). Comparative studies between different hormonal suppression treatment strategies are lacking.

Lastly, assuming that endometriosis is a chronic inflammatory condition, the effect of several anti-inflammatory, immunomodulatory and antioxidant agents has been investigated in the context of inflammation and altered redox balance in the follicular fluid microenvironment and of suspected impaired oocyte quality (340). Pentoxifilline has been the most studied anti-inflammatory and antioxidant agent in endometriosis-associated infertility, and it has also been shown to enhance sperm motility and improve semen parameters in men with oligoasthenospermia. However, according to a recent Cochrane review (341) including 5 RCT, there is no conclusive evidence on its effectiveness and safety in endometriosis-associated-infertility.

Emerging treatment strategies

By elucidating the cellular and molecular mechanisms involved in endometriosis-associated infertility and based on the assumption that current hormonal suppression-based treatment cause important side effects rather than effectively improve fertility (342), there is a fundamental need to identify potential signaling pathways for non-hormonal targets for endometriosis associated with infertility.

Non-coding RNAs (ncRNAs) have rapidly emerged as important regulatory molecules in cancer and several reproductive diseases such as recurrent pregnancy loss and endometriosis (95). The use of ncRNAs as a therapeutic tool is still in its infancy; however, the US-FDA has recently approved three RNAi therapies (343, 344). In the context of impaired endometrial receptivity and progesterone resistance, Petracco et al. (345) identified a putative miR135 binding site in HOXA10 gene showing that miR135a and miR135b are expressed in normal endometrium and increased in the endometrium of women with endometriosis; they likely act by regulating targets of progesterone action in the endometrium. Furthermore, miR-451 was found to be the most highly downregulated in the mid-secretory phase of eutopic endometrium of baboons (346) and women with endometriosis (347) compared to controls, leading to an increased expression of transcription factors involved in regulation and mediation of progesterone signaling such as

GATA2 and YWHAZ. Similarly, H19 is one of the first long noncoding RNA identified; it is expressed in a menstrual cycle-dependent fashion, confined to the stroma, and acts as a decoy for several tumor-suppressor miRNA. Moreover, it is positively regulated by E2 and negatively regulated by progesterone in the mouse. It was recently found that in the eutopic endometrium of patients with endometriosis, downregulation of H19 will increase let-7 activity, contributing to a decreased proliferation of endometrial stromal cells (through IGFR1 expression inhibition) and contributing to the impaired endometrial preparation and receptivity (through reduction of stromal cell proliferation) (348). Finally, further studies have investigated the role of certain miRNA within progesterone resistance during the luteal phase in women with endometriosis; they reveal that miR-30b, miR-30d, miR-29c and miR-194-3p are up-regulated whereas mi-494 and miR-923 are down regulated in receptive endometrium (230, 349) of both humans and baboons (350). Lastly, upregulation of miR-196 and MEK/ERK signaling proteins was reported in infertile women with minimal/mild endometriosis mediating downregulation of PGR expression and decidualization in eutopic endometrium (351). MicroRNA based therapies are promising new fertility treatments.

In the context of a pro-inflammatory endometrium, one recent study (352) investigated the pharmacological effects of selective inhibition of prostaglandin receptors (EP2/EP4) by using a chimeric mouse model of endometriosis and found that endometrial functional receptivity can potentially be restored the interaction among prostaglandin E_2 (PGE_2), estrogens and progesterone. The results indicate that inhibition of EP2/EP4 decreases PGE_2, estrogen biosynthesis and signaling, pro-inflammatory cytokine production, and increases P_4 signaling in eutopic endometrium of women with endometriosis.

Stem cell therapy has shown to be promising as a new therapeutic target for infertility, especially Asherman's syndrome (353). The interaction between BMDSC and endometrial MSC has generated considerable interest because of their tropism toward inflamed foci.

Stem cell properties of self-renewal and differentiation made attractive their use to replace potential damaged tissues and inflammation by reducing intrauterine adhesions and fibrosis (354, 355), improving endometrial thickness (356) and promote endometrial regeneration. To date, the use of stem cell for treating endometriosis, and in particular endometriosis-associated infertility offers an attractive option because of its tropic and immunomodulatory proprieties.

Endometriotic lesions recruit stem cells away from the uterus resulting in inadequate endometrial repair and regeneration as endometriosis more effective in recruiting BMDSCs than eutopic endometrium (357). Lesions highly express CXCL12, a chemoattractant for BMDSCs expressed in many organs, as well as by endometrial stromal cells (357). Inhibiting its receptor (CXCR4)was shown to impact the migration of BMDSCs to the uterus (358). Endometriosis related-chronic inflammation likely acts by continually recruiting BMDSCs to the lesions as demonstrated in animal (52). Moreover, physiologic estradiol levels can increase CXCL12 and CXCR4 expression by endometrial stromal cells and BMDSCs respectively *in vitro*, thereby increasing the chemoattractiveness between the two and consequently, migration (358).

Interestingly, Badoxifene, a selective antagonist ER antagonist, showed to reduce both ectopic endometriotic lesions and the BMDCs engraftment to them, redirecting these cells to the eutopic endometrium (54, 359, 360). This phenomenon might be able to create a new endometrium partially free of epigenetic defects.

The route of stem cell administration will be a crucial component of any stem cell based therapy. Significantly greater levels of stem cell incorporate in uteri of mice when cells were administered systemically by intravenous injection as compared with local injections into the uterus (361). Interestingly, mice that received a systemic infusion of BMDSCs prior to uterine injury were also more likely than twice to achieve a pregnancy, suggesting functional repair of damaged endometrium was due to BMDSs activity (355). BMDSCs maybe superior to ESC in the treatment of uterine injury; they may allow a more complete repair due to their superior versatility, developing into a large number of cell types required for endometrial function (355).

Fertility preservation

The need for reproductive counseling utilizing a multidisciplinary medical team has become more evident in endometriosis not only prior to surgery but also at diagnosis, based on the assumption that fertility is likely to be compromised in these women.

Several options are currently available to preserve fertility, including embryo or oocyte cryopreservation and ovarian tissue cryopreservation which are no longer considered experimental procedures (362). Vitrification or planned oocyte cryopreservation technology has grown enormously during the last few years. Several meta-analyses demonstrated that clinical outcomes after vitrification are superior to the standard slow-freezing/thawing. Moreover, comparable results between vitrificated and fresh oocytes were reported (363, 364).

Many questions in terms of efficiency, effectiveness and risks remain unanswered, and the strength of evidence to support fertility preservation in endometriosis, regardless disease severity, is still limited (365). Systematically offering FP to patients with endometriosis might have a dramatic effect on the public healthcare expenditure and may expose patient to unnecessary clinical risks (366, 367). In the context of ovarian endometriosis, subgroups that would particularly benefit from

fertility preservation are women with bilateral endometriomas and those scheduled for surgery for contralateral recurrence after unilateral endometrioma surgery or in whom spontaneous conception is unlikely after ovarian surgery (365–368). One of the advantages related with an earlier approach is the opportunity to preserve oocyte at a young age. However, lack of reliable data regarding the effectiveness limit full scale adoption. Women at young age may have a greater risk of recurrence, and when there is not an immediate desire for pregnancy, offering FP could be a beneficial option. Ovarian tissue cryopreservation represents an alternative in case where ovarian hyperstimulation is contraindicated. The first case of autologous ovarian transplantation with cryopreserved tissue was reported in 2000 by Oktay et al. (369). Later updates by the same group in 2010 (370) and Donnez et al. in 2005 (371) reported a similar successful approach in endometriosis cases and to date, other few cases have been reported (372–378).

Fertility sparing surgery

Optimizing fertility in patients with endometriosis requires reducing potential iatrogenic harm to the ovarian reserve. In this context, the role of adnexal sparing surgery, when indicated, is crucial for treating symptomatic women with endometriosis-associated pain for improving accessibility of follicles prior to ART due to endometriosis-associated infertility. Thus, skilled surgeons with expertise in reproductive pathophysiology are required to avoid potential insults to the healthy parenchyma and ovarian vascular network.

Decrease of normal ovarian cortex (ovarian reserve) and disease recurrence are the two main risks associated with surgery for endometrioma, regardless the surgical technique. Several surgical approaches for endometrioma have been proposed: Excisional, ablative or a combination of both. Lower rates of spontaneous pregnancy and higher rates of recurrence are associated with ablative surgery compared with cystectomy, whereas cystectomy was found to be deleterious for residual ovarian function (162, 379, 380). Since the meta-analysis of Dan et al. in 2013 (380), RCTs comparing ablative versus excisional techniques in terms of ovarian reserve markers and ovarian residual volume have been performed (381–386). Data from animal studies (387) and RCT (381, 382, 388) on the use of laser and plasma energy is encouraging and may result in less inadvertent tissue removal and thermal injury compared with cystectomy and bipolar electrosurgery. Fertility sparing surgery performed in the context of endometriosis-associated infertility also requires the systematic evaluation and optimization of tubal anatomy and patency. Concomitant adhesiolysis with restoration of pelvic anatomy is also recommended when anatomical distortion is present.

Conclusions

The mechanisms involved in endometriosis-associated infertility are still not completely understood and this condition is multifactorial. Endometriosis-associated pain and inflammation, altered pelvic anatomy and adhesions, disrupted ovarian function, and compromised endometrial receptivity all play a major role in endometriosis infertility in women with endometriosis. Identifying innovative, non-invasive diagnostic tools in endometriosis that also predict a higher risk of infertility remains one of the major research and clinical priorities in this disease; delayed diagnosis allows for disease progression which is clearly detrimental from the perspective of fertility.

Treatment options of infertility associated with endometriosis are still limited. Surgery and ART remain the mainstay of effective therapy. All medical therapies currently approved for use in this disease prevent or diminish fertility and therefore are not helpful in treating this condition. Future non-hormonal medical therapies are needed that can enhance fertility by interfering with the pathways outlined above.

Endometriosis-associated infertility requires a multidisciplinary, personalized, shared and holistic approach based on patient's unique characteristics, endometriosis subtype and level of impairment.

Author contributions

GB and HT contributed to manuscript writing and editing. HT revised the manuscript for important intellectual content; all authors approved the final version of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Taylor HS, Kotlyar AM, Flores VA. Endometriosis is a chronic systemic disease: clinical challenges and novel innovations. *Lancet* (2021) 397:839–52. doi: 10.1016/S0140-6736(21)00389-5

2. Nezhat C, Nezhat F, Nezhat C. Endometriosis: Ancient disease, ancient treatments. *Fertil Steril* (2012) 98:6S. doi: 10.1016/j.fertnstert.2012.08.001

3. Macer ML, Taylor HS. Endometriosis and infertility: A review of the pathogenesis and treatment of endometriosis-associated infertility. *Obstet Gynecol Clin North Am* (2012) 39:535–49. doi: 10.1016/j.ogc.2012.10.002

4. Healy DL, Trounson AO, Andersen AN. Female infertility: causes and treatment. *Lancet* (1994) 343(8912):1539–44. doi: 10.1016/s0140-6736(94)92941-6

5. Agarwal SK, Chapron C, Giudice LC, Laufer MR, Leyland N, Missmer SA, et al. Clinical diagnosis of endometriosis: A call to action. *Am J Obstet Gynecol* (2019) 220:354.e1–354.e12. doi: 10.1016/j.ajog.2018.12.039

6. Eisenberg VH, Decter DH, Chodick G, Shalev V, Weil C. Burden of endometriosis: Infertility, comorbidities, and healthcare resource utilization. *J Clin Med* (2022) 11:1133. doi: 10.3390/jcm11041133

7. Koninckx PR, Meuleman C, Demeyere S, Lesaffre E, Cornillie FJ. Suggestive evidence that pelvic endometriosis is a progressive disease, whereas deeply infiltrating endometriosis is associated with pelvic pain. *Fertil Steril* (1991) 55:759–65. doi: 10.1016/s0015-0282(16)54244-7

8. Sampson JA. Perforating hemorrhage (chocolate) cysts of the ovary: Their importance and especially their relation to pelvic adenomas of endometrial type ("adenomyoma" of the uterus, rectovaginal septum, sigmoid, etc.). *Arch Surg* (1921) 3:245–323. doi: 10.1001/archsurg.1921.011100800030011

9. Sampson JA. Peritoneal endometriosis due to the menstrual dissemination of endometrial tissue into the peritoneal cavity. *Am J Obstet Gynecol* (1927) 14:422–69. doi: 10.1016/S0002-9378(15)30003-X

10. Sampson JA. The development of the implantation theory for the origin of peritoneal endometriosis. *Am J Obstet Gynecol* (1940) 40:549–57. doi: 10.1016/S0002-9378(40)91238-8

11. Halme J, Hammond MG, Hulka JF, Raj SG, Talbert LM. Retrograde menstruation in healthy women and in patients with endometriosis. *Obstet Gynecol* (1984) 64:151–4.

12. Kruitwagen RF, Poels LG, Willemsen WN, de Ronde IJ, Jap PH, Rolland R. Endometrial epithelial cells in peritoneal fluid during the early follicular phase. *Fertil Steril* (1991) 55:297–303. doi: 10.1016/s0015-0282(16)54119-3

13. Koks CAM, Dunselman GAJ, de Goeij AFPM, Arends JW, Evers JLH. Evaluation of a menstrual cup to collect shed endometrium for. *Vitro Stud Fertil Steril* (1997) 68:560–4. doi: 10.1016/s0015-0282(97)00250-1

14. Leyendecker G, Kunz G, Herbertz M, Beil D, Huppert P, Mall G, et al. Uterine peristaltic activity and the development of endometriosis. *Ann N Y Acad Sci* (2004) 1034:338–55. doi: 10.1196/annals.1335.036

15. Leyendecker G, Kunz G, Wildt L, Beil D, Deininger H. Uterine hyperperistalsis and dysperistalsis as dysfunctions of the mechanism of rapid sperm transport in patients with endometriosis and infertility. *Hum Reprod* (1996) 11:1542–51. doi: 10.1093/oxfordjournals.humrep.a019435

16. Rakhila H, Bourcier N, Akoum A, Pouliot M. Abnormal expression of prostaglandins E2 and F2α receptors and transporters in patients with endometriosis. *BioMed Res Int* (2015) 2015:808146. doi: 10.1155/2015/808146

17. Kawano Y, Hirakawa T, Nishida M, Yuge A, Yano M, Nasu K, et al. Functioning endometrium and endometrioma in a patient with Mayer- rokitanski-Kuster-Hauser syndrome. *Jpn Clin Med* (2014) 5:43–5. doi: 10.4137/JCM.S12611

18. Tal A, Tal R, Pluchino N, Taylor HS. Endometrial cells contribute to preexisting endometriosis lesions in a mouse model of retrograde menstruation. *Biol Reprod* (2019) 100:1453–60. doi: 10.1093/biolre/ioz039

19. Lee B, Du H, Taylor HS. Experimental murine endometriosis induces DNA methylation and altered gene expression in eutopic endometrium. *Biol Reprod* (2009) 80:79–85. doi: 10.1095/biolreprod.108.070391

20. D'Hooghe TM. Clinical relevance of the baboon as a model for the study of endometriosis. *Fertil Steril* (1997) 68:613–25. doi: 10.1016/s0015-0282(97)00277-x

21. Fazleabas AT, Brudney A, Gurates B, Chai D, Bulun S. A modified baboon model for endometriosis. *Ann N Y Acad Sci* (2002) 955:308–17. doi: 10.1111/j.1749-6632.2002.tb02791.x

22. Vercellini P, Abbiati A, Viganò P, Somigliana ED, Daguati R, Meroni F, et al. Asymmetry in distribution of diaphragmatic endometriotic lesions: Evidence in favour of the menstrual reflux theory. *Hum Reprod* (2007) 22:2359–67. doi: 10.1093/humrep/dem224

23. Batt RE, Smith RA, Buck GL, Martin DC, Chapron C, Koninckx PR, et al. Müllerianosis. *Histol Histopathol* (2007) 22:1161–6. doi: 10.14670/HH-22.1161

24. Signorile PG, Baldi F, Bussani R, D'Armiento M, De Falco M, Baldi A. Ectopic endometrium in human foetuses is a common event and sustains the theory of müllerianosis in the pathogenesis of endometriosis, a disease that predisposes to cancer. *J Exp Clin Cancer Res* (2009) 28:49. doi: 10.1186/1756-9966-28-49

25. Batt RE, Mitwally MFM. Endometriosis from thelarche to midteens: Pathogenesis and prognosis, prevention and pedagogy. *J Pediatr Adolesc Gynecol* (2003) 16:337–47. doi: 10.1016/j.jpag.2003.09.008

26. Tandoi I, Somigliana E, Riparini J, Ronzoni S, Vigano' P, Candiani M. High rate of endometriosis recurrence in young women. *J Pediatr Adolesc Gynecol* (2011) 24:376–9. doi: 10.1016/j.jpag.2011.06.012

27. Gruenwald P. Origin of endometriosis from the mesenchyme of the celomic walls. *Am J Obstet Gynecol* (1942) 44:470–4. doi: 10.1016/S0002-9378(42)90484-8

28. Suginami H. A reappraisal of the coelomic metaplasia theory by reviewing, endometriosis occurring in unusual sites and instances. *Am J Obstet Gynecol* (1991) 165:214–8. doi: 10.1016/0002-9378(91)90254-o

29. Nawroth F, Rahimi G, Nawroth C, Foth D, Ludwig M, Schmidt T. Is there an association between septate uterus and endometriosis? *Hum Reprod* (2006) 21:542–4. doi: 10.1093/humrep/dei344

30. Mok-Lin EY, Wolfberg A, Hollinquist H, Laufer MR. Endometriosis in a patient with Mayer-Rokitansky-Küster-Hauser syndrome and complete uterine agenesis: Evidence to support the theory of coelomic metaplasia. *J Pediat Adolesc Gynecol* (2010) 23:e35–7. doi: 10.1016/j.jpag.2009.02.010

31. Troncon JK, Zani ACT, Vieira ADD, Poli-Neto OB, Nogueira AA, Rosa-E-Silva JC. Endometriosis in a patient with mayer-rokitansky-küster-hauser syndrome. *Case Rep Obstet Gynecol* (2014) 2014:376231. doi: 10.1155/2014/376231

32. Asencio FA, Ribeiro HA, Ribeiro PA, Mario M, Adamyan L, Ussia A, et al. Case reports and systematic review of estrogen independent symptomatic postmenopausal endometriosis. *Gynecol Surg* (2018) 16:3. doi: 10.1186/s10397-019-1056-x

33. Giannarini G, Scott CA, Moro U, Grossetti B, Pomara G, Selli C. Cystic endometriosis of the epididymis. *Urology* (2006) 68:203.e1–3. doi: 10.1016/j.urology.2006.01.017

34. Jabr FI, Mani V. An unusual cause of abdominal pain in a male patient: Endometriosis. *Avicenna J Med* (2014) 4:99–101. doi: 10.4103/2231-0770.140660

35. Rei C, Williams T, Feloney M. Endometriosis in a man as a rare source of abdominal pain: A case report and review of the literature. *Case Rep Obstet Gynecol* (2018) 2018:2083121. doi: 10.1155/2018/2083121

36. Martin JD, Hauck AE. Endometriosis in the male. *Am Surg* (1985) 51:426–30.

37. Sampson JA. Metastatic or embolic endometriosis, due to the menstrual dissemination of endometrial tissue into the venous circulation. *Am J Pathol* (1927) 3:93–110.43.

38. Vinatier D, Orazi G, Cosson M, Dufour P. Theories of endometriosis. *Eur J Obstet Gynecol Reprod Biol* (2001) 96:21–34. doi: 10.1016/s0301-2115(00)00405-x

39. Samani EN, Mamillapalli R, Li F, Mutlu L, Hufnagel D, Krikun G, et al. Micrometastasis of endometriosis to distant organs in a murine model. *Oncotarget* (2017) 10:2282–91. doi: 10.18632/oncotarget.16889

40. Mechsner S, Weichbrodt M, Riedlinger WF, Bartley J, Kaufmann AM, Schneider A, et al. Estrogen and progestogen receptor positive endometriotic lesions and disseminated cells in pelvic sentinel lymph nodes of patients with deep infiltrating rectovaginal endometriosis: A pilot study. *Hum Reprod* (2008) 23:2202–9. doi: 10.1093/humrep/den259

41. Taylor HS. Endometrial cells derived from donor stem cells in bone marrow transplant recipients. *JAMA* (2004) 292(1):81–5. doi: 10.1001/jama.292.1.81

42. Mints M, Jansson M, Sadeghi B, Westgren M, Uzunel M, Hassan M, et al. Endometrial endothelial cells are derived from donor stem cells in a bone marrow transplant recipient. *Hum Reprod* (2008) 23:139–43. doi: 10.1093/humrep/dem342

43. Ikoma T, Kyo S, Maida Y, Ozaki S, Takakura M, Nakao S, et al. Bone marrow-derived cells from male donors can compose endometrial glands in female transplant recipients. *Am J Obstet Gynecol* (2009) 201(6):608.e1–8. doi: 10.1016/j.ajog.2009.07.026

44. Gargett CE, Schwab KE, Deane JA. Endometrial stem/progenitor cells: The first 10 years. *Hum Reprod Update* (2016) 22:137–63. doi: 10.1093/humupd/dmv051

45. Leyendecker G, Herbertz M, Kunz G, Mall G. Endometriosis results from the dislocation of basal endometrium. *Hum Reprod* (2002) 17:2725–36. doi: 10.1093/humrep/17.10.2725

46. Figueira PG, Abrão MS, Krikun G, Taylor HS. Stem cells in endometrium and their role in the pathogenesis of endometriosis. *Ann N Y Acad Sci* (2011) 1221:10–7. doi: 10.1111/j.1749-6632.2011.05969.x

47. Du H, Taylor HS. Contribution of bone marrow-derived stem cells to endometrium and endometriosis. *Stem Cells* (2007) 25:2082–6. doi: 10.1634/stemcells.2006-0828

48. Nezhat C, King LP, Paka C, Odegaard J, Beygui R. Bilateral thoracic endometriosis affecting the lung and diaphragm. *JSLS* (2012) 16:140–2. doi: 10.4293/108680812X13291597716384

49. Ciriaco P, Muriana P, Carretta A, Ottolina J, Candiani M, Negri G. Catamenial pneumothorax as the first expression of thoracic endometriosis syndrome and pelvic endometriosis. *J Clin Med* (2022) 11:1200. doi: 10.3390/jcm11051200

50. Hwang SM, Lee CW, Lee BS, Park JH. Clinical features of thoracic endometriosis: A single center analysis. *Obstet Gynecol Sci* (2015) 58:223–31. doi: 10.5468/ogs.2015.58.3.223

51. Sarma D, Iyengar P, Marotta TR, terBrugge KG, Gentili F, Halliday W. Cerebellar endometriosis. *AJR* (2004) 182:1543–6. doi: 10.2214/ajr.182.6.1821543

52. Du H, Naqvi H, Taylor HS. Ischemia/reperfusion injury promotes and granulocyte-colony stimulating factor inhibits migration of bone marrow-derived stem cells to endometrium. *Stem Cells Dev* (2012) 21:3324–31. doi: 10.1089/scd.2011.0193

53. Kulak J Jr, Fischer C, Komm B, Taylor HS. Treatment with bazedoxifene, a selective estrogen receptor modulator, causes regression of endometriosis in a mouse model. *Endocrinology* (2011) 152:3226–32. doi: 10.1210/en.2010-1010

54. Santamaria X, Massasa EE, Taylor HS. Migration of cells from experimental endometriosis to the uterine endometrium. *Endocrinology* (2012) 153:5566–74. doi: 10.1210/en.2012-1202

55. Kennedy SH, Mardon H, Barlow DH. Familial endometriosis. *J Assist Reprod Genet* (1995) 12:32–4. doi: 10.1007/BF02214126

56. Hadfield RM, Yudkin PL, Coe CL, Scheffler J, Uno H, Barlow DH, et al. Risk factors for endometriosis in the rhesus monkey (Macaca mulatta): A case- control study. *Hum Reprod Update* (1997) 3:109–15. doi: 10.1093/humupd/3.2.109

57. Zondervan KT, Weeks DE, Colman R, Cardon LR, Hadfield R, Schleffler J, et al. Familial aggregation of endometriosis in a large pedigree of rhesus macaques. *Hum Reprod* (2004) 19:448–55. doi: 10.1093/humrep/deh052

58. Hadfield RM, Mardon HJ, Barlow DH, Kennedy SH. Endometriosis in monozygotic twins. *Fertil Steril* (1997) 68:941–2. doi: 10.1016/s0015-0282(97)00359-2

59. Saha R, Pettersson HJ, Svedberg P, Olovsson M, Bergqvist A, Marions L, et al. Heritability of endometriosis. *Fertil Steril* (2015) 104:947–52. doi: 10.1016/j.fertnstert.2015.06.035

60. Treloar SA, O'Connor DT, O'Connor VM, Martin NG. Genetic influences on endometriosis in an Australian twin sample. *Fertil Steril* (1999) 71:701–10. doi: 10.1016/s0015-0282(98)00540-8

61. Dalsgaard T, Hjordt Hansen MV, Hartwell D, Lidegaard O. Reproductive prognosis in daughters of women with and without endometriosis. *Hum Reprod* (2013) 28:2284–8. doi: 10.1093/humrep/det231

62. Malinak LR, Buttram VCJr, Elias S, Simpson JL. Heritage aspects of endometriosis. II. clinical characteristics of familial endometriosis. *Am J Obstet Gynecol* (1980) 137:332–37. doi: 10.1016/0002-9378(80)90918-7

63. Uno S, Kyo S, Maida Y, Ozaki S, Takakura M, Nakao S, et al. A genome-wide association study identifies genetic variants in the CDKN2BAS locus associated with endometriosis in Japanese. *Nat Genet* (2010) 42:707–10. doi: 10.1038/ng.612

64. Painter JN, Anderson CA, Nyholt DR, Macgregor S, Lin J, Lee SH, et al. Genome-wide association study identifies a locus at 7p15.2 associated with endometriosis. *Nat Genet* (2011) 43:51–4. doi: 10.1038/ng.731

65. Albertsen HM, Chettier R, Farrington P, Ward K. Genome-wide association study link novel loci to endometriosis. *PloS One* (2013) 8:e58257. doi: 10.1371/journal.pone.0058257

66. Nyholt DR, Low SK, Anderson CA, Painter JN, Uno S, Morris AP, et al. Genome-wide association meta-analysis identifies new endometriosis risk loci. *Nat Genet* (2012) 44:1355–9. doi: 10.1038/ng.2445

67. Sapkota Y, Steinthorsdottir V, Morris AP, Fassbender A, Rahmioglu N, De Vivo I, et al. Meta-analysis identifies five novel loci associated with endometriosis highlighting key genes involved in hormone metabolism. *Nat Commun* (2017) 8:15539. doi: 10.1038/ncomms15539

68. Pagliardini L, Gentilini D, Sanchez AM, Candiani M, Viganò P, Di Blasio AM. Replication and meta-analysis of previous genome-wide association studies confirm vezatin as the locus with the strongest evidence for association with endometriosis. *Hum Reprod* (2015) 30:987–93. doi: 10.1093/humrep/dev022

69. Montgomery GW, Nyholt DR, Zhao ZZ, Treloar SA, Painter JN, Missmer SA, et al. The search for genes contributing to endometriosis risk. *Hum Reprod Update* (2008) 14:447–57. doi: 10.1093/humupd/dmn016

70. Bianco B, Loureiro FA, Trevisan CM, Peluso C, Christofolini DM, Montagna E, et al. Effects of *FSHR* and *FSHB* variants on hormonal profile and reproductive outcomes of infertile women with endometriosis. *Front Endocrinol (Lausanne)* (2021) 12:760616. doi: 10.3389/fendo.2021.760616

71. Naqvi H, Ilagan Y, Krikun G, Taylor HS. Altered genome-wide methylation in endometriosis. *Reprod Sci* (2014) 21:1237–43. doi: 10.1177/1933719114532841

72. Guo SW. Epigenetics of endometriosis. *Mol Hum Reprod* (2009) 15:587–607. doi: 10.1093/molehr/gap064

73. Nasu K, Kawano Y, Tsukamoto Y, Takano M, Takai N, Li H, et al. Aberrant DNA methylation status of endometriosis: Epigenetics as the pathogenesis, biomarker and therapeutic target. *J Obstet Gynaecol Res* (2011) 37:683–95. doi: 10.1111/j.1447-0756.2011.01663.x

74. Wu Y, Halverson G, Basir Z, Strawn E, Yan P, Guo SW. Aberrant methylation at HOXA10 may be responsible for its aberrant expression in the endometrium of patients with endometriosis. *Am J Obstet Gynecol* (2005) 193:371–80. doi: 10.1016/j.ajog.2005.01.034

75. Taylor HS, Bagot C, Kardana A, Olive D, Arici A. HOX gene expression is altered in the endometrium of women with endometriosis. *Hum Reprod* (1999) 14 (5):1328–31. doi: 10.1093/humrep/14.5.1328

76. Kulp JL, Mamillapalli R, Taylor HS. Aberrant HOXA10 methylation in patients with common gynecologic disorders: Implications for reproductive outcomes. *Reprod Sci* (2016) 23:455–63. doi: 10.1177/1933719116630427

77. Wu Y, Strawn E, Basir Z, Halverson G, Guo SW. Promoter hypermethylation of progesterone receptor isoform b (PR-b) in endometriosis. *Epigenetics* (2006) 1:106–11. doi: 10.4161/epi.1.2.2766

78. Nie J, Liu X, Guo SW. Promoter hypermethylation of progesterone receptor isoform b (PR-b) in adenomyosis and its rectification by a histone deacetylase inhibitor and a demethylation agent. *Reprod Sci* (2010) 17:995–1005. doi: 10.1177/1933719110377118

79. Attia GR, Zeitoun K, Edwards D, Johns A, Carr BR, Bulun SE. Progesterone receptor isoform a but not b is expressed in endometriosis. *J Clin Endocrinol Metab* (2000) 85:2897–902. doi: 10.1210/jcem.85.8.6739

80. Rubel CA, Wu SP, Lin L, Wang T, Lanz RB, Li X, et al. A Gata2-dependent transcription network regulates uterine progesterone responsiveness and endometrial function. *Cell Rep* (2016) 17:1414–25. doi: 10.1016/j.celrep.2016.09.093

81. Xue Q, Lin Z, Yin P, Milad MP, Cheng YH, Confino E, et al. Transcriptional activation of steroidogenic factor-1 by hypomethylation of the 5′ CpG island in endometriosis. *J Clin Endocrinol Metab* (2007) 92:3261–7. doi: 10.1210/jc.2007-0494

82. Dyson MT, Roqueiro D, Monsivais D, Ercan CM, Pavone ME, Brooks DC, et al. Genome-wide DNA methylation analysis predicts an epigenetic switch for GATA factor expression in endometriosis. *PloS Genet* (2014) 10:e1004158. doi: 10.1371/journal.pgen.1004158

83. Wu Y, Strawn E, Basir Z, Halverson G, Guo SW. Aberrant expression of deoxyribonucleic acid methyltransferases DNMT1, DNMT3A, and DNMT3B in women with endometriosis. *Fertil Steril* (2007) 87:24–32. doi: 10.1016/j.fertnstert.2006.05.077

84. Dyson MT, Kakinuma T, Pavone ME, Monsivais D, Navarro A, Malpani SS, et al. Aberrant expression and localization of deoxyribonucleic acid methyltransferase 3B in endometriotic stromal cells. *Fertil Steril* (2015) 104:953–63. doi: 10.1016/j.fertnstert.2015.06.046

85. Wu Y, Starzinski-Powitz A, Guo SW, Trichostatin A. A histone deacetylase inhibitor, attenuates invasiveness and reactivates e-cadherin expression in immortalized endometriotic cells. *Reprod Sci* (2007) 14:374–82. doi: 10.1177/1933719107302913

86. Meixell DA, Mamillapalli R, Taylor HS. Methylation of microribonucleic acid let-7b regulatory regions in endometriosis. *F S Sci* (2022) 3(2):197–203. doi: 10.1016/j.xfss.2022.03.001

87. Kawano Y, Nasu K, Li H, Tsuno A, Abe W, Takai N, et al. Application of the histone deacetylase inhibitors for the treatment of endometriosis: Histone modifications as pathogenesis and novel therapeutic target. *Hum Reprod* (2011) 26:2486–98. doi: 10.1093/humrep/der203

88. Wilting RH, Yanover E, Heideman MR, Jacobs H, Horner J, van der Torre J, et al. Overlapping functions of Hdac1 and Hdac2 in cell cycle regulation and haematopoiesis. *EMBO J* (2010) 29:2586–97. doi: 10.1038/emboj.2010.136

89. Colón-Díaz M, Báez-Vega P, García M, Ruiz A, Monteiro JB, Fourquet J, et al. {HDAC}1 and HDAC 2 are differentially expressed in endometriosis. *Reprod Sci* (2012) 19:483–92. doi: 10.1177/1933719111432870

90. Kim TH, Yoo JY, Choi KC, Shin JH, Leach RE, Fazleabas AT, et al. Loss of HDAC3 results in nonreceptive endometrium and female infertility. *Sci Transl Med* (2019) 11:eaaf7533. doi: 10.1126/scitranslmed.aaf7533

91. Samartzis EP, Noske A, Samartzis N, Fink D, Imesch P. The expression of histone deacetylase 1, but not other class I histone deacetylases, is significantly

increased in endometriosis. *Reprod Sci* (2013) 20:1416–22. doi: 10.1177/1933719113488450

92. Monteiro JB, Colón-Díaz M, García M, Gutierrez S, Colón M, Seto E, et al. Endometriosis is characterized by a distinct pattern of histone 3 and histone 4 lysine modifications. *Reprod Sci* (2014) 21:305–18. doi: 10.1177/1933719113497267

93. La Ferlita A, Battaglia R, Andronico F, Caruso S, Cianci A, Purrello M, et al. Non-coding RNAs in endometrial physiopathology. *Int J Mol Sci* (2018) 19:2120. doi: 10.3390/ijms19072120

94. Fehlmann T, Ludwig N, Backes C, Meese E, Keller A. Distribution of microRNA biomarker candidates in solid tissues and body fluids. *RNA Biol* (2016) 13:1084–8. doi: 10.1080/15476286.2016.1234658

95. Santamaria X, Taylor H. MicroRNA and gynecological reproductive diseases. *Fertil Steril* (2014) 101:1545–51. doi: 10.1016/j.fertnstert.2014.04.044

96. Bjorkman S, Taylor HS. MicroRNAs in endometriosis: Biological function and emerging biomarker candidates. *Biol Reprod* (2019) 100:1135–46. doi: 10.1093/biolre/ioz014

97. Wei S, Xu H, Kuang Y. Systematic enrichment analysis of microRNA expression profiling studies in endometriosis. *Iran J Basic Med Sci* (2015) 18:423–9.

98. Haikalis ME, Wessels JM, Leyland NA, Agarwal SK, Foster WG. MicroRNA expression pattern differs depending on endometriosis lesion type. *Biol Reprod* (2018) 98:623–33. doi: 10.1093/biolre/ioy019

99. Kuokkanen S, Chen B, Ojalvo L, Benard L, Santoro N, Pollard JW. Genomic profiling of microRNAs and messenger RNAs reveals hormonal regulation in microRNA expression in human endometrium. *Biol Reprod* (2010) 82:791–801. doi: 10.1095/biolreprod.109.081059

100. Sha AG, Liu JL, Jiang XM, Ren JZ, Ma CH, Lei W, et al. Genome-wide identification of micro-ribonucleic acids associated with human endometrial receptivity in natural and stimulated cycles by deep sequencing. *Fertil Steril* (2011) 96:150.e5–5.e5. doi: 10.1016/j.fertnstert.2011.04.072

101. Yang YM, Yang WX. Epithelial-to-mesenchymal transition in the development of endometriosis. *Oncotarget* (2017) 8:41679–89. doi: 10.18632/oncotarget.16472

102. Viganò P, Ottolina J, Bartiromo L, Bonavina G, Schimberni M, Villanacci R, et al. Cellular components contributing to fibrosis in endometriosis: A literature review. *J Minim Invasive Gynecol* (2020) 27:287–95. doi: 10.1016/j.jmig.2019.11.011

103. Saare M, Rekker K, Laisk-Podar T, Rahmioglu N, Zondervan K, Salumets A, et al. Challenges in endometriosis miRNA studies — From tissue heterogeneity to disease specific miRNAs. *Biochim Biophys Acta Mol Basis Dis* (2017) 1863:2282–92. doi: 10.1016/j.bbadis.2017.06.018

104. Weber JA, Baxter DH, Zhang S, Huang DY, How Huang K, Jen Lee M, et al. The microRNA spectrum in 12 body fluids. *Clin Chem* (2010) 56:1733–41. doi: 10.1373/clinchem.2010.147405

105. Panir K, Schjenken JE, Robertson SA, Hull ML. Non-coding RNAs in endometriosis: A narrative review. *Hum Reprod Update* (2018) 24:497–515. doi: 10.1093/humupd/dmy014

106. Grümmer R. Translational animal models to study endometriosis-associated infertility. *Semin Reprod Med* (2013) 31(2):125–32. doi: 10.1055/s-0032-1333477

107. Story L, Kennedy S. Animal studies in endometriosis: A review. *ILAR J* (2004) 45(2):132–8. doi: 10.1093/ilar.45.2.132

108. Dick EJJr, Hubbard GB, Martin LJ, Leland MM. Record review of baboons with histologically confirmed endometriosis in a large established colony. *J Med Primatol* (2003) 32:39–47. doi: 10.1034/j.1600-0684.2003.00008.x

109. TE LINDE RW, SCOTT RB. Experimental endometriosis. *Am J Obstet Gynecol* (1950) 60:1147–73. doi: 10.1016/0002-9378(50)90517-5

110. D'Hooghe TM, Bambra CS, Suleman MA, Dunselman GA, Evers HL, Koninckx PR. Development of a model of retrograde menstruation in baboons (*Papio anubis*). *Fertil Steril* (1994) 62:635–8.

111. Kaplan CR, Eddy CA, Olive DL, Schenken RS. Effect of ovarian endometriosis on ovulation in rabbits. *Am J Obstet Gynecol* (1989) 160:40–4. doi: 10.1016/0002-9378(89)90083-5

112. Hayashi S, Nakamura T, Motooka Y, Ito F, Jiang L, Akatsuka S, et al. Novel ovarian endometriosis model causes infertility via iron-mediated oxidative stress in mice. *Redox Biol* (2020) 37:101726. doi: 10.1016/j.redox.2020.101726

113. Grechukhina O, Petracco R, Popkhadze S, Massasa E, Paranjape T, Chan E, et al. A polymorphism in a let-7 microRNA binding site of KRAS in women with endometriosis. *EMBO Mol Med* (2012) 4:206–17. doi: 10.1002/emmm.201100200

114. Yong PJ, Sadownik L, Brotto LA. Concurrent deep-superficial dyspareunia: prevalence, associations, and outcomes in a multidisciplinary vulvodynia program. *J Sex Med* (2015) 12:219 –27. doi: 10.1111/jsm.12729

115. Mabrouk M, Del Forno S, Spezzano A, Raimondo D, Arena A, Zanello M, et al. Painful love: Superficial dyspareunia and three dimensional transperineal ultrasound evaluation of pelvic floor muscle in women with endometriosis. *J Sex Marital Ther* (2020) 46:187–196.14. doi: 10.1080/0092623X.2019.1676852

116. Wahl KJ, Orr NL, Lisonek M, Noga H, Bedaiwy MA, Williams C, et al. Deep dyspareunia, superficial dyspareunia, and infertility concerns among women with endometriosis: A cross-sectional study. *Sex Med* (2020) 8:274–81. doi: 10.1016/j.esxm.2020.01.002

117. Vercellini P, Meana M, Hummelshoj L, Somigliana E, Vigano` P, Fedele L. Priorities for endometriosis research: A proposed focus on deep dyspareunia. *Reprod Sci* (2011) 18:114–8. doi: 10.1177/1933719110382921

118. Vercellini P, Somigliana E, Consonni D, Frattaruolo MP, De Giorgi O, Fedele L. Surgical versus medical treatment for endometriosis-associated severe deep dyspareunia: I. effect on pain during intercourse and patient satisfaction. *Hum Reprod* (2012) 27:3450–9. doi: 10.1093/humrep/des313

119. Denny E, Mann C. Endometriosis-associated dyspareunia: The impact on women's lives. *J Fam Plann Reprod Health Care* (2007) 33:189–93. doi: 10.1783/147118907781004831

120. Barbara G, Facchin F, Buggio L, Somigliana E, Berlanda N, Kustermann A, et al. What is known and unknown about the association between endometriosis and sexual functioning: A systematic review of the literature. *Reprod Sci* (2017) 24:1566–76. doi: 10.1177/1933719117707054

121. Barbara G, Facchin F, Meschia M, Berlanda N, Frattaruolo MP, Vercellini P. When love hurts. A systematic review on the effects of surgical and pharmacological treatments for endometriosis on female sexual functioning. *Acta Obstet Gynecol Scand* (2017) 96:668–87. doi: 10.1111/aogs.13031

122. Pluchino N, Wenger JM, Petignat P, Tal R, Bolmont M, Taylor HS, et al. Sexual function in endometriosis patients and their partners: Effect of the disease and consequences of treatment. *Hum Reprod Update* (2016) 22:762–74. doi: 10.1093/humupd/dmw031

123. Schenken RS, Asch RH, Williams RF, Hodgen GD. Etiology of infertility in monkeys with endometriosis: luteinized unruptured follicles, luteal phase defects, pelvic adhesions, and spontaneous abortions. *Fertil Steril* (1984) 41:122–30. doi: 10.1016/s0015-0282(16)47552-7

124. Gleicher N, Weghofer A, Barad DH. Defining ovarian reserve to better understand ovarian aging. *Reprod Biol Endocrinol* (2011) 9:23. doi: 10.1186/1477-7827-9-23

125. Nelson SM. Biomarkers of ovarian response: current and future applications. *Fertil Steril* (2013) 99:963–9. doi: 10.1016/j.fertnstert.2012.11.051

126. Santoro N. Using antimüllerian hormone to predict fertility. *JAMA* (2017) 318:1333–4. doi: 10.1001/jama.2017.14954

127. Sanchez AM, Papaleo E, Corti L, Santambrogio P, Levi S, Viganò P, et al. Iron availability is increased in individual human ovarian follicles in close proximity to an endometrioma compared with distal ones. *Hum Reprod* (2014) 29:577–83. doi: 10.1093/humrep/det466

128. Sanchez AM, Viganò P, Somigliana E, Panina-Bordignon P, Vercellini P, Candiani M. The distinguishing cellular and molecular features of the endometriotic ovarian cyst: From pathophysiology to the potential endometrioma-mediated damage to the ovary. *Hum Reprod Update* (2014) 20:217–30. doi: 10.1093/humupd/dmt053

129. Matsuzaki S, Schubert B. Oxidative stress status in normal ovarian cortex surrounding ovarian endometriosis. *Fertil Steril* (2010) 93:2431–2. doi: 10.1016/j.fertnstert.2009.08.068

130. Maneschi F, Marasá L, Incandela S, Mazzarese M, Zupi E. Ovarian cortex surrounding benign neoplasms: A histologic study. *Am J Obstet Gynecol* (1993) 169:388–93. doi: 10.1016/0002-9378(93)90093-x

131. Kitajima M, Dolmans MM, Donnez O, Masuzaki H, Soares M, Donnez J. Enhanced follicular recruitment and atresia in cortex derived from ovaries with endometriomas. *Fertil Steril* (2014) 101:1031–7. doi: 10.1016/j.fertnstert.2013.12.049

132. Kuroda M, Kuroda K, Arakawa A, Fukumura Y, Kitade M, Kikuchi I, et al. Histological assessment of impact of ovarian endometrioma and laparoscopic cystectomy on ovarian reserve. *J Obstet Gynaecol Res* (2012) 38:1187–93. doi: 10.1111/j.1447-0756.2012.01845.x

133. Pavone ME, Hirshfeld-Cytron J, Tingen C, Thomas C, Thomas J, Lowe MP, et al. Human ovarian tissue cortex surrounding benign and malignant lesions. *Reprod Sci* (2014) 2:582–9. doi: 10.1177/1933719113506498

134. Schubert B, Canis M, Darcha C, Artonne C, Pouly JL, Déchelotte P, et al. Human ovarian tissue from cortex surrounding benign cysts: A model to study ovarian tissue cryopreservation. *Hum Reprod* (2005) 20:1786–92. doi: 10.1093/humrep/dei002

135. Dolmans M-M, Martinez-Madrid B, Gadisseux E, Guiot Y, Yuan WY, Torre A, et al. Short-term transplantation of isolated human ovarian follicles and cortical tissue into nude mice. *Reproduction* (2007) 134(2):253–62. doi: 10.1530/REP-07-0131

136. Takeuchi A, Koga K, Satake E, Makabe T, Taguchi A, Miyashita M, et al. Endometriosis triggers excessive activation of primordial follicles *via* PI3K-PTEN-

Akt- Foxo3 pathway. *J Clin Endocrinol Metab* (2019) 104:5547–54. doi: 10.1210/jc.2019-00281

137. Sanchez AM, Somigliana E, Vercellini P, Pagliardini L, Candiani M, Vigano P. Endometriosis as a detrimental condition for granulosa cell steroidogenesis and development: From molecular alterations to clinical impact. *J Steroid Biochem Mol Biol* (2016) 155:35–46. doi: 10.1016/j.jsbmb.2015.07.023

138. Di Nisio V, Rossi G, Di Luigi G, Palumbo P, D'Alfonso A, Iorio R, et al. Increased levels of proapoptotic markers in normal ovarian cortex surrounding small endometriotic cysts. *Reprod Biol* (2019) 19:225–9. doi: 10.1016/j.repbio.2019.08.002

139. Thombre Kulkarni M, Shafrir A, Farland LV, Terry KL, Whitcomb BW, Eliassen AH, et al. Association between laparoscopically confirmed endometriosis and risk of early natural menopause. *JAMA Netw Open* (2022) 5:e2144391. doi: 10.1001/jamanetworkopen.2021.44391

140. Muzii L, Di Tucci C, Di Feliciantonio M, Galati G, Di Donato V, Musella A, et al. Antimüllerian hormone is reduced in the presence of ovarian endometriomas: A systematic review and meta-analysis. *Fertil Steril* (2018) 110:932–40.e1. doi: 10.1016/j.fertnstert.2018.06.025

141. Tian Z, Zhang Y, Zhang C, Wang Y, Zhu HL. Antral follicle count is reduced in the presence of endometriosis: A systematic review and meta-analysis. *Reprod BioMed Online* (2021) 42:237–47. doi: 10.1016/j.rbmo.2020.09.014

142. Kasapoglu I, Ata B, Uyaniklar O, Seyhan A, Orhan A, Yildiz Oguz S, et al. Endometrioma-related reduction in ovarian reserve (ERROR): A prospective longitudinal study. *Fertil Steril* (2018) 110:122–7. doi: 10.1016/j.fertnstert.2018.03.015

143. Gupta S, Agarwal A, Agarwal R, Loret de Mola JR. Impact of ovarian endometrioma on assisted reproduction outcomes. *Reprod BioMed Online* (2006) 13:349–60. doi: 10.1016/s1472-6483(10)61439-3

144. Hamdan M, Dunselman G, Li TC, Cheong Y. The impact of endometrioma on IVF/ICSI outcomes: A systematic review and meta-analysis. *Hum Reprod Update* (2015) 21:809–25. doi: 10.1093/humupd/dmv035

145. Alshehre SM, Narice BF, Fenwick MA, Metwally M. The impact of endometrioma on *in vitro* fertilisation/intra-cytoplasmic injection IVF/ICSI reproductive outcomes: A systematic review and meta-analysis. *Arch Gynecol Obstet* (2021) 303:3–16. doi: 10.1007/s00404-020-05796-9

146. Yang C, Geng Y, Li Y, Chen C, Gao Y. Impact of ovarian endometrioma on ovarian responsiveness and IVF: A systematic review and meta-analysis. *Reprod BioMed Online* (2015) 31:9–19. doi: 10.1016/j.rbmo.2015.03.005

147. Benaglia L, Pasin R, Somigliana E, Vercellini P, Ragni G, Fedele L. Unoperated ovarian endometriomas and responsiveness to hyperstimulation. *Hum Reprod* (2011) 26:1356–61. doi: 10.1016/j.fertnstert.2013.01.110

148. Almog B, Shehata F, Sheizaf B, Tan SL, Tulandi T. Effects of ovarian endometrioma on the number of oocytes retrieved for *in vitro* fertilization. *Fertil Steril* (2011) 95:525–7. doi: 10.1016/j.fertnstert.2010.03.011

149. Esinler I, Bozdag G, Arikan I, Demir B, Yarali H. Endometrioma ≤3 cm in diameter per se does not affect ovarian reserve in intracytoplasmic sperm injection cycles. *Gynecol Obstet Invest* (2012) 74:261–4. doi: 10.1159/000339630

150. Filippi F, Benaglia L, Paffoni A, Restelli L, Vercellini P, Somigliana E, et al. Ovarian endometriomas and oocyte quality: Insights from in vitro fertilization cycles. *Fertil Steril* (2014) 101:988–93.e1. doi: 10.1016/j.fertnstert.2014.01.008

151. Benaglia L, Bermejo A, Somigliana E, Faulisi S, Ragni G, Fedele L, et al. *In vitro* fertilization outcome in women with unoperated bilateral endometriomas. *Fertil Steril* (2013) 99:1714–9. doi: 10.1016/j.fertnstert.2013.01.110

152. Ferrero S, Scala C, Tafi E, Racca A, Venturini PL, Leone Roberti Maggiore U. Impact of large ovarian endometriomas on the response to superovulation for *in vitro* fertilization: A retrospective study. *Eur J Obstet Gynecol Reprod Biol* (2017) 213:17–21. doi: 10.1016/j.ejogrb.2017.04.003

153. Karadağ C, Yoldemir T, Demircan Karadağ S, Turgut A. The effects of endometrioma size and bilaterality on ovarian reserve. *J Obstet Gynaecol* (2020) 40:531–6. doi: 10.1080/01443615.2019.1633518

154. Muzii L, Bianchi A, Crocè C, Manci N, Panici PB. Laparoscopic excision of ovarian cysts: Is the stripping technique a tissue-sparing procedure? *Fertil Steril* (2002) 77:609–14. doi: 10.1016/s0015-0282(01)03203-4

155. Hachisuga T, Kawarabayashi T. Histopathological analysis of laparoscopically treated ovarian endometriotic cysts with special reference to loss of follicles. *Hum Reprod* (2002) 17:432–5. doi: 10.1093/humrep/17.2.432

156. Roman H, Tarta O, Pura I, Opris I, Bourdel N, Marpeau L, et al. Direct proportional relationship between endometrioma size and ovarian parenchyma inadvertently removed during cystectomy, and its implication on the management of enlarged endometriomas. *Hum Reprod* (2010) 25:1428–32. doi: 10.1093/humrep/deq069

157. Muzii L, Marana R, Angioli R, Bianchi A, Cucinella G, Vignali M, et al. Histologic analysis of specimens from laparoscopic endometrioma excision performed by different surgeons: Does the surgeon matter? *Fertil Steril* (2011) 95:2116–9. doi: 10.1016/j.fertnstert.2011.02.034

158. Biacchiardi CP, Piane LD, Camanni M, Deltetto F, Delpiano EM, Marchino GL, et al. Laparoscopic stripping of endometriomas negatively affects ovarian follicular reserve even if performed by experienced surgeons. *Reprod BioMed Online* (2011) 23:740–6. doi: 10.1016/j.rbmo.2011.07.014

159. Garcia-Velasco JA, Somigliana E. Management of endometriomas in women requiring IVF: To touch or not to touch. *Hum Reprod* (2009) 24:496–501. doi: 10.1093/humrep/den398

160. Somigliana E, Berlanda N, Benaglia L, Viganò P, Vercellini P, Fedele L. Surgical excision of endometriomas and ovarian reserve: A systematic review on serum antimüllerian hormone level modifications. *Fertil Steril* (2012) 98:1531–8. doi: 10.1016/j.fertnstert.2012.08.009

161. Raffi F, Metwally M, Amer S. The impact of excision of ovarian endometrioma on ovarian reserve: A systematic review and meta-analysis. *J Clin Endocrinol Metab* (2012) 97:3146–54. doi: 10.1210/jc.2012-1558

162. Younis JS, Shapso N, Ben-Sira Y, Nelson SM, Izhaki I. Endometrioma surgery-a systematic review and meta-analysis of the effect on antral follicle count and anti-müllerian hormone. *Am J Obstet Gynecol* (2022) 226:33–51.e7. doi: 10.1016/j.ajog.2021.06.102

163. Nankali A, Kazeminia M, Jamshidi PK, Shohaimi S, Salari N, Mohammadi M, et al. The effect of unilateral and bilateral laparoscopic surgery for endometriosis on anti-mullerian hormone (AMH) level after 3 and 6 months: A systematic review and meta-analysis. *Health Qual Life Outcomes* (2020) 18:314. doi: 10.1186/s12955-020-01561-3

164. Moreno-Sepulveda J, Romeral C, Niño G, Pérez-Benavente A. The effect of laparoscopic endometrioma surgery on anti-müllerian hormone: A systematic review of the literature and meta-analysis. *JBRA Assist Reprod* (2022) 26:88–104. doi: 10.5935/1518-0557.20210060

165. Younis JS, Shapso N, Fleming R, Ben-Shlomo I, Izhaki I. Impact of unilateral versus bilateral ovarian endometriotic cystectomy on ovarian reserve: A systematic review and meta-analysis. *Hum Reprod Update* (2019) 25:375–91. doi: 10.1093/humupd/dmy049

166. Muzii L, Di Tucci C, Di Feliciantonio M, Marchetti C, Perniola G, Panici PB. The effect of surgery for endometrioma on ovarian reserve evaluated by antral follicle count: A systematic review and meta-analysis. *Hum Reprod* (2014) 29:2190–8. doi: 10.1093/humrep/deu199

167. Wu CQ, Albert A, Alfaraj S, Taskin O, Alkusayer GM, Havelock J, et al. Live birth rate after surgical and expectant management of endometriomas after *In vitro* fertilization: A systematic review, meta-analysis, and critical appraisal of current guidelines and previous meta-analyses. *J Minim Invasive Gynecol* (2019) 26:299–311.e3. doi: 10.1016/j.jmig.2018.08.029

168. Tao X, Chen L, Ge S, Cai L. Weigh the pros and cons to ovarian reserve before stripping ovarian endometriomas prior to IVF/ICSI: A meta-analysis. *PloS One* (2017) 12:e0177426. doi: 10.1371/journal.pone.0177426

169. Nickkho-Amiry M, Savant R, Majumder K, Edi-O'sagie E, Akhtar M. The effect of surgical management of endometrioma on the IVF/ICSI outcomes when compared with no treatment? a systematic review and meta-analysis. *Arch Gynecol Obstet* (2018) 297:1043–57. doi: 10.1007/s00404-017-4640-1

170. Ban Frangež H, Vrtacnik Bokal E, Štimpfel M, Divjak Budihna T, Gulino FA, Garzon S, et al. Reproductive outcomes after laparoscopic surgery in infertile women affected by ovarian endometriomas, with or without *in vitro* fertilisation: Results from the SAFE (surgery and ART for endometriomas) trial. *J Obstet Gynaecol* (2022) 42(5):1293–300. doi: 10.1080/01443615.2021.1959536

171. Dongye H, Ji X, Ma X, Song J, Yan L. The impact of endometriosis on embryo quality in *in-vitro* Fertilization/Intracytoplasmic sperm injection: A systematic review and meta-analysis. *Front Med (Lausanne)* (2021) 8:669342. doi: 10.3389/fmed.2021.669342

172. Barnhart K, Dunsmoor-Su R, Coutifaris C. Effect of endometriosis on *in vitro* fertilization. *Fertil Steril* (2002) 77:1148–55. doi: 10.1016/s0015-0282(02)03112-6

173. Harb HM, Gallos ID, Chu J, Harb M, Coomarasamy A. The effect of endometriosis on *in vitro* fertilisation outcome: A systematic review and meta-analysis. *BJOG* (2013) 120:1308–20. doi: 10.1111/1471-0528.12366

174. Hamdan M, Omar SZ, Dunselman G, Cheong Y. Influence of endometriosis on assisted reproductive technology outcomes: A systematic review and meta-analysis. *Obstet Gynecol* (2015) 125:79–88. doi: 10.1097/AOG.0000000000000592

175. Barbosa MA, Teixeira DM, Navarro PA, Ferriani RA, Nastri CO, Martins WP. Impact of endometriosis and its staging on assisted reproduction outcome: systematic review and meta-analysis. *Ultrasound Obstet Gynecol* (2014) 44:261–78. doi: 10.1002/uog.13366

176. Freis A, Dietrich JE, Binder M, Holschbach V, Strowitzki T, Germeyer A. Relative morphokinetics assessed by time-lapse imaging are altered in embryos from patients with endometriosis. *Reprod Sci* (2018) 25:1279–85. doi: 10.1177/1933719117741373

177. Díaz I, Navarro J, Blasco L, Simón C, Pellicer A, Remohí J. Impact of stage III-IV endometriosis on recipients of sibling oocytes: Matched case-control study. *Fertil Steril* (2000) 74:31–4. doi: 10.1016/s0015-0282(00)00570-7

178. Simón C, Gutiérrez A, Vidal A, de los Santos MJ, Tarín JJ, Remohí J, et al. Outcome of patients with endometriosis in assisted reproduction: Results from *in-vitro* fertilization and oocyte donation. *Hum Reprod* (1994) 9:725–9. doi: 10.1093/oxfordjournals.humrep.a138578

179. Remohí J, Ardiles G, García-Velasco JA, Gaitán P, Simón C, Pellicer A. Endometrial thickness and serum oestradiol concentrations as predictors of outcome in oocyte donation. *Hum Reprod* (1997) 12:2271–6. doi: 10.1093/humrep/12.10.2271

180. Gomes FM, Navarro PA, de Abreu LG, Ferriani RA, dos Reis RM, de Moura MD. Effect of peritoneal fluid from patients with minimal/mild endometriosis on progesterone release by human granulosa-lutein cells obtained from infertile patients without endometriosis: A pilot study. *Eur J Obstet Gynecol Reprod Biol* (2008) 138:60–5. doi: 10.1016/j.ejogrb.2007.12.008

181. Skrzypczak J. Morphology and steroidogenesis of cultured granulosa cells from endometrioidally changed ovaries. *Exp Clin Endocrinol Diabetes* (1995) 103:228–32. doi: 10.1055/s-0029-1211355

182. Du YB, Gao MZ, Shi Y, Sun ZG, Wang J. Endocrine and inflammatory factors and endometriosis-associated infertility in assisted reproduction techniques. *Arch Gynecol Obstet* (2013) 287:123–30. doi: 10.1007/s00404-012-2567-0

183. Regiani T, Cordeiro FB, da Costa Ldo V, Salgueiro J, Cardozo K, Carvalho VM, et al. Follicular fluid alterations in endometriosis: label-free proteomics by MS (E) as a functional tool for endometriosis. *Syst Biol Reprod Med* (2015) 61:263–76. doi: 10.3109/19396368.2015.1037025

184. Da Broi MG, de Albuquerque FO, de Andrade AZ, Cardoso RL, Jordão Junior AA, Navarro PA. Increased concentration of 8-hydroxy-2'-deoxyguanosine in follicular fluid of infertile women with endometriosis. *Cell Tissue Res* (2016) 366:231–42. doi: 10.1007/s00441-016-2428-4

185. Da Broi MG, Malvezzi H, Paz CC, Ferriani RA, Navarro PA. Follicular fluid from infertile women with mild endometriosis may compromise the meiotic spindles of bovine metaphase II oocytes. *Hum Reprod* (2014) 29:315–23. doi: 10.1093/humrep/det378

186. Nasiri N, Moini A, Eftekhari-Yazdi P, Karimian L, Salman-Yazdi R, Arabipoor A. Oxidative stress statues in serum and follicular fluid of women with endometriosis. *Cell J* (2017) 18:582–7. doi: 10.22074/cellj.2016.4724

187. Prieto L, Quesada JF, Cambero O, Pacheco A, Pellicer A, Codoceo R, et al. Analysis of follicular fluid and serum markers of oxidative stress in women with infertility related to endometriosis. *Fertil Steril* (2012) 98:126–30. doi: 10.1016/j.fertnstert.2012.03.052

188. Giacomini E, Sanchez AM, Sarais V, Beitawi SA, Candiani M, Viganò P. Characteristics of follicular fluid in ovaries with endometriomas. *Eur J Obstet Gynecol Reprod Biol* (2017) 209:34–8. doi: 10.1016/j.ejogrb.2016.01.032

189. Paffoni A, Bolis V, Ferrari S, Benaglia L, Vercellini P, Somigliana E. The gametotoxic effects of the endometrioma content: Insights from a parthenogenetic human model. *Reprod Sci* (2019) 26:573–9. doi: 10.1177/1933719118777637

190. Singh AK, Dutta M, Chattopadhyay R, Chakravarty B, Chaudhury K. Intrafollicular interleukin-8, interleukin-12, and adrenomedullin are the promising prognostic markers of oocyte and embryo quality in women with endometriosis. *J Assist Reprod Genet* (2016) 33:1363–72. doi: 10.1007/s10815-016-0782-5

191. Choi YS, Cho S, Seo SK, Park JH, Kim SH, Lee BS. Alteration in the intrafollicular thiol-redox system in infertile women with endometriosis. *Reproduction* (2015) 149:155–62. doi: 10.1530/REP-14-0438

192. Xu H, Schultze-Mosgau A, Agic A, Diedrich K, Taylor RN, Hornung D. Regulated upon activation, normal T cell expressed and secreted (RANTES) and monocyte chemotactic protein 1 in follicular fluid accumulate differentially in patients with and without endometriosis undergoing *in vitro* fertilization. *Fertil Steril* (2006) 86:1616–20. doi: 10.1016/j.fertnstert.2006.05.043

193. Mansour G, Abdelrazik H, Sharma RK, Radwan E, Falcone T, Agarwal A. L-carnitine supplementation reduces oocyte cytoskeleton damage and embryo apoptosis induced by incubation in peritoneal fluid from patients with endometriosis. *Fertil Steril* (2009) 91:2079–86. doi: 10.1016/j.fertnstert.2008.02.097

194. Mansour G, Sharma RK, Agarwal A, Falcone T. Endometriosis-induced alterations in mouse metaphase II oocyte microtubules and chromosomal alignment: A possible cause of infertility. *Fertil Steril* (2010) 94:1894–9. doi: 10.1016/j.fertnstert.2009.09.043

195. Jianini BTGM, Giorgi VS, Da Broi MG, de Paz CC, Rosa E Silva JC, Ferriani RA, et al. Peritoneal fluid from infertile women with Minimal/Mild endometriosis compromises the meiotic spindle of metaphase II bovine oocytes. *Reprod Sci* (2017) 24:1304–11. doi: 10.1177/1933719116687658

196. Hahn DW, Carraher RP, Foldesy RG, McGuire JL. Experimental evidence for failure to implant as a mechanism of infertility associated with endometriosis. *Am J Obstet Gynecol* (1986) 155:1109–13. doi: 10.1016/0002-9378(86)90360-1

197. Steinleitner A, Lambert H, Kazensky C, Danks P. Peritoneal fluid from endometriosis patients affects reproductive outcome in an in vivo model. *Fertil Steril* (1990) 53(5):926–9. doi: 10.1016/s0015-0282(16)53533-x

198. Illera MJ, Juan L, Stewart CL, Cullinan E, Ruman J, Lessey BA. Effect of peritoneal fluid from women with endometriosis on implantation in the mouse model. *Fertil Steril* (2000) 74:41–8. doi: 10.1016/s0015-0282(00)00552-5

199. Aeby TC, Huang T, Nakayama RT. The effect of peritoneal fluid from patients with endometriosis on human sperm function *in vitro*. *Am J Obstet Gynecol* (1996) 174:1779–83. doi: 10.1016/S0002-9378(96)70210-7

200. Liu Y, Luo L, Zhao H. Changes of cytokines levels in peritoneal fluids of patients with endometriosis and its effect on reproductive activity. *J Tongji Med Univ* (2000) 20:163–5. doi: 10.1007/BF02887062

201. Mansour G, Aziz N, Sharma R, Falcone T, Goldberg J, Agarwal A. The impact of peritoneal fluid from healthy women and from women with endometriosis on sperm DNA and its relationship to the sperm deformity index. *Fertil Steril* (2009) 92:61–7. doi: 10.1016/j.fertnstert.2008.05.048

202. Said TM, Agarwal A, Falcone T, Sharma RK, Bedaiwy MA, Li L. Infliximab may reverse the toxic effects induced by tumor necrosis factor alpha in human spermatozoa: An *in vitro* model. *Fertil Steril* (2005) 83:1665–73. doi: 10.1016/j.fertnstert.2004.11.068

203. Oral E, Arici A, Olive DL, Huszar G. Peritoneal fluid from women with moderate or severe endometriosis inhibits sperm motility: The role of seminal fluid components. *Fertil Steril* (1996) 66:787–92. doi: 10.1016/s0015-0282(16)58637-3

204. Reeve L, Lashen H, Pacey AA. Endometriosis affects sperm-endosalpingeal interactions. *Hum Reprod* (2005) 20:448–51. doi: 10.1093/humrep/deh606

205. Arumugam K. Endometriosis and infertility: Raised iron concentration in the peritoneal fluid and its effect on the acrosome reaction. *Hum Reprod* (1994) 9:1153–7. doi: 10.1093/oxfordjournals.humrep.a138649

206. Benaglia L, Somigliana E, Vercellini P, Abbiati A, Ragni G, Fedele L. Endometriotic ovarian cysts negatively affect the rate of spontaneous ovulation. *Hum Reprod* (2009) 24:2183–6. doi: 10.1093/humrep/dep202

207. Leone Roberti Maggiore U, Scala C, Venturini PL, Remorgida V, Ferrero S. Endometriotic ovarian cysts do not negatively affect the rate of spontaneous ovulation. *Hum Reprod* (2015) 30:299–307. doi: 10.1093/humrep/deu308

208. Dmowski WP, Rao R, Scommegna A. The luteinized unruptured follicle syndrome and endometriosis. *Fertil Steril* (1980) 33:30–4. doi: 10.1016/s0015-0282(16)44473-0

209. Cahill DJ, Hull MG. Pituitary-ovarian dysfunction and endometriosis. *Hum Reprod Update* (2000) 6:56–66. doi: 10.1093/humupd/6.1.56

210. Schenken RS, Werlin LB, Williams RF, Prihoda TJ, Hodgen GD. Histologic and hormonal documentation of the luteinized un-ruptured follicle syndrome. *Am J Obstet Gynecol* (1986) 154(4):839–47. doi: 10.1016/0002-9378(86)90469-2

211. Moon CE, Bertero MC, Curry TE, London SN, Muse KN, Sharpe KL, et al. The presence of luteinized unruptured follicle syndrome and altered folliculogenesis in rats with surgically induced endometriosis. *Am J Obstet Gynecol* (1993) 169:676–82. doi: 10.1016/0002-9378(93)90642-v

212. Pal AK, Biswas S, Goswami SK, Kabir SN. Effect of pelvic endometrial implants on overall reproductive functions of female rats. *Biol Reprod* (1999) 60:954–8. doi: 10.1095/biolreprod60.4.954

213. Horton J, Sterrenburg M, Lane S, Maheshwari A, Li TC, Cheong Y. Reproductive, obstetric, and perinatal outcomes of women with adenomyosis and endometriosis: A systematic review and meta-analysis. *Hum Reprod Update* (2019) 25:592–632. doi: 10.1093/humupd/dmz012

214. Pellicer A, Oliveira N, Ruiz A, Remohí J, Simon C. Exploring the mechanism(s) of endometriosis-related infertility: An analysis of embryo development and implantation in assisted reproduction. *Hum Reprod* (1995) 10:91–7. doi: 10.1093/humrep/10

215. Arici A, Oral E, Bukulmez O, Duleba A, Olive DL, Jones EE. The effect of endometriosis on implantation: Results from the Yale university *in vitro* fertilization and embryo transfer program. *Fertil Steril* (1996) 65:603–7. doi: 10.1016/s0015-0282(16)58162-x

216. Miravet-Valenciano J, Ruiz-Alonso M, Gómez E, Garcia-Velasco JA. Endometrial receptivity in eutopic endometrium in patients with endometriosis: It is not affected, and let me show you why. *Fertil Steril* (2017) 108:28–31. doi: 10.1016/j.fertnstert.2017.06.002

217. Lessey BA, Kim JJ. Endometrial receptivity in the eutopic endometrium of women with endometriosis: It is affected, and let me show you why. *Fertil Steril* (2017) 108(1):19–27. doi: 10.1016/j.fertnstert.2017.05.031

218. Bishop LA, Gunn J, Jahandideh S, Devine K, Decherney AH, Hill MJ. Endo- metriosis does not impact live-birth rates in frozen embryo transfers of euploid blastocysts. *Fertil Steril* (2021) 115:416–22. doi: 10.1016/j.fertnstert.2020.07.050

219. Kamath MS, Subramanian V, Antonisamy B, Sunkara SK. Endometriosis and oocyte quality: An analysis of 13614 donor oocyte recipient and autologous IVF cycles. *Hum Reprod Open* (2022) 2022:hoac025. doi: 10.1093/hropen/hoac025

220. May KE, Villar J, Kirtley S, Kennedy SH, Becker CM. Endometrial alterations in endometriosis: A systematic review of putative biomarkers. *Hum Reprod Update* (2011) 17:637–53. doi: 10.1093/humupd/dmr013

221. Aghajanova L, Velarde MC, Giudice LC. Altered gene expression profiling in endometrium: Evidence for progesterone resistance. *Semin Reprod Med* (2010) 28:51–8. doi: 10.1055/s-0029-1242994

222. Igarashi TM, Bruner-Tran KL, Yeaman GR, Lessey BA, Edwards DP, Eisenberg E, et al. Reduced expression of progesterone receptor-b in the endometrium of women with endometriosis and in cocultures of endometrial cells exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Fertil Steril* (2005) 84:67–74. doi: 10.1016/j.fertnstert.2005.01.113

223. Park JS, Lee JH, Kim M, Chang HJ, Hwang KJ, Chang KH. Endometrium from women with endometriosis shows increased proliferation activity. *Fertil Steril* (2009) 92:1246–9. doi: 10.1016/j.fertnstert.2009.04.025

224. Fazleabas AT. Progesterone resistance in a baboon model of endometriosis. *Semin Reprod Med* (2010) 28:75–80. doi: 10.1055/s-0029-1242997

225. Jones CJ, Denton J, Fazleabas AT. Morphological and glycosylation changes associated with the endometrium and ectopic lesions in a baboon model of endometriosis. *Hum Reprod* (2006) 21:3068–80. doi: 10.1093/humrep/del310

226. Al-Sabbagh M, Lam EW, Brosens JJ. Mechanisms of endometrial progesterone resistance. *Mol Cell Endocrinol* (2012) 358:208–15. doi: 10.1016/j.mce.2011.10.035

227. Patel BG, Rudnicki M, Yu J, Shu Y, Taylor RN. Progesterone resistance in endometriosis: Origins, consequences and interventions. *Acta Obstet Gynecol Scand* (2017) 96:623–32. doi: 10.1111/aogs.13156

228. Bedaiwy MA, Dahoud W, Skomorovska-Prokvolit Y, Yi L, Liu JH, Falcone T, et al. Abundance and localization of progesterone receptor isoforms in endometrium in women with and without endometriosis and in peritoneal and ovarian endometriotic implants. *Reprod Sci* (2015) 22:1153–61. doi: 10.1177/1933719115585145

229. Wölfler MM, Küppers M, Rath W, Buck VU, Meinhold-Heerlein I, Classen-Linke I. Altered expression of progesterone receptor isoforms a and b in human eutopic endometrium in endometriosis patients. *Ann Anat* (2016) 206:1–6. doi: 10.1016/j.aanat.2016.03.004

230. Pei T, Liu C, Liu T, Xiao L, Luo B, Tan J, et al. miR-194-3p represses the progesterone receptor and decidualization in eutopic endometrium from women with endometriosis. *Endocrinology* (2018) 159:2554–62. doi: 10.1210/en.2018-00374

231. Moberg C, Bourlev V, Ilyasova N, Olovsson M. Levels of oestrogen receptor, progesterone receptor and αB-crystallin in eutopic endometrium in relation to pregnancy in women with endometriosis. *Hum Fertil (Camb)* (2015) 18:30–7. doi: 10.3109/14647273.2014.922705

232. Lessey BA, Palomino WA, Apparao KB, Young SL, Lininger RA. Estrogen receptor-alpha (ER-alpha) and defects in uterine receptivity in women. *Reprod Biol Endocrinol* (2006) 4 Suppl:S9. doi: 10.1186/1477-7827-4-S1-S9

233. Osiński M, Wirstlein P, Wender-Ożegowska E, Mikołajczyk M, Jagodziński PP, Szczepańska M. HSD3B2, HSD17B1, HSD17B2, ESR1, ESR2 and AR expression in infertile women with endometriosis. *Ginekol Pol* (2018) 89:125–34. doi: 10.5603/GP.a2018.0022

234. Coutifaris C, Myers ER, Guzick DS, Diamond MP, Carson SA, Legro RS, et al. Histological dating of timed endometrial biopsy tissue is not related to fertility status. *Fertil Steril* (2004) 82:1264–72. doi: 10.1016/j.fertnstert.2004.03.069

235. Murray MJ, Meyer WR, Zaino RJ, Lessey BA, Novotny DB, Ireland K, et al. A critical analysis of the accuracy, reproducibility, and clinical utility of histologic endometrial dating in fertile women. *Fertil Steril* (2004) 81:1333–43. doi: 10.1016/j.fertnstert.2003.11.030

236. Gonen Y, Casper RF. Prediction of implantation by the sonographic appearance of the endometrium during controlled ovarian stimulation for *in vitro* fertilization (IVF). *J In Vitro Fert Embryo Transf* (1990) 7:146–52. doi: 10.1007/BF01135678

237. Isaacs JDJr, Wells CS, Williams DB, Odem RR, Gast MJ, Strickler RC. Endometrial thickness is a valid monitoring parameter in cycles of ovulation induction with menotropins alone. *Fertil Steril* (1996) 65:262–6. doi: 10.1016/s0015-0282(16)58082-0

238. Oliveira JB, Baruffi RL, Mauri AL, Petersen CG, Borges MC, Franco JGJr. Endometrial ultrasonography as a predictor of pregnancy in an *in-vitro* fertilization programme after ovarian stimulation and gonadotrophin-releasing hormone and gonadotrophins. *Hum Reprod* (1997) 12:2515–8. doi: 10.1093/humrep/12.11.2515

239. Reuter KL, Cohen S, Furey L, Baker S. Sonographic appearance of the endometrium and ovaries during cycles stimulated with human menopausal gonadotropin. *J Reprod Med* (1996) 41:509–14.

240. Momeni M, Rahbar MH, Kovanci E. A meta-analysis of the relationship between endometrial thickness and outcome of *in vitro* fertilization cycles. *J Hum Reprod Sci* (2011) 4:130–7. doi: 10.4103/0974-1208.92287

241. Kao LC, Germeyer A, Tulac S, Lobo S, Yang JP, Taylor RN, et al. Expression profiling of endometrium from women with endometriosis reveals candidate genes for disease-based implantation failure and infertility. *Endocrinology* (2003) 144:2870–81. doi: 10.1210/en.2003-0043

242. Du H, Taylor HS. The role of hox genes in female reproductive tract development, adult function, and fertility. *Cold Spring Harb Perspect Med* (2015) 6:a023002. doi: 10.1101/cshperspect.a023002

243. Zanatta A, Rocha AM, Carvalho FM, Pereira RM, Taylor HS, Motta EL, et al. The role of the Hoxa10/HOXA10 gene in the etiology of endometriosis and its related infertility: A review. *J Assist Reprod Genet* (2010) 27:701–10. doi: 10.1007/s10815-010-9471-y

244. Gendron RL, Paradis H, Hsieh-Li HM, Lee DW, Potter SS, Markoff E. Abnormal uterine stromal and glandular function associated with maternal reproductive defects in hoxa-11 null mice. *Biol Reprod* (1997) 56:1097–105. doi: 10.1095/biolreprod56.5.1097

245. Esfandiari F, Favaedi R, Heidari-Khoei H, Chitsazian F, Yari S, Piryaei A, et al. Insight into epigenetics of human endometriosis organoids: DNA methylation analysis of HOX genes and their cofactors. *Fertil Steril* (2021) 115:125–37. doi: 10.1016/j.fertnstert.2020.08.1398

246. Kim JJ, Taylor HS, Lu Z, Ladhani O, Hastings JM, Jackson KS, et al. Altered expression of HOXA10 in endometriosis: Potential role in decidualization. *Mol Hum Reprod* (2007) 13:323–32. doi: 10.1093/molehr/gam005

247. Daftary GS, Taylor HS. EMX2 gene expression in the female reproductive tract and aberrant expression in the endometrium of patients with endometriosis. *J Clin Endocrinol Metab* (2004) 89:2390–6. doi: 10.1210/jc.2003-031389

248. Lessey BA, Castelbaum AJ, Sawin SW, Buck CA, Schinnar R, Bilker W, et al. Aberrant integrin expression in the endometrium of women with endometriosis. *J Clin Endocrinol Metab* (1994) 79:643–9. doi: 10.1210/jcem.79.2.7519194

249. Joshi NR, Kohan-Ghadr HR, Roqueiro DS, Yoo JY, Fru K, Hestermann E, et al. Genetic and epigenetic changes in the eutopic endometrium of women with endometriosis: Association with decreased endometrial αvβ3 integrin expression. *Mol Hum Reprod* (2021) 27:gaab018. doi: 10.1093/molehr/gaab018

250. Shih AJ, Adelson RP, Vashistha H, Khalili H, Nayyar A, Puran R, et al. Single-cell analysis of menstrual endometrial tissues defines phenotypes associated with endometriosis. *BMC Med* (2022) 20:315. doi: 10.1186/s12916-022-02500-3

251. Klemmt PA, Carver JG, Kennedy SH, Koninckx PR, Mardon HJ. Stromal cells from endometriotic lesions and endometrium from women with endometriosis have reduced decidualization capacity. *Fertil Steril* (2006) 85:564–72. doi: 10.1016/j.fertnstert.2005.08.046

252. Yin X, Pavone ME, Lu Z, Wei J, Kim JJ. Increased activation of the PI3K/AKT pathway compromises decidualization of stromal cells from endometriosis. *J Clin Endocrinol Metab* (2012) 97:E35–43. doi: 10.1210/jc.2011-1527

253. Osteen KG, Bruner-Tran KL, Eisenberg E. Reduced progesterone action during endometrial maturation: A potential risk factor for the development of endometriosis. *Fertil Steril* (2005) 83:529–37. doi: 10.1016/j.fertnstert.2004.11.026

254. Ahn JI, Yoo JY, Kim TH, Kim YI, Ferguson SD, Fazleabas AT, et al. cAMP-response element–binding 3–like protein 1 (CREB3L1) is required for decidualization and its expression is decreased in women with endometriosis. *Curr Mol Med* (2016) 16:276–87. doi: 10.2174/1566524016666160225153659

255. Aghajanova L, Hamilton A, Kwintkiewicz J, Vo KC, Giudice LC. Steroidogenic enzyme and key decidualization marker dysregulation in endometrial stromal cells from women with versus without endometriosis. *Biol Reprod* (2008) 80:105–14. doi: 10.1095/biolreprod.108.070300

256. Su RW, Strug MR, Joshi NR, Jeong JW, Miele L, Lessey BA, et al. Decreased notch pathway signaling in the endometrium of women with endometriosis impairs decidualization. *J Clin Endocrinol Metab* (2015) 100:E433–42. doi: 10.1210/jc.2014-3720

257. Campbell RA, Bhat-Nakshatri P, Patel NM, Constantinidou D, Ali S, Nakshatri H. Phosphatidylinositol 3-kinase/AKT–mediated activation of es- trogen receptor alpha: A new model for antiestrogen resistance. *J Biol Chem* (2001) 276:9817–24. doi: 10.1074/jbc.M010840200

258. Sanchez M, Sauve K, Picard N, Tremblay A. The hormonal response of estrogen receptor beta is decreased by the phosphatidylinositol 3-kinase/Akt pathway *via* a phosphorylation-dependent release of CREB-binding protein. *J Biol Chem* (2007) 282:4830–40. doi: 10.1074/jbc.M607908200

259. Vallvé-Juanico J, Houshdaran S, Giudice LC. The endometrial immune environment of women with endometriosis. *Hum Reprod Update* (2019) 25:564–91. doi: 10.1093/humupd/dmz018

260. Berbic M, Schulke L, Markham R, Tokushige N, Russell P, Fraser IS. Macrophage expression in endometrium of women with and without endometriosis. *Hum Reprod* (2009) 24:325–32. doi: 10.1093/humrep/den393

261. Khan KN, Masuzaki H, Fujishita A, Kitajima M, Sekine I, Ishimaru T. Differential macrophage infiltration in early and advanced endometriosis and adjacent peritoneum. *Fertil Steril* (2004) 81:652–61. doi: 10.1016/j.fertnstert.2003.07.037

262. Takebayashi A, Kimura F, Kishi Y, Ishida M, Takahashi A, Yamanaka A, et al. Subpopulations of macrophages within eutopic endometrium of endometriosis patients. *Am J Reprod Immunol* (2015) 73:221–31. doi: 10.1111/aji.12331

263. Giuliani E, Parkin KL, Lessey BA, Young SL, Fazleabas AT. Characterization of uterine NK cells in women with infertility or recurrent pregnancy loss and associated endometriosis. *Am J Reprod Immunol* (2014) 72:262–9. doi: 10.1111/aji.1225

264. Odukoya O, Bansal A, Cooke I. Serum endometrial IgG antibodies and soluble CD23 concentrations in patients with endometriosis. *Acta Obstet Gynecol Scand* (1996) 75:927–31. doi: 10.3109/00016349609055030

265. Hey-Cunningham AJ, Riaz A, Fromm PD, Kupresanin F, Markham R, McGuire HM. Circulating and endometrial regulatory T cell and related populations in endometriosis and infertility: Endometriosis is associated with blunting of endometrial cyclical effects and reduced proportions in moderate-severe disease. *Reprod Sci* (2022) 29:229–42. doi: 10.1007/s43032-021-00658-4

266. Capezzuoli T, Vannuccini S, Fantappie F, Orlandi G, Rizzello F, Coccia ME, et al. Ultrasound findings in infertile & women with endometriosis: Evidence of concomitant uterine disorders. *Gynecol Endocrinol* (2020) 36:808–812. doi: 10.1080/09513590.2020.1736027

267. Kunz G, Beil D, Huppert P, Noe M, Kissler S, Leyendecker G. Adenomyosis in endometriosis–prevalence and impact on fertility. Evidence from magnetic resonance imaging. *Hum Reprod* (2005) 20:2309–16. doi: 10.1093/humrep/dei021

268. Barrier BF, Malinowski MJ, Dick EJ Jr, Hubbard GB, Bates GW. Adenomyosis in the baboon is associated with primary infertility. *Fertil Steril;* (2004) 82:1091–4. doi: 10.1016/j.fertnstert.2003.11.065

269. Campo S, Campo V, Benagiano G. Adenomyosis and infertility. *Reprod BioMed Online* (2012) 24(1):35–46. doi: 10.1016/j.rbmo.2011.10.003

270. Benagiano G, Brosens I, Habiba M. Structural and molecular features of the endomyometrium in endometriosis and adenomyosis. *Hum Reprod Update* (2014) 20(3):386–402. doi: 10.1093/humupd/dmt052

271. Fischer CP, Kayisili U, Taylor HS. HOXA10 expression is decreased in endometrium of women with adenomyosis. *Fertil Steril* (2011) 95(3):1133–6. doi: 10.1016/j.fertnstert.2010.09.060

272. Nirgianakis K, Kalaitzopoulos DR, Schwartz ASK, Spaanderman M, Kramer BW, Mueller MD, et al. Fertility, pregnancy and neonatal outcomes of patients with adenomyosis: A systematic review and meta-analysis. *Reprod BioMed Online* (2021) 42(1):185–206. doi: 10.1016/j.rbmo.2020.09.023

273. Liang T, Zhang W, Pan N, Han B, Li R, Ma C. Reproductive outcomes of *In vitro* fertilization and fresh embryo transfer in infertile women with adenomyosis: A retrospective cohort study. *Front Endocrinol (Lausanne)* (2022) 13:865358. doi: 10.3389/fendo.2022.865358

274. Bazot M, Darai E. Role of transvaginal sonography and magnetic resonance imaging in the diagnosis of uterine adenomyosis. *Fertil Steril* (2018) 109:389–97. doi: 10.1016/j.fertnstert.2018.01.024

275. Klatsky PC, Tran ND, Caughey AB, Fujimoto VY. Fibroids and reproductive outcomes: A systematic literature review from conception to delivery. *Am J Obstet Gynecol* (2008) 198:357–66. doi: 10.1016/j.ajog.2007.12.039

276. Pritts EA, Parker WH, Olive DL. Fibroids and infertility: An updated system- atic review of the evidence. *Fertil Steril* (2009) 91:1215–23. doi: 10.1016/j.fertnstert.2008.01.051

277. Hirsch M, Begum MR, Paniz É, Barker C, Davis CJ, Duffy J. Diagnosis and management of endometriosis: A systematic review of international and national guidelines. *BJOG* (2018) 125:556–64. doi: 10.1111/1471-0528.14838

278. Practice Committee of the American Society for Reproductive Medicine. Endometriosis and infertility: A committee opinion. *Fertil Steril* (2012) 98:591–8. doi: 10.1016/j.fertnstert.2012.05.031

279. Becker CM, Bokor A, Heikinheimo O, Horne A, Jansen F, Kiesel L, et al. ESHRE guideline: Endometriosis. *Hum Reprod Open* (2022) 2:hoac009. doi: 10.1093/hropen/hoac009

280. Adamson GD, Pasta DJ. Endometriosis fertility index: The new, validated endometriosis staging system. *Fertil Steril* (2010) 94:1609–15. doi: 10.1016/j.fertnstert.2009.09.035

281. Vesali S, Razavi M, Rezaeinejad M, Maleki-Hajiagha A, Maroufizadeh S, Sepidarkish M. Endometriosis fertility index for predicting non-assisted reproductive technology pregnancy after endometriosis surgery: A systematic review and meta-analysis. *BJOG* (2020) 127:800–9. doi: 10.1111/1471-0528.16107

282. Tomassetti C, Bafort C, Meuleman C, Welkenhuysen M, Fieuws S, D'Hooghe T. Reproducibility of the endometriosis fertility index: A prospective inter-/intra-rater agreement study. *BJOG* (2020) 127:107–14. doi: 10.1111/1471-0528.15880

283. Practice Committee of the American Society for Reproductive Medicine and Practice Committee of the American Society for Reproductive Medicine. Fertility evaluation of infertile women: A committee opinion. *Fertil Steril* (2021) 116:1255–65. doi: 10.1016/j.fertnstert.2021.08.038

284. Kliman HJ, Honig S, Walls D, Luna M, McSweet JC, Copperman AB. Optimization of endometrial preparation results in a normal endometrial function test (EFT) and good reproductive outcome in donor ovum recipients. *J Assist Reprod Genet* (2006) 23:299–303. doi: 10.1007/s10815-006-9061-1

285. Díaz-Gimeno P, Horcajadas JA, Martínez-Conejero JA, Esteban FJ, Alama P, Pellicer A, et al. A genomic diagnostic tool for human endometrial receptivity based on the transcriptomic signature. *Fertil Steril* (2011) 95:50–60. doi: 10.1016/j.fertnstert.2010.04.063

286. Díaz-Gimeno P, Ruiz-Alonso M, Blesa D, Bosch N, Martínez-Conejero JA, Alamá P, et al. The accuracy and reproducibility of the endometrial receptivity array is superior to histology as a diagnostic method for endometrial receptivity. *Fertil Steril* (2013) 99:508–17. doi: 10.1016/j.fertnstert.2012.09.046

287. Nezhat C, Rambhatla A, Miranda-Silva C, Asiaii A, Nguyen K, Eyvazzadeh A, et al. BCL-6 overexpression as a predictor for endometriosis in patients undergoing *In vitro* fertilization. *JSLS* (2020) 24:e2020.00064. doi: 10.4293/JSLS.2020.00064

288. Almquist LD, Likes CE, Stone B, Brown KR, Savaris R, Forstein DA, et al. Endometrial BCL6 testing for the prediction of in vitro fertilization outcomes: A cohort study. *Fertil Steril* (2017) 108:1063–9. doi: 10.1016/j.fertnstert.2017.09.017

289. Evans-Hoeker E, Lessey BA, Jeong JW, Savaris RF, Palomino WA, Yuan L, et al. Endometrial BCL6 overexpression in eutopic endometrium of women with endometriosis. *Reprod Sci* (2016) 23:1234–41. doi: 10.1177/1933719116649711

290. Sansone AM, Hisrich BV, Young RB, Abel WF, Bowens Z, Blair BB, et al. Evaluation of BCL6 and SIRT1 as non-invasive diagnostic markers of endometriosis. *Curr Issues Mol Biol* (2021) 43:1350–60. doi: 10.3390/cimb43030096

291. Muyldermans M, Cornillie FJ, Koninckx PR. CA125 and endometriosis. *Hum Reprod Update* (1995) 1:173–87. doi: 10.1093/humupd/1.2.173

292. Barbieri RL, Niloff JM, Bast RC Jr, Scaetzl E, Kistner RW, Knapp RC. Elevated serum concentrations of CA-125 in patients with advanced endometriosis. *Fertil Steril* (1986) 45:630–4. doi: 10.1016/s0015-0282(16)49333-7

293. Hirsch M, Duffy J, Davis CJ, Nieves Plana M, Khan KS. Diagnostic accuracy of cancer antigen 125 for endometriosis: A systematic review and meta-analysis. *BJOG* (2016) 123:1761–8. doi: 10.1111/1471-0528.14055

294. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* (2007) 9:654–9. doi: 10.1038/ncb1596

295. Schmittgen TD, Lee EJ, Jiang J, Sarkar A, Yang L, Elton TS, et al. Real-time PCR quantification of precursor and mature microRNA. *Methods* (2008) 44:31–8. doi: 10.1016/j.ymeth.2007.09.006

296. 't Hoen PA, Ariyurek Y, Thygesen HH, Vreugdenhil E, Vossen RH, de Menezes RX, et al. Deep sequencing-based expression analysis shows major advances in robustness, resolution and inter-lab portability over five microarray platforms. *Nucleic Acids Res* (2008) 36:e141. doi: 10.1093/nar/gkn705

297. Cho S, Mutlu L, Grechukhina O, Taylor HS. Circulating microRNAs as potential biomarkers for endometriosis. *Fertil Steril* (2015) 103:1252–60.e1. doi: 10.1016/j.fertnstert.2015.02.013

298. Suryawanshi S, Vlad AM, Lin HM, Mantia-Smaldone G, Laskey R, Lee M, et al. Plasma microRNAs as novel biomarkers for endometriosis and endometriosis-associated ovarian cancer. *Clin Cancer Res* (2013) 19:1213–24. doi: 10.1158/1078-0432.CCR-12-2726

299. Jia SZ, Yang Y, Lang J, Sun P, Leng J. Plasma miR-17-5p, miR-20a and miR-22 are down-regulated in women with endometriosis. *Hum Reprod* (2013) 28:322–30. doi: 10.1093/humrep/des413

300. Moustafa S, Burn M, Mamillapalli R, Nematian S, Flores V, Taylor HS. Accurate diagnosis of endometriosis using serum microRNAs. *Am J Obstet Gynecol* (2020) 223:557.e1–557.e11. doi: 10.1016/j.ajog.2020.02.050

301. Wang L, Huang W, Ren C, Zhao M, Jiang X, Fang X, et al. Analysis of serum microRNA profile by solexa sequencing in women with endometriosis. *Reprod Sci* (2016) 23:1359–70. doi: 10.1177/1933719116641761

302. Cosar E, Mamillapalli R, Ersoy GS, Cho S, Seifer B, Taylor HS. Serum microRNAs as diagnostic markers of endometriosis: A comprehensive array-based analysis. *Fertil Steril* (2016) 106:402–9. doi: 10.1016/j.fertnstert.2016.04.013

303. Rekker K, Saare M, Roost AM, Kaart T, Sõritsa D, Karro H, et al. Circulating miR-200-family micro-RNAs have altered plasma levels in patients with endometriosis and vary with blood collection time. *Fertil Steril* (2015) 104:938–46.e2. doi: 10.1016/j.fertnstert.2015.06.029

304. Hsu CY, Hsieh TH, Tsai CF, Tsai HP, Chen HS, Chang Y, et al. miRNA-199a-5p regulates VEGFA in endometrial mesenchymal stem cells and contributes to the pathogenesis of endometriosis. *J Pathol* (2014) 232:330–43. doi: 10.1002/path.4295

305. Wang WT, Zhao YN, Han BW, Hong SJ, Chen YQ. Circulating microRNAs identified in a genome-wide serum microRNA expression analysis as noninvasive biomarkers for endometriosis. *J Clin Endocrinol Metab* (2013) 98:281–289. doi: 10.1210/jc.2012-2415

306. Misir S, Hepokur C, Oksasoglu B, Yildiz C, Yanik A, Aliyazicioglu Y. Circulating serum miR-200c and miR-34a-5p as diagnostic biomarkers for endometriosis. *J Gynecol Obstet Hum Reprod* (2021) 50:102092. doi: 10.1016/j.jogoh.2021.102092

307. Hossein Razi M, Eftekhar M, Ghasemi N, Hasan Sheikhha M, Dehghani Firoozabadi A. Expression levels of circulatory mir-185-5p, vascular endothelial growth factor, and platelet-derived growth factor target genes in endometriosis. *Int J Reprod BioMed* (2020) 18:347–58. doi: 10.18502/ijrm.v13i5.7155

308. Papari E, Noruzinia M, Kashani L, Foster WG. Identification of candidate microRNA markers of endometriosis with the use of next-generation sequencing and quantitative real-time polymerase chain reaction. *Fertil Steril* (2020) 113:1232–41. doi: 10.1016/j.fertnstert.2020.01.026

309. Pateisky P, Pils D, Szabo L, Kuessel L, Husslein H, Schmitz A, et al. Hsa-miRNA-154-5p expression in plasma of endometriosis patients is a potential diagnostic marker for the disease. *Reprod BioMed Online* (2018) 37:449–66. doi: 10.1016/j.rbmo.2018.05.007

310. Bashti O, Noruzinia M, Garshasbi M, Abtahi M. MiR-31 and miR-145 as potential non-invasive regulatory biomarkers in patients with endometriosis. *Cell J* (2018) 20:84–9. doi: 10.22074/cellj.2018.4915

311. Nothnick WB, Falcone T, Joshi N, Fazleabas AT, Graham A. Serum miR-451a levels are significantly elevated in women with endometriosis and recapitulated in baboons (Papio anubis) with experimentally-induced disease. *Reprod Sci* (2017) 24:1195–202. doi: 10.1177/1933719116681519

312. Nisenblat V, Sharkey DJ, Wang Z, Evans SF, Healey M, Ohlsson Teague EMC, et al. Plasma miRNAs display limited potential as diagnostic tools for endometriosis. *J Clin Endocrinol Metab* (2019) 104:1999–2022. doi: 10.1210/jc.2018-01464

313. Agrawal S, Tapmeier T, Rahmioglu N, Kirtley S, Zondervan K, Becker C. The miRNA mirage: How close are we to finding a non-invasive diagnostic biomarker in endometriosis? A systematic review. *Int J Mol Sci* (2018) 19:599. doi: 10.3390/ijms19020599

314. Leonova A, Turpin VE, Agarwal SK, Leonardi M, Foster WG. A critical appraisal of the circulating levels of differentially expressed microRNA in endometriosis. *Biol Reprod* (2021) 105:1075–85. doi: 10.1093/biolre/ioab134

315. Seifer BJ, Su D, Taylor HS. Circulating miRNAs in murine experimental endometriosis. *Reprod Sci* (2017) 24:376–81. doi: 10.1177/1933719116667228

316. Hodgson RM, Lee HL, Wang R, Mol BW, Johnson N. Interventions for endometriosis-related infertility: A systematic review and network meta-analysis. *Fertil Steril* (2020) 113:374–382.e2. doi: 10.1016/j.fertnstert.2019.09.031

317. Jin X, Ruiz Beguerie J. Laparoscopic surgery for subfertility related to endometriosis: A meta-analysis. *Taiwan J Obstet Gynecol* (2014) 53:303–8. doi: 10.1016/j.tjog.2013.02.004

318. Somigliana E, Infantino M, Candiani M, Vignali M, Chiodini A, Busacca M, et al. Association rate between deep peritoneal endometriosis and other forms of the disease: Pathogenetic implications. *Hum Reprod* (2004) 19:168–71. doi: 10.1093/humrep/deg513

319. Meuleman C, Tomassetti C, D'Hoore A, Van Cleynenbreugel B, Penninckx F, Vergote I, et al. Surgical treatment of deeply infiltrating endometriosis with colorectal involvement. *Hum Reprod Update* (2011) 17:311–26. doi: 10.1093/humupd/dmq057

320. Vercellini P, Somigliana E, Viganò P, Abbiati A, Barbara G, Crosignani PG. Surgery for endometriosis-associated infertility: A pragmatic approach. *Hum Reprod* (2009) 24:254–69. doi: 10.1093/humrep/den379

321. Vercellini P, Barbara G, Buggio L, Frattaruolo MP, Somigliana E, Fedele L. Effect of patient selection on estimate of reproductive success after surgery for rectovaginal endometriosis: literature review. *Reprod BioMed Online* (2012) 24:389–95. doi: 10.1016/j.rbmo.2012.01.003

322. Iversen ML, Seyer-Hansen M, Forman A. Does surgery for deep infiltrating bowel endometriosis improve fertility? A systematic review. *Acta Obstet Gynecol Scand* (2017) 96:688–93. doi: 10.1111/aogs.13152

323. Casals G, Carrera M, Domínguez JA, Abrão MS, Carmona F. Impact of surgery for deep infiltrative endometriosis before *In vitro* fertilization: A systematic review and meta-analysis. *J Minim Invasive Gynecol* (2021) 28:1303–12.e5. doi: 10.1016/j.jmig.2021.02.007

324. Somigliana E, Garcia-Velasco JA. Treatment of infertility associated with deep endometriosis: Definition of therapeutic balances. *Fertil Steril* (2015) 104:764–70. doi: 10.1016/j.fertnstert.2015.08.003

325. Douay-Hauser N, Yazbeck C, Walker F, Luton D, Madelenat P, Koskas M. Infertile women with deep and intraperitoneal endometriosis: Comparison of fertility outcome according to the extent of surgery. *J Minim Invasive Gynecol* (2011) 18:622–8. doi: 10.1016/j.jmig.2011.06.004

326. Alborzi S, Zahiri Sorouri Z, Askari E, Poordast T, Chamanara K. The success of various endometrioma treatments in infertility: A systematic review and meta-analysis of prospective studies. *Reprod Med Biol* (2019) 18:312–22. doi: 10.1002/rmb2.12286

327. Tummon IS, Asher LJ, Martin JS, Tulandi T. Randomized controlled trial of superovulation and insemination for infertility associated with minimal or mild endometriosis. *Fertil Steril* (1997) 68:8–12. doi: 10.1016/s0015-0282(97)81467-7

328. Werbrouck E, Spiessens C, Meuleman C, D'Hooghe T. No difference in cycle pregnancy rate and in cumulative live-birth rate between women with surgically treated minimal to mild endometriosis and women with unexplained infertility after controlled ovarian hyperstimulation and intrauterine insemination. *Fertil Steril* (2006) 86:566–71. doi: 10.1016/j.fertnstert.2006.01.044

329. Vassilopoulou L, Matalliotakis M, Zervou MI, Matalliotaki C, Spandidos DA, Matalliotakis I. Endometriosis and *in vitro* fertilisation. *Exp Ther Med* (2018) 16:1043–51. doi: 10.3892/etm.2018.6307

330. Pabuccu R, Onalan G, Kaya C. GnRH agonist and antagonist protocols for stage I-II endometriosis and endometrioma in *in vitro* fertilization/intracytoplasmic sperm injection cycles. *Fertil Steril* (2007) 88:832–9. doi: 10.1016/j.fertnstert.2006.12.046

331. Hughes E, Brown J, Collins JJ, Farquhar C, Fedorkow DM, Vandekerckhove P. Ovulation suppression for endometriosis. *Cochrane Database Syst Rev* (2007) 2007:CD000155. doi: 10.1002/14651858.CD000155.pub2

332. Chen I, Veth VB, Choudhry AJ, Murji A, Zakhari A, Black AY, et al. Pre- and postsurgical medical therapy for endometriosis surgery. *Cochrane Database Syst Rev* (2020) 11:Cd003678. doi: 10.1002/14651858.CD003678.pub3

333. Georgiou EX, Melo P, Baker PE, Sallam HN, Arici A, Garcia-Velasco JA, et al. Long-term GnRH agonist therapy before in vitro fertilisation (IVF) for improving fertility outcomes in women with endometriosis. *Cochrane Database Syst Rev* (2019) 2019:CD013240. doi: 10.1002/14651858.CD013240.pub2

334. Cao X, Chang HY, Xu JY, Zheng Y, Xiang YG, Xiao B, et al. The effectiveness of different down-regulating protocols on *in vitro* fertilization-embryo transfer in endometriosis: A meta-analysis. *Reprod Biol Endocrinol* (2020) 18:16. doi: 10.1186/s12958-020-00571-6

335. Tomassetti C, Beukeleirs T, Conforti A, Debrock S, Peeraer K, Meuleman C, et al. The ultra-long study: A randomized controlled trial evaluating long-term GnRH downregulation prior to ART in women with endometriosis. *Hum Reprod* (2021) 36:2676–86. doi: 10.1093/humrep/deab163

336. Kaponis A, Chatzopoulos G, Paschopoulos M, Georgiou I, Paraskevaidis V, Zikopoulos K, et al. Ultralong administration of gonadotropin-releasing hormone agonists before *in vitro* fertilization improves fertilization rate but not clinical pregnancy rate in women with mild endometriosis: A prospective, randomized, controlled trial. *Fertil Steril* (2020) 113:828–35. doi: 10.1016/j.fertnstert.2019.12.018

337. Taylor H, Li HJ, Carson S, Flores V, Pal L, Robbins J, et al. Pre-IVF treatment with a GnRH antagonist in women with endometriosis (PREGNANT): Study protocol for a prospective, double-blind, placebo-controlled trial. *BMJ Open* (2022) 12(6):e052043. doi: 10.1136/bmjopen-2021-052043

338. Tamura H, Yoshida H, Kikuchi H, Josaki M, Mihara Y, Shirafuta Y, et al. The clinical outcome of dienogest treatment followed by *in vitro* fertilization and embryo transfer in infertile women with endometriosis. *J Ovarian Res* (2019) 12:123. doi: 10.1186/s13048-019-0597-y

339. Guo H, Li J, Shen X, Cong Y, Wang Y, Wu L, et al. Efficacy of different progestins in women with advanced endometriosis undergoing controlled ovarian hyperstimulation for *in vitro* fertilization-a single-center non-inferiority randomized controlled trial. *Front Endocrinol (Lausanne)* (2020) 11:129. doi: 10.3389/fendo.2020.00129

340. Corachán A, Pellicer N, Pellicer A, Ferrero H. Novel therapeutic targets to improve IVF outcomes in endometriosis patients: A review and future prospects. *Hum Reprod Update* (2021) 27:923–72. doi: 10.1093/humupd/dmab014

341. Grammatis AL, Georgiou EX, Becker CM. Pentoxifylline for the treatment of endometriosis-associated pain and infertility. *Cochrane Database Syst Rev* (2021) 8:CD007677. doi: 10.1002/14651858

342. Taylor HS. Emerging therapies for endometriosis. *Fertil Steril* (2021) 115:317–8. doi: 10.1016/j.fertnstert.2020.11.005

343. Adams D, Gonzalez-Duarte A, O'Riordan WD, Yang CC, Ueda M, Kristen AV, et al. Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis. *N Engl J Med* (2018) 379:11–21. doi: 10.1056/NEJMoa1716153

344. Balwani M, Sardh E, Ventura P, Peiró PA, Rees DC, Stölzel U, et al. Phase 3 trial of RNAi therapeutic givosiran for acute intermittent porphyria. *N Engl J Med* (2020) 382:2289–301. doi: 10.1056/NEJMoa1913147

345. Petracco R, Grechukhina O, Popkhadze S, Massasa E, Zhou Y, Taylor HS. MicroRNA 135 regulates HOXA10 expression in endometriosis. *J Clin Endocrinol Metab* (2011) 96:E1925–33. doi: 10.1210/jc.2011-1231

346. Joshi NR, Su RW, Chandramouli GV, Khoo SK, Jeong JW, Young SL, et al. Altered expression of microRNA-451 in eutopic endometrium of baboons (Papio anubis) with endometriosis. *Hum Reprod* (2015) 30:2881–91. doi: 10.1093/humrep/dev229

347. Gao S, Liu S, Gao ZM, Deng P, Wang DB. Reduced microRNA-451 expression in eutopic endometrium contributes to the pathogenesis of endometriosis. *World J Clin cases* (2019) 7:2155–64. doi: 10.12998/wjcc.v7.i16.2155

348. Ghazal S, McKinnon B, Zhou J, Mueller M, Men Y, Yang L, et al. H19 lncRNA alters stromal cell growth *via* IGF signaling in the endometrium of women with endometriosis. *EMBO Mol Med* (2015) 7:996–1003. doi: 10.15252/emmm.201505245

349. Altmäe S, Martinez-Conejero JA, Esteban FJ, Ruiz-Alonso M, Stavreus-Evers A, Horcajadas JA, et al. MicroRNAs miR-30b, miR-30d, and miR-494 regulate human endometrial receptivity. *Reprod Sci* (2013) 20:308–17. doi: 10.1177/1933719112453507

350. Joshi NR, Miyadahira EH, Afshar Y, Jeong JW, Young SL, Lessey BA, et al. Progesterone resistance in endometriosis is modulated by the altered expression of MicroRNA-29c and FKBP4. *J Clin Endocrinol Metab* (2017) 102:141–9. doi: 10.1210/jc.2016-2076

351. Zhou M, Fu J, Xiao L, Yang S, Song Y, Zhang X, et al. miR-196a overexpression activates the MEK/ERK signal and represses the progesterone receptor and decidualization in eutopic endometrium from women with endometriosis. *Hum Reprod* (2016) 31:2598–608. doi: 10.1093/humrep/dew223

352. Arosh JA, Lee J, Balasubbramanian D, Stanley JA, Long CR, Meagher MW, et al. Molecular and preclinical basis to inhibit PGE2 receptors EP2 and EP4 as a novel nonsteroidal therapy for endometriosis. *Proc Natl Acad Sci U.S.A.* (2015) 112:9716–21. doi: 10.1073/pnas.1507931112

353. Simoni M, Taylor HS. Therapeutic strategies involving uterine stem cells in reproductive medicine. *Curr Opin Obstet Gynecol* (2018) 30:209–16. doi: 10.1097/GCO.0000000000000457

354. Nagori CB, Panchal SY, Patel H. Endometrial regeneration using autologous adult stem cells followed by conception by *in vitro* fertilization in a patient of severe asherman's syndrome. *J Hum Reprod Sci* (2011) 4:43–8. doi: 10.4103/0974-1208.82360

355. Alawadhi F, Du H, Cakmak H, Taylor HS. Bone marrow-derived stem cell (BMDSC) transplantation improves fertility in a murine model of asherman's syndrome. *PloS One* (2014) 9:e96662. doi: 10.1371/journal.pone.0096662

356. Santamaria X, Cabanillas S, Cervelló I, Arbona C, Raga F, Ferro J, et al. Autologous cell therapy with CD133+ bone marrow-derived stem cells for refractory asherman's syndrome and endometrial atrophy: A pilot cohort study. *Hum Reprod* (2016) 31:1087–96. doi: 10.1093/humrep/dew042

357. Moridi I, Mamillapalli R, Cosar E, Ersoy GS, Taylor HS. Bone marrow stem cell chemotactic & activity is induced by elevated CXCl12 in endometriosis. *Reprod Sci* (2017) 24:526–533. doi: 10.1177/1933719116672587

358. Wang X, Mamillapalli R, Mutlu L, Du H, Taylor HS. Chemoattraction of bone marrow- derived stem cells towards human endometrial stromal cells is mediated by estradiol regulated CXCL12 and CXCR4 expression. *Stem Cell Res* (2015) 15:14–22. doi: 10.1016/j.scr.2015.04.004

359. Sakr S, Naqvi H, Komm B, Taylor HS. Endometriosis impairs bone marrow-derived stem cell recruitment to the uterus whereas bazedoxifene treatment leads to endometriosis regression and improved uterine stem cell engraftment. *Endocrinology* (2014) 155:1489–97. doi: 10.1210/en.2013-1977

360. Naqvi H, Sakr S, Presti T, Krikun G, Komm B, Taylor HS. Treatment with bazedoxifene and conjugated estrogens results in regression of endome- triosis in a murine model. *Biol Reprod* (2014) 90:121. doi: 10.1095/biolreprod.113.114165

361. Liu Y, Tal R, Pluchino N, Mamillapalli R, Taylor HS. Systemic administration of bone marrow-derived cells leads to better uterine engraftment than use of uterine-derived cells or local injection. *J Cell Mol Med* (2018) 22:67–76. doi: 10.1111/jcmm.13294

362. Practice Committee of the American Society for Reproductive Medicine. Electronic address: Asrm@asrm.org. Fertility preservation in patients undergoing gonadotoxic therapy or gonadectomy: A committee opinion. *Fertil Steril* (2019) 112:1022–33. doi: 10.1016/j.fertnstert.2019.09.013

363. Potdar N, Gelbaya TA, Nardo LG. Oocyte vitrification in the 21st century and post-warming fertility outcomes: A systematic review and meta-analysis. *Reprod BioMed Online* (2014) 29:159–76. doi: 10.1016/j.rbmo.2014.03.024

364. Rienzi L, Gracia C, Maggiulli R, LaBarbera AR, Kaser DJ, Ubaldi FM, et al. Oocyte, embryo and blastocyst cryopreservation in ART: systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. *Hum Reprod Update* (2017) 23:139–55. doi: 10.1093/humupd/dmw038

365. Calagna G, Della Corte L, Giampaolino P, Maranto M, Perino A. Endometriosis and strategies of fertility preservation: A systematic review of the literature. *Eur J Obstet Gynecol Reprod Biol* (2020) 254:218–25. doi: 10.1016/j.ejogrb.2020.09.045

366. Somigliana E, Vigano P, Filippi F, Papaleo E, Benaglia L, Candiani M, et al. Fertility preservation in women with endometriosis: For all, for some, for none? *Hum Reprod* (2015) 30:1280–6. doi: 10.1093/humrep/dev078

367. Streuli I, Benard J, Hugon-Rodin J, Chapron C, Santulli P, Pluchino N. Shed- ding light on the fertility preservation debate in women with endometriosis: A SWOT analysis. *Eur J Obstet Gynecol Reprod Biol* (2018) 229:172–8. doi: 10.1016/j.ejogrb.2018.08.577

368. Cobo A, García-Velasco JA, Remohí J, Pellicer A. Oocyte vitrification for fertility preservation for both medical and nonmedical reasons. *Fertil Steril* (2021) 115:1091–101. doi: 10.1016/j.fertnstert.2021.02.006

369. Oktay K, Karlikaya G. Ovarian function after transplantation of frozen, banked autologous ovarian tissue. *N Engl J Med* (2000) 342:1919. doi: 10.1056/NEJM200006223422516

370. Oktay K, Oktem O. Ovarian cryopreservation and transplantation for fertility preservation for medical indications: Report of an ongoing experience. *Fertil Steril* (2010) 93:762–8. doi: 10.1016/j.fertnstert.2008.10.006

371. Donnez J, Squifflet J, Dolmans MM, Martinez-Madrid B, Jadoul P, Van Langendonckt A. Orthotopic transplantation of fresh ovarian cortex: A report of two cases. *Fertil Steril* (2005) 84:1018. doi: 10.1016/j.fertnstert.2005.06.011

372. Elizur SE, Chian RC, Holzer HE, Gidoni Y, Tulandi T, Tan SL. Cryopreservation of oocytes in a young woman with severe and symptomatic endometriosis: A new indication for fertility preservation. *Fertil Steril* (2009) 91:293.e1–3. doi: 10.1016/j.fertnstert.2007.06.040

373. Garcia-Velasco JA, Domingo J, Cobo A, Martínez M, Carmona L, Pellicer A. Five years' experience using oocyte vitrification to preserve fertility for medical and nonmedical indications. *Fertil Steril* (2013) 99:1994–9. doi: 10.1016/j.fertnstert.2013.02.004

374. Raad J, Sonigo C, Tran C, Sifer C, Durnerin IC, Grynberg M. Oocyte vitrification for preserving fertility in patients with endometriosis: First observational cohort study… and many unresolved questions. *Letter to Editor Eur J Obstet Gynecol Reprod Biol* (2018) 220:140–1. doi: 10.1016/j.ejogrb.2017.12.001

375. Kuroda K, Ikemoto Y, Ochiai A, Ozaki R, Matsumura Y, Nojiri S, et al. Combination treatment of preoperative embryo cryopreservation and endoscopic surgery (Surgery-ART hybrid therapy) in infertile women with diminished ovarian reserve and uterine myomas or ovarian endometriomas. *J Minim Invasive Gynecol* (2019) 26:1369–75. doi: 10.1016/j.jmig.2019.02.008

376. Cobo A, Giles J, Paolelli S, Pellicer A, Remohí J, García-Velasco JA. Oocyte vitrification for fertility preservation in women with endometriosis: An observational study. *Fertil Steril* (2020) 113:836–44. doi: 10.1016/j.fertnstert.2019.11.017

377. Kim SJ, Kim SK, Lee JR, Suh CS, Kim SH. Oocyte cryopreservation for fertility preservation in women with ovarian endometriosis. *Reprod BioMed Online* (2020) 40:827–34. doi: 10.1016/j.rbmo.2020.01.028

378. Hong YH, Lee HK, Kim SK, Lee JR, Suh CS. The significance of planned fertility preservation for women with endometrioma before an expected ovarian cystectomy. *Front Endocrinol (Lausanne)* (2021) 12:794117. doi: 10.3389/fendo.2021.794117

379. Hart RJ, Hickey M, Maouris P, Buckett W. Excisional surgery versus ablative surgery for ovarian endometriomata. *Cochrane Database Syst Rev* (2008) 2008:CD004992. doi: 10.1002/14651858.CD004992.pub3

380. Dan H, Limin F. Laparoscopic ovarian cystectomy versus fenestration/coagulation or laser vaporization for the treatment of endometriomas: A meta-analysis of randomized controlled trials. *Gynecol Obstet Invest* (2013) 76:75–82. doi: 10.1159/000351165

381. Candiani M, Ottolina J, Posadzka E, Ferrari S, Castellano LM, Tandoi I, et al. Assessment of ovarian reserve after cystectomy versus "one-step" laser vaporization in the treatment of ovarian endometrioma: A small randomized clinical trial. *Hum Reprod* (2018) 33:2205–11. doi: 10.1093/humrep/dey305.

382. Rius M, Gracia M, Ros C, Martínez-Zamora MÁ, deGuirior C, Quintas L, et al. Impact of endometrioma surgery on ovarian reserve: A prospective, randomized, pilot study comparing stripping with CO_2 laser vaporization in patients with bilateral endometriomas. *J Int Med Res* (2020) 48:300060520927627. doi: 10.1177/0300060520927627

383. Shaltout MF, Elsheikhah A, Maged AM, Elsherbini MM, Zaki SS, Dahab S, et al. A randomized controlled trial of a new technique for laparoscopic management of ovarian endometriosis preventing recurrence and keeping ovarian reserve. *J Ovarian Res* (2019) 20:12:66. doi: 10.1186/s13048-019-0542-0

384. Sweed MS, Makled AK, El-Sayed MA, Shawky ME, Abd-Elhady HA, Mansour AM, et al. Ovarian reserve following laparoscopic ovarian cystectomy vs cyst deroofing for endometriomas. *J Minim Invasive Gynecol* (2019) 26:877–82. doi: 10.1016/j.jmig.2018.06.022

385. Muzii L, Achilli C, Bergamini V, Candiani M, Garavaglia E, Lazzeri L, et al. Comparison between the stripping technique and the combined excisional/ablative technique for the treatment of bilateral ovarian endometriomas: A multicentre RCT. *Hum Reprod* (2016) 31:339–44. doi: 10.1093/humrep/dev31

386. Araujo RSDC, Maia SB, Baracat CMF, Fernandes CQBA, Ribeiro HSAA, Ribeiro PAAG. Ovarian function following use of various hemostatic techniques during treatment for unilateral endometrioma: A randomized controlled trial. *Int J Gynaecol Obstet* (2021) 20:410. doi: 10.1002/ijgo.13912

387. Pedroso J, Gutierrez M, Volker KW. Comparative thermal effects of J-plasma, monopolar, argon and laser electrosurgery in a porcine tissue model. *J Minim Invasive Gynecol* (2014) 21:S59. doi: 10.1016/j.jmig.2014.08.210

388. Tsolakidis D, Pados G, Vavilis D, Athanatos D, Tsalikis T, Giannakou A, et al. The impact on ovarian reserve after laparoscopic ovarian cystectomy versus three-stage management in patients with endometriomas: A prospective randomized study. *Fertil Steril* (2010) 94:71–7. doi: 10.1016/j.fertnstert.2009.01.138

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Overexpression of hypoxia-inducible factor 1α and excessive vascularization in the peri-implantation endometrium of infertile women with chronic endometritis

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Objective: Chronic endometritis (CE) contributes to impaired endometrial receptivity and is closely associated with poor *in vitro* fertilization (IVF) outcomes. However, the mechanisms underlying CE are unclear. Here, we investigated the role of the hypoxic microenvironment and endometrial vascularization in the peri-implantation endometrium of infertile women with CE.

Methods: This retrospective study involved 15 fertile women and 77 infertile patients diagnosed with CE based on CD138+ ≥1/10 high-power fields (HPFs). The CE patients were divided into Group 1 (CD138+ 1–4/10 HPFs, 53 cases) and Group 2 (CD138+ ≥5/10 HPFs, 24 cases). The expression levels of hypoxia-inducible factor 1α (HIF1α), vascular endothelial growth factor A (VEGFA), and vascular endothelial growth factor receptor 2 (VEGFR2) in peri-implantation endometrium were assessed by qRT-PCR and western blot analyses. Spatial levels of HIF1α, VEGFA, and VEGFR2 in various endometrial compartments was determined using immunohistochemistry and *H*-score analysis. Microvascular density (MVD) was determined using CD34 staining and scored using Image J. Finally, we used qRT-PCR to assess changes in the expression of HIF1α, VEGFA, and VEGFR2 in CE patients after treatment with first-line antibiotics.

Result(s): Relative to Group 1 and control group, during the implantation window, protein and mRNA levels of HIF1α, VEGFA, and VEGFR2 were markedly high in Group 2 ($P<0.05$). *H*-score analysis showed that HIF1α, VEGFA, and VEGFR2 in the luminal, glandular epithelium, and stromal compartments were markedly elevated in Group 2, comparing to control group and Group 1 ($P<0.05$). Moreover, markedly elevated MVD levels were observed in Group 2. Notably, the above indexes did not differ significantly in

the control group versus Group 1. Treatment with antibiotics significantly suppressed the endometrial HIF1α and VEGFA levels in CE-cured patients.

Conclusion(s): Here, we for the first time report the upregulation of HIF1α, VEGFA, and VEGFR2, as well as excessive endometrial vascularization in the peri-implantation endometrium of CE patients. Our findings offer new insights into reduced endometrial receptivity in CE-associated infertility.

KEYWORDS

chronic endometritis, *in vitro* fertilization, hypoxia-inducible factor 1α, VEGFA, infertility, endometrial receptivity, endometrial vascularization

Introduction

Chronic endometritis (CE) refers to the continuous and subtle infections of the endometrial mucosa (1), which are mainly thought to be caused by various bacteria, such as *Escherichia coli, Staphylococcus* spp., *Mycoplasma, Chlamydia, Streptococcus* spp., *and Ureaplasma*. However, CE etiology remains unclear (2). Currently, the gold standard for CE diagnosis is fluid hysteroscopy combined with endometrial biopsy and histopathological identification based on immunostaining for syndecan-1 (CD138), a plasma cell marker (3–5). However, consensus on the cutoff values for identifying clinical CE is lacking.

Mounting evidence indicates that CE adversely affects reproductive health and pregnancy outcomes after *in vitro* fertilization and embryonic transfer (IVF-ET), such as repeated implantation failure (RIF) (6–8), recurrent miscarriage (RM) (9–11), and preterm labor (12). Several studies have reported that treatment with oral antibiotics significantly improves IVF outcomes in women with CE (10, 13–15). Recent findings indicate that CE influences the level and population of immunocompetent cells that infiltrate the endometrium (7, 16, 17). Additionally, CE dysregulates the expressions of various endometrial receptivity-associated genes in proliferative and mid-luteal phases (18, 19). CE also adversely affects endometrial receptivity by negatively impacting normal endometrial decidualization by modifying the expression of sex steroid receptors (20) and shifting cytokine milieu towards proinflammatory immune responses by impairing autophagy (21). Recent studies involving endometrial receptivity analysis (ERA) have reported that CE modifies the individual window of implantation (WOI), leading to embryo–endometrial asynchrony (22). But, the pathomechanisms by which CE exerts its negative effects on implantation and pregnancy, especially endometrial angiogenesis dysregulation, are unclear.

During the peri-implantation period (cycle days 20–24), harmonious endometrial angiogenesis is critical for the supply of oxygen and nutrients for cell growth and successful embryo implantation (23). During this time, the endometrial microenvironment has low oxygen levels and normal oxygen homeostasis is vital for early development of the trophoblast (24, 25). Hypoxia-inducible factor-1α (HIF1α) is key in adaptation of cell to hypoxia and is expressed exclusively during the menstrual and secretory phases (26). HIF1α mediates inflammatory response and vasculogenesis by activating the transcription of angiogenic genes (27). We have previously reported that overweight PCOS is associated with significantly reduced HIF1α expressions in mid–secretory phases of the human endometrium, which may be associated with reduced endometrial receptivity (28). Chen, X., et al. reported that HIF −1α levels in endometrial luminal epithelium and stroma in women with recurring miscarriage are high, relative to fertile controls. However, the role of HIF1α in CE pathogenesis is unknown.

Vascular endothelial growth factor A (VEGFA), a HIF1α target, is the main stimulator of angiogenesis and normal VEGFA levels are necessary for endometrial receptivity (29). To exert its effects, VEGFA binds the three tyrosine kinase receptors, VEGFR-1, -2, and -3 (29). VEGFR2, which exhibits the highest proangiogenic activity, is mainly expressed in luminal epithelial as well as in endometrial glandular cells, and in myometrial smooth muscle cells (30). Additionally, CD34 is a widely used immunohistochemical indicator of microvascular density (MVD: number of vessel profiles/mm^2) in endometrial sections (31, 32). Endometrial vascularity has also been measured by quantifying the above indexes in multiple situations, such as recurrent miscarriage and recurrent implantation failure (23, 32, 33). Notably, the role of angiogenesis and vascular distribution at the time of embryo implantation in CE patients is unclear.

To improve our understanding of CE and uncover potential therapeutic strategies, a systemic investigation of endometrial oxygen homeostasis and vascular pathology is required. To the best of our knowledge, our study is the first to investigate the

expression of HIF1α, VEGFA, and VEGFR2, as well as endometrial vascular features during the precisely timed implantation window in CE patients versus fertile women without CE.

Materials and methods

Subjects

Ethical approval for this retrospective case control study was granted by the ethical committee of Yantai Yuhuangding Hospital Affiliated to Qingdao University. Study participants were required to sign written informed consents after receiving details on the study during their first appointment. A total of 77 infertile women with CE diagnosed using hysteroscopy, histological identification of plasma cells and CD138 immunohistochemistry were involved into the study. These participants had been trying to conceive for ≥1 year and were referred to the reproduction medical center of Yantai Yuhuangding Hospital Affiliated to Qingdao University between July 2019 and February 2022 with unexplained infertility. During the same period, 15 healthy age-matched women who had conceived naturally, had ≥1 live births, and had no history of spontaneous early pregnancy loss, were recruited as the control group during clinic visits for physical examination or contraception counseling. Other inclusion criteria were, a) age of 22–38 years, b) regular menstrual cycles (26–33 days), c) no history of endometriosis or adenomyosis, d) no autoimmune or thrombotic disease, e) no previous tuberculosis diagnosis, and f) no immunotherapy, steroid hormone treatment, or antibiotic management during the preceding three months.

Hysteroscopy and endometrial biopsy

To detect luteinization hormone (LH) surge, from day 9 of the menstrual cycle, all participants underwent a urine dipstick test daily. Hysteroscopy using a forward-oblique 30° hysteroscope (Karl Storz GmbH &Co KG, Tuttlingen, Germany; sheath diameter: 5mm) for diagnosis was done during the "implantation window" (5–7 days after ultrasound-confirmed ovulation) by experienced senior physicians and the uterine cavity examined thoroughly. Macroscopic signs of CE included the manifestation of diffuse or focal endometrial hyperemia, micropolyps (diameter <1 mm) and stromal edema (34) (Figure 1). Thickness of the endometrium was measured using transvaginal Doppler ultrasound. On the hysteroscopy day, blood samples were obtained for analysis of serum progesterone (P_4) and estradiol (E_2) levels. Instantly after hysteroscopy, all women were subjected to endometrial biopsy by inserting the tip of Pipelle sampler (Prodimed) into uterine fundus and moving it back and forth 4 times, each with a 90 degree rotation. Endometrial samples were rinsed thrice using sterile phosphate buffered-saline (PBS) and each sample separated into 2 aliquots, one for histological and immunohistochemistry assaying, another for our Biospecimen Bank which was stored at -80°C until use.

Histology and Immunohistochemistry for CE diagnosis

Endometrial samples were fixed in neutral formaldehyde solution and later embedded in paraffin for histological analysis. The micro-sections were stained with hematoxylin and eosin. The histological examinations were performed by the single operator who was unaware of the hysteroscopic findings. Histological

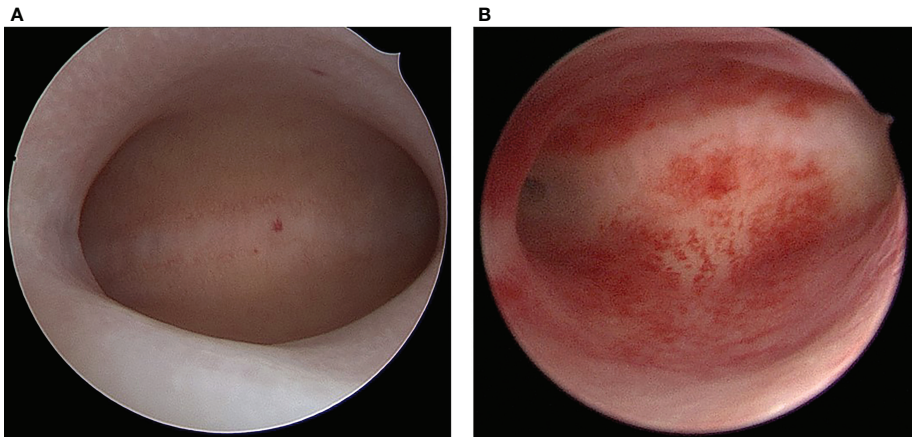

FIGURE 1
Hysteroscopic finding of normal endometrium **(A)** and chronic endometritis **(B)**.

diagnosis of CE was following: superficial stromal edema, increased stromal density, pleomorphic stromal inflammatory infiltrate

dominated by lymphocytes, and plasma cells. Sections (5 µm) were cut and incubated with a 1:250 dilution of mouse anti-CD138 antibody (Gene Tech, clone B-A38 Shanghai, China).

We included only women with signs of CE at hysteroscopy whose diagnosis was confirmed by histology and immunohistochemistry for CD138.

CE diagnosis was based on microscopic observation of ≥1 CD138+ plasma cell/10 randomly selected high power fields (HPFs) in the stromal layer described before (1). However, the clinical significance of CD138+ 1–4/10 HPFs is controversial. A recent study found no significant difference in pregnancy outcomes in the CD138+ 1–4/10 HPFs group versus the control group (35). Another study found that the clinical pregnancy rate in patients with CD138+ ≥5/10 HPFs was markedly lower relative to patients with CD138+ 0–4/10 HPFs (64.6% vs. 44.74%; P=0.01) (5). However, no studies have examined the mechanisms underlying the effects of CE on pregnancy outcomes in patients with various levels of CD138+ plasma cells. Here, CE patients were classified into Group 1 (CD138+ 1–4/10 HPFs) and Group 2 (CD138+ ≥5/10 HPFs).

Real time quantitative PCR

Isolation of total RNA from endometrial tissues was performed by a Trizol reagent (TaKaRa, Dalian, China). NanoDrop 2000 (Thermo Scientific-USA) was used to assess RNA purity. The HiScript III RT SuperMix (Vazyme Biotech Co., Ltd, Nangjing, China) was used for cDNA synthesis. ChamQ universal SYBR qPCR master mix (Vazyme Biotech Co.,Ltd, Nangjing, China) was used for real time quantitative PCR. The primers used in this study were: HIF1α: F-5'-atctcatccaagaagccctaac-3', R-5'-gatcgtctggctgctgtaata-3'; VEGFA: F-5'-atcgagtacatcttcaagccat-3', R-5'-gtgaggtttgatcc gcataatc-3'; VEGFR2: F-5'-ggagcttaagaatgcatccttg-3', R-5'-gatgctttccccaatacttgtc-3'; GAPDH (F-5'-ccgcctggagaaacc tgccaag-3', R-5'- caccaccctgttgctgtagccg-3') (Sangon Biotech Co. Ltd., Shanghai, China). Relative gene expressions were determined by the $2^{-\Delta\Delta Ct}$ approach with GAPDH as reference gene. Assays were performed in triplicates.

Western blot analysis

Endometrial tissues were lysed using RIPA buffer (Sparkjade, Jinan, China) and protein concentration evaluated by a BCA assay kit (Thermo Fisher Scientific). Equal concentrations of proteins were then separated on 10% SDS-PAGE gels, transferred onto polyvinylidene difluoride fluoride (PVDF) membrane (Sparkjade, Jinan, China) that were then blocked for 1 h using 5% skimmed milk in Tris-buffered saline with 0.05% at room temperature (RT). Thereafter, overnight incubation of membranes was done with rabbit primary antibodies against HIF1α, VEGFA, and VEGFR2 (all from Sangon Biotech, Shanghai, China) at 4°C. They were then incubated for 2 h with HRP-conjugated secondary antibodies at RT and signal developed by an ECL kit (Thermo Fisher Scientific). GAPDH was used as loading control. Image J (NIH, MD, USA) was used to measure band intensities.

Immunohistochemistry staining

Immunohistochemical staining was done on 4-µm thick endometrial tissue sections fixed in formaldehyde and paraffin–embedded as previously reported (28). Briefly, xylene was used to deparaffinize the tissue sections while graded ethanol series was used to rehydrate them. Endogenous peroxidase activities were blocked using hydrogen peroxide (3%). To block non-specific binding, sections were incubated in 5% bovine serum albumin at RT. Next, incubation of the sections was done overnight with primary polyclonal rabbit antibodies against HIF1α, VEGFA, and VEGFR2 (all from Sangon Biotech, Shanghai, China) at 4°C. Then, they were incubated with conjugated anti-rabbit IgG secondary antibodies (Sangon Biotech, Shanghai, China) for 1 h at RT. Sections were then counterstained with Harris' hematoxylin. For negative staining control samples, the primary antibodies were excluded. Sections were assessed by light microscopy (Leica) at a ×20 magnification. CD34 immunohistochemistry analysis using an anti-CD34 monoclonal antibody (Sangon Biotech, Shanghai, China, 1:100) was used to detect endothelial cells.

Histochemical score

The levels of HIF1α, VEGFA, and VEGFR2 in endometrial sections was graded based on H-score as: H score $=\sum P_i\ (i+1)$. Whereby i is staining intensity (1, 2 and 3 respectively denote weak, moderate and strong) while P_i is the % of stained cells at every intensity (0%–100%). H scores were separately determined in stroma, luminal epithelium and glandular epithelium. Sections were independently scored by two examiners and any discrepancies between them, resolved through reexamination and consensus. Final HIF1α, VEGFA, and VEGFR2 staining scores were obtained from the mean score of three tests on each endometrial compartment (glandular epithelium, stroma, and luminal epithelium).

Microvessel counting

CD34 staining was independently assessed under a light microscope (Leica; magnification: ×200, 0.754 mm^2 per field) by

three independent examiners. The 1st field to be imaged was selected randomly while guaranteeing that it had a luminal epithelial border. Successive fields were acquired by moving one field to the right or left of original fields, keeping luminal epithelial borders in view to standardize endometrial area depth for counting micro-vessels. This process was repeated to capture 5 fields. Every brown-stained cell cluster or cell that had been clearly detached from neighboring microvessels and other connective tissue elements was taken as one countable vessel. Endometrial stroma area was measured in the same field in which blood vessel counts was determined and blood vessel counts presented per mm^2 of the stroma. An observer-related mean was determined for every histologic section with the mean of the three observer-related means reported as final MVD for every sample.

Statistical analysis

Statistical analyses were done on GraphPad Prism 8. Comparisons of normally distributed continuous variables was done by one-way ANOVA followed by Tukey's *post hoc* test. The non-parametric Kruskal–Wallis test with Dunn's multiple comparisons test was used to compare non-normally distributed data. Categorical variables were compared using Kruskal–Wallis χ^2 test. Effects of antibiotics on HIF1α, VEGFA, and VEGFR2 expressions before and after treatment were compared using paired Student *t* test. $p<0.05$ indicated significant differences.

Results

Demographics

The participants demographic features are summarized on Table 1. The study group exhibited relatively high homogeneity. Age, body mass index (BMI), and menstrual cycle length did not differ markedly across all groups. Levels of E_2 and P_4, and endometrial thickness at the midluteal phase did not differ across the three groups.

The mRNA and protein expressions of HIF–1α, VEGFA, and VEGFR2 during implantation

Compared with the control group and Group 1, the mRNA levels of HIF1α, VEGFA, and VEGFR2 were markedly high in Group 2 ($P<0.05$) but did not differ significantly between Group 1 and control group ($P>0.05$, Figures 2A–C). Similarly, western blot analysis showed that relative to control group and group 1, protein levels of HIF1α, VEGFA, and VEGFR2 were markedly higher in Group 2 ($P<0.05$), but did not differ between group 1 and control group ($P>0.05$, Figures 2D–G).

Spatial levels of HIF1α, VEGFA, and VEGFR2 in peri-implantation endometrium

Immunohistochemical analysis revealed positive HIF1α, VEGFA, and VEGFR2 staining in all endometrial compartments in women with CE, as well as the fertile controls. HIF1α, VEGFA, and VEGFR2 staining was most intense in glandular epithelium, with the three groups exhibiting similar staining patterns (Figure 3). *H*-score analysis revealed that when compared with the $CD138^-$ group and CD138+ 1–4/HPF group, the intensities of HIF1α, VEGFA, and VEGFR2 staining in luminal epithelium, glandular epithelium, and stroma were markedly higher in women with CD138+ ≥5/HPF ($P<0.05$). However, the *H*-scores for HIF1α, VEGFA, and VEGFR2 staining did not differ markedly between $CD138^-$ and CD138+ 1–4/HPF groups (Table 2).

TABLE 1 Demographic characteristics in women with CE or fertile controls.

	Control group (n=15)	Group 1 (n=53)	Group 2 (n=24)	*P* value
Age (y)	34.91 ± 1.67	36.51 ± 2.59	35.83 ± 2.31	0.291
BMI (Kg/m^2)	22.32 ± 2.32	22.42 ± 1.54	21.92 ± 1.99	0.574
Menstrual cycle length (d)	28.10 ± 1.44	29.30 ± 1.52	30.20 ± 2.02	0.883
Endometrial thickness (mm)	8.93 ± 1.12	8.99 ± 1.54	8.87 ± 0.99	0.991
E_2 (pg/mL)	209.66 ± 19.54	198.32 ± 16.97	188.11 ± 22.32	0.337
P_4 (ng/mL)	8.77 ± 1.02	9.38 ± 2.03	8.99 ± 0.98	0.363
Infertility				
Primarily (n/%)	0	29	13	0.871[a]
Secondarily (n/%)	0	24	11	

Data are expressed as mean ± SD or n.
[a]Differences between groups were compared by χ^2 test.
BMI, body mass index; E_2, Estradiol; P_4, Progesterone.

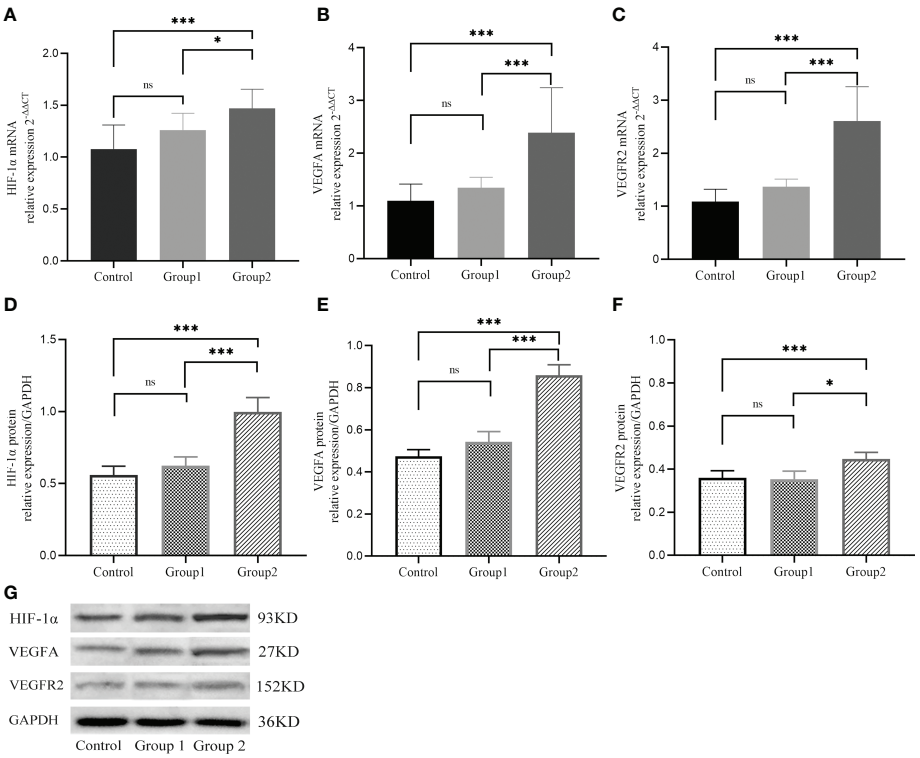

FIGURE 2
The mRNA **(A-C)** and protein **(D-G)** expressions of HIF–1α, VEGFA, and VEGFR2 during implantation in three groups. *, P < 0.05; ***, P < 0.01; ns, P > 0.05.

Microvascular density (MVD)

Brown-staining for CD34 indicated endothelial cells or endothelial cell clusters in the endometrial stromal layer, which was considered to be a single, countable vessel. Image J analysis showed that the density of CD34-positive cells in Group 2 was higher than in control group and Group 1. However, the density of CD34-positive cells did not markedly differ between Group 1 and control group (P>0.05, Figures 4A-C). The CD138+ ≥5/HPF group exhibited the highest MVD values (Figure 4D), indicating that inflammation level influences endometrial angiogenesis.

Changes of HIF–1α, VEGFA and VEGFR2 in CE patients after antibiotics therapy

CE patients were orally treated with the first-line antibiotics, metronidazole (400 mg, once daily for 14 days) and doxycycline (100 mg, twice daily). Following the first course of antibiotics, 65 of 74 participants (87.8%) who underwent a second endometrial biopsy (in the next "implantation window" phase) were found to be recovered, as revealed by the absence of stromal plasma cells. The nine (12.2%) patients who still presented with CD138+/10 HPFs were categorized as having persistent CE. RT-qPCR analyzed the impact of antibiotics on the expression of HIF1α, VEGFA, and VEGFR2 in CE patients, and showed that levels of HIF1α and VEGFA were markedly reduced in patients who recovered from CE when compared with paired patients before treatment (P<0.001 and P=0.011, respectively, Figures 5A, B). Nevertheless, levels of HIF1α and VEGFA did not differ markedly between patients with persistent CE (P=0.41 and P=0.49, respectively, Figure 5D, E). VEGFR2 levels did not differ considerably prior to and after antibiotic treatment (P=0.16 and P=0.21, respectively, Figures 5C, F).

Discussion

Although CE is well known to adversely affect endometrial receptivity, the underlying mechanisms are poorly understood. Appropriate angiogenesis is considered to be an important marker of endometrial receptivity. Furthermore, HIF1α, an indicator of oxygen homeostasis, regulates the endometrial microenvironment and drives the transcription of angiogenic and metabolic genes. This is the first study to explore the effects of CE on HIF1α expression and endometrial vascularity.

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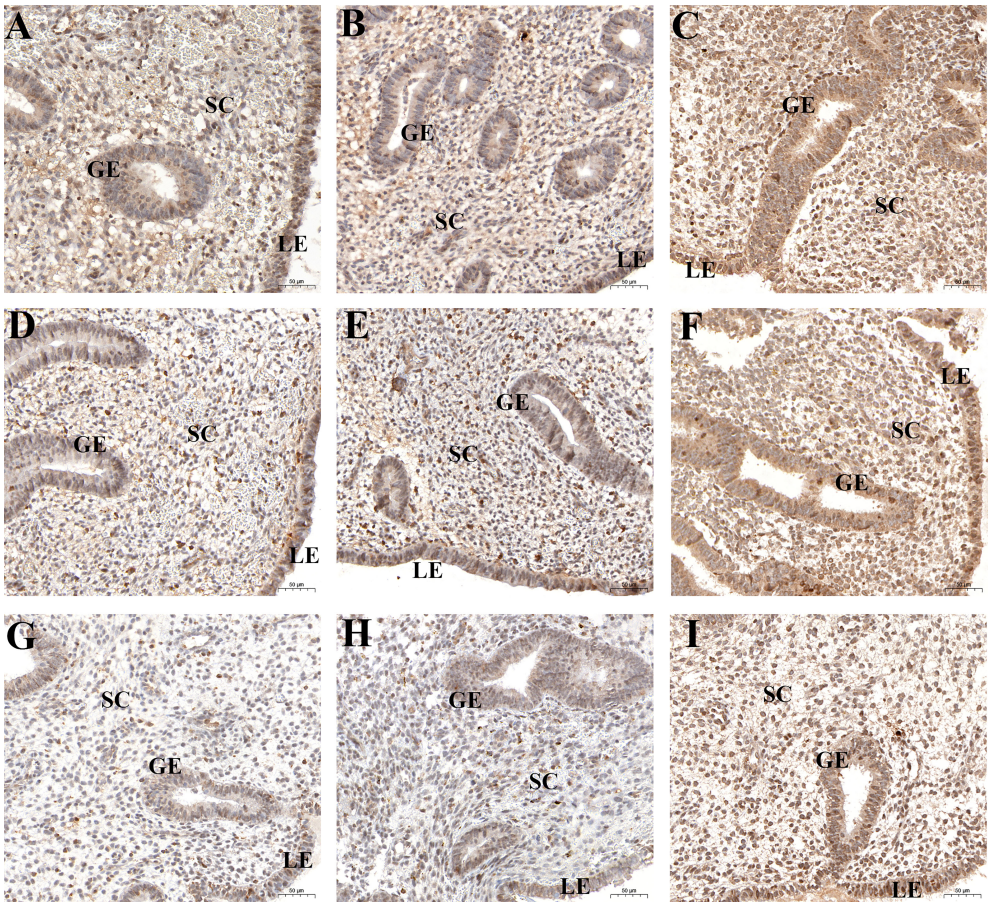

FIGURE 3
Spatial levels of HIF1α **(A-C)**, VEGFA **(D-F)**, and VEGFR2 **(G-I)** in peri-implantation endometrium from the fertile controls **(A, D, G)**, CD138+ 1–4/HPF group **(B, E, H)**, and CD138+ ≥5/HPF group **(C, F, I)**. Original magnification, ×200. Scale bar = 50μm. GE: glandular epithelium; LE: luminal epithelium; SC: stromal cell.

TABLE 2 The *H*-scores for HIF-1α, VEGFA and VEGFR2 in peri-implantation endometrium from women with CE or fertile controls.

				P value		
	Control group (n=15)	Group 1 (n=53)	Group 2 (n=24)	Control*Vs* Group 1	Group 1*Vs*Group 2	Control*Vs*Group 2
Luminal epithelium						
HIF-1α	41 (29-122)	48 (33-129)	107 (61-278)	0.521	0.012	0.005
VEGFA	55 (36-131)	53 (37-136)	111 (55-262)	0.613	0.024	0.011
VEGFR2	24 (15-82)	22 (29-92)	94 (18-190)	0.732	0.197	0.037
Glandular epithelium						
HIF-1α	167 (103-302)	173 (99-306)	247 (114-339)	0.651	0.032	0.028
VEGFA	162 (107-305)	179 (104-302)	263 (109-344)	0.729	0.044	0.036
VEGFR2	101 (59-207)	108 (86-218)	214 (93-302)	0.596	0.038	0.014
Stroma						
HIF-1α	36 (22-99)	38 (19-110)	72 (18-130)	0.577	0.049	0.042
VEGFA	43 (29-114)	49 (31-126)	83 (20-138)	0.693	0.044	0.022
VEGFR2	19 (17-97)	21 (18-92)	84 (58-164)	0.805	0.031	0.012

Data are expressed as median (range). Groups were compared by non-parametric Kruskal-Wallis test with Dunn's multiple comparisons test.

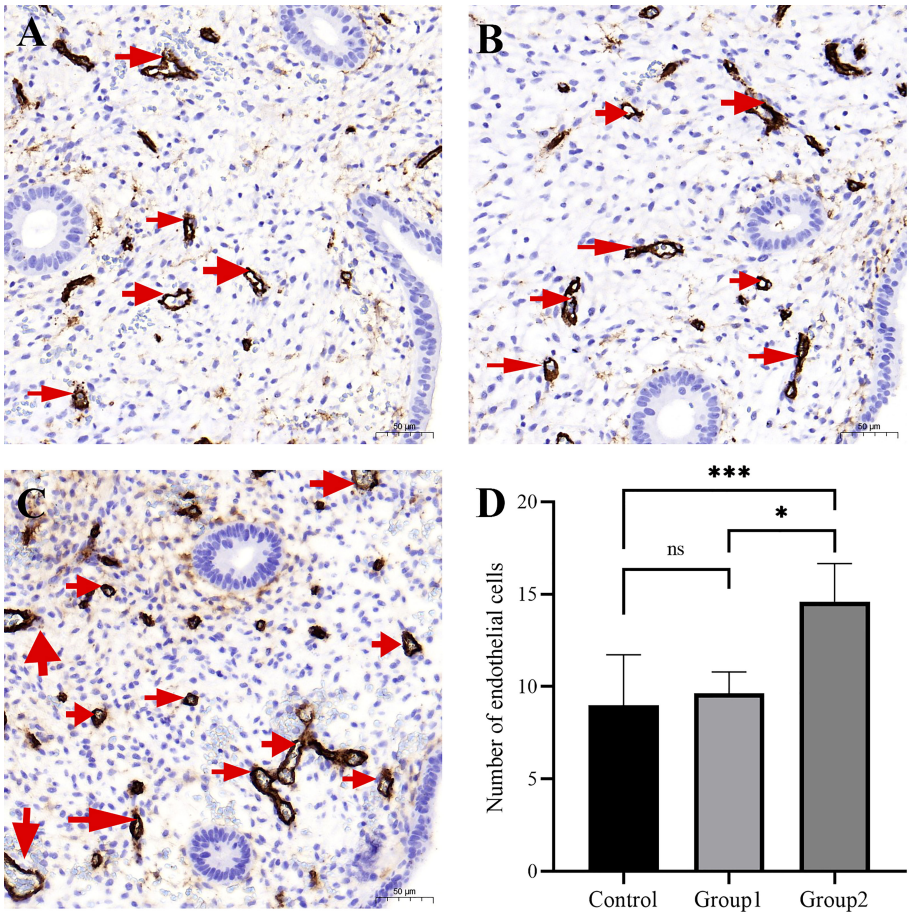

FIGURE 4
The micro-blood vessels in endometrial biopsies were determined by CD34−stained sections. Representative images of blood vessels localized in endometrium (arrows) from the fertile controls **(A)**, CD138+ 1–4/10 HPFs group **(B)**, and CD138+ ≥5/10 HPFs group **(C)** are shown. Original magnification, ×200. Scale bar = 50μm. The CD138+ ≥5/10 HPFs group exhibited the highest MVD values **(D)**. *, P < 0.05; ***, P < 0.01; ns, P > 0.05.

Here, we find that endometrial HIF1α mRNA and protein levels were markedly elevated in infertile CE patients with CD138 ≥5/10 HPFs, but did not differ significantly between normal fertile women without CE and CE patients with CD138 1–4/10 HPFs. This observation is consistent with findings from other CE-associated disorders like endometriosis (36) and recurrent miscarriage. In endometriosis, Zhang et al. reported significantly higher HIF1α levels in the eutopic endometrium when compared with the normal endometrium (37). Another study observed in women with recurrent miscarriage, endometrial HIF1α protein levels increased significantly around the time of embryo implantation (38). The endometrium has complex oxygen requirements that change after implantation. Prior to implantation, dissolved oxygen concentrations are relatively low on endometrium surfaces, relative to inner portions of the endometrium and myometrium (23). Oxygen concentration diminishes with distance from blood vessels (39). Furthermore, before implantation, development of the embryo occurs in hypoxic environments (25). Thus, hypoxic environments of pre-implantation endometrium are appropriate for human embryogenesis. However, after implantation and angiogenesis, endometrial oxygen levels rises significantly (40), leading to an aerobic environment with adequate oxygen levels for embryonic development. Thus, the initial hypoxic endometrial microenvironment changes over time, which results in dysregulated physiological functions. Based on our findings and previous reports, we hypothesize that in CE patients, endometrial oxygen levels remain low after embryonic implantation, resulting in a hypoxic microenvironment without sufficient oxygen for normal embryonic development, and which negatively affects endometrial receptivity for the embryo. However, the exact mechanisms by which CE contributes

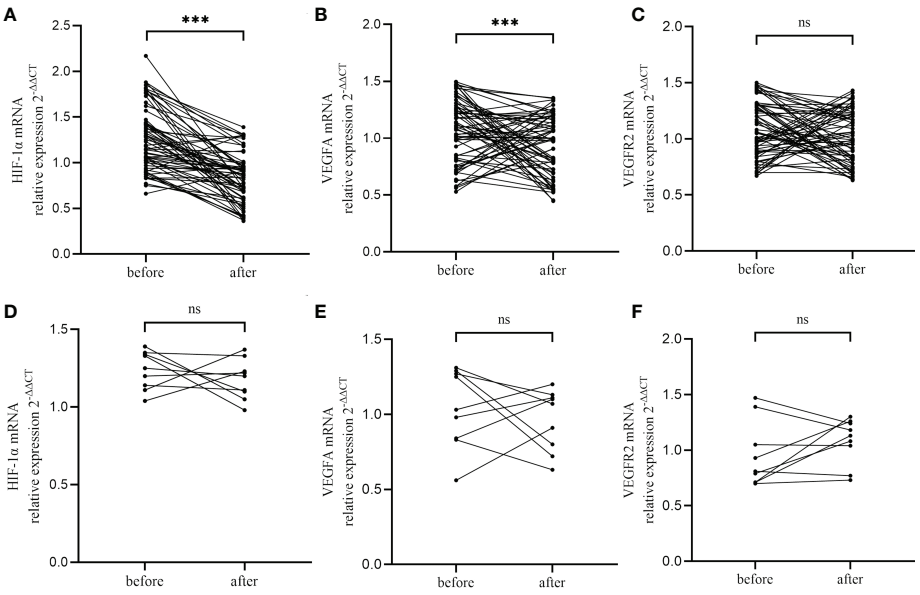

FIGURE 5
Changes of HIF–1α **(A, D)**, VEGFA **(B, E)** and VEGFR2 **(C, F)** in CE patients after antibiotics therapy, including CE-cured patients **(A-C)** and CE-persistent patients **(D-F)**. ***, P < 0.01; ns, P > 0.05.

to a persistently hypoxic microenvironment requires further investigation.

Our findings also revealed that relative to women without CE and CE patients with CD138 1–4/10 HPFs, CE patients with CD138 ≥5/10 HPFs had significantly higher levels of VEGFA and its receptor, VEGFR2. Consistent with our findings, Cicinelli, E. et al. found that when compared with patients without CE, VEGFA mRNA levels were markedly elevated in proliferative endometria of CE patients. However, in contrast with our study, using high-throughput RT PCR, Cicinelli, E. et al. did not establish marked differences in endometrial VEGFA mRNA levels during the implantation window in CE patients versus controls. The discrepancy may be because of differences in CE definition based on fluid hysteroscopy and histology, small sample size, and ethnic differences in the study by Cicinelli, E. et al. Our study, involving a relatively large sample size and various experimental techniques, found that VEGFA and its main receptor, VEGFR2, were markedly upregulated in CE patients, implying that CE may be associated with excessive endometrial angiogenesis. Moreover, IHC analysis confirmed that CE patients have superfluous endometrial vascular density (CD34-positive cells), which in turn, impairs endometrial receptivity. Based on ERA testing, a recent study reported that the individual WOI of 62.5% (10/16 cases) CE patients might be brought forward by the CE (22). Taken together, these findings indicate that the upregulation of VEGFA and VEGFR2, and increased endometrial MVD in CE patients may cause embryo–endometrial asynchrony.

We noted a significant increase in levels of endometrial HIF1α, VEGFA, and VEGFR2 in all compartments, including the luminal epithelium, glandular epithelium, and endometrial stroma in women with CD138 ≥5/10 HPFs. Elevated levels of plasma cells in endometrial tissue are correlated with a marked increase in expressions of master regulators of oxygen homeostasis and vasculogenesis, HIF–1α, VEGFA and VEGFR2. It is known that successful implantation, placentation, and gestation need synchronized vascular development and adaptation in every endometrial compartment. Thus, we postulated that persistent chronic inflammation may cause endometrial luminal, stromal cells, and glands to undergo holistic changes that disrupt normal endometrial physiology, thereby impairing reproductive capacity in CE patients. Additionally, CE may often be asymptomatic, or it may be accompanied by irregular vaginal bleeding and/or increased leukorrhea. Moreover, endometrial hyperemia, the presence of diffuse micropolyps (diameter <1mm), or polypoid endometria are frequently observed in CE patients during hysteroscopy. Therefore, the prevalence of known spatial HIF1α, VEGFA, and VEGFR2 overexpression may explain the impaired endometrial receptivity associated with CE and support the hypothesis that CE is pathologically involved in the development of various endometrial disorders, including abnormal bleeding and proliferative diseases like hyperemia, micropolyps, and hyperplastic lesions.

Currently, standardized guidelines for CE diagnosis are lacking. Particularly, consensus on the minimum number of stromal plasma cells indicative of clinical CE is lacking. Different reproductive medical centers use different criteria to determine the density of

plasmacytes. Some studies have applied the stringent criterion of >5 CD138+ cells in at least 1 of 3 section levels in endometrial samples (5, 17). However, most studies apply ≥1 CD138+ cells in 1 microscopic HPF as the diagnostic criterion (9, 36, 41, 42). Studies have not determined if variations in CD138+ cells/HPF are responsible for the different pathomechanisms in CE patients. We established that endothelial MVD and expression patterns of HIF1α, VEGFA, and VEGFR2 do not differ between women without CE and CE patients with CD138 1–4/10 HPFs, but we did find significant elevations in CE patients with CD138+ ≥5/10 HPFs. These findings are consistent with previous findings that pregnancy outcomes are not affected in women with CD138+ 1–4/10 HPFs (35). Based on the finding above, we postulate that different numbers of endometrial CD138+ cells may have different pathological mechanisms. This possibility warrants further investigation.

Notably, we found that a single antibiotic treatment course significantly reduced the expression of HIF1α and VEGFA in cured CE patients. However, for patients with persistent CE, the expression levels of HIF1α and VEGFA did not differ significantly after antibiotic treatment when compared to before treatment. Additionally, antibiotics did not appear to affect VEGFR2 expression, whether or not CE was cured. These observations indicate that first line antibiotics (doxycycline plus metronidazole) mainly influence HIF−1α and VEGFA but not VEGFR2. Most studies indicate that antibiotics are effective against CE and therefore improve reproductive outcomes (43). Thus, the recovery of endometrial HIF1α and VEGFA expression levels in cured CE patients may result from successful treatment and may improve pregnancy outcomes.

This study has limitations. Because this is a retrospective, single-center study, clinical and demographic data of the study participants were incomplete. Therefore, some factors that may confound our conclusions may have been missed. Thus, prospective multicenter clinical studies are needed to validate our observations. Additionally, because this is the first study in this field, we only used representative factors from the hypoxia-inducible factor family and the vascular endothelial growth factor family to study the status of hypoxia and vascularization. Hence, other important factors may be involved. Finally, the participants' pregnancy outcomes (e.g., implantation rate and pregnancy rate) were not assessed after antibiotic treatment. Thus, we did not establish the clinical prognostic value of treatment with antibiotics.

Conclusion

Here, we report that endometrial upregulation of HIF1α, VEGFA, and VEGFR2, as well as excessive vascularization in the peri-implantation endometrium, may reduce endometrial receptivity in infertile CE patients. Notably, although endometrial hypoxic and angiogenic patterns in patients with CD138+ 1–4/HPF were similar to those of participants without CE, they were markedly aberrant in women with CD138+ ≥5/HPF. Finally, we report that antibiotics-mediated remodeling of hypoxic and angiogenic homeostasis in the endometrium may improve reproductive outcomes. Our findings offer new insights into reduced endometrial receptivity in CE-associated infertility.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the ethical committee of Yantai Yuhuangding Hospital Affiliated to Qingdao University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

ZL, XL, and FL conceived and designed the study. YS and LY performed the real time quantitative PCR and the western blot. PZ and WZ contributed to the statistical analysis. DM completed immunohistochemistry staining. XW and SL analyzed histochemical score, and DM, XW and SL completed microvessel counting. ZL, XL, and HB wrote the manuscript. HB was responsible for the revision of articles. All authors reviewed the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Cicinelli E, Resta L, Loizzi V, Pinto V, Santarsiero C, Cicinelli R, et al. Antibiotic therapy versus no treatment for chronic endometritis: A case-control study. *Fertil Steril* (2021) 115(6):1541–8. doi: 10.1016/j.fertnstert.2021.01.018

2. Drizi A, Djokovic D, Lagana AS, van Herendael B. Impaired inflammatory state of the endometrium: A multifaceted approach to endometrial inflammation. current insights and future directions. *Prz Menopauzalny* (2020) 19(2):90–100. doi: 10.5114/pm.2020.97863

3. Crum CP, Egawa K, Fenoglio CM, Richart RM. Chronic endometritis: the role of immunohistochemistry in the detection of plasma cells. *Am J Obstet Gynecol* (1983) 147(7):812–5. doi: 10.1016/0002-9378(83)90045-5

4. Moreno I, Cicinelli E, Garcia-Grau I, Gonzalez-Monfort M, Bau D, Vilella F, et al. The diagnosis of chronic endometritis in infertile asymptomatic women: a comparative study of histology, microbial cultures, hysteroscopy, and molecular microbiology. *Am J Obstet Gynecol* (2018) 218(6):602.e601–2.e616. doi: 10.1016/j.ajog.2018.02.012

5. Li Y, Xu S, Yu S, Huang C, Lin S, Chen W, et al. Diagnosis of chronic endometritis: How many CD138(+) cells/HPF in endometrial stroma affect pregnancy outcome of infertile women? *Am J Reprod Immunol* (2021) 85(5): e13369. doi: 10.1111/aji.13369

6. Johnston-MacAnanny EB, Hartnett J, Engmann LL, Nulsen JC, Sanders MM, Benadiva CA. Chronic endometritis is a frequent finding in women with recurrent implantation failure after *in vitro* fertilization. *Fertil Steril* (2010) 93(2):437–41. doi: 10.1016/j.fertnstert.2008.12.131

7. Kitaya K, Tada Y, Hayashi T, Taguchi S, Funabiki M, Nakamura Y. Comprehensive endometrial immunoglobulin subclass analysis in infertile women suffering from repeated implantation failure with or without chronic endometritis. *Am J Reprod Immunol* (2014) 72(4):386–91. doi: 10.1111/aji.12277

8. Liu Y, Chen X, Huang J, Wang CC, Yu MY, Laird S, et al. Comparison of the prevalence of chronic endometritis as determined by means of different diagnostic methods in women with and without reproductive failure. *Fertil Steril* (2018) 109 (5):832–9. doi: 10.1016/j.fertnstert.2018.01.022

9. McQueen DB, Bernardi LA, Stephenson MD. Chronic endometritis in women with recurrent early pregnancy loss and/or fetal demise. *Fertil Steril* (2014) 101(4):1026–30. doi: 10.1016/j.fertnstert.2013.12.031

10. McQueen DB, Perfetto CO, Hazard FK, Lathi RB. Pregnancy outcomes in women with chronic endometritis and recurrent pregnancy loss. *Fertil Steril* (2015) 104(4):927–31. doi: 10.1016/j.fertnstert.2015.06.044

11. Bouet PE, El Hachem H, Monceau E, Gariepy G, Kadoch IJ, Sylvestre C. Chronic endometritis in women with recurrent pregnancy loss and recurrent implantation failure: Prevalence and role of office hysteroscopy and immunohistochemistry in diagnosis. *Fertil Steril* (2016) 105(1):106–10. doi: 10.1016/j.fertnstert.2015.09.025

12. Morimune A, Kimura F, Nakamura A, Kitazawa J, Takashima A, Amano T, et al. The effects of chronic endometritis on the pregnancy outcomes. *Am J Reprod Immunol* (2021) 85(3):e13357. doi: 10.1111/aji.13357

13. Cicinelli E, Matteo M, Tinelli R, Lepera A, Alfonso R, Indraccolo U, et al. Prevalence of chronic endometritis in repeated unexplained implantation failure and the IVF success rate after antibiotic therapy. *Hum Reprod* (2015) 30(2):323–30. doi: 10.1093/humrep/deu292

14. Kitaya K, Matsubayashi H, Takaya Y, Nishiyama R, Yamaguchi K, Takeuchi T, et al. Live birth rate following oral antibiotic treatment for chronic endometritis in infertile women with repeated implantation failure. *Am J Reprod Immunol* (2017) 78(5). doi: 10.1111/aji.12719

15. Song D, Feng X, Zhang Q, Xia E, Xiao Y, Xie W, et al. Prevalence and confounders of chronic endometritis in premenopausal women with abnormal bleeding or reproductive failure. *Reprod BioMed Online* (2018) 36(1):78–83. doi: 10.1016/j.rbmo.2017.09.008

16. Kitaya K, Yasuo T. Aberrant expression of selectin e, CXCL1, and CXCL13 in chronic endometritis. *Mod Pathol* (2010) 23(8):1136–46. doi: 10.1038/modpathol.2010.98

17. Li Y, Yu S, Huang C, Lian R, Chen C, Liu S, et al. Evaluation of peripheral and uterine immune status of chronic endometritis in patients with recurrent reproductive failure. *Fertil Steril* (2020) 113(1):187–96.e181. doi: 10.1016/j.fertnstert.2019.09.001

18. Di Pietro C, Cicinelli E, Guglielmino MR, Ragusa M, Farina M, Palumbo MA, et al. Altered transcriptional regulation of cytokines, growth factors, and apoptotic proteins in the endometrium of infertile women with chronic endometritis. *Am J Reprod Immunol* (2013) 69(5):509–17. doi: 10.1111/aji.12076

19. Cicinelli E, Vitagliano A, Loizzi V, De Ziegler D, Fanelli M, Bettocchi S, et al. Altered gene expression encoding cytochines, grow factors and cell cycle regulators in the endometrium of women with chronic endometritis. *Diagnostics (Basel)* (2021) 11(3):471. doi: 10.3390/diagnostics11030471

20. Wu D, Kimura F, Zheng L, Ishida M, Niwa Y, Hirata K, et al. Chronic endometritis modifies decidualization in human endometrial stromal cells. *Reprod Biol Endocrinol* (2017) 15(1):16. doi: 10.1186/s12958-017-0233-x

21. Wang WJ, Zhang H, Chen ZQ, Zhang W, Liu XM, Fang JY, et al. Endometrial TGF-beta, IL-10, IL-17 and autophagy are dysregulated in women with recurrent implantation failure with chronic endometritis. *Reprod Biol Endocrinol* (2019) 17(1):2. doi: 10.1186/s12958-018-0444-9

22. Kuroda K, Horikawa T, Moriyama A, Nakao K, Juen H, Takamizawa S, et al. Impact of chronic endometritis on endometrial receptivity analysis results and pregnancy outcomes. *Immun Inflammation Dis* (2020) 8(4):650–8. doi: 10.1002/iid3.354

23. Chen X, Man GCW, Liu Y, Wu F, Huang J, Li TC, et al. Physiological and pathological angiogenesis in endometrium at the time of embryo implantation. *Am J Reprod Immunol* (2017) 78(2). doi: 10.1111/aji.12693

24. Kingdom JC, Kaufmann P. Oxygen and placental vascular development. *Adv Exp Med Biol* (1999) 474:259–75. doi: 10.1007/978-1-4615-4711-2_20

25. Red-Horse K, Zhou Y, Genbacev O, Prakobphol A, Foulk R, McMaster M, et al. Trophoblast differentiation during embryo implantation and formation of the maternal-fetal interface. *J Clin Invest* (2004) 114(6):744–54. doi: 10.1172/JCI22991

26. Critchley HO, Osei J, Henderson TA, Boswell L, Sales KJ, Jabbour HN, et al. Hypoxia-inducible factor-1alpha expression in human endometrium and its regulation by prostaglandin e-series prostanoid receptor 2 (EP2). *Endocrinology* (2006) 147(2):744–53. doi: 10.1210/en.2005-1153

27. Semenza GL. Expression of hypoxia-inducible factor 1: mechanisms and consequences. *Biochem Pharmacol* (2000) 59(1):47–53. doi: 10.1016/s0006-2952(99)00292-0

28. Zhao D, Qu D, Dai H, Liu Y, Jiang L, Huang X, et al. Effects of hypoxiainducible factor1alpha on endometrial receptivity of women with polycystic ovary syndrome. *Mol Med Rep* (2018) 17(1):41–21. doi: 10.3892/mmr.2017.7890

29. Guo X, Yi H, Li TC, Wang Y, Wang H, Chen X. Role of vascular endothelial growth factor (VEGF) in human embryo implantation: Clinical implications. *Biomolecules* (2021) 11(2):253. doi: 10.3390/biom11020253

30. Walter LM, Rogers PA, Girling JE. Differential expression of vascular endothelial growth factor-a isoforms in the mouse uterus during early pregnancy. *Reprod BioMed Online* (2010) 21(6):803–11. doi: 10.1016/j.rbmo.2010.07.005

31. Rogers PA, Martinez F, Girling JE, Lederman F, Cann L, Farrell E, et al. Influence of different hormonal regimens on endometrial microvascular density and VEGF expression in women suffering from breakthrough bleeding. *Hum Reprod* (2005) 20(12):3341–7. doi: 10.1093/humrep/dei239

32. Chen L, Quan S, Ou XH, Kong L. Decreased endometrial vascularity in patients with antiphospholipid antibodies-associated recurrent miscarriage during midluteal phase. *Fertil Steril* (2012) 98(6):1495–502.e1491. doi: 10.1016/j.fertnstert.2012.08.006

33. Chen X, Jin X, Liu L, Man CW, Huang J, Wang CC, et al. Differential expression of vascular endothelial growth factor angiogenic factors in different endometrial compartments in women who have an elevated progesterone level before oocyte retrieval, during *in vitro* fertilization-embryo transfer treatment. *Fertil Steril* (2015) 104(4):1030–6. doi: 10.1016/j.fertnstert.2015.06.021

34. Cicinelli E, Vitagliano A, Kumar A, Lasmar RB, Bettocchi S, Haimovich S, et al. Unified diagnostic criteria for chronic endometritis at fluid hysteroscopy:

proposal and reliability evaluation through an international randomized-controlled observer study. *Fertil Steril* (2019) 112(1):162–73.e162. doi: 10.1016/j.fertnstert.2019.03.004

35. Xiong Y, Chen Q, Chen C, Tan J, Wang Z, Gu F, et al. Impact of oral antibiotic treatment for chronic endometritis on pregnancy outcomes in the following frozen-thawed embryo transfer cycles of infertile women: a cohort study of 640 embryo transfer cycles. *Fertil Steril* (2021) 116(2):413–21. doi: 10.1016/j.fertnstert.2021.03.036

36. Cicinelli E, Trojano G, Mastromauro M, Vimercati A, Marinaccio M, Mitola PC, et al. Higher prevalence of chronic endometritis in women with endometriosis: a possible etiopathogenetic link. *Fertil Steril* (2017) 108(2):289–95.e281. doi: 10.1016/j.fertnstert.2017.05.016

37. Zhang L, Xiong W, Li N, Liu H, He H, Du Y, et al. Estrogen stabilizes hypoxia-inducible factor 1alpha through G protein-coupled estrogen receptor 1 in eutopic endometrium of endometriosis. *Fertil Steril* (2017) 107(2):439–47. doi: 10.1016/j.fertnstert.2016.11.008

38. Chen X, Jiang L, Wang CC, Huang J, Li TC. Hypoxia inducible factor and microvessels in peri-implantation endometrium of women with recurrent miscarriage. *Fertil Steril* (2016) 105(6):1496–502.e1494. doi: 10.1016/j.fertnstert.2016.02.032

39. Rodesch F, Simon P, Donner C, Jauniaux E. Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. *Obstet Gynecol* (1992) 80(2):283–5.

40. Matsumoto L, Hirota Y, Saito-Fujita T, Takeda N, Tanaka T, Hiraoka T, et al. HIF2alpha in the uterine stroma permits embryo invasion and luminal epithelium detachment. *J Clin Invest* (2018) 128(7):3186–97. doi: 10.1172/JCI98931

41. Cicinelli E, Matteo M, Tinelli R, Pinto V, Marinaccio M, Indraccolo U, et al. Chronic endometritis due to common bacteria is prevalent in women with recurrent miscarriage as confirmed by improved pregnancy outcome after antibiotic treatment. *Reprod Sci* (2014) 21(5):640–7. doi: 10.1177/1933719113508817

42. Yang R, Du X, Wang Y, Song X, Yang Y, Qiao J. The hysteroscopy and histological diagnosis and treatment value of chronic endometritis in recurrent implantation failure patients. *Arch Gynecol Obstet* (2014) 289(6):1363–9. doi: 10.1007/s00404-013-3131-2

43. Vitagliano A, Saccardi C, Noventa M, Di Spiezio Sardo A, Saccone G, Cicinelli E, et al. Effects of chronic endometritis therapy on *in vitro* fertilization outcome in women with repeated implantation failure: a systematic review and meta-analysis. *Fertil Steril* (2018) 110(1):103–12.e101. doi: 10.1016/j.fertnstert.2018.03.017

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Uterine cytokine profiles after low-molecular-weight heparin administration are associated with pregnancy outcomes of patients with repeated implantation failure

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Introduction: Low molecular-weight heparin (LMWH) plays a role in repeated implantation failure (RIF), but outcomes are controversial. LMWH can potentially modulate local immune responses associated with the establishment and maintenance of pregnancy. The study aimed to explore the effects of LWMH in uterine inflammatory cytokine profiles and pregnancy outcomes of patients with repeated implantation failure (RIF) but without thrombophilia.

Methods: We compared clinical characteristics and reproductive outcomes among 326 patients with RIF, but not thrombophilia, undergoing frozen embryo transfer (FET) cycle with or without LMWH treatment. Endometrium secretions were aspirated from both groups after 3 days of progesterone administration before and after LMWH treatment. Cytokine mRNA expression was analyzed in primary endometrial cells in vitro.

Results: The clinical and ongoing pregnancy rates did not significantly differ between the groups (31.5% vs. 24.4%, p = 0.15; 29.6% vs. 20.7%, p = 0.06). Concentrations of IL-6 and granulocyte-colony stimulating factor (G-CSF) in uterine secretions were significantly increased in the LWMH group, regardless of pregnancy outcomes ($P < 0.05$). And, in all patients treated with LWMH, those of secreted IL-6, IL-15 and G-CSF were significantly increased in pregnant group ($P < 0.05$). The expression of mRNA for G-CSF and IL-6 was significantly increased in human endometrial stromal cells in vitro ($P < 0.05$) after stimulation with LWMH (10 IU/mL).

Conclusions: Uterine cytokine profiles after LMWH administration are associated with pregnancy outcomes and LMWH may be beneficial for patients with three implantation failures who do not have coagulation disorders.

KEYWORDS

cytokine, low-molecular-weight heparin, pregnancy, repeated implantation failure, embryo transfer

Introduction

Repeated implantation failure (RIF) is mainly defined as the inability to achieve a clinical pregnancy after three consecutive *in vitro* fertilization (IVF) attempts, involving the transfer of one or two high-quality embryos per cycle (1). Implantation failure has been associated with lifestyle habits (i.e. smoking and obesity), low quality of embryos, thrombophilia, uterine factors such as congenital uterine anomalies, endometrial polyps, and adnexal pathologies (i.e. hydrosalpinx). patients with RIF usually request further adjuvant therapies which generally can be grouped in four categories: uterine interventions (e.g. hysteroscopy, endometrial local injury); laboratory technologies and interventions(e.g. blastocyst culture, sequential embryo transfer, preimplantation genetic testing for aneuploidies); immunomodulatory or anticoagulant therapies (e.g. subcutaneous or intrauterine granulocyte colony stimulating factor administration, low-molecular-weight heparin,aspirin; prednisolone) and treatments enhancing endometrial receptivity(e.g. intramuscular growth hormone, endometrial receptivity array). Until now, the phenomenon of RIF presents a frustrating challenge for clinicians.

The administration of low-molecular-weight heparin (LMWH) is one of several methods that are currently being applied clinically to overcome implantation failure (2, 3). As an anticoagulant by facilitating the effects of antithrombin, LMWH might also modulate some of the mechanisms that underlie the successful implantation and penetration of developing embryos through their ability to interact with various adhesion molecules, growth factors, cytokines, and enzymes (4, 5). Heparin certainly improves pregnancy rates among women with repeated IVF failure and thrombophilia (6, 7). However, the improvement of pregnancy outcomes in patients without thrombophilia remains ambiguous. We previously assessed uterine cytokine profiles under different treatments or types of patients (8, 9). The present study aimed to define the effects of LMWH on uterine inflammatory cytokine profiles and its association with pregnancy outcomes of patients with RIF but without thrombophilia.

Materials and methods

Patients

We enrolled 393 women undergoing frozen embryo transfer at the Reproductive Medical Center of Ruijin Hospital affiliated to the Medical School, Shanghai Jiao Tong University. The inclusion criteria comprised: no ongoing pregnancy lasting beyond 10 weeks (dated from the day of ET) despite a cumulative total of at least six fresh or frozen embryos transferred on day 3 or day 5 of previous transfer cycles, age <38 years, body mass index (BMI) ≥ 19 and ≤ 25 kg/m^2, and basal FSH ≤ 12 IU/L.

The exclusion criteria comprised a history of endocrine or metabolic disorders, ovarian cystectomy or oophorectomy, or pathology affecting the endometrial cavity and/or receptivity, and clinical and/or laboratory markers of hereditary or acquired thrombophilia and/or autoimmune disorders. The exclusion of thrombophilia and autoimmune disease was based on the two published meta-analysis (10, 11), including the laboratory test of protein C, protein S, antithrombin III activity, antiphospholipid antibodies, beta-2 glycoprotein I, Homocysteine, antinuclear antibodies and lupus antibody.

Study design

All eligible participants underwent a test cycle with standard hormone replacement therapy (HRT) to prepare the endometrium before frozen embryo transfer (FET) cycles. Endometrial secretions aspirated on day 4 of progesterone administration were stored at -196°C. One or two months later, the patients were randomly assigned to receive either the standard HRT protocol with LMWH added (study group), or the standard HRT protocol alone (control group) from the initiation of the FET cycle. All patients provided written informed consent to participate after receiving a detailed explanation about the study and before the start of the treatment cycles. Endometrial secretions aspirated from all participants immediately before embryo transfer were stored at -196°C. Figure 1 describes a flowchart of the study. Endometrial secretions were aspirated from 393 women with RIF during the test cycles and from 344 of them again during the FET cycles. We excluded 67 patients due to unsatisfactory samples (n = 41) and the absence of qualified embryos (n = 26).

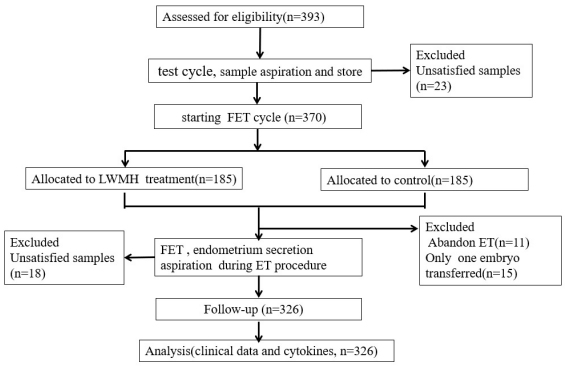

FIGURE 1
Flow chart of study . FET, frozen embryo transfer; LMWH, low molecular weight heparin.

Endometrium preparation and FET

Briefly, incremental doses of oral estradiol (4–6 mg/day) started from days 2 or 3 of the menstrual cycle and continued for 12–20 days. Thereafter, the absence of folliculogenesis and an endometrial thickness of ≥8 mm was confirmed by transvaginal ultrasonography. Subsequently Vaginal progesterone (90 mg/day) was subsequently started, and FET proceeded on day 4 of progesterone administration.

The study group was administered with LMWH (enoxaparin sodium, Clexane, 4000 anti-Xa IU; Sanofi-Aventis, Paris, France) at a standard dose of 40 mg/0.4 mL/day starting on the day of progesterone administration. The patients subcutaneously self-administered the LMWH for 10 weeks thereafter if they tested positive for pregnancy, and discontinued if it was negative.

On day 4 of progesterone administration, morphologically selected embryos were thawed, then transferred *in utero*. The scores of embryos were evaluated according to our published standard (12) and only patients with 2 cleaved transferred embryos were included in the study. All patients received oral estradiol and vaginal progesterone supplement until 12 weeks of pregnant, or discontinued if test negative.

Endometrial sampling and processing

Patients were placed in the lithotomy position, and then a speculum was inserted through the cervix, which was then cleansed. An embryo transfer catheter (CCD Laboratories, Paris, France) was transcervically introduced, and suction was gradually applied with a 2 ml syringe. The tips of the catheter were cut off and snap frozen in liquid nitrogen and stored at -80° C. Blood contamination with endometrial secretions can affect measurement of some cytokines; thus, samples that were moderately or severely contaminated were excluded from the analyses.

Multiplex immunoassay

The samples of endometrial secretions were analyzed using multiplex immunoassays as we described (8). According to a published study (13), key soluble implantation regulators were identified as candidate mediators for inclusion in the assays. Mediators without appropriate antibodies or having cross interference were excluded. The final panel included interleukins (IL)-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IL-18, leukemia inhibitory factor (LIF), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), granulocyte macrophage-colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), vascular endothelial growth factor (VEGF), macrophage inhibitory protein-1β (MIP-1b), and monocyte chemotactic protein-1 (MCP-1).

Primary endometrium cell isolation and culture

We used primary human endometrial epithelial (HEEC) and stromal (HESC) cells as study models *in vitro*. The isolation procedure was based on a previous protocol (14) with slight modifications. Briefly, both primary cell types were isolated from fresh endometrial biopsies obtained from six women just following endometrial secretions aspiration in test cycles. The tissues were minced and digested, then primary stromal and epithelial cells were isolated, seeded on dishes in Dulbecco's modified Eagle's medium (DMEM) Complete Medium/F12 containing 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 10% (v/v) fetal bovine serum (Thermo Fisher), and incubated at 37°C under a 5% CO_2 atmosphere for 48 h for RNA isolation.

RNA isolation and RT-qPCR

After immunocytochemically verifying the cells, human endometrial epithelial cells (HEEC) and stromal cells (HESC) were incubated with low (1 IU/mL) and high (10 IU/mL) concentrations of LMWH (Invitrogen, Carlsbad, CA, USA) for 48 h, and then extracted RNA using Trizol. The RNA was reverse transcribed using a cDNA Reverse Transcription Kit (Toyobo, Japan) and cDNA was synthesized using PrimeScript RT Master Mix (RR036A Takara Bio Inc., Kusatsu, Japan). The cDNA was stored at −20°C. The cDNA was reverse-transcribed using RT-qPCR with SYBR Premix Ex Taq kit (RR420A; Takara). Each reaction included an initial denaturation at 95°C for 30 min, 40 amplification cycles at 95°C for 5 s and annealing at 60°C for 34 s. Gene expression was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Fold change (FC) was calculated according to the $2^{-\Delta\Delta Ct}$ method and values with $P < 0.05$ were considered statistically significant. All cell samples were analyzed in triplicate.

Pregnancy outcome measures

The primary outcome measure was ongoing pregnancy rates. Secondary outcome measures comprised biochemical (positive β-hCG), and clinical pregnancy rates. Ongoing pregnancy rates were defined as the presence of at least one fetal heart pulse on ultrasound beyond 20 weeks and clinical pregnancy was defined as ultrasound confirmation of a gestational sac at 4 weeks after embryo transfer. Outcome measures were not changed after the trial commenced.

Sample size calculation

The sample size was calculated using Stat version 13 (StataCorp, College Station, TX). The pregnancy rate of patients with RIF in our center has been rather low (<20%). Considering the scenario where intervention increases clinical pregnancy rates from 20% to 40%, then 100 participants would be required in each arm to provide a significance level of 0.05 and a power of 0.8 in the analyses of outcomes.

Statistical analysis

Non-normally distributed data regarding uterine cytokine profiles were log-transformed before analysis. Continuous variables are expressed as medians with ranges and were compared using nonparametric Mann–Whitney U-tests. Proportions of categorical variables were compared using Chi-square and Fisher exact tests. All tests were two-sided and values were considered statistically significant at $p < 0.05$. All data were statistically analyzed using SPSS 26.0 (IBM Corp., Armonk, NY, USA).

Results

Baseline and cycle characteristics

The final analysis included 326 patients. Age, BMI, ovarian reservation, and duration of infertility did not significantly differ between patients treated with or without LMWH. The median numbers of previous implantation failures were 4 (3–6) in both groups and none of the patient smoked cigarettes. Table 1 shows the clinical characteristics of the groups.

Treatment outcomes

Table 2 summarizes the pregnancy outcomes.

TABLE 1 Baseline and demographic characteristics of the participants.

	LMWH (N = 162)	Control (N = 164)
Age (y)	33 (27–38)	32 (26–38)
BMI (kg/m^2)	22.4 (20.6–25.4)	21.9 (19.1–25.7)
Basal FSH (IU/L)	8.2 (6.5–11.2)	8.7 (6.3–11.7)
AFC	8 (6–15)	9 (5–15)
Duration of infertility (y)	5 (1–8)	4 (1–9)
Smoking (n, %)	0	0
No of previous implantation Failures	4 (3–6)	4 (3–6)
Primary subfertility (n, %)	122,75.3%	129,78.7%
The history of spontaneous abortion (n, %)	10,6.2%	12,7.3%

All values are $P > 0.05$. AFC, antral follicle count; BMI, body mass index; FSH, follicle stimulating hormone.

TABLE 2 Summary of treatment and pregnancy outcomes of LMWH and control groups.

	LMWH (N = 162)	Control (N = 164)	p
Endometrium thickness (mm)	9.2 ± 1.3	9.4 ± 1.1	0.32
Hormone level on ET day			
E2 (pg/ml)	122.6 ± 33.7	133.9 ± 31.2	0.19
P (ng/ml)	7.2 ± 1.5	8.1 ± 1.3	0.11
Embryos transferred (n)	2.0	2.0	> 0.99
Average scores of transferred embryos	7.1 ± 1.2	7.3 ± 1.5	0.14
Positive pregnancy test (n,%)	55 (34.0%)	45 (27.4%)	0.20
Clinical pregnancy (n,%)	51 (31.5%)	40 (24.4%)	0.15
Ongoing pregnancy (n,%)	48 (29.6%)	34 (20.7%)	0.06
Implantation (n,%)	55 (17.0%)	42 (12.8%)	0.14
Miscarriage (n,%)	3 (5.9%)	6 (15.0%)	0.21
LMWH side effects	0	0	> 0.99
LMWH infection	0	0	> 0.99

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All patients were transferred with two cleaved embryos on day3 of the FET cycle. Cycle characteristics, including endometrium thickness, hormone level, and average scores of transferred embryos did not significantly differ between the groups. Rates of positive results in pregnancy tests (biochemical pregnancy) and clinical pregnancies were similar in both groups (34.0% vs. 27.7%, p = 0.2, and 31.5% vs. 24.4%, p = 0.15).

Miscarriage and ongoing pregnancy rates were also similar between the control and LWMH groups (5.9% vs. 15.0%, p = 0.21 and 29.6% vs. 20.7%, p = 0.06, respectively) although the ongoing pregnancy rate tended to increase in the LWMH group. All miscarriages occurred between 7 and 10 weeks of gestation and among the 9 patients of miscarriage, two and four patients in the control and treatment groups, respectively, had spontaneous abortion at least once. None of the patients reported discomfort or side effects.

Endometrial cytokine profiles

Table 3 shows a comparison of the medians and 25th and 75th percentiles of concentrations of each mediator in the endometrium at the test and FET cycles between the two groups. Endometrium cytokine concentrations did not significantly differ between the test and FET cycles in the control group. Cytokines that significantly differed between the two cycles in the LWMH group comprised IL-6 (P < 0.05) and G-CSF (P < 0.05). Concentrations of IL-8, IL-10, IL-15, TNF-α, GM-CSF, VEGF, MIP-1b and MCP-1, differed in the LWMH group with P value >0.05 but < 0.1

Intra-patient comparison of endometrial cytokines between two FET cycles

Following the changes in the cytokine profiles induced LWMH treatment, we compared the cytokine concentrations between test and FET cycles in each patient of the LWMH group to determine the relationship between cytokine changes and pregnancy outcomes. Figure 2 depicts the endometrial cytokine concentration ratios between the two cycles in individual patients based on whether they became clinically pregnant. The expression of all tested soluble mediators did not differ significantly between pregnant and non-pregnant women (Figure 2A), whereas the concentrations of IL-6, IL-15, and G-

TABLE 3 Table III. Comparison of uterine cytokine concentrations in endometrial secretions between LMWH and control groups.

Cytokine concentrations (pg/mg total protein)				
	LMWH (N = 162)		Control (N=164)	
	Test cycle	FET cycle	Test cycle	FET cycle
IL-1b	19.5 (13.6;30.9)	17.2 (11.5;29.6)	17.6 (11.2;31.9)	19.3 (10.2;31.5)
IL-2	16.3 (10.8; 26.1)	15.6 (11.2;28.9)	18.9 (9.2;22.3)	17.1 (10.2;25.6)
IL-4	11.1 (7.8;16.8)	12.1 (10.2;20.5)	10.2 (8.1;17.2)	11.9 (9.1;18.2)
IL-5	12.3 (8.8;19.6)	14.2 (7.2;22.8)	10.4 (8.2;18.5)	12.1 (7.2;23.5)
IL-6[a]	18.1 (12.3;31.7)	28.5 (19.8;45.3)	16.2 (11.9;32.6)	18.3 (10.7;33.8)
IL-7	13.3 (8.7;19.9)	11.2 (9.6;20.3)	12.4 (8.5;19.9)	12.9 (8.1;20.3)
IL-8[b]	396.6 (197.1;892.2)	498.2 (244.6; 887.1)	371.2 (200.6;906.3)	369.3 (191.3;813.4)
IL-10	27.3 (19.4;44.7)	25.3 (20.8;45.6)	24.2 (18.5;42.9)	26.9 (17.6;50.3)
IL-12	13.8 (10.2;22.4)	12.1 (9.8;22.4)	15.8 (11.3;20.9)	16.1 (10.9;23.2)
IL-13	9.9 (7.0;15.3)	11.2 (6.8;16.2)	11.2 (7.8;16.8)	12.8 (7.9;18.4)
IL-15[b]	10.3 (6.4;18.2)	13.4 (7.9;21.2)	11.3 (5.8;19.4)	10.8 (6.3;18.9)
IL-17	17.3 (11.6;28.3)	15.2 (10.9;27.2)	18.1 (10.2;29.9)	16.2 (9.2;26.9)
LIF	79.1 (59.2;115.6)	88.9 (62.3;130.8)	69.2 (55.4;120.9)	78.7 (60.9;129.6)
IL-18	9.5 (7.0;15.2)	11.4 (8.0;18.7)	10.1 (7.2;14.1)	12.0 (7.5;19.6)
MCP-1[b]	419.0 (150.1;866.7)	302.3 (198.3;812.4)	388.2 (191.3;992.3)	416.8 (167.2;813.3)
MIP-1b[b]	125.8 (76.1;227.0)	100.3 (77.2;250.1)	119.3 (56.2;238.9)	131.4 (62.9;277.1)
TNF-a[b]	17.6 (13.3;30.2)	13.4 (10.2;25.3)	16.2 (12.9;33.2)	18.1 (11.8;34.1)
VEGF[b]	13.2 (8.4;22.7)	16.2 (8.9;23.8)	14.9 (9.2;25.4)	15.3 (9.1;27.2)
G-CSF[a]	90.5 (53.6;117.4)	133.6 (62.2;160.3)	100.3 (50.2;128.4)	112.6 (59.9;138.7)
GM-CSF[b]	18.2 (13.4;31.9)	14.1 (11.2;32.9)	17.1 (11.8;32.4)	16.2 (9.8;33.7)
IFN-g[b]	18.1 (12.3;28.9)	22.1 (11.2;29.6)	19.4 (14.2;30.7)	17.9 (12.8;31.5)

Values are shown as medians (25th; 75th percentiles). Two-tailed paired t-tests on log transformed data. [a]p < 0.05, [b]p < 0.1.

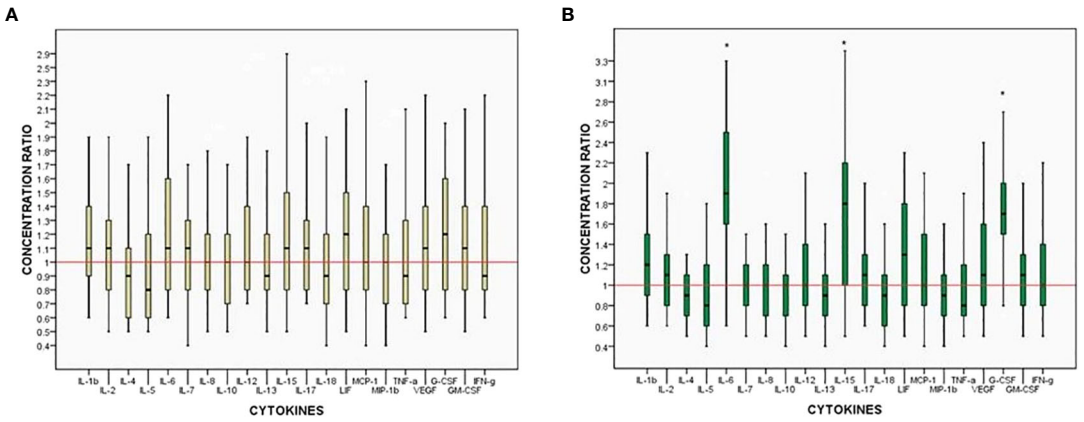

FIGURE 2
Ratios of intra-patient endometrium cytokine concentrations between two cycles. Box: median, 25%–75% interquartile range (IQR). Whiskers 10%–90% IQR. **(A)** Non-pregnant women. **(B)** Pregnant women. *p<0.05.

CSF were all significantly higher in pregnant patients treated with LWMH (P < 0.05; Figure 2B)

Changes in cytokine expression induced by LWMH in primary endometrial cells

We investigated the influence of LWMH on cytokine expression in primary endometrium cells *in vitro*. Because the results *in vivo* indicated that LWMH altered the concentrations of IL-6, IL-8, IL-10, IL-15, TNF-α, GM-CSF, G-CSF, VEGF, MIP-1b and MCP-1, we measured their levels of mRNA expression of *in vitro*. Figure 3A shows that stimulation with LWMH (10 IU/ml) significantly increased the mRNA expression of G-CSF and IL-6 in HESC (P < 0.05), whereas the expression of all other cytokines in HEEC did not obviously differ (P > 0.05; Figure 3B).

Discussion

Although RIF has not been unanimously defined, it is characterized by failure to achieve pregnancy after multiple embryo transfers. The incomplete understanding of RIF and its diverse etiologies pose therapeutic challenges. When a good-quality embryo fails to result in pregnancy, the ability of the endometrium to allow effective embryo apposition and adhesion is frequently suspected. However, non-organic uterine

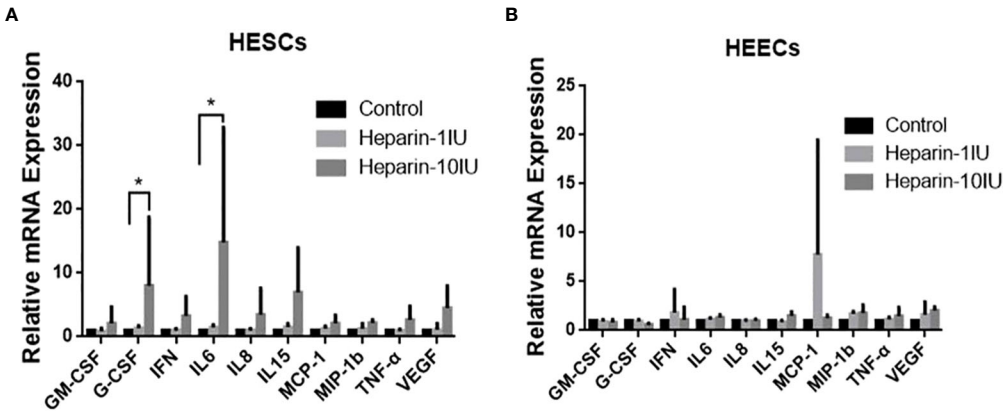

FIGURE 3
Cytokine mRNA levels are altered in HESC and HEEC incubated with LWMH for 48h. Data are presented as means ±SEM and *P<0.05. **(A)** Human endometrial stromal cells. **(B)** Human endometiral epithelial cells.

aberrations are difficult to detect in the clinical setting. The active, and especially the innate immune systems, are important components of this process, and uterine immune disequilibrium could have a negative impact on embryo implantation. Several immunological abnormalities have been linked to RIF and recurrent pregnancy loss (RPL) (15–17).

LWMH can prevent thrombin-induced pregnancy loss in women with antiphospholipid antibodies (APA) (6, 7). Interaction studies between LWMH and implantation-related uterine factors have revealed potential benefits of LWMH during very early pregnancy (18, 19). However, whether LWMH can confer benefits on patients with RIF but no thrombophilia has remained unclear.

The clinical parameters in FET cycles revealed that FET with or without LMWH treatment did not result in statistically significant differences in clinical pregnancy, ongoing pregnancy, and miscarriage rates among 326 patients with three unsuccessful IVF/ICSI cycles enrolled in the study. Several recent trials have found a trend toward a positive effect of LWMH in women with RIF but no APS, but the evidence is still inconclusive (20, 21). According to a meta-analysis by Akhtar et al. (20) that included three RCT (386 women), analyses of fixed, but not random effects have linked LWMH to higher rates of live birth and pregnancy. However, only one of the three RCT studies has investigated women who had at least two failed IVF cycles. Drakakis et al. (22) conducted a multicenter cohort study included 230 women who had at least two failed fresh IVF/ICSI cycles. They found no statistically significant differences in clinical pregnancy and miscarriage rates between those who received LWMH and those who did not. The current and previously published findings indicate that the evidence supporting the routine administration of LWMH to RIF patients remains weak. Furthermore, it is still not recommended for general patients undergoing IVF.

For embryo attachment and invasion, a local immune biological reaction should be orchestrated and balanced. A pro-inflammatory environment is critical during the pre-implantation period, and uterine cytokines play a role as intercellular messengers in this immune remodeling. To the best of our knowledge, this was the first study to look at the effects of LWMH treatment on uterine secretion cytokines *in vivo*. Here, LWMH administration was found to be associated with increased uterine concentrations of IL-6 and G-CSF in RIF women. Furthermore, LWMH administration increased IL-8, IL-15, VEGF, IFN- and decreased MCP-1, MIP-1b, and TNF-a levels in uterine secretions. The most intriguing finding of the study was that LWMH administration significantly increased IL-6, IL-15, and G-CSF concentrations in pregnant patients but not in non-pregnant patients. We hypothesized that the variable role of LMWH in women with RIF is dependent on the individual uterine immune microenvironment and its response to LWMH stimulation, based on the variety of LWMH effects and their association with successful embryo implantation.

In immunology effects, IL-6 and IL-15 were generally acknowledged as pro-inflammatory cytokines. The mid-secretory phase of the cycle is when the concentration of IL-6 produced by glandular and luminal epithelial cells in human endometrium peaks, indicating that IL-6 plays a role in embryo implantation (23, 24). It was shown that IL-6 induces the production of metalloproteinases (MMP)-14, MMP-11, and leptin secretion to alter endometrial tissues to permit trophoblast invasion (25). Additionally, through controlling trophoblast cell proliferation, differentiation, and hCG synthesis, it is a crucial regulator of early placental development (26, 27). Insufficient local IL-6 may contribute to fetal loss, since IL-6 expression is reduced during the mid-secretory phase of endometrial of women prone to recurrent spontaneous abortion (RSA) (28). Consistent with the role of IL-6 in key reproductive events, IL-6 null mutant mice exhibit elevated fetal resorption and delayed parturition, while exogenous IL-6 can correct the fetal loss (29).

However, it should be noted that excessive IL-6 expression may negatively impact early pregnancy in a number of cell signaling pathways. In a recent study, we demonstrated that uterine IL-6 concentrations are elevated in adenomyosis patients and that this is associated with decreased endometrial receptivity (8). Women with endometriosis and unexplained infertility have been observed by other investigators to have higher endometrial IL-6 concentrations (30). As a result, the individualized uterine environmental setting of the woman should determine the beneficial effect of LMWH on embryo implantation and growth.

Interleukin-15, a different cytokine associated with LMWH therapy, is expressed in both epithelial and stromal cells, and its expression rises in the latter following decidualization (31). Studies conducted *in vitro* have demonstrated that IL-15 has a role in the endometrial recruitment of peripheral blood NK cells and subsequent differentiation into uterine NK cells (uNK cells) (32). Additionally, IL-15 is a potent stimulator of uNK cell activation and proliferation (33). Conclusions regarding stromal expression of IL-15 in RIF-affected women were contentious (34, 35). Actually, uNK cells have dual functions. While proper uNK cell activation is essential for optimal embryo implantation, in a Th1-dominant environment, uNK cells transform into cytotoxic killer cells that recognize and reject trophoblastic cells as non-self. A study by Lédée et al. (36) identified low IL-15/fibroblast growth factor-inducible molecule 14 (Fn-14) mRNA as a biomarker of insufficient uNK cell activation/maturation in RIF patients. This ratio was raised with higher pregnancy rates in the subsequent embryo transfer cycle following individualized care, such as endometrial local injury. The current study discovered that successful pregnancies in RIF women were associated with increased IL-15 secretion caused by LWMH medication, suggesting that LWMH may act as a possible immunological regulator in some RIF women.

Unlike IL-6 or IL-15, G-CSF usually plays role in the initiation and maintenance of pregnancy by temporarily suppressing immune response through its effects on lymphocytes, macrophages, and Th-2 type cells. Clinically, G-CSF was found to be more abundant in the placental villous tissues of normal pregnancy women than in those with RSA (37).. Gleicher et al. first described a potential benefit of G-CSF for women with thin endometrium who undergo frozen embryo transfer in 2014 (38). According to Kamath (39), G-CSF was associated with a significantly higher clinical pregnancy rate with a pooled risk ratio of 2.51 (95% CI, 1.36-4.63) in a RIF population compared to patients who received no intervention. However, a recent randomized controlled trial found that intrauterine G-CSF administration had no effect on pregnancy outcomes in RIF patients with normal endometrium (40). The discovery that LWMH stimulated uterine G-CSF secretion provides a new explanation and strategy for improving endometrial receptivity in RIF patients regardless of endometrial thickness.

In vitro, we investigated the effects of LWMH on cytokine production in HEEC and HESC. None of the candidate cytokines were affected by LWMH exposure at either 1 or 10 IU/mL in HEEC cells. G-CSF and IL-6 mRNA expression was significantly increased in HESC cells treated with 10 IU/mL LWMH for 48 hours. Although LWMH therapy increased uterine IL-15 secretion in pregnant patients, we were unable to replicate this effect in cultured cells *in vitro*.

The function of LWMH on cultured HEEC and HESC *in vitro* is subject to debate. LWMH is commonly described as an anti-inflammatory and immunoregulatory agent that inhibits NK cell cytotoxicity (41) or as a leukocyte recruitment and vascular adhesion inhibitor (42). For example, it has been reported that LMWH can inhibit TNF-induced IL-6 expression by interacting with the transcription factor nuclear factor of transcription (NF) B in human ESCs (43). In contrast to previous research, we discovered that LWMH increased the expression of IL-6, a pro-inflammatory cytokine, but had no effect on TNF-expression in HESC. Variations in the content of various LWMH, which is a mixture of polysulfated glycosaminoglycans, could explain this phenomenon. Spratte et al. found that the ability of LMWH to regulate cytokines in HESC depends on its molecular size (19).

Quao et al. (44) reported the upregulation of G-CSF induced by LWMH stimulation in basal cultures of human HEEC, which we confirmed in both vivo and *in vitro* experiments. Given the effects of G-CSF on trophoblast invasion, its increased expression may play a role in RIF patients' embryo implantation. However, the elevated IL-15 levels in pregnant patients given LWMH were not confirmed *in vitro*, implying that the molecular mechanisms underlying LWMH's influence on uterine cytokine signaling pathways are complicated.

The current study discovered unique uterine cytokine profiles in the presence of LMWH. We discovered that increased endometrial IL-6, IL-15, and G-CSF secretion caused by LMWH is linked to better pregnancy outcomes in RIF patients. Although clinical outcomes did not differ significantly between the LWMH and control groups, our findings suggested that LMWH may be beneficial for patients with three implantation failures who do not have coagulation disorders. The findings of this study suggest that large randomized placebo-controlled trials are needed to assess the role(s) of LMWH in the regulation of uterine local immunity *in vivo*. Furthermore, because the molecular weight, half-life, and activities of LMWH prepared using different methods vary, comparative studies are required to evaluate their ability to improve embryo implantation in women with RIF.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by The ethics committee of Ruijin Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

ZN and AZ conceived and designed the experiments, and composed the paper. MZ performed the cell experiments and interpreted the results. LX and SZ participated in study execution. All authors participated in the revision and final approval of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Simon A, Laufer N. Assessment and treatment of repeated implantation failure (RIF). *J Assist Reprod Genet* (2012) 29:1227–39. doi: 10.1007/s10815-012-9861-4

2. Coughlan C, Ledger W, Wang Q, Liu F, Demirol A, Gurgan T, et al. Recurrent implantation failure: definition and management. *Reprod BioMed Online* (2014) 28:14–38. doi: 10.1016/j.rbmo.2013.08.011

3. Berker B, Taskin S, Kahraman K, Taskin EA, Atabekoglu C, Sonmezer M. The role of low-molecular-weight heparin in recurrent implantation failure: a prospective, quasi-randomized, controlled study. *Fertil Steril* (2011) 95:2499–502. doi: 10.1016/j.fertnstert.2010.12.033

4. Cristian Z, Anna N, Domenica G, Marilisa B, Alessandra Z, Tullia T, et al. The HMGB1/RAGE pro-inflammatory axis in the human placenta: Modulating effect of low molecular weight heparin. *Molecules* (2017) 22:1997. doi: 10.3390/molecules22111997

5. Bruno V, Svensson-Arvelund J, Ruber M, Berg G, Piccione E, Jenmalm MC, et al. Effects of low molecular weight heparin on the polarization and cytokine profile of macrophages and T helper cells *in vitro*. *Sci Rep* (2018) 8:4166. doi: 10.1038/s41598-018-22418-2

6. Qublan H, Amarin Z, Dabbas M, Farraj AE, Beni-Merei Z, Al-Akash H, et al. Low-molecular-weight heparin in the treatment of recurrent IVF-ET failure and thrombophilia: a prospective randomized placebo-controlled trial. *Hum Fertil (Camb)* (2008) 11:246–53. doi: 10.1080/14647270801995431

7. Khizroeva J, Makatsariya A, Bitsadze V, Makatsariya N, Khamani N. *In vitro* fertilization outcomes in women with antiphospholipid antibodies circulation. *J Matern Fetal Neonatal Med* (2020) 33:1988–93. doi: 10.1080/14767058.2018.1535586

8. Zhihong N, Yun F, Pinggui Z, Sulian Z, Zhang A. Cytokine profiling in the eutopic endometrium of adenomyosis during the implantation window after ovarian stimulation. *Reprod Sci* (2016) 23:124–33. doi: 10.1177/1933719115597761

9. Zhu X, Niu Z, Ye Y, Xia L, Chen Q, Feng Y. Endometrium cytokine profiles are altered following ovarian stimulation but almost not in subsequent hormone replacement cycles. *Cytokine* (2019) 114:6–10. doi: 10.1016/j.cyto.2018.11.002

10. van Dijk MM, Kolte AM, Limpens J, Kirk E, Quenby S, van Wely M, et al. Recurrent pregnancy loss: diagnostic workup after two or three pregnancy losses? a systematic review of the literature and meta-analysis. *Hum Reprod Update* (2020) 26:356–67. doi: 10.1093/humupd/dmz048

11. Simopoulou M, Sfakianoudis K, Maziotis E, Grigoriadis S, Giannelou P, Rapani A, et al. The impact of autoantibodies on IVF treatment and outcome: A systematic review. *Int J Mol Sci* (2019) 20:892. doi: 10.3390/ijms20040892

12. Xia L, Zhao S, Xu H, Wu X, Zhang A, Niu Z. Miscarriage rate is high with frozen-thawed blastocysts arising from poor-quality cleavage stage embryos. *Front Endocrinol (Lausanne)* (2020) 11:561085. doi: 10.3389/fendo.2020.561085

13. Pantos K, Grigoriadis S, Maziotis E, Pistola K, Xystra P, Pantou A, et al. The role of interleukins in recurrent implantation failure: A comprehensive review of the literature. *Int J Mol Sci* (2022) 23:2198. doi: 10.3390/ijms23042198

14. Xu B, Zhou M, Wang J, Zhang D, Guo F, Si C, et al. Increased AIF-1-mediated TNF-alpha expression during implantation phase in IVF cycles with GnRH antagonist protocol. *Hum Reprod* (2018) 33:1270–80. doi: 10.1093/humrep/dey119

15. Beer AE, Kwak JY, Ruiz JE. Immunophenotypic profiles of peripheral blood lymphocytes in women with recurrent pregnancy losses and in infertile women with multiple failed *in vitro* fertilization cycles. *Am J Reprod Immunol* (1996) 35:376–82. doi: 10.1111/j.1600-0897.1996.tb00497.x

16. Ledee N, Petitbarat M, Prat-Ellenberg L, Dray G, Cassuto GN, Chevrier L, et al. Endometrial immune profiling: a method to design personalized care in assisted reproductive medicine. *Front Immunol* (2020) 11:1032. doi: 10.3389/fimmu.2020.01032

17. Alecsandru D, Klimczak AM, Garcia Velasco JA, Pirtea P, Franasiak JM. Immunologic causes and thrombophilia in recurrent pregnancy loss. *Fertil Steril* (2021) 115:561–6. doi: 10.1016/j.fertnstert.2021.01.017

18. Di Simone N, Di Nicuolo F, Castellani R, Veglia M, Tersigni C, Silano M, et al. Low-molecular-weight heparins induce decidual heparin-binding epidermal growth factor-like growth factor expression and promote survival of decidual cells undergoing apoptosis. *Fertil Steril* (2012) 97:169–77.e161. doi: 10.1016/j.fertnstert.2011.10.021

19. Spratte J, Schonborn M, Treder N, Bornkessel F, Zygmunt M, Fluhr H. Heparin modulates chemokines in human endometrial stromal cells by interaction with tumor necrosis factor alpha and thrombin. *Fertil Steril* (2015) 103:1363–9. doi: 10.1016/j.fertnstert.2015.02.023

20. Akhtar MA, Sur S, Raine-Fenning N, Jayaprakasan K, Thornton JG, Quenby S. Heparin for assisted reproduction. *Cochrane Database Syst Rev* (2013) 17:CD009452. doi: 10.1002/14651858.CD009452

21. Lodigiani C, Di Micco P, Ferrazzi P, Libre L, Arfuso V, Polatti F, et al. Low-molecular-weight heparin in women with repeated implantation failure. *Womens Health (Lond)* (2011) 7:425–31. doi: 10.2217/whe.11.38

22. Siristatidis C, Dafopoulos K, Salamalekis G, Galazios G, Christoforidis N, Moustakarias T, et al. Administration of low-molecular-weight heparin in patients with two or more unsuccessful IVF/ICSI cycles: a multicenter cohort study. *Gynecol Endocrinol* (2018) 34:747–51. doi: 10.1080/09513590.2018.1442426

23. Tabibzadeh S, Kong QF, Babaknia A, May LT. Progressive rise in the expression of interleukin-6 in human endometrium during menstrual cycle is initiated during the implantation window. *Hum Reprod* (1995) 10:2793–9. doi: 10.1093/oxfordjournals.humrep.a135793

24. Dubinsky V, Junovich G, Gentile T, Gutiérrez G. IL-6 as a regulatory factor of the humoral response during pregnancy. *Am J Reprod Immunol* (2008) 60:197–203. doi: 10.1111/j.1600-0897.2008.00614.x

25. Desai N, Scarrow M, Lawson J, Kinzer D, Goldfarb J. Evaluation of the effect of interleukin-6 and human extracellullar matrix on embryonic development. *Hum Reproduction* (1999) 14:1588–92. doi: 10.1093/humrep/14.6.1588

26. Li Y, Matsuzaki N, Masuhiro K, Kameda T, Taniguchi T, Saji F, et al. Trophoblast-derived tumor necrosis factor-alpha induces release of human chorionic gonadotropin using interleukin-6 (IL-6) and IL-6-receptor-dependent system in the normal human trophoblasts. *J Clin Endocrinol Metab* (1992) 74:184–91. doi: 10.1210/jcem.74.1.1727819

27. Jiang XY, Lu TM, Shu WH, Zhou HY. Correlation between IL-6 and invasiveness of ectoderm cells of embryo in early pregnancy. *J Biol Regul Homeost Agents* (2016) 30:559–63.

28. Jasper MJ, Tremellen KP, Robertson SA. Reduced expression of IL-6 and IL-1alpha mRNAs in secretory phase endometrium of women with recurrent miscarriage. *J Reprod Immunol* (2007) 73:74–84. doi: 10.1016/j.jri.2006.06.003

29. Robertson SA. Immune regulation of conception and embryo implantation-all about quality control? *J Reprod Immunol* (2010) 85:51–7. doi: 10.1016/j.jri.2010.01.008

30. Demir B, Guven S, Guven ES, Atamer Y, Gul T. Serum IL-6 level may have role in the pathophysiology of unexplained infertility. *Am J Reprod Immunol* (2009) 62:261–7. doi: 10.1111/j.1600-0897.2009.00734

31. Dimitriadis E, White CA, Jones RL, Salamonsen LA. Cytokines, chemokines and growth factors in endometrium related to implantation. *Hum Reprod Update* (2005) 11:613–30. doi: 10.1093/humupd/dmi023

32. Kitaya K, Yamaguchi T, Honjo H. Central role of interleukin-15 in postovulatory recruitment of peripheral blood CD16(-) natural killer cells into human endometrium. *J Clin Endocrinol Metab* (2005) 90:2932–40. doi: 10.1210/jc.2004-2447

33. Kitaya K, Yasuda J, Yagi I, Tada Y, Fushiki S, Honjo H. IL-15 expression at human endometrium and decidua. *Biol Reprod* (2000) 63:683–7. doi: 10.1095/biolreprod63.3.683

34. Mariee N, TC Li, Laird SM. Expression of leukaemia inhibitory factor and interleukin 15 in endometrium of women with recurrent implantation failure after IVF; correlation with the number of endometrial natural killer cells. *Hum Reprod* (2012) 27:1946–54. doi: 10.1093/humrep/des134

35. Lédée-Bataille N, Bonnet-Chea K, Hosny G, Dubanchet S, Frydman R, Chaouat G. Role of the endometrial tripod interleukin-18, -15, and -12 in inadequate uterine receptivity in patients with a history of repeated *in vitro* fertilization-embryo transfer failure. *Fertil Steril* (2005) 83:598–605. doi: 10.1016/j.fertnstert.2004.11.021

36. Lédée N, Petitbarat M, Chevrier L, Vitoux D, Vezmar K, Rahmati M, et al. The uterine immune profile may help women with repeated unexplained embryo implantation failure after in vitro fertilization. *Am J Reprod Immunol* (2016) 75:388–401. doi: 10.1111/aji.12483

37. Ding J, Yang C, Zhang Y, Wang J, Zhang S, Guo D, et al. M2 macrophage-derived G-CSF promotes trophoblasts EMT, invasion and migration via activating PI3K/Akt/Erk1/2 pathway to mediate normal pregnancy. *J Cell Mol Med* (2021) 25:2136–47. doi: 10.1111/jcmm.16191

38. Barad DH, Yu Y, Kushnir VA, Shohat-Tal A, Lazzaroni E, Lee HJ, et al. A randomized clinical trial of endometrial perfusion with granulocyte colony-stimulating factor in *in vitro* fertilization cycles: impact on endometrial thickness and clinical pregnancy rates. *Fertil Steril* (2014) 101:710–5. doi: 10.1016/j.fertnstert.2013.12.016

39. Kamath MS, Chittawar PB, Kirubakaran R, Mascarenhas M. Use of granulocyte-colony stimulating factor in assisted reproductive technology: A systematic review and meta-analysis. *Eur J Obstet Gynecol Reprod Biol* (2017) 214:16–24. doi: 10.1016/j.ejogrb.2017.04.022

40. Kalem Z, Namli Kalem M, Bakirarar B, Kent E, Makrigiannakis A, Gurgan T. Intrauterine G-CSF administration in recurrent implantation failure (RIF): An rct. *Sci Rep* (2020) 10:5139. doi: 10.1038/s41598-020-61955-7

41. Spratte J, Schönborn M, Treder N, Bornkessel F, Zygmunt M, Fluhr H. Heparin modulates chemokines in human endometrial stromal cells by interaction with tumor necrosis factor α and thrombin. *Fertil Steril* (2015) 103:1363–9. doi: 10.1016/j.fertnstert.2015.02.023

42. Sheikhansari G, Pourmoghadam Z, Danaii S, Mehdizadeh A, Yousefi M. Etiology and management of recurrent implantation failure: A focus on intra-uterine PBMC-therapy for RIF. *J Reprod Immunol* (2020) 139:103121. doi: 10.1016/j.jri.2020.103121

43. Spratte J, Meyer zu Schwabedissen H, Endlich N, Zygmunt M, Fluhr H. Heparin inhibits TNF-α signaling in human endometrial stromal cells by interaction with NF-κB. *Mol Hum Reprod* (2013) 19:227–36. doi: 10.1093/molehr/gas060

44. Quao ZC, Tong M, Bryce E, Guller S, Chamley LW, Abrahams VM. Low molecular weight heparin and aspirin exacerbate human endometrial endothelial cell responses to antiphospholipid antibodies. *Am J Reprod Immunol* (2018) 79:10. doi: 10.1111/aji.12785

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Bromocriptine inhibits proliferation in the endometrium from women with adenomyosis

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Objective: Bromocriptine treatment has been shown to reduce menstrual bleeding and pain in women with adenomyosis in a pilot clinical trial. The underlying mechanism contributing to the treatment effect is however unknown. The purpose of this study was to explore the effect of bromocriptine on the proliferation and migration properties of the endometrium in women with adenomyosis, by assessing cellular and molecular changes after six months of vaginal bromocriptine treatment.

Methods: Endometrial specimens were collected during the proliferative phase from women with adenomyosis (n=6) before (baseline) and after six months of treatment with vaginal bromocriptine. Immunohistochemistry was used to determine changes in the protein expression of Ki67 in the endometrium of women with adenomyosis. Primary endometrial stromal cells isolated at baseline were expanded *in vitro* and exposed to different doses of bromocriptine to determine the optimal half-maximum inhibitory concentration (IC50) using CellTiter-Blue® Cell Viability Assay. Cell proliferation was assessed by bromodeoxyuridine ELISA assay and Ki67 gene expression was checked by real-time PCR. The migratory ability of endometrial stromal cells was determined by wound healing and transwell migration assays. Small RNA sequencing was applied on tissues collected from women with adenomyosis before and after bromocriptine treatment to identify differentially expressed microRNAs (miRNAs) after bromocriptine treatment. Bioinformatic methods were used for target gene prediction and the identification of biological pathways by enrichment procedures.

Results: Vaginal bromocriptine treatment reduced the Ki67 protein expression in the endometrium of women with adenomyosis and did not change the prolactin mRNA expression and protein concentration of prolactin in endometrial tissues. Bromocriptine significantly inhibited the proliferative and migrative abilities of endometrial stromal cells derived from women with adenomyosis *in vitro*. Moreover, small RNA sequencing revealed 27 differentially expressed miRNAs

between the endometrium of women with adenomyosis before and after six months of vaginal bromocriptine treatment. KEGG pathway analysis on targeted genes of 27 miRNAs showed that several signaling pathways associated with cell proliferation and apoptosis were enriched after bromocriptine treatment.

Conclusion: Bromocriptine treatment exhibits an anti-proliferative effect in the endometrium of women with adenomyosis *in vivo* and *in vitro*. Bromocriptine might inhibit the proliferation of endometrial tissue in adenomyosis in part through the regulation of dysregulated microRNAs and proliferation-associated signaling pathways.

KEYWORDS

adenomyosis, bromocriptine, endometrium, proliferation, microRNAs

Introduction

Adenomyosis is a benign uterine disorder, defined by the presence of endometrial glands and stroma infiltration within the myometrium, resulting in uterine enlargement (1). Women with adenomyosis usually present with heavy menstrual bleeding, dysmenorrhea, and chronic pelvic pain (2, 3). Furthermore, accumulating evidence shows that adenomyosis has a negative impact on reproductive outcomes (4). Various treatment options are applied in treating adenomyosis from a complete hysterectomy to non-invasive medications and options in between such as adenomyomectomy, high-intensity focused ultrasound, etc. (5). Current medications include oral contraceptive pills, progestogens, GnRH agonists, and so on. These hormonal treatments typically only relieve symptoms to a variable and unpredictable extent and side effects are usually observed. Novel drugs such as oxytocin antagonists (6), dopamine agonists (7, 8), and anti-platelet therapy (9) have been brought up and holds promising in treating adenomyosis. Unraveling the therapeutic mechanism of those novel drugs is needed in the future. An in-depth investigation of the etiology and pathogenesis of adenomyosis could also accelerate novel drug development.

Despite several hypotheses proposed so far including microtrauma of the junctional zone, metaplasia of stem cells in the myometrium, and invagination of endometrial tissue into the myometrium, the precise pathogenesis of adenomyosis remains unknown (10, 11). Previously, common features were discovered including enhanced proliferation and high invasive capacity of endometrial cells derived from women with adenomyosis (12, 13). A recent study utilizing single-cell transcriptomic technology revealed that enhanced endometrial cell proliferation, migration, and resistance to apoptosis contribute to the invasion of the endometrium into the myometrium in women with adenomyosis (14). Moreover, an increasing number of molecular studies have reported abnormal activation of canonical pathways in the endometrium of women with adenomyosis such as eukaryotic initiation of factor-2 (EIF2) signaling, oxidative phosphorylation, mTOR, IL-6, ERK/MAPK, and TGF-beta signaling as well as altered expression of estrogen and progesterone receptors (15–18). Studies have also reported that dysregulation of long non-coding RNAs and microRNAs may contribute to the aberrant gene functions observed in the endometrial and myometrial compartments of adenomyosis patients (15, 16).

It has been proposed that increased levels of prolactin (PRL) may contribute to the development of adenomyosis. PRL is mainly produced and secreted by the pituitary gland but also in small amounts in the human endometrium and myometrium and the decidua during pregnancy (17, 18). In murine uteri, minimal elevation of serum PRL was sufficient to cause adenomyosis (19, 20). Several animal models have shown increased uterine concentrations of PRL to be a risk factor for adenomyosis (21, 22).

Bromocriptine is a dopamine receptor agonist that is commonly used to treat hyperprolactinemia by activating dopamine D2 receptors and augmenting low hypothalamic dopamine secretions, therefore restraining high PRL levels through a negative feedback loop (23, 24). Our previous clinical trial showed that vaginal bromocriptine treatment for six months significantly improves symptoms of patients with diffuse adenomyosis including menstrual bleeding and pelvic pain (7, 8) and resulted in imaging characteristics changes following treatment (7). However, the underlying mechanism of bromocriptine in reducing adenomyosis-associated symptoms remains unknown.

The objective of the study was to investigate the effect of bromocriptine on endometrial tissue from women with adenomyosis *in vivo* and primary endometrial stromal cells from the same women *in vitro*. To achieve this, we tested the expression of a proliferation marker and prolactin on endometrial tissue before and after bromocriptine treatment, cultured primary endometrial stromal cells from women with adenomyosis, and assessed their proliferation and migration ability while exposing the cells to bromocriptine treatment *in vitro*. To further explore the molecular mechanism of action of bromocriptine, small RNA sequencing was conducted on endometrial tissue to identify differentially expressed microRNAs. Predicted target genes of differentially expressed microRNAs were further analyzed using KEGG functional pathway enrichment.

Materials and methods

Specimen collection

The study was approved by the regional ethics board at Karolinska Institutet, Stockholm, Sweden (2013/2060-31/1) and registered at Eudract.ema.europa.eu (EudraCT 2013-004409-14). Informed consent was given to and signed by the study participants before commencing any study-related activity. In total, six patients diagnosed with diffuse adenomyosis without other known gynecological disorders such as endometriosis or myomas were recruited in this study. All patients had regular menstrual cycles. A daily dose of 5 mg bromocriptine was provided to patients *via* the vaginal route of administration for six months, as described in the clinical trial (8). Diffuse adenomyosis was clinically diagnosed based on symptoms and transvaginal sonography and magnetic resonance imaging. All the endometrial biopsies from women with adenomyosis before and after bromocriptine treatment were collected during the proliferative phase according to the last menstrual period. The collected fresh specimens were immediately fixed, frozen, and isolated for the following experiments.

Immunohistochemistry staining

Formalin-fixed and paraffin-embedded specimens were used for immunohistochemistry staining. Six baseline samples and four after-treatment samples were included. Fewer after-treatment samples were included due to sample loss. For each sample, three replicates were prepared. 5 µm sections of paraffin-embedded endometrial tissues were prepared and immunohistochemistry staining was performed using a standardized protocol (25). Primary antibody against Ki67 (catalog no. NB110-89717, Novus biologicals, Biotechne, USA) were diluted in 1:200 using diluent DaVinci Green (Biocare Medical, Concord, CA) and incubated overnight at 4 °C. Rabbit MACH 3TM Probe and its respective HRP polymer (Biocare Medical, Concord, CA) and Betazoid DAB Chromogen (Biocare Medical, Concord, CA) were used to detect the antibody. Finally, tissue sections were counterstained using hematoxylin (Vector Laboratories, Inc., Burlingame, CA) and mounted using the xylene-based medium Pertex® (Histolab, Gothenburg, Sweden). Immunopositivity for Ki67 stained areas (six randomly selected areas per slide) was analyzed at 20x magnification using Image J software.

Endometrial stromal cell isolation and identification

Primary endometrial stromal cells from women with adenomyosis were isolated according to a protocol with minor modifications (25). Briefly, endometrial tissues were homogenized and treated in sequential steps of pancreatin-0.05% trypsin enzymatic solution, collagenase 4 (0.1 U/ml), and DNase I (16 µg/ml) solution in Ca2+/Mg2+-free PBS (Gibco® Thermo Fisher Scientific, Sweden) and incubated for 30 minutes at each step. Enzymatically digested cell suspensions were filtered through a 100 µM cell strainer to remove larger debris while collecting the flowthrough containing both the epithelial and stromal fractions. Later, these cell suspensions were adherent and expanded *in vitro* for two-three generations, frozen, and stored in liquid nitrogen for the following experiments. The isolated ESC was cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) and 1% Penicillin-Streptomycin solutions (Gibco, USA). When the cells reached 80-90% confluency, the cells were passaged, and the medium was changed every 2-3 days. Cyto-immunofluorescent staining for vimentin (Abcam, ab16700) and pan-cytokeratin (Abcam, ab86734) was applied for confirmation of stromal cell phenotype.

Cell viability assay

Cell viability assay was assessed on ESC at baseline for determining the optimal half-maximum inhibitory concentration (IC50) under the treatment of bromocriptine (2-bromo-a-ergocryptine methane sulfonate salt). Bromocriptine was purchased from Sigma-Aldrich (Sweden, AB). 7.5mg bromocriptine powder was dissolved in 1 ml of absolute ethanol to get a 10 mM as stock concentration. Cell complete medium was used to prepare a bromocriptine working solution. CellTiter-Blue® Cell Viability kit (Catalog no.G8081, Promega Biotech AB, Stockholm, Sweden) was used to detect the fluorescence intensity after 48 hours of bromocriptine treatment. Bromocriptine dissolved in ethanol was added to the culture medium of controlled cells. The kit applies a fluorescence-based method by detecting the reduction of the indicator dye, resazurin. The fluorescent signal from the CellTiter-Blue® Reagent is proportional to the number of viable cells. Briefly, cells were seeded in 96-well culture plates at a concentration of 5000 cells/100ul per well. After cell adherence, various concentrations of bromocriptine (5, 10, 20, 40, 80, 160, 320 µM) were added accordingly in a 96-well plate for 48 hours. CellTiter-Blue® reagents were added 4 hours before the end of treatment. A 96-well filter-based multi-mode microplate reader FLUOstar Omega (BMG LABTECH, Ortenberg, Germany) was used to detect fluorescence intensity at 590 nm, normalized to the respective vehicle treatment. The drug dose-response inhibitory curve was plotted by GraphPad 9.0 to capture the IC50 value.

Cell proliferation assay

The proliferative ability of ESC after treatment with bromocriptine was assessed using the BrdU cell proliferation ELISA kit (catalog no. ab126556; Abcam) in accordance with the manufacturer's protocol. Briefly, cells were seeded in a 96-well plate at a concentration of 5000 cells/well and incubated for 24 hours. Cells were then treated with bromocriptine for 48 hours. 20µL BrdU was added at least 2 hours before the termination of treatment. Next, cells were fixed for 30 minutes and incubated with the human anti-BrdU antibody for 1 hour at room temperature. After washing with washing buffer three times, 100 ul peroxidase goat anti-mouse IgG was added to each well and incubated for 30 minutes. In the end, absorbance was

measured at 450 nm wavelength by a microplate reader FLUOstar Omega (BMG LABTECH, Ortenberg, Germany).

Migration assay

To test the impact of bromocriptine on cell migration *in vitro*, a wound healing assay, and transwell migration assay was utilized to evaluate the horizontal and vertical migratory ability respectively. For the wound healing assay, cells were seeded in Culture-Insert 2 Well in a μ-Dish35mm, high (ibidi, 80206, Germany). After attachment, the inserts were gently removed to create gaps, and cells were treated with or without bromocriptine. Images were taken at different time points after insert removal (0, 12, 24, 36h). The percentage of the area covered by migrated cells was calculated by ImageJ software. For the transwell migration assay, 2×104 cells were seeded on 8.0 μm transwell migration (catalog no.353097, Corning) in a 24-well plate (catalog no.353504, Corning) containing a concentration of 2% fetal bovine serum (FBS) in DMEMF12 medium. The lower chamber contained 10% FBS as a chemoattractant. After 12, 24, and 36 hours, inserts were taken out and fixed with methanol, and washed with PBS. The migrated cells on the bottom of the inserts were stained with crystal violet. The non-migrated cells on top of the inserts were removed by gently wiping them with a cotton swab. The number of stained cells was counted in four representative areas in each well under 4X magnification using a light microscope (Nikon TS100).

Real-time PCR

Total RNA was extracted from the bromocriptine-treated ESC as well as frozen tissues at baseline and after bromocriptine treatment using a Zymo Quick-RNA microprep kit (Zymo, USA) according to manufacturers' protocol. The cell RNA was only extracted from three samples due to the remaining three samples were of higher passage. The eluted RNA was used immediately for cDNA conversion. cDNA was generated using SuperScript® VILO™ kit (Invitrogen®, Thermo Fisher Scientific, Waltham, USA). Shortly, the protocol required the preparation of an RNA-free master mix which was added to 0.2 ml PCR tubes along with RNA from each sample. The following thermocycling conditions were used to generate cDNA: 10 minutes at 25 degrees, 60 minutes at 42 degrees, and finally 5 minutes at 85 degrees. The Taqman® gene probe for Ki67 (Hs01032443_m1) was used for real-time PCR. The real-time PCR reaction condition consists of 20 seconds of holding stage at 95 degrees and 40 cycles of a one-second denaturation at 95 degrees and then 20 seconds of annealing and extension at 60 degrees. Gene expressions were performed and quantified by a StepOne Plus Real-time PCR instrument (Applied Biosystems, Foster City, CA, USA). Experiments were performed in triplicates. Ribosomal RNA 18s (4319413E) was used as a housekeeping gene to normalize the expression of the target gene. Fold change was calculated using the comparative Ct method.

Enzyme-linked immunosorbent assay

Protein lysates were extracted from endometrial tissues collected at baseline and bromocriptine treatment using Pierce ® RIPA lysis and extraction buffer (catalog no. 89900; Thermo Fisher Scientific, Waltham, USA) supplemented with complete™, Mini, EDTA-free protease inhibitor cocktail (catalog no. 4693159001; Sigma-Aldrich, USA). Total protein was quantified using a Qubit protein assay kit. All samples were diluted to 50 ng/ml total protein. PRL levels were then measured using the Prolactin ELISA kit (catalog no. ab108679; Abcam, UK), according to the manufacturer's protocol. Briefly, this kit uses a competitive ELISA method wherein, the plate is pre-coated with capture antibody. Tissue lysate from endometrial samples and standards provided in the kit were dispensed as duplicates to each well along with Prolactin-HRP conjugates followed by 60 minutes of incubation. Later, they were detected using a TMB-substrate solution by measuring the absorbance at 450 nm. On the cellular level, the conditioned medium of cells was collected after 48 hours of treatment with 113.3 μM bromocriptine and centrifuged at 12000 rpm for 5 minutes at 4°C to remove cell debris. The supernatant was then stored in a -80°C freezer for the following assays. A Prolactin ELISA kit (catalog no. EHIAPRL, Invitrogen) was used to quantify the PRL level in the conditioned medium. Briefly, the standards were diluted accordingly, and samples were diluted 50-fold from the original conditioned medium. 50 μl diluted medium was added per well. After adding the stop solution, the absorbance value was measured by a microplate reader FLUOstar Omega (BMG LABTECH, Ortenberg, Germany) at the wavelength of 450nm.

Small RNA sequencing

Library preparation: Total RNA was extracted from tissue stored in RNA later using RNeasy total RNA Kit (Qiagen, Hilden, Germany) as per the manufacturer's protocol. cDNA libraries for small RNA sequencing were constructed according to a highly sensitive small RNA sequencing protocol (26) with 1ng of total RNA as starting material. Each sample was indexed using customized barcodes (IDT Technologies, Germany), and the 10ng library from each is pooled and sequenced. A thermal cycler from BIOER Life Touch (Techtum, China) was used in all the steps. The final cDNA libraries quality was checked on a High Sensitivity DNA chip (Agilent Technologies, USA) using Agilent 2100 Bioanalyzer System (Agilent Technologies, USA). DNA quantity was determined with Qubit Flex Fluorometer (Invitrogen, Singapore) using the Qubit 1X dsDNA High Sensitivity Kit (Invitrogen, Oregon, USA). Sequencing was performed on the Illumina NextSeq 550 platform with 1x75 basepairs, and single-end reads at the Bioinformatics and Expression Analysis core facility at Karolinska University Hospital, Sweden.

Bioinformatics analysis

The bioinformatics analysis pipeline of small RNA sequencing is mainly adopted from a previously published pipeline with minor

modifications (27). Briefly, quality control of the FASTQ files was done using the FastQC (v0.11.9) software. In the following preprocessing steps, UMI sequences were removed and appended to the read's header for later UMI analysis. Reads were further trimmed for Illumina 3'adapter and the two cytosine-adenine (CA) bases linked to the UMI. The trimmed reads were aligned to the hg38 genome using STAR (version 2.7.2a). A read length filter of maximal 40nt was subsequently applied to the aligned reads (28). PCR duplicates were removed to create absolute molecule counts, and precursor molecule reads were filtered (27) and annotated using Mirbase (miRNAs) and GtRNAdb (tRNAs). The Bioconductor software DESeq (version 1.24.0) was used to identify differentially expressed miRNAs between the groups. Differentially expressed miRNAs with a false discovery rate (FDR) < 0.05 and a fold change (FC) of < -2 or > 2 were considered biologically and statistically significant. Raw data files are deposited in NCBI's Gene Expression Omnibus and are accessible using the GEO Series accession number: GSE207522. Experimentally validated target genes for selected miRNAs of interest were identified using MiRTarBase (Release 8.0) (26). Target genes with both strong and weak experimental evidence were included in the downstream analysis. Biological pathways enriched among the miRNA target genes were analyzed using the web tool g:Profiler (version e101_eg48_p14_baf17f0) (29) by utilizing the default g:SCS algorithm. An adjusted p-value of <0.05 was considered statistically significant.

Statistical analysis

GraphPad Prism 9 (GraphPad Software Inc., USA) was used for statistical analysis and graphical illustrations. The continuous variables were described as the mean ± standard deviation (SD). Two-sided Mann-Whitney Test was performed to test the Ki67 protein and gene level difference, and BrdU proliferation level difference. Two-sided Wilcoxon matched-pairs signed rank test was performed to test the tissue PRL level. IC50 was obtained by generating a dose-response curve. Two-sided paired t-test was performed to determine the PRL level in the conditioned medium. P<0.05 was considered statistically significant.

Results

Bromocriptine exhibits an anti-proliferative effect on endometrium from women with adenomyosis

As shown in the study flow chart (Figure 1), we determined the expression level of the proliferative marker, Ki67 in the endometrial tissue from baseline and after bromocriptine treatment. To further investigate the anti-proliferative effect of bromocriptine. The primary endometrial stromal cells (Figure 2A) of women with adenomyosis were isolated and identified by vimentin and cytokeratin immunofluorescent staining (Figure 2B). Cells were treated with different doses of bromocriptine (5, 10, 20, 40, 80, 160, 320 μM) for 48 hours, and cell viability decreased in a dose-dependent manner, showing a cytotoxic effect. The half-maximal inhibitory concentration (IC50) value of bromocriptine towards ESC was 113.3 μM (Figure 2C). The results showed a significant reduction of Ki67 protein expression in endometrial tissue from women with adenomyosis after bromocriptine treatment (Figures 3A, B). Furthermore, the BrdU ELISA assay showed that the proliferative ability of ESC decreased significantly after treatment with a 113.3 μM dose of bromocriptine for 48 hours (Figure 2D). Ki67 gene expression was also reduced in the ESC treated with bromocriptine *in vitro* (Figure 2E). In summary, we found that bromocriptine could inhibit endometrial proliferation in adenomyosis both *in vivo* and *in vitro*.

Bromocriptine reduces the migratory ability of endometrial stromal cells in adenomyosis

It has been reported that the high invasiveness of adenomyosis endometrium may be involved in the development of adenomyosis (12). To explore the effect of bromocriptine on the migratory ability of ESC, the cells were cultured in DMEM/F12 medium with 2% fetal bovine serum and 113.3 μM bromocriptine. DMEM/F12 with 2% fetal bovine serum was used as the control group. After 24 hours and 36 hours of treatment, the horizontal migratory ability of cells was significantly decreased in the 113.3 μM bromocriptine group compared with the control group (Figure 4A, B). For the transwell migration assay, DMEM/F12 medium with 10% fetal bovine serum was added to the lower chamber as an attractant to induce cell migration. In line with the above finding, the vertical migratory ability of adenomyotic ESC was also significantly lower in the bromocriptine group compared to the control group (Figure 4C, D). So, bromocriptine inhibits the migratory ability of endometrial stromal cells in adenomyosis *in vitro*.

Local prolactin level does not correlate with the clinical effects of bromocriptine

Several animal studies have shown that uterine PRL may contribute to the pathogenesis of adenomyosis (21, 22). To study the influence of bromocriptine on local PRL secretion, protein and RNA were extracted from the endometrial tissue of women with adenomyosis before and after bromocriptine treatment. No significant difference was observed in either PRL mRNA expression level or concentrations of PRL in endometrial tissue after bromocriptine treatment (Figures 3C, D). To explore whether bromocriptine affects the PRL secretion of adenomyotic ESC *in vitro*, PRL levels in a conditioned medium of ESC were measured after treatment with bromocriptine for 48 hours by ELISA. In line with the *in-vivo* data, no significant difference was identified between the bromocriptine-treated and untreated control groups (Figure S1). These results indicate that the effect of bromocriptine in treating adenomyosis-related symptoms may not correlate with local PRL levels in endometrial tissue from women with adenomyosis.

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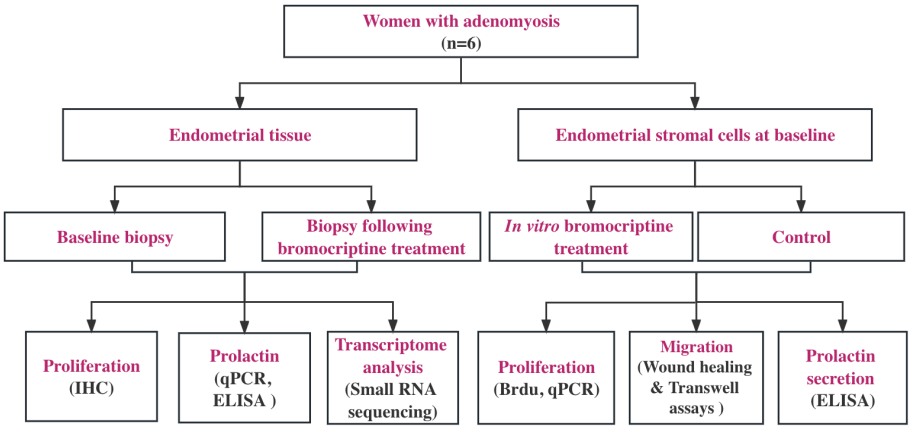

FIGURE 1
Study flow chart. Six women with diffuse adenomyosis who were previously enrolled in our pilot study (Andersson et al., 2019) were enrolled for the assessment of cellular and molecular changes induced by vaginal bromocriptine treatment. All the endometrial biopsies were collected during the proliferative phase according to the last menstrual period. The biological action of the bromocriptine was explored *in vivo* and *in vitro* separately. Proliferation analysis on tissues at baseline and after treatment was performed using immunohistochemistry (IHC). Local prolactin concentration and mRNA expression level were measured by ELISA and real-time PCR. Small RNA sequencing was performed, and the data were analyzed using bioinformatic software (Partek, R) to understand bromocriptine-induced changes on the transcriptomic level. For *in vitro* experiment, after determining the optimal half-maximal inhibitory concentration of bromocriptine, cell proliferation, and migration assays were used to evaluate the effect of bromocriptine on the cultured cells. The prolactin level in the conditioned medium of cells was measured by ELISA.

Upregulated microRNAs after bromocriptine treatment are predicted to affect proliferation-associated pathways

To better understand the molecular mechanism of bromocriptine in adenomyosis, small RNA sequencing was conducted on endometrium from women with adenomyosis before and after six months of vaginal bromocriptine treatment. In total, 27 miRNAs (-2 < FC < 2; *P*-value <0.05) were significantly upregulated after bromocriptine treatment (Table 1). To explore how the differentially expressed miRNAs may be involved in the treatment effect of bromocriptine, we predicted target genes of the differentially

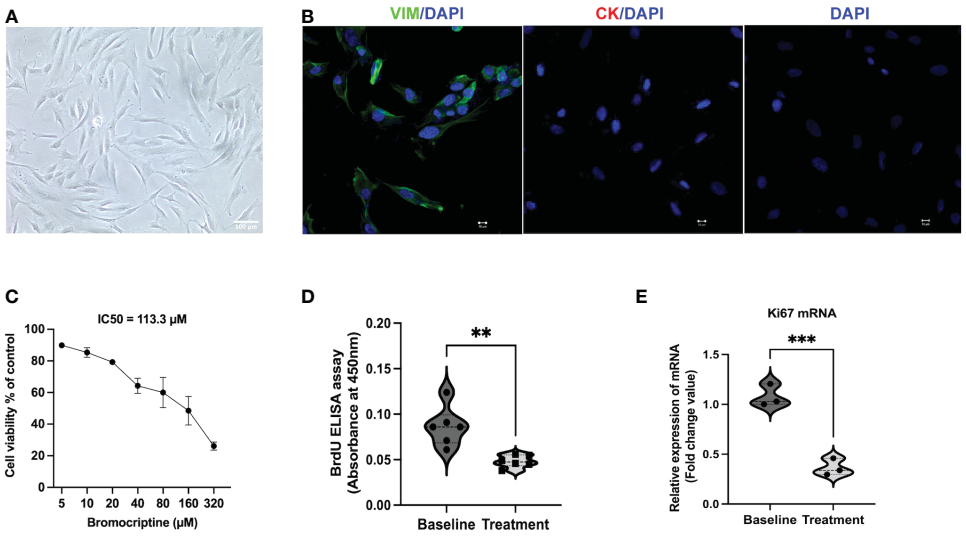

FIGURE 2
Identification of endometrial stromal cells derived from women with adenomyosis and the effect of bromocriptine on cell proliferative ability *in vitro*. **(A)** The primary endometrial stromal cells in adenomyosis showed a typical spindle-shaped morphology under a light microscope (Magnification of 20×, scale bar:100 μm). **(B)** Cultured cells were positive for vimentin (Green) and negative for cytokeratin (Red) by immunofluorescent staining. **(C)** Effect of various doses of bromocriptine (5, 10, 20, 40, 80, 160, 320 μM) on endometrial stromal cells (n=3). After 48 hours treatment of with bromocriptine, the viability of the cells decreased dose-dependently with an IC50 value of 113.3 μM. **(D)** Bromocriptine inhibited the proliferation of endometrial stromal cells by BrdU proliferation assay. **(E)** Effect of 113.3 μM bromocriptine treatment for 48 hours on Ki67 mRNA expression in cultured ADS-ESCs. 18sRNA was used as a reference gene. The analysis and plot were generated by GraphPad 9.0. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences.

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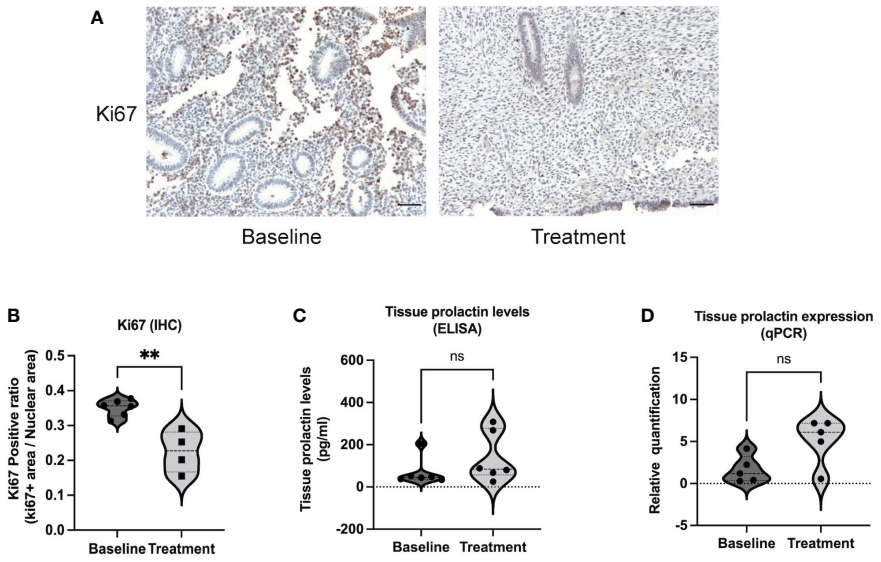

FIGURE 3
Ki67 and prolactin expression in endometrial tissue between baseline and bromocriptine treatment in women with adenomyosis. **(A)** Representative images of Ki67 immunohistochemistry staining at baseline and after treatment (Magnification of 20×, scale bar:100 μm). **(B)** The Ki67 positive ratio in endometrial tissue was quantified by ImageJ. The protein levels of Ki67 decreased significantly after the treatment of bromocriptine (n=6 for the baseline and n=4 for the treatment). **(C)** Prolactin concentration in endometrial tissue was evaluated by ELISA (n=6 both for the baseline and treatment). **(D)** Tissue prolactin mRNA expression levels were determined using real-time PCR (n=5 both for the baseline and treatment). The analysis and plot were generated by GraphPad 9.0. Two-tailed Mann-Whitney U test was performed. $^{**}P < 0.01$ indicates a significant difference. ns indicates no significant difference.

expressed miRNAs followed by KEGG pathway enrichment analysis. In total, 27 miRNAs were predicted to target 572 genes. Pathway enrichment analysis further showed that the predicted target genes of the miRNAs were significantly enriched in 122 KEGG pathways. Out of these 122 pathways, 21 signaling pathways were associated with cell proliferation and apoptosis (Figure 5).

Discussion

In the present study, we investigated the cellular and molecular changes of the endometrium after bromocriptine treatment in women with adenomyosis. Our results revealed that bromocriptine inhibits the proliferation of endometrial cells in

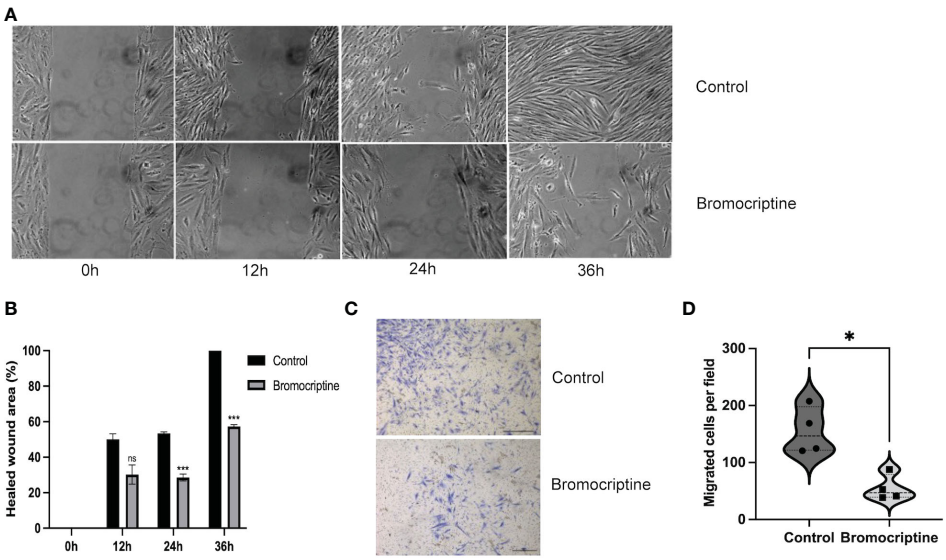

FIGURE 4
Effect of bromocriptine on cell migration ability. **(A)** Representative bright-field images of the wound healing area at 0h, 12h, 24h, and 36h (Magnification of 20×). **(B)** Statistical analysis of healed wound area in bromocriptine and control groups. A dose of 113.3 μM bromocriptine treatment significantly inhibited the cell's horizontal migratory ability at 24h and 36h. **(C)** Representative images of the bottom surface of a transwell migration assay in endometrial stromal cells (Magnification of 4×, scale bar:500 μm). **(D)** Quantification of migrated cells. The number of migrated cells in the bromocriptine group reduced significantly compared to the control group. The analysis and plot were generated by GraphPad 9.0. $^{*}P < 0.05$, $^{***}P < 0.001$, ns indicates no significant difference.

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TABLE 1 27 upregulated differentially expressed microRNAs in women with adenomyosis after bromocriptine treatment compared to before bromocriptine treatment.

Differentially expressed microRNAs (Treatment Vs Baseline)	Mean counts (Baseline, B)	Mean counts (Treatment, T)	Fold change (T Vs B)	Adjusted P value (T Vs B)
hsa-let-7a-3p	2	21	7,42	0,01543418
hsa-miR-210-3p	3	25	5,94	0,03466447
hsa-miR-486-3p	2	12	5,31	0,04993067
hsa-miR-106b-3p	20	135	5,04	0,01543418
hsa-miR-324-5p	1	6	4,79	0,03483399
hsa-miR-451a	54	291	4,38	0,03483399
hsa-miR-486-5p	649	3394	4,20	0,034833989
hsa-miR-497-5p	4	16	3,67	0,02510916
hsa-miR-185-5p	89	408	3,55	0,02510916
hsa-miR-1307-3p	5	20	3,31	0,049930675
hsa-miR-25-3p	112	473	3,20	0,034833989
hsa-miR-199a-3p	36	146	3,19	0,02510916
hsa-miR-3184-5p	6	23	3,11	0,034833989
hsa-miR-99b-3p	2	9	3,05	0,049930675
hsa-miR-106b-5p	3	12	3,01	0,034833989
hsa-miR-221-3p	25	96	2,92	0,034833989
hsa-miR-22-3p	101	365	2,82	0,027744514
hsa-miR-21-5p	293	1031	2,71	0,034833989
hsa-miR-425-5p	11	40	2,69	0,015462837
hsa-miR-31-5p	18	61	2,64	0,015434175
hsa-miR-30b-5p	7	23	2,50	0,034833989
hsa-miR-363-3p	50	166	2,49	0,034833989
hsa-miR-181a-5p	162	476	2,38	0,015434175
hsa-miR-361-3p	31	83	2,19	0,034833989
hsa-miR-103b	62	165	2,17	0,034664469
hsa-miR-30e-5p	228	650	2,16	0,049930675
hsa-let-7d-3p	14	36	2,10	0,034833989

women with adenomyosis both *in vitro* and *in vivo*. Furthermore, bromocriptine also reduces the migratory ability of ESC *in vitro*. In line with these results, small RNA sequencing data further showed that bromocriptine treatment upregulated the expression of several miRNAs which were predicted to affect biological pathways associated with cell proliferation. Overall, the findings of this study lay the foundation for further exploration of the potential clinical utility of bromocriptine as an effective treatment for adenomyosis.

Common features of adenomyosis include the increased capacity of endometrial cells to proliferate, migrate and invade the myometrium (11). Several studies suggested that the enhanced proliferative and migratory properties of endometrial cells derived from adenomyosis may contribute to the infiltration of endometrial cells in the myometrium as a critical step in disease development (30–32). The cell proliferation marker, Ki67 was significantly decreased after bromocriptine treatment *in vivo*. Moreover, we found that bromocriptine inhibited the proliferation of ESC cultured *in vitro* in a dose-dependent way. 113.3 μM was found to be the optimal dosage for bromocriptine to exert inhibitory effects. Future *in vivo* studies for the selection of the optimal dose of bromocriptine for future clinical use is required. In addition to cell proliferation, enhanced cell migratory ability could also contribute to the development of adenomyosis. Hence, the effect of bromocriptine on the migratory ability of stromal cells was evaluated by scratch and transwell migration assays. Our results suggest that bromocriptine decreases the proliferation and migration potential of endometrial cells in adenomyosis.

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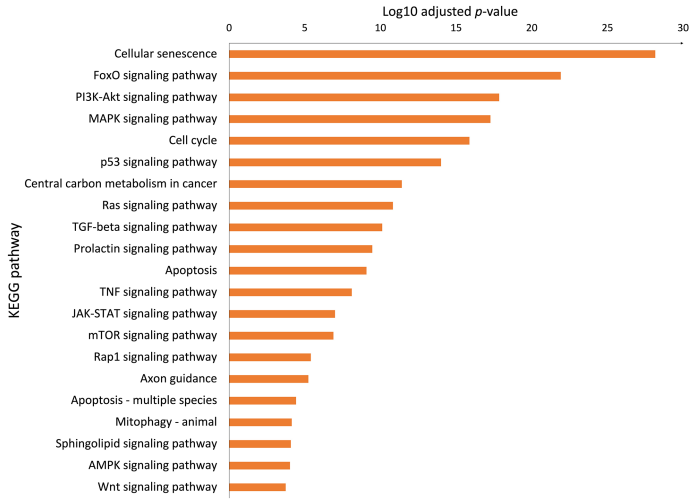

FIGURE 5
Biological pathways associated with cell proliferation and apoptosis. The Bar chart showed significant KEGG pathways involved in cell proliferation and apoptosis enriched the upregulated microRNAs (adjusted P-value < 0.05, selected from 122 pathways).

MicroRNAs are a group of small nucleotides that usually act as gene silencers. They play important roles in regulating gene expression, exerting many biological actions such as anti-proliferative, anti-migrative, and pro-apoptotic effects. Adenomyosis has been linked to changes in the miRNA profile of the endometrium (16). In the above study, several differentially expressed miRNAs expressed in the endometrium of adenomyosis patients have been suggested as diagnostic markers with relatively high sensitivity and specificity. In our study, we speculate that bromocriptine exerts an anti-cell proliferation action by silencing specific gene expression by activating several miRNAs expression. Sheu et al. reported that upregulation of hsa-miR-486-3p results in the downregulation of four genes (DDX11, E2F1, NPTX1, and PLXNA4), which may contribute to decreased proliferation of fibroblasts from idiopathic pulmonary fibrosis (33). In line with the above study, our study showed that hsa-miR-486-3p was one of the top three upregulated miRNAs in the endometrium which may be involved in the decreased cell proliferation after the bromocriptine treatment. Another study indicated that 15 upregulated miRNAs in senescent human fibroblast cells may act as promoters of cell cycle arrest and telomere degradation, resulting in a senescent phenotype in the end (34). Among the above 15 miRNAs, hsa-miR-181a-5p and hsa-miR-221-3p overlapped with our results. The two overlapping miRNAs may reflect the possible role of cellular senescence in the bromocriptine-induced changes in the endometrium of women with adenomyosis. This was further supported by the biological pathway analysis which showed that cellular senescence was among the most significantly enriched pathways. Previously, studies have reported that aberrant activation of key growth signaling pathways such as PI3K-AKT signaling (15) and ERK/MAPK signaling (30) were present in the endometrium of women with adenomyosis. In line with the above reports, we found that PI3K-AKT, Cell cycle, JAK-STAT, and several signaling pathways associated with cell proliferation are involved in the bromocriptine effects on adenomyosis-related symptoms. Besides the proliferation pathways, we do see several KEGG pathways associated with cell migratory function such as TGF-beta, and Wnt signaling pathways. Those signaling pathways are not at the top of the KEGG ranking. The key regulators may intertwine with proliferation pathways and might involve many cell biology functions and therefore may facilitate the invasion of stromal cells to the myometrium.

As a key reproductive hormone, PRL contributes to many physiological functions in maintaining women's health such as breast development and lactation. Except for the principal site of PRL production, the anterior pituitary gland, several new potential sites have been found such as endometrial and myometrial tissue (35, 36). PRL is synthesized in the human endometrium during the late luteal phase and pregnancy. Progesterone stimulates PRL secretion and its receptor expression when endometrial stromal cells differentiate into decidual cells (37, 38). However, PRL local secretion pattern in healthy human endometrium during the remaining menstrual cycle is unclear so far. It is reported that uterine prolactin has the same protein structure as pituitary prolactin but does have a different 5' regulatory region (18). The prolactin receptor is present in the uterus and uterine prolactin also appears to act as a local growth factor and a smooth muscle cell mitogen (39). The tissue-specific secretion of PRL may also be influenced by serum PRL circulation, other hormones, and pathological conditions. As far as we know, the current evidence for the prolactin hypothesis was promoted by murine models of adenomyosis (19, 40). Subsequent studies suggested there may be evidence for the role of prolactin in human adenomyosis (41). To test the prolactin hypothesis, we compared the local tissue PRL level

before and after bromocriptine treatment. However, we did not observe a significant reduction in PRL level in the endometrium in women with adenomyosis after bromocriptine treatment. Furthermore, the PRL level in the conditioned medium of bromocriptine-treated stromal cells remained unchanged as well. These negative results might indicate that bromocriptine may not be involved in local PRL regulation in endometrial tissue. However, a small sample size can compromise the conclusions drawn from the study. Though PRL receptor was discovered both in the myometrium and endometrium indicating a possible role of PRL in the baboon uterus (40), the action-targeted locations and cell types within the uterus induced by PRL remain unclear. As far as we know, the current evidence for the prolactin hypothesis is mainly derived from animal studies. Hence, considering the complex pathologies of adenomyosis, more well-designed human studies including healthy controls are needed to elucidate PRL expression patterns in endometrial tissue, as well as the role of dopamine in PRL regulation in endometrial tissue in humans. Based on the current evidence from this study, we could conclude that the local endometrial PRL level does not correlate with the anti-proliferation effect of bromocriptine in adenomyosis.

In our previous pilot trial, we chose the vaginal route to obtain a high local uterine concentration of bromocriptine and to minimize side effects. Six months duration allowed the bromocriptine to fully exert its function, reflected in the improvement of symptoms. In the follow-up study now, the exploration of the *in vivo* and *in vitro* effects of bromocriptine treatment on adenomyosis shows that vaginal bromocriptine improved symptoms in adenomyosis could potentially be explained by inhibiting the proliferation of endometrial cells. However, there are several limitations of our study including the small sample size and lack of primary epithelial cells. The pathogenesis of adenomyosis is unclear, so far there is no single hypothesis that could explain all pathological alterations. Currently, many studies do observe higher proliferative and migratory properties of the endometrium of women diagnosed with adenomyosis. However, whether such endometrial changes are the cause or consequences of adenomyosis is still debated. Besides, other factors such as inflammation, hormonal imbalance, and abnormal neoangiogenesis may also interweave with endometrial alteration during the development of adenomyosis (42).

Study limitations include small sample size, the lack of functional experiments on endometrial epithelial cells, and the lack of in-depth downstream analysis of deregulated miRNAs, which are to be addressed in future studies. Our study is a small self-controlled trial, we focused on studying the changes in pathological endometrial tissue under the influence of bromocriptine in adenomyosis. In the future, more human studies are required to elucidate the prolactin hypothesis in adenomyosis and investigate the effect of bromocriptine on healthy endometrial tissue as well.

Current treatment options are limited and invasive for adenomyosis due to the unclear pathophysiology. More drugs should be developed specifically for adenomyosis. As a pilot study, the findings of our study revealed that bromocriptine could suppress the proliferation of endometrium in women with adenomyosis both *in vitro* and *in vivo*. Moreover, several upregulated miRNAs may be involved in the antiproliferative action of bromocriptine. Further research into the underlying roles of miRNAs, signaling pathways, and molecular actions of bromocriptine in the treatment of adenomyosis are needed in the future. We consider that this mechanism may partially explain the improved symptoms observed in women with adenomyosis after bromocriptine treatment.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE207522, GSE207522.

Ethics statement

The studies involving human participants were reviewed and approved by the regional ethics board at Karolinska Institutet, Stockholm, Sweden. The patients/participants provided their written informed consent to participate in this study

Author contributions

ES was responsible for the clinical trial hypothesis. JA and KG-D were responsible for the clinical trial conduct including regulatory approvals. SP-S, YT, NB, and KG-D formulated the experimental study design. SP-S, YT, and CF developed the secondary study objectives and experimental approaches. JA recruited the study participants and collected the endometrial biopsies. SP-S processed clinical samples and performed preparatory steps for small RNA sequencing. YT performed *in vitro* experiments. NB and CF prepared the small RNA libraries. NB, SP-S, CF, and EK performed the bioinformatic analysis of the small RNA data. YT and CF performed functional downstream analysis and interpreted the results. YT and SP-S wrote the manuscript. SP-S, CF, ES, JA, EK, DP, PL, and KG-D critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

ES receives active research support regarding adenomyosis R01HD105714 from the Eunice Kennedy Shriver Institute of Child Health and Human Development of the National Institutes of Health, Bethesda, MD USA, and serves as an Associate Editor for *Frontiers in Reproductive Health*.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1026168/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Prolactin level in conditioned medium. ns indicates no significant difference between the group with adding bromocriptine and without adding bromocriptine.

References

1. Upson K, Missmer SA. Epidemiology of adenomyosis. *Semin Reprod Med* (2020) 38:089–107. doi: 10.1055/S-0040-1718920

2. Halvorson LM, Giudice LC, Stewart EA. Eye to the future in adenomyosis research. *Semin Reprod Med* (2020) 38:197–200. doi: 10.1055/S-0040-1721503

3. Cockerham AZ. Adenomyosis: A challenge in clinical gynecology. *J Midwifery Womens Health* (2012) 57:212–20. doi: 10.1111/j.1542-2011.2011.00117.x

4. Cozzolino M, Tartaglia S, Pellegrini L, Troiano G, Rizzo G, Petraglia F. The effect of uterine adenomyosis on IVF outcomes: A systematic review and meta-analysis. *Reprod Sci (Thousand Oaks, Calif.)* (2022) 29:3177–93. doi: 10.1007/s43032-021-00818-6

5. Sharara FI, Kheil MH, Feki A, Rahman S, Klebanoff JS, Ayoubi JM, et al. Review_ current and prospective treatment of adenomyosis. *J Clin Med* (2021) 10:1–12. doi: 10.3390/jcm10153410

6. Mechsner S, Grum B, Gericke C, Loddenkemper C, Dudenhausen JW, Ebert AD. Possible roles of oxytocin receptor and vasopressin-1α receptor in the pathomechanism of dysperistalsis and dysmenorrhea in patients with adenomyosis uteri. *Fertil Steril* (2010) 94:2541–6. doi: 10.1016/J.FERTNSTERT.2010.03.015

7. Andersson JK, Pozzi Mucelli R, Epstein E, Stewart EA, Gemzell-Danielsson K. Vaginal bromocriptine for treatment of adenomyosis: Impact on magnetic resonance imaging and transvaginal ultrasound. *Eur J Obstet Gynecol Reprod Biol* (2020) 254:38–43. doi: 10.1016/J.EJOGRB.2020.08.040

8. Andersson JK, Khan Z, Weaver AL, Vaughan LE, Gemzell-Danielsson K, Stewart EA. Vaginal bromocriptine improves pain, menstrual bleeding and quality of life in women with adenomyosis: A pilot study. *Acta Obstet Gynecol Scand* (2019) 98:1341–50. doi: 10.1111/AOGS.13632

9. Zhu B, Chen Y, Shen X, Liu X, Guo SW. Anti-platelet therapy holds promises in treating adenomyosis: Experimental evidence. *Reprod Biol Endocrinol* (2016) 14:1–16. doi: 10.1186/S12958-016-0198-1/FIGURES/8

10. Zhai J, Vannuccini S, Petraglia F, Giudice LC. Review_ adenomyosis: Mechanisms and pathogenesis. *Semin Reprod Med* (2020) 38:129–43. doi: 10.1055/s-0040-1716687

11. Benagiano G, Brosens I, Habiba M. Structural and molecular features of the endomyometrium in endometriosis and adenomyosis. *Hum Reprod Update* (2014) 20:386–402. doi: 10.1093/humupd/dmt052

12. Mehasseb MK, Taylor AH, Pringle JH, Bell SC, Habiba M. Enhanced invasion of stromal cells from adenomyosis in a three-dimensional coculture model is augmented by the presence of myocytes from affected uteri. *Fertil Steril* (2010) 94:2547–51. doi: 10.1016/J.FERTNSTERT.2010.04.016

13. Li J, Yanyan M, Mu L, Chen X, Zheng W. The expression of Bcl-2 in adenomyosis and its effect on proliferation, migration, and apoptosis of endometrial stromal cells. *Pathol Res Pract* (2019) 215(8):152477. doi: 10.1016/J.PRP.2019.152477

14. Liu Z, Sun Z, Liu H, Niu W, Wang X, Liang N, et al. Single-cell transcriptomic analysis of eutopic endometrium and ectopic lesions of adenomyosis. *Cell Biosci* (2021) 11:51. doi: 10.1186/s13578-021-00562-z

15. Jiang JF, Sun AJ, Xue W, Deng Y, Wang YF. Aberrantly expressed long noncoding RNAs in the eutopic endometria of patients with uterine adenomyosis. *Eur J Obstet Gynecol Reprod Biol* (2016) 199:32–7. doi: 10.1016/J.EJOGRB.2016.01.033

16. Borisov E, Knyazeva M, Novak V, Zabegina L, Prisyazhnaya T, Karizkiy A, et al. Analysis of reciprocally dysregulated miRNAs in eutopic endometrium is a promising approach for low invasive diagnostics of adenomyosis. *Diagnostics* (2020) 10(10):782. doi: 10.3390/diagnostics10100782

17. Stewart EA, Jain P, Penglase MD, Friedman AJ, Nowak RA. The myometrium of postmenopausal women produces prolactin in response to human chorionic gonadotropin and alpha-subunit. *vitro. Fertil Steril* (1995) 64:972–6. doi: 10.1016/s0015-0282(16)57912-6

18. Gellersen B, Bonhoff A, Hunt N, Bohnet HG. Decidual-type prolactin expression by the human myometrium. *Endocrinology* (1991) 129:158–68. doi: 10.1210/ENDO-129-1-158

19. Fiçicioglu C, Tekin HI, Arioglu PF, Okar I. A murine model of adenomyosis: the effects of hyperprolactinemia induced by fluoxetine hydrochloride, a selective serotonin reuptake inhibitor, on adenomyosis induction in wistar albino rats. *Acta Eur Fertil* (1995) 26:75–9.

20. Singtripop T, Mori T, Min Kyun P, Sakamoto S, Kawashima S. Development of uterine adenomyosis after treatment with dopamine antagonists in mice. *Life Sci* (1991) 49:201–6. doi: 10.1016/0024-3205(91)90004-U

21. Mori T, Singtripop T, Kawashima S. Animal model of uterine adenomyosis: Is prolactin a potent inducer of adenomyosis in mice? *Am J Obstet Gynecol* (1991) 165:232–4. doi: 10.1016/0002-9378(91)90258-S

22. Łupicka M, Socha BM, Szczepańska AA, Korzekwa AJ. Prolactin role in the bovine uterus during adenomyosis. *Domest Anim Endocrinol* (2017) 58:1–13. doi: 10.1016/j.domaniend.2016.07.003

23. Besser GM, Pfeiffer RF, Thorner MO. Review_ ANNIVERSARY REVIEW: 50 years since the discovery of bromocriptine. *Eur J Endocrinol* (2018) 179:R69–75. doi: 10.1530/EJE-18-0378

24. Kok P, Roelfsema F, Frölich M, van Pelt J, Stokkel MPM, Meinders AE, et al. Activation of dopamine D2 receptors simultaneously ameliorates various metabolic features of obese women. *Am J Physiol Endocrinol Metab* (2006) 291(2006):E1038-43. doi: 10.1152/AJPENDO.00567.2005

25. Boggavarapu NR, Lalitkumar S, Joshua V, Kasvandik S, Salumets A, Lalitkumar PG, et al. Compartmentalized gene expression profiling of receptive endometrium reveals progesterone regulated ENPP3 is differentially expressed and secreted in glycosylated form. *Sci Rep* (2016) 6:33811. doi: 10.1038/SREP33811

26. Chou CH, Shrestha S, Yang CD, Chang NW, Lin YL, Liao KW, et al. miRTarBase update 2018: a resource for experimentally validated microRNA-target interactions. *Nucleic Acids Res* (2018) 46:D296–302. doi: 10.1093/NAR/GKX1067

27. Hagemann-Jensen M, Abdullayev I, Sandberg R, Faridani OR. Small-seq for single-cell small-RNA sequencing. *Nat Protoc* (2018) 13:2407–24. doi: 10.1038/s41596-018-0049-y

28. Faridani OR, Abdullayev I, Hagemann-Jensen M, Schell JP, Lanner F, Sandberg R. Single-cell sequencing of the small-RNA transcriptome. *Nat Biotechnol 2016 34:12* (2016) 34:1264–6. doi: 10.1038/nbt.3701

29. Raudvere U, Kolberg L, Kuzmin I, Arak T, Adler P, Peterson H, et al. g:Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). *Nucleic Acids Res* (2019) 47:W191–8. doi: 10.1093/NAR/GKZ369

30. Xiang Y, Sun Y, Yang B, Yang Y, Zhang Y, Yu T, et al. Transcriptome sequencing of adenomyosis eutopic endometrium: A new insight into its pathophysiology. *J Cell Mol Med* (2019) 23:8381–91. doi: 10.1111/jcmm.14718

31. Yuan B, Wang W, Zhao H, Wang L. Role of lncRNA TUG1 in adenomyosis and its regulatory mechanism in endometrial epithelial cell functions. *Endocrinology* (2022) 163:1–12. doi: 10.1210/endocr/bqac033

32. Wang Y-Y, Duan H, Wang S, Quan Y-J, Huang J-H, Guo Z-C. Elevated circular RNA PVT1 promotes eutopic endometrial cell proliferation and invasion of adenomyosis *via* miR-145/Talin1 axis. *Biomed Res Int* (2021) 2021, 8868700. doi: 10.1155/2021/8868700

33. Sheu CC, Chang WA, Tsai MJ, Liao SH, Chong IW, Kuo PL. Gene expression changes associated with nintedanib treatment in idiopathic pulmonary fibrosis fibroblasts: A next-generation sequencing and bioinformatics study. *J Clin Med* (2019) 8:308. doi: 10.3390/jcm8030308

34. Markopoulos GS, Roupakia E, Tokamani M, Vartholomatos G, Tzavaras T, Hatziapostolou M, et al. Senescence-associated microRNAs target cell cycle regulatory genes in normal human lung fibroblasts. *Exp Gerontol* (2017) 96:110–22. doi: 10.1016/j.exger.2017.06.017

35. Maslar IA, Riddick DH. Prolactin production by human endometrium during the normal menstrual cycle. *Am J Obstet Gynecol* (1979) 135:751–4. doi: 10.1016/0002-9378(79)90386-7

36. Walters CA, Daly DC, Chapitis J, Kuslis ST, Prior JC, Kusmik WF, et al. Human myometrium: a new potential source of prolactin. *Am J Obstet Gynecol* (1983) 147:639–44. doi: 10.1016/0002-9378(83)90441-6

37. Wetendorf M, DeMayo FJ. The progesterone receptor regulates implantation, decidualization, and glandular development *via* a complex paracrine signaling network. *Mol Cell Endocrinol* (2012) 357:108–18. doi: 10.1016/J.MCE.2011.10.028

38. Brosens JJ, Hayashi N, White JO. Progesterone receptor regulates decidual prolactin expression in differentiating human endometrial stromal cells. *Endocrinology* (1999) 140:4809–20. doi: 10.1210/ENDO.140.10.7070

39. Nowak RA, Mora S, Diehl T, Rhoades AR, Stewart EA. Prolactin is an autocrine or paracrine growth factor for human myometrial and leiomyoma cells. *Gynecol Obstet Invest* (1999) 48:127–32. doi: 10.1159/000010154

40. Frasor J, Gaspar CA, Donnelly KM, Gibori G, Fazleabas AT. Expression of prolactin and its receptor in the baboon uterus during the menstrual cycle and pregnancy. *J Clin Endocrinol Metab* (1999) 84:3344–50. doi: 10.1210/JCEM.84.9.5948

41. Taran FA, Weaver AL, Coddington CC, Stewart EA. Understanding adenomyosis: a case-control study. *Fertil Steril* (2010) 94:1223–8. doi: 10.1016/J.FERTNSTERT.2009.06.049

42. Zhai J, Vannuccini S, Petraglia F, Giudice LC. Adenomyosis: Mechanisms and pathogenesis. *Semin Reprod Med* (2020) 38:129. doi: 10.1055/S-0040-1716687

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Endometrial whole metabolome profile at the receptive phase: influence of Mediterranean Diet and infertility

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Introduction: Several metabolite classes have been identified in human endometrium, including lipids, nucleotides, amino acids, organic acids, and sugars. The first studies suggest the importance of metabolites in endometrial functions, as imbalance in uterine metabolites has been associated with low implantation rate and endometriosis. Nevertheless, most of studies have put emphasis on specific metabolite classes, and we lack the knowledge of the whole metabolome composition in human uterus. Further, a healthy dietary pattern has been shown to potentially protect against different endometrial dysfunctions and is a potential modulator of metabolomic composition and, consequently, the intrauterine microenvironment. The Mediterranean Diet (MD), characterized by a high intake of fruits, vegetables, cereals, nuts, legumes, fish, and olive oil, and a low consumption of meat, dairy products, and processed foods, has been associated with a wide range of benefits for health. Indeed, the MD pattern has displayed a beneficial role in endometriosis management and fertility; however, the relationship between the MD and the endometrial metabolome is still unknown. In our study, we set out to analyze receptive-phase endometrial metabolome profiles among women with infertility and their associations with MD.

Methods: The study included women with male factor infertility (n=8), unexplained infertility (n=10), recurrent implantation failure (n=14), and

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endometriosis (n=13). The endometrial metabolome was analyzed with ultrahigh-performance liquid chromatography-tandem mass spectroscopy (UPLC–MS/MS). The MD adherence of the participants was assessed using the 14-point MEDAS questionnaire of adherence to the MD.

Results: We provide the whole metabolome profile of the endometrium, where 925 different metabolites were identified. Among these metabolites, lipids comprised the largest part, where polyunsaturated fatty acids (PUFAs) prevailed. Women with endometriosis and recurrent implantation failure were found to have lower levels of PUFAs compared to women with male factor and unexplained infertility (i.e., no clear endometrial alterations), identifying a metabolome profile associated with infertility diagnoses where altered endometrial functions are suspected. Moreover, MD adherence seemed to be associated with the endometrial metabolomic profile in a manner dependent on the health status of the uterus.

Conclusion: The study findings provide insight into the molecular background of female infertility and lead to identification of potential molecular biomarkers and possibilities for modulating the endometrial microenvironment and, thereby, endometrial functions involved in embryo implantation and infertility.

KEYWORDS

endometriosis, endometrium, folic acid, lifestyle, metabolomics, Mediterranean diet, recurrent implantation failure, unexplained infertility

1 Introduction

The human endometrium is a dynamic tissue that undergoes continuous cycles of shed, repair, regeneration, and remodeling to prepare for the embryo implantation (1). Due to the complexity of the involved mechanisms and the relative lack of information, embryo implantation process in humans remains the "black box" (2). Unfavorable endometrial microenvironment could lead to endometrial dysfunction promoting different disorders including embryo implantation failure and infertility, abnormal uterine bleeding, endometriosis, and endometrial cancer among others (1, 3). In this sense, the novel "omics" techniques based on the massive molecular characterization of DNA, RNA, proteins, and other small molecules synthesized have allowed to define the molecular phenotype of the receptive-phase endometrium at the mid-secretory phase (so-called "window of implantation") and the genomic, epigenomic, proteomic, and metabolomic methods have helped to gain insight into the molecular markers of the mid-secretory endometrial functions in health and disease (3–8).

Metabolomics has emerged as a powerful tool to identify small molecules with a molecular mass below 1200Da that participate as intermediates and final downstream products of cellular processes (i.e., metabolites), which contribute to a close real-time understanding of the functional phenotype (9). Despite the growing number of metabolomic studies, the use of the whole metabolite profile analysis related to uterine health is still scarce. The first endometrial metabolome studies have analyzed women with endometriosis and have suggested several potential biomarkers of the disease (10–12). The whole metabolome profiling in the uterus of fertile and infertile women undergoing assisted reproductive techniques showed that specific metabolomic signatures contribute to beneficial endometrial microenvironment to achieve pregnancy (13–15). These are the first findings indicating that metabolomics is a powerful tool in unravelling uterine health and understanding receptive-phase endometrial functions to achieve successful embryo implantation.

Factors that may influence uterine microenvironment include nutrition together with other lifestyle factors. It has been previously shown that a healthy dietary pattern might protect against different endometrial dysfunctions such as infertility, endometriosis, polycystic ovary syndrome, dysmenorrhea, or endometrial cancer (16, 17). In fact, food groups, such as fruits and vegetables, could promote fertility; while other dietary elements, including trans-fatty acids, alcohol, and caffeine, have been associated with adverse effects on fertility (18). The Mediterranean Diet (MD), characterized by a high consumption of fruits, vegetables, cereals, nuts, legumes, fish, and olive oil, and a low consumption of meat, dairy products, and processed foods, has been associated with a wide range of benefits for health (19). Indeed, the MD has been shown to have a beneficial role in endometriosis management (20), fertility (21, 22), and polycystic ovary syndrome (23). However, although lifestyle interventions have shown to influence the uterine proteome (24), the relationship between the MD and the endometrial metabolome is still unknown. In the current study, we set out to analyze

receptive-phase endometrial metabolome profiles among women with infertility and their associations with the MD.

2 Materials and methods

2.1 Study subjects and design

This cross-sectional study was approved by the Ethics Committee of the Junta de Andalucía (CEIM/CEI 0463-M1-18r). All procedures carried out in the present study were in accordance with the last revised ethical guidelines of the Declaration of Helsinki and the legally enforced Spanish regulation, which regulates the clinical investigation of human beings (RD 223/04). Written informed consent was obtained from all subjects prior to inclusion.

Forty-five women of infertile couples were recruited at the Reproductive Unit (Virgen de las Nieves University Hospital, Granada) between March 2019 and April 2021. All participants attended the Reproductive Unit after at least 1 year of unprotected sex and not being able to conceive. The inclusion criteria involved women entering an infertility treatment. Infertility causes comprised endometriosis, recurrent implantation failure (RIF), unexplained infertility, or male factor infertility. Endometriosis was diagnosed by laparotomy or laparoscopic surgery and histological confirmation, and RIF was defined as repeated implantation failure after the transfer of 3 good-quality embryos. Unexplained infertility was considered when the medical examination yielded no information about reproductive complications in the couple. Lastly, male factor was defined considering the reference values of the World Health Organization manual for semen analysis (25). Patients with age ≥43 years old, with hormone therapy, gynecological tumors, systemic diseases, pelvic inflammatory disease, or other pelvic pathological conditions were excluded from the study.

Participants were invited to complete an extensive lifestyle questionnaire to collect information including demographic characteristics. Body mass index (BMI) was calculated from the measured weight and height data. Ovulation day was estimated using a digital ovulation test (ClearBlue, Swiss Precision Diagnostics GmbH, Geneva, Switzerland) to precisely schedule the endometrial biopsy collection 7-9 days after the luteinizing hormone (LH) peak (day LH+7-9, window of implantation). After insertion of the speculum, the endometrial tissue sample was collected using the endometrial curette device (Gynétics, Medical Products, Hamont-Achel, Belgium). All biopsies were placed in cryovials, were snap-frozen in the gas phase of liquid nitrogen and stored at -80°C until analysis.

2.2 Endometrial metabolomic profiling

The untargeted metabolomics of endometrial samples were analyzed at Metabolon Inc. (Morrisville, NC, USA) on a platform consisting of four independent ultrahigh-performance liquid chromatography-tandem mass spectroscopy (UPLC–MS/MS) instruments as previously described (26). Briefly, samples were prepared using the automated MicroLab STAR® system from the Hamilton Company. Several recovery standards were added prior to the first step in the extraction process for quality-control (QC) purposes. To recover chemically diverse metabolites, proteins and other macromolecules were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting supernatant was divided into 4 aliquots, then placed briefly in a TurboVap (Zymark, Hopkinton, MA, USA) to remove the organic solvent and stored overnight under nitrogen before preparation for analysis. The sample extract was then reconstituted in solvents compatible with each of the four methods. Thus, each aliquot was run using a different method: 1) acidic positive-ion conditions chromatographically optimized for more hydrophilic compounds (reverse phase [RP]/UPLC-MS/MS Pos Early); 2) acidic positive-ion conditions chromatographically optimized for more hydrophobic compounds (RP/UPLC-MS/MS Pos Late); 3) basic negative-ion-optimized conditions (RP/UPLC-MS/MS Neg). These methods used separate acid- and base-dedicated C18 columns (Waters UPLC BEH C18, 2.1×100 mm, 1.7 µm). The fourth aliquot was analyzed via negative ionization after elution from a hydrophilic interaction liquid chromatographic (HILIC) column (Waters UPLC BEH Amide, 2.1x150 mm, 1.7 µm) (HILIC/UPLC-MS/MS Polar). The details of the used solvents and chromatography are described before (27). All UPLC-MS/MS methods used a Waters ACQUITY UPLC (Waters, Milford, MA, USA) coupled to a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer (Thermo Fischer, Waltham, MA, USA) equipped with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer. The MS analysis alternated between MS and data-dependent MS^n scans using dynamic exclusion. The MS scan range varied slightly between methods but covered 70-1000 m/z and operated at 35,000 mass resolution.

Three types of controls were analyzed along with the experimental samples: 1) a pooled sample generated from a small volume of each experimental sample (i.e., technical replicate); 2) extracted water (i.e., blank); and 3) a cocktail of QC standards spiked into every analyzed sample for instrument performance monitoring. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the internal standards that were added to each sample prior to injection into the mass spectrometers (median RSDs = 3%). Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the pooled matrix samples, which were technical replicates of pooled samples (median RSD = 7%). Experimental samples and controls were randomized across the platform run.

2.3 Endometrial metabolome detection and identification

Raw data, peak-identified, and QC was extracted and processed using Metabolon's hardware and software as described previously (28). Endometrial metabolites were identified by comparison of the ion features in the experimental samples to reference libraries of

authenticated standards with known chromatographic retention index (RI), mass to charge ratios (m/z), and MS/MS spectral fragmentation signatures. Further, biochemical identification was based on 3 criteria: RI within a narrow RI window (150 RI units [≈10s]), accurate mass match (± 10ppm), and quality of MS/MS fragmentation match. While there may be similarities between these molecules based on one of these factors, the use of all three data points allows to distinguish and differentiate biochemicals. To ensure high quality of the dataset, QC and curation processes were subsequently used to confirm accurate and consistent chemical assignment and remove system artefacts and background noise. Peaks were quantified through area-under-the-curve analysis and these peak area values allowed the determination of relative quantification among samples (29).

Some compounds were detected by multiple methods (i.e., RP/UPLC-MS/MS Pos Early, RP/UPLC-MS/MS Pos Late, RP/UPLC-MS/MS Neg, or HILIC/UPLC-MS/MS Polar). The UPLC–MS/MS method used to evaluate each compound was pre-selected based on sensitivity and reproducibility criteria ("Platform" column of Supplementary Table S1).

2.4 Adherence to Mediterranean Diet

Adherence to the MD was assessed using the 14-point MEDAS questionnaire of adherence to the MD as previously reported and validated (30). The MEDAS questionnaire includes 12 questions related to frequency intake of key foods, and 2 questions related to specific dietary habits of the MD (Supplementary Table S2). Each question scores 0 or 1 point. The global score ranges from 0 to 14, being 0 points null adherence and 14 points complete adherence to the MD. Then, participants were categorized in high adherence (≥9 points) or low adherence (<9 points) to the MD, following previous categorization (30). The consumption of folic acid supplement was also registered among the participants with an additional question together with the MEDAS questionnaire (Supplementary Table S2).

2.5 Statistical analyses

The normal distribution assumption was tested using the Shapiro-Wilk test, visual histograms, and Q-Q plots. Non-normally distributed variables (i.e., endometrial metabolomic signature) were $\log_{10}$-transformed before further analysis. The baseline characteristics and outcomes of the study participants were expressed as mean ± standard deviation (unless otherwise stated). Differences in demographic characteristics between the subjects of our study were analyzed using independent sample *t*-test or chi-square tests where appropriate.

Multivariate statistical analyses (i.e., partial least squares discriminant analysis [PLS-DA]) were performed on the entire metabolomics dataset using the MetaboAnalyst 5.0 (https://www.metaboanalyst.ca) to investigate potential difference in the metabolomic signature between women with low and high adherence to MD, different infertility diagnoses, and between women supplemented with folic acid and women with no supplementation. To increase the importance of low abundance ions without significant amplification of noise, raw data were normalized by Pareto scaling (31).

To find further differences in metabolite composition between the different groups, one-way analysis of covariance (ANCOVA) controlling for age and BMI was applied. Besides, we conducted Pearson partial correlation analyses to examine the relationship between the adherence to the MD and the endometrial metabolomic signature adjusting for age and BMI. The correlation analyses were performed pooling all participants together and splitting the cohort based on the results of the uterine examination (see results section).

Normality and descriptive analyses were performed in Statistical Package for the Social Sciences (SPSS 28.0, IBM Corp, Armonk, NY, USA). All statistical and correlation analyses were performed using the R software (V.3.6.0; R Foundation for Statistical Computing). Whisker's plot and volcano plots were built using GraphPad Prism software v.9 (GraphPad Software, San Diego, CA, USA). All *p*-values were corrected by controlling for the multiple testing, False Discovery Rate (FDR) (32). Statistical significance was set p-value<0.05 after FDR correction.

3 Results

3.1 Study subjects

The study cohort included a total of 45 women with infertility. The characteristics of the participants are presented in Table 1. Participants were primarily Caucasian, with just one Hispanic woman.

Based on the MEDAS score, the participants were categorized as high adherence (n=25) or low adherence (n=20) to the MD. We also studied the differences in metabolome profiles depending on the infertility diagnosis to relate the uterine microenvironment with endometrial dysregulation. To increase the statistical power of our analysis and based on the thorough clinical examination, 27 patients with endometriosis and RIF were grouped as endometrial-factor infertile (EFI) and 18 women with male factor infertility and unexplained infertility were grouped together as no endometrial-factor infertility (NEFI) was detected. This grouping was further supported by the metabolome signatures detected (Figure 1), where women with RIF or endometriosis demonstrated similar metabolite profiles while women with male factor infertility and unexplained infertility grouped together. Statistical tests revealed that the different groups were age- and BMI-matched (Table 1).

3.2 Metabolome composition

Nine hundred twenty-five metabolites from diverse chemical classes were identified from endometrial tissue samples using an untargeted metabolomic approach. These 925 metabolites included amino acids, lipids, nucleotides, carbohydrates, and xenobiotics among others. The list of identified compounds is provided in

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TABLE 1 Patient's demographics based on the adherence to Mediterranean Diet (MD).

	Total (n=45)	High adherence to the MD (n=25)	Low adherence to the MD (n=20)	*p*-value[a]
Age (years)	34.62 ± 3.67	34.56 ± 3.82	34.7 ± 3.57	0.901
BMI (kg/m^2)	24.62 ± 4.19	25.13 ± 4.62	23.99 ± 3.6	0.368
Infertility diagnosis, n (%)				
Male factor	8 (18)	3 (12)	5 (25)	0.3
Unexplained infertility	10 (22)	8 (32)	2 (10)	
Recurrent implantation failure	14 (31)	7 (28)	7 (35)	
Endometriosis	13 (29)	7 (28)	6 (30)	
Uterine examination, n (%)				
No endometrial-factor infertility (NEFI)	18 (40)	11 (44)	7 (35)	0.54
Endometrial-factor infertility (EFI)	27 (60)	14 (56)	13 (65)	
Folic acid supplementation*, n (%)				
Yes	26 (58)	16 (64)	10 (50)	0.355
No	19 (42)	9 (36)	10 (50)	

Data values are presented as n (%) or mean ± standard deviation for continuous traits.
*The number of women varies due to nonresponse to this item: high adherence to the MD (N=23), low adherence to the MD (N=18).
[a]t-test (continuous variable) or Pearson's chi-square test of independence.
BMI, body mass index.

Supplementary Table S1. The most abundant metabolites in the entire cohort were N1, N12-diacetylspermine, adenosine 5'-monophosphate (AMP), 4-ethylphenylsulfate, guanosine 5'-monophosphate (5'-GMP), 2-hydroxy-3-methylvalerate, and UDP- glucuronate.

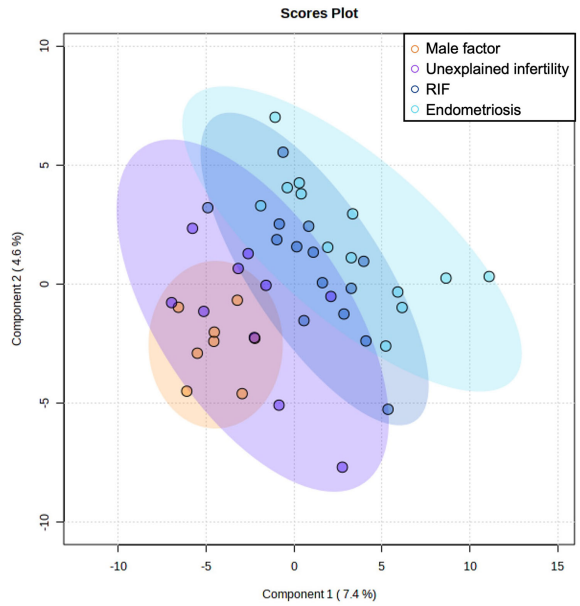

FIGURE 1
Multivariate Partial Least Squares Discriminant Analysis scores plot from women with male factor infertility, unexplained infertility, endometriosis, and recurrent implantation failure (RIF).

3.2.1 Metabolite profiles and MD adherence

When we compared metabolome based on adherence to diet, the PLS-DA scores plot did not show any different metabolomic signature between the study groups with high and low adherence to the MD (Figure 2A) and no significant differences were detected after correction between the metabolites and the different groups (Supplementary Table S3).

When focusing on metabolite classes, the adherence to the MD showed statistically significant correlations with amino acids and lipids levels (all *p*-values<0.05 after FDR correction; Figure 2B). Specifically, the adherence to the MD was positively correlated with amino acids related to the histidine metabolism (1-methyl-5-imidazoleacetate and 1-ribosyl-imidazoleacetate), to the methionine, cysteine, SAM, and taurine metabolism (N-acetylmethionine sulfoxide, 5-methylthioribose, and 3-sulfo-L-alanine), and the thyroxine amino acid (Figure 2B, Supplementary Table S4). In contrast, we observed that the adherence to the MD was negatively correlated with different amino acid metabolites (argininate, ornithine, betaine, spermidine, and aspartate) and with carnitines (e.g., deoxycarnitine, carnitine, malonylcarnitine, and 2-methylhexanoylcarnitine) (Figure 2B, Supplementary Table S4).

3.2.2 Metabolite profiles and endometrial-factor infertility

Comparing groups based on the uterine examination, the PLS-DA scores plot did not discriminate the metabolomic signature between patients with EFI and second group with NEFI (Figure 3A). However, dihomolinolenate (20:3 n-3 or n-6), linolenate (18:3 n-3 or n-6), and linoleate (18:2 n-6) metabolites related with the

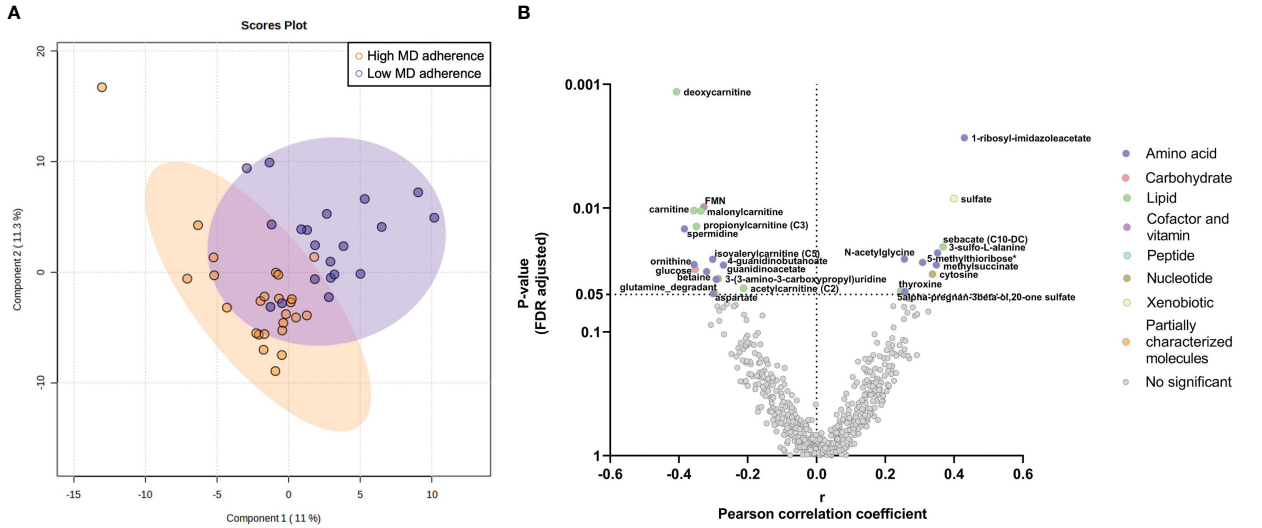

FIGURE 2
Endometrial metabolomic signature analysis in women with high and low adherence to Mediterranean Diet (MD). **(A)** Multivariate Partial Least Squares Discriminant Analysis scores plot from women with high and low MD adherence. **(B)** Partial correlation analyses between the MD global score and the endometrial metabolome. The X-axis represents Pearson partial correlations coefficients, whereas the Y-axis represents the FDR-adjusted *p*-values of the correlations. Grey dots represent non-significant correlations, whereas colored dots represent statistically significant correlations (*p*-value<0.05 after FDR correction).

polyunsaturated fatty acids (PUFAs) pathway were significantly increased in NEFI women compared to EFI women (all *p*-values<0.001 after FDR correction; Figure 3B, Supplementary Table S5).

Next, we performed the correlation analyses in parallel among patient group of women with EFI and among group of women with NEFI and we observed a higher number of significant correlations between the adherence to the MD and metabolites in women with EFI than in women with no detectable endometrial dysfunction (Figure 4). In the NEFI group, up to 31 metabolites correlated to MD. On one hand, the adherence to the MD was positively correlated to different fatty acids (2S,3R-dihydroxybutyrate, 2-hydroxystearate, and 2-hydroxypalmitate), to progestin steroids (pregnanediol-3-glucuronide, and 5alpha-pregnan-3beta,20beta-diol monosulfate) while was negatively correlated to metabolites involved in the xanthine metabolism (1,3,7-trimethylurate, 5-acetylamino-6-amino-3-methyluracil, caffeine, 1,7-dimethylurate, and 1-methylurate) (Figure 4A, Supplementary Table S4).

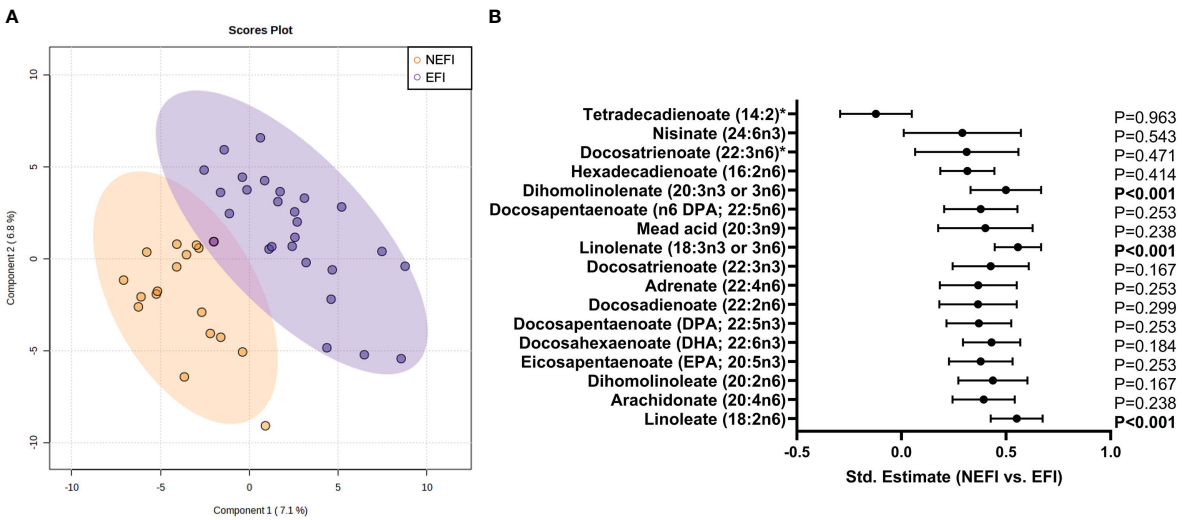

FIGURE 3
Endometrial metabolomic signature analysis in women with no detectable endometrial dysfunction in comparison to patient group with endometriosis and recurrent implantation failure. **(A)** Multivariate Partial Least Squares Discriminant Analysis scores plot from women with no detectable endometrial dysfunction and women with endometrial-factor infertility. **(B)** Statistical differences in long chain polyunsaturated fatty acid metabolism between women with no detectable endometrial dysfunction and women with endometrial-factor infertility. Statistical significance was set *p*-value<0.05 after FDR correction. NEFI, no endometrial-factor infertility; EFI, endometrial-factor infertility.

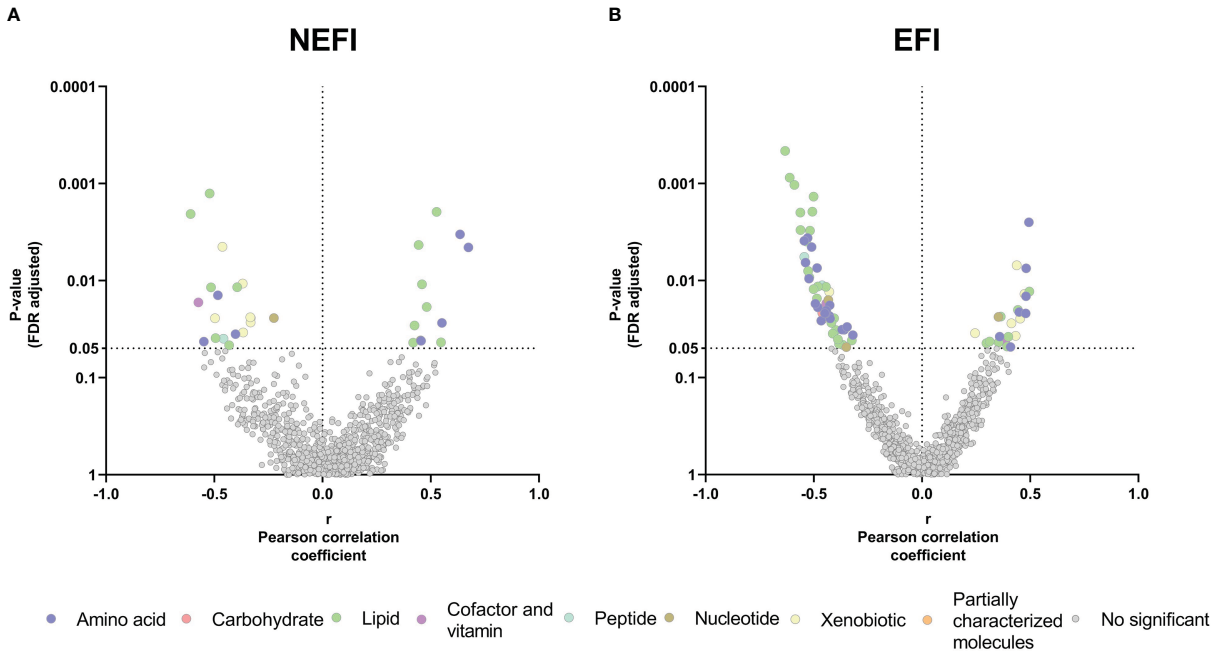

FIGURE 4
Relationship between the Mediterranean Diet adherence and the endometrial metabolome in **(A)** women with no detectable endometrial dysfunction and **(B)** patient group with endometriosis and recurrent implantation failure. The X-axis represents Pearson partial correlations coefficients, whereas the Y-axis represents the FDR-adjusted *p*-values of the correlations. Grey dots represent non-significant correlations, whereas colored dots represent statistically significant correlations (*p*-value<0.05 after FDR correction). NEFI, no endometrial-factor infertility; EFI, endometrial-factor infertility.

Contrary, in patients with EFI, a total of 73 metabolites correlated to MD. We observed a positive correlation between the adherence to the MD and metabolites involved in the xanthine metabolism (i.e., 5-acetylamino-6-amino-3-methyluracil, 1,3,7-trimethylurate, 1-methylxanthine, and paraxanthine) and in the metabolism of ceramides (i.e., dihydroceramides, hexosylceramides, and lactosylceramides) (Figure 4B, Supplementary Table S4). We also found in women with EFI, a negative correlation between the adherence to the MD and carnitine metabolites (e.g., carnitine, deoxycarnitine, or acyl-carnitines), primary bile acid metabolites (i.e., cholate, glycochenodeoxycholate 3-sulfate, glycochenodeoxycholate, and chenodeoxycholate), and different amino acid metabolites (e.g., aspartate, argininate, ornithine, orotidie, spermidine, betaine, or homocysteine) (Figure 4B, Supplementary Table S4).

3.2.3 Metabolite profiles and combination of MD adherence and endometrial factor

Additionally, we combined the 2 classification features (i.e., MD adherence and endometrial-factor infertility) for comparisons and 4 groups were established: high MD adherence and NEFI (n=11), high MD adherence and EFI (n=14), low MD adherence and NEFI (n=7), and low MD adherence and EFI (n=13) (Table 1). When we compared metabolome profiles among these groups, linolenate metabolite was significantly more present among women with high MD adherence and NEFI compared to women with low MD adherence and EFI (*p*-value=0.014 after FDR correction, Supplementary Figure S1).

3.2.4 Metabolite profiles and folic acid intake

Regarding the folic acid supplementation, the PLS-DA scores plot did not show any different clusters (Figure 5) nor were

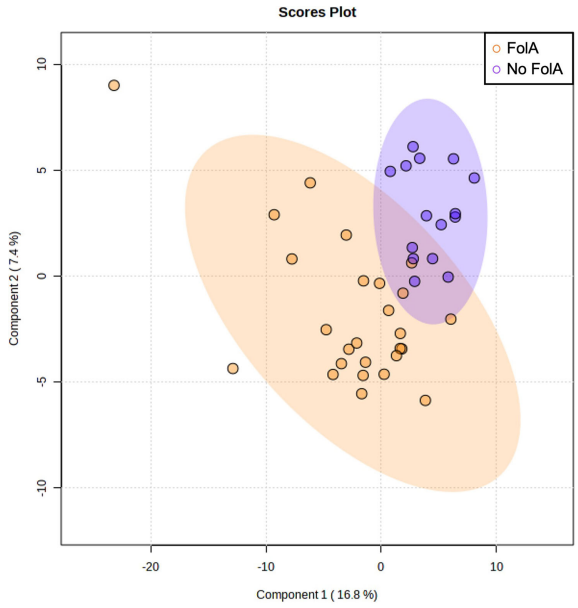

FIGURE 5
Multivariate Partial Least Squares Discriminant Analysis scores plot from women supplemented with folic acid (FolA) and women with no supplementation.

significant differences observed in the metabolomic profile when comparing the groups of women who were supplemented with folic acid or not (Supplementary Table S6).

4 Discussion

In this cross-sectional study, we used an untargeted metabolomics approach to profile the endometrial metabolome and to assess possible differences in the metabolite composition between women with low and high adherence to MD, different infertility diagnoses (i.e., male factor infertility, unexplained infertility, RIF, and endometriosis), and taking also into account folic acid supplementation. We provide the whole metabolome profile of the endometrium, where 925 different metabolites were identified. Among these metabolites, lipids comprised the largest percentage (40%), where PUFAs such as mead acid, dihomo-linolenate, and docosatrienoate prevailed. Our main results indorse those women with endometrium-related infertility (i.e., endometriosis or RIF) have lower levels of PUFAs in the endometrium compared to women with no clear endometrial factor infertility (i.e., male factor and unexplained infertility). Further, supporting our grouping based on infertility diagnosis, male factor and unexplained infertility demonstrated closer metabolome patterns while RIF and endometriosis were more similar. Moreover, adherence to MD seemed to be associated with the endometrial metabolomic profile, differing between women with distinct infertility diagnoses. In this way, adherence to MD could be related to the levels of different fatty acids, progestin steroids, xanthine metabolites, carnitines, amino acids, and bile acids in a manner dependent on the health status of the uterus.

In our study, PUFAs prevailed in all the women studied. When comparing the study groups based on the uterine examination, dihomolinolenate, linoleate, and linolenate were detected at lower levels in the endometrium of women with RIF or endometriosis. PUFAs are fatty acids with multiple double bonds in their structures which cannot be synthesized by the human body, being considered essential fatty acids (33). Linoleate and linolenate are the conjugated acids of linoleic acid (LA) and α-linolenic acid (ALA), respectively, which are the primary PUFAs in Western diets (34). PUFAs are shown to play a substantial role in the regulation of body homeostasis and are considered crucial for reproductive health (35). These lipids participate in female fertility at different reproductive phases, including oocyte maturation and quality, and embryo implantation (36–39). Our study results indicate that microenvironment in the uteri of women with RIF or endometriosis could be lacking in PUFAs, which could negatively impact the endometrial functions.

We detected low levels of linolenate in women with EFI compared to women with NEFI. Interestingly, when we combined MD adherence and endometrial-factor infertility features, the results did support that linolenate was less present in the endometrium of women with low MD adherence and EFI compared to women with high MD adherence and NEFI. Previous studies show contradicting results, as negative correlations between ALA levels in different biological fluids (i.e., serum, follicular fluid) of women undergoing infertility treatment and pregnancy rates (40) and number of metaphase II oocytes (41) have been found. Similarly, serum ALA levels have shown positive association with the presence of endometriosis (40). These discrepancies could be due to the different analyzed specimens (serum *versus* endometrial biopsy). Some evidence also manifests that LA has negative effects on oocyte maturation (33), which could be mediated by the role of lipids in the physical properties and biological functions of membranes (13). Other authors measured the LA : ALA ratio (n-6 to n-3) and they found that women with higher LA : ALA ratios had greater embryo implantation and pregnancy rates compared to women with reduced LA : ALA ratios (42, 43), suggesting a potential role for increased LA to enhance endometrial inflammation and receptivity (43) and for elevated ALA to disturb embryo implantation (35). It is worth mentioning that LA is a precursor to docosapentaenoic acid (n-6). Thus, our data seem to support the previous results as we found that the docosapentaenoate (i.e., conjugated acid of docosapentaenoic acid) is less present in the endometrium of women with EFI. Nevertheless, another study revealed that docosapentaenoic acid was higher in women with no implantation compared to women with successful implantation (44). In this way, further investigation is required to unravel the role of n-6 and n-3 PUFAs in endometrial receptivity as this ratio imbalance could lead to aberrant endometrial functions.

Likewise, we observed a positive correlation between adherence to MD and metabolites involved in the metabolism of ceramides in women with EFI. In line, we noticed that ceramide metabolites were higher in women with no endometrial dysfunction. Although with no statistical significance, these results agree with a previous study where ceramides were detected at lower amount in the endometrial fluid of women with endometriosis compared to women without endometriosis (10).

Another family of metabolites that we found dysregulated in women with endometrium-related infertility were progestin steroids (i.e., intermediates of progesterone metabolism) which are shown to contribute to fertility and the maintenance of pregnancy (45). We revealed that lower adherence to MD was related to lower endometrial levels of these hormonal intermediates in women with endometrium-related infertility. A recent work demonstrated that premature rise in the serum progesterone level in the late follicular phase led to a disrupted endometrial lipid profile in the endometrium during the peri-implantation period (44), suggesting that the altered lipid pattern could desynchronize endometrial receptivity and early embryo implantation. There seems to be a link between progesterone metabolism and lipid profiles, which needs further investigation.

Additional interesting result of our study is that a higher adherence to MD correlated with lower levels of carnitine metabolites within the whole cohort. Accordingly, the patient group of women with EFI exhibited a negative correlation between MD adherence and acylcarnitines. It could be that MD confers protection against endometriosis since increased carnitine levels were observed in women with endometriosis (10) and correlated with the cytokine and cellular profile of endometriosis (46). Moreover, high levels of acylcarnitines have also been

associated with beta-oxidation dysfunction, participating in inflammation processes (47). Further, we detected a negative correlation of MD adherence with bile acid metabolites in women with EFI, which corroborates that high levels of bile acids are a negative factor for health (48). All these results related to lipid metabolism endorse that lipidome is an essential aspect of the complex process of endometrium receptivity and embryo implantation (35) and alterations in lipid metabolism could lead to endometrial dysfunctions.

We also found a negative correlation between the MD adherence and xanthine metabolites (i.e., products of the purine and xenobiotics degradation) in women with NEFI and a positive correlation in women with EFI. A healthy diet in women without endometrial abnormal functioning has been associated with lower levels of these metabolites, which were found to be elevated in previous studies in women with endometrial disorders (e.g., caffeine (49), xanthosine (11)). However, studies involving xanthines in female reproduction have been mainly focused on their use in oocyte *in vitro* maturation (49) and other reproductive outcomes need to be considered.

Some aspects of the current study should be considered as limitations. Firstly, the cross-sectional design does not allow the establishment of causality. Secondly, our results should be interpreted as preliminary due to the limited sample size, which might have underpowered the detection of statistical differences in metabolite composition between the studied groups. Further studies investigating a MD intervention on the endometrial metabolomic signature are thus required to confirm our results.

Despite these shortcomings, the strength of our study is the focus on a very narrow time-frame of the cycle, the mid-secretory phase, and all the samples were collected at the day LH+7, minimizing thereby the heterogeneity and it enabled us to study the receptive-phase uterine microenvironment. Further, existing metabolomic studies of female reproductive health have mainly been performed in serum samples and assessed a preestablished groups of metabolites, while in our study we analyzed for the first time the endometrial whole metabolic signature with an untargeted method.

This study presents the endometrial whole metabolome profile, demonstrating that PUFAs are prevailing in the human uterus. In conclusion, we showed that women with RIF or endometriosis displayed lower levels of PUFAs in the endometrium compared to women with no clear endometrial alterations, identifying a metabolomic profile associated with infertility diagnoses where altered endometrial functions are suspected (i.e., endometriosis and RIF). In addition, adherence to the MD was associated with the endometrial metabolic profile in a manner dependent on the health status of the uterus. Our study findings help to understand the molecular background of female infertility, which could allow to identify potential molecular biomarkers and take personalized medicine into the reproductive clinical practices. With a healthy diet, endometrial microenvironment could be changed from unfavorable to favorable, which could have an influence on endometrial functions.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of the Junta de Andalucía (CEIM/CEI 0463-M1-18r). The patients/participants provided their written informed consent to participate in this study.

Author contributions

NM, LJ-F, AS-L, RS-L, and SA conceived and designed the study. NM, AS-L, JF, and SR-D recruited the participants and obtained the samples. LJ-F and RS-L processed the diet data. NM and LJ-F analyzed the data and drafted the manuscript. AS-L, RS-L, AC-G, JF, SR-D, CA, and SA revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1120988/full#supplementary-material

SUPPLEMENTARY TABLE 1
Dataset comprising the average peak intensity and detection platform of each identified metabolite in women with high and low adherence to the Mediterranean Diet.

SUPPLEMENTARY TABLE 2
Quantitative score (14-item) of adherence to the Mediterranean Diet. The participants were asked to choose the best option for each question. '<' means less than. The option marked with grey color is the one used as criteria for 1 point. The participants also self-reported their folic acid intake.

SUPPLEMENTARY TABLE 3
Statistical differences in metabolites between groups based on Mediterranean Diet (MD) adherence (i.e., high and low MD adherence).

SUPPLEMENTARY TABLE 4
Correlation analyses between the Mediterranean Diet global score and the endometrial metabolome.

SUPPLEMENTARY TABLE 5
Statistical differences in metabolites between groups based on uterine examination (i.e., women with no endometrial-factor infertility and patients with endometrial-factor infertility).

SUPPLEMENTARY TABLE 6
Statistical differences in metabolites between groups based on folic acid supplementation.

SUPPLEMENTARY FIGURE 1
Statistical difference in linolenate metabolite between women with high Mediterranean Diet (MD) adherence and no endometrial-factor infertility and women with low MD adherence and endometrial-factor infertility. Statistical significance was set p-value<0.05 after FDR correction. EFI, endometrial-factor infertility; NEFI, no endometrial-factor infertility.

References

1. Evans J, Salamonsen LA, Winship A, Menkhorst E, Nie G, Gargett CE, et al. Fertile ground: human endometrial programming and lessons in health and disease. *Nat Rev Endocrinol* (2016) 12:654–67. doi: 10.1038/nrendo.2016.116

2. Ban Z, Knöspel F, Schneider MR. Shedding light into the black box: advances in *in vitro* systems for studying implantation. *Dev Biol* (2020) 463:1–10. doi: 10.1016/j.ydbio.2020.04.003

3. Garcia-Alonso L, Handfield L-F, Roberts K, Nikolakopoulou K, Fernando RC, Gardner L, et al. Mapping the temporal and spatial dynamics of the human endometrium *in vivo* and *in vitro*. *Nat Genet* (2021) 53:1698–711. doi: 10.1038/s41588-021-00972-2

4. Tokarz J, Adamski J, Rižner TL. Metabolomics for diagnosis and prognosis of uterine diseases? a systematic review. *J Pers Med* (2020) 10:294. doi: 10.3390/jpm10040294

5. Suhorutshenko M, Kukushkina V, Velthut-Meikas A, Altmäe S, Peters M, Mägi R, et al. Endometrial receptivity revisited: endometrial transcriptome adjusted for tissue cellular heterogeneity. *Hum Reprod* (2018) 33:2074–86. doi: 10.1093/humrep/dey301

6. Sola-Leyva A, Andrés-León E, Molina NM, Terron-Camero LC, Plaza-Díaz J, Sáez-Lara MJ, et al. Mapping the entire functionally active endometrial microbiota. *Hum Reprod* (2021) 36:1021–31. doi: 10.1093/humrep/deaa372

7. Altmäe S, Koel M, Võsa U, Adler P, Suhorutšenko M, Laisk-Podar T, et al. Meta-signature of human endometrial receptivity: a meta-analysis and validation study of transcriptomic biomarkers. *Sci Rep* (2017) 7:10077. doi: 10.1038/s41598-017-10098-3

8. Altmäe S, Esteban FJ, Stavreus-Evers A, Simón C, Giudice L, Lessey BA, et al. Guidelines for the design, analysis and interpretation of "omics" data: focus on human endometrium. *Hum Reprod Update* (2014) 20:12–28. doi: 10.1093/humupd/dmt048

9. Wishart DS. Emerging applications of metabolomics in drug discovery and precision medicine. *Nat Rev Drug Discovery* (2016) 15:473–84. doi: 10.1038/nrd.2016.32

10. Domínguez F, Ferrando M, Díaz-Gimeno P, Quintana F, Fernández G, Castells I, et al. Lipidomic profiling of endometrial fluid in women with ovarian endometriosis†. *Biol Reprod* (2017) 96:772–9. doi: 10.1093/biolre/iox014

11. Li J, Guan L, Zhang H, Gao Y, Sun J, Gong X, et al. Endometrium metabolomic profiling reveals potential biomarkers for diagnosis of endometriosis at minimal-mild stages. *Reprod Biol Endocrinol* (2018) 16:42. doi: 10.1186/s12958-018-0360-z

12. Li J, Gao Y, Guan L, Zhang H, Sun J, Gong X, et al. Discovery of phosphatidic acid, phosphatidylcholine, and phosphatidylserine as biomarkers for early diagnosis of endometriosis. *Front Physiol* (2018) 9:14. doi: 10.3389/fphys.2018.00014

13. Matorras R, Martinez-Arranz I, Arretxe E, Iruarrizaga-Lejarreta M, Corral B, Ibañez-Perez J, et al. The lipidome of endometrial fluid differs between implantative and non-implantative IVF cycles. *J Assist Reprod Genet* (2020) 37:385–94. doi: 10.1007/s10815-019-01670-z

14. Hong L, Zhu YC, Liu S, Wu T, Li Y, Ye L, et al. Multi-omics reveals a relationship between endometrial amino acid metabolism and autophagy in women with recurrent miscarriage. *Biol Reprod* (2021) 105:393–402. doi: 10.1093/biolre/ioab101

15. Yurci A, Dokuzeylul Gungor N, Gurbuz T. Spectroscopy analysis of endometrial metabolites is a powerful predictor of success of embryo transfer in women with implantation failure: a preliminary study. *Gynecol Endocrinol* (2021) 37:415–21. doi: 10.1080/09513590.2021.1883584

16. Ciebiera M, Esfandyari S, Siblini H, Prince L, Elkafas H, Wojtyła C, et al. Nutrition in gynecological diseases: current perspectives. *Nutrients* (2021) 13:1178. doi: 10.3390/nu13041178

17. Gaskins AJ, Nassan FL, Chiu Y-H, Arvizu M, Williams PL, Keller MG, et al. Dietary patterns and outcomes of assisted reproduction. *Am J Obstet Gynecol* (2019) 220:567.e1–567.e18. doi: 10.1016/j.ajog.2019.02.004

18. Hsiao PY, Fung JL, Mitchell DC, Hartman TJ, Goldman MB. Dietary quality, as measured by the alternative healthy eating index for pregnancy (AHEI-p), in couples planning their first pregnancy. *Public Health Nutr* (2019) 22:3385–94. doi: 10.1017/S1368980019001290

19. Guasch-Ferré M, Willett WC. The Mediterranean diet and health: a comprehensive overview. *J Intern Med* (2021) 290:549–66. doi: 10.1111/joim.13333

20. Ott J, Nouri K, Hrebacka D, Gutschelhofer S, Huber JC, Wenzl R. Endometriosis and nutrition-recommending a Mediterranean diet decreases endometriosis-associated pain: an experimental observational study. *J Aging Res Clin Pract* (2012) 1:162–6.

21. Skoracka K, Ratajczak AE, Rychter AM, Dobrowolska A, Krela-Kaźmierczak I. Female fertility and the nutritional approach: the most essential aspects. *Adv Nutr* (2021) 12:2372–86. doi: 10.1093/advances/nmab068

22. Muffone ARMC, de Oliveira Lübke PDP, Rabito EI. Mediterranean Diet and infertility: a systematic review with meta-analysis of cohort studies. *Nutr Rev* (2022). doi: 10.1093/nutrit/nuac087

23. Mei S, Ding J, Wang K, Ni Z, Yu J. Mediterranean Diet combined with a low-carbohydrate dietary pattern in the treatment of overweight polycystic ovary syndrome patients. *Front Nutr* (2022) 9:876620. doi: 10.3389/fnut.2022.876620

24. Abdulkhalikova D, Sustarsic A, Vrtačnik Bokal E, Jancar N, Jensterle M, Burnik Papler T. The lifestyle modifications and endometrial proteome changes of women with polycystic ovary syndrome and obesity. *Front Endocrinol (Lausanne)* (2022) 13:888460. doi: 10.3389/fendo.2022.888460

25. World Health Organization. *WHO laboratory manual for the examination and processing of human semen, 6th ed*. Geneva: World Health Organization (2021).

26. Evans AM, Bridgewater B, Qiang L, Mitchell M, Robinson R, Dai H, et al. High resolution mass spectrometry improves data quality and quality as compared to unit mass resolution mass spectrometry in high throughput profiling metabolomics. *Metabolomics* (2014) 4:132. doi: 10.4172/2153-0769.1000132

27. Visconti A, Le Roy CI, Rosa F, Rossi N, Martin TC, Mohney RP, et al. Interplay between the human gut microbiome and host metabolism. *Nat Commun* (2019) 10:4505. doi: 10.1038/s41467-019-12476-z

28. DeHaven CD, Evans AM, Dai H, Lawton KA. Organization of GC/MS and LC/MS metabolomics data into chemical libraries. *J Cheminform* (2010) 2:9. doi: 10.1186/1758-2946-2-9

29. Evans AM, DeHaven CD, Barrett T, Mitchell M, Milgram E. Integrated, nontargeted ultrahigh performance liquid chromatography/electrospray ionization tandem mass spectrometry platform for the identification and relative quantification of the small-molecule complement of biological systems. *Anal Chem* (2009) 81:6656–67. doi: 10.1021/ac901536h

30. Schröder H, Fitó M, Estruch R, Martínez-González MA, Corella D, Salas-Salvadó J, et al. A short screener is valid for assessing Mediterranean diet adherence among older Spanish men and women. *J Nutr* (2011) 141:1140–5. doi: 10.3945/jn.110.135566

31. Eriksson L, Kettaneh-Wold N, Wold S. Scaling: introduction to multi- and megavariate data analysis using projection methods (PCA & PLS). *Umetrics* (1999), 213–25.

32. Benjamini Y, Krieger AM, Yekutieli D. Adaptive linear step-up procedures that control the false discovery rate. *Biometrika* (2006) 93:491–507. doi: 10.1093/biomet/93.3.491

33. Wang R, Feng Y, Chen J, Chen Y, Ma F. Association between polyunsaturated fatty acid intake and infertility among American women aged 20–44 years. *Front Public Health* (2022) 10:938343. doi: 10.3389/fpubh.2022.938343

34. Simopoulos AP. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed Pharmacother* (2002) 56:365–79. doi: 10.1016/S0753-3322(02)00253-6

35. Yang T, Zhao J, Liu F, Li Y. Lipid metabolism and endometrial receptivity. *Hum Reprod Update* (2022) 28:858–89. doi: 10.1093/humupd/dmac026

36. Sturmey R, Reis A, Leese H, McEvoy T. Role of fatty acids in energy provision during oocyte maturation and early embryo development. *Reprod Domest Anim* (2009) 44:50–8. doi: 10.1111/j.1439-0531.2009.01402.x

37. Norwitz ER, Schust DJ, Fisher SJ. Implantation and the survival of early pregnancy. *New Engl J Med* (2001) 345:1400–8. doi: 10.1056/NEJMra000763

38. Nehra D, Le HD, Fallon EM, Carlson SJ, Woods D, White YA, et al. Prolonging the female reproductive lifespan and improving egg quality with dietary omega-3 fatty acids. *Aging Cell* (2012) 11:1046–54. doi: 10.1111/acel.12006

39. Hammiche F, Vujkovic M, Wijburg W, de Vries JHM, Macklon NS, Laven JSE, et al. Increased preconception omega-3 polyunsaturated fatty acid intake improves embryo morphology. *Fertil Steril* (2011) 95:1820–3. doi: 10.1016/j.fertnstert.2010.11.021

40. Jungheim ES, Macones GA, Odem RR, Patterson BW, Moley KH. Elevated serum alpha-linolenic acid levels are associated with decreased chance of pregnancy after *in vitro* fertilization. *Fertil Steril* (2011) 96:880–3. doi: 10.1016/j.fertnstert.2011.07.1115

41. Mirabi P, Chaichi MJ, Esmaeilzadeh S, Ali Jorsaraei SG, Bijani A, Ehsani M, et al. The role of fatty acids on ICSI outcomes: a prospective cohort study. *Lipids Health Dis* (2017) 16:18. doi: 10.1186/s12944-016-0396-z

42. Jungheim ES, Frolova AI, Jiang H, Riley JK. Relationship between serum polyunsaturated fatty acids and pregnancy in women undergoing *In Vitro* fertilization. *J Clin Endocrinol Metab* (2013) 98:E1364–8. doi: 10.1210/jc.2012-4115

43. Moran L, Tsagareli V, Noakes M, Norman R. Altered preconception fatty acid intake is associated with improved pregnancy rates in overweight and obese women undertaking in vitro fertilisation. *Nutrients* (2016) 8:10. doi: 10.3390/nu8010010

44. Li J, Gao Y, Guan L, Zhang H, Chen P, Gong X, et al. Lipid profiling of peri-implantation endometrium in patients with premature progesterone rise in the late follicular phase. *J Clin Endocrinol Metab* (2019) 104:5555–65. doi: 10.1210/jc.2019-00793

45. di Renzo GC, Mattei A, Gojnic M, Gerli S. Progesterone and pregnancy. *Curr Opin Obstet Gynecol* (2005) 17:598–600. doi: 10.1097/01.gco.0000191899.84567.4d

46. Dionyssopoulou E, Vassiliadis S, Evangeliou A, Koumantakis EE, Athanassakis I. Constitutive or induced elevated levels of l-carnitine correlate with the cytokine and cellular profile of endometriosis. *J Reprod Immunol* (2005) 65:159–70. doi: 10.1016/j.jri.2004.12.002

47. Vouk K, Hevir N, Ribič-Pucelj M, Haarpaintner G, Scherb H, Osredkar J, et al. Discovery of phosphatidylcholines and sphingomyelins as biomarkers for ovarian endometriosis. *Hum Reprod* (2012) 27:2955–65. doi: 10.1093/humrep/des152

48. Osuna-Prieto FJ, Rubio-Lopez J, Di X, Yang W, Kohler I, Rensen PCN, et al. Plasma levels of bile acids are related to cardiometabolic risk factors in young adults. *J Clin Endocrinol Metab* (2022) 107:715–23. doi: 10.1210/clinem/dgab773

49. Minelli A, Bellezza I. Methylxanthines and reproduction. *Handb Exp Pharmacol* (2011) 200:349–72. doi: 10.1007/978-3-642-13443-2_13

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A novel therapeutic approach for endometriosis using adipose-derived stem cell-derived conditioned medium- A new hope for endometriotic patients in improving fertility

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Introduction: Endometriosis is defined as the growth of endometrial glands and stromal cells in a heterotopic location with immune dysregulation. It usually leads to chronic pelvic pain and subfertility. Although various treatments are available, the recurrence rate remains high. Adipose tissue is an abundant source of multipotent mesenchymal adipose-derived stem cells (ADSCs). ADSCs display effects on not only tissue regeneration, but also immune regulation. Thus, the current study aims to test the effects of ADSCs on the growth of endometriosis.

Methods: ADSCs isolated from lipoaspiration-generated adipose tissue and their conditioned medium (ADSC-CM) were subjected to quality validation, including karyotyping as well as growth promotion and sterility tests for microbial contamination under Good Tissue Practice and Good Manufacturing Practice regulations. An autologous endometriosis mouse model was established by suturing endometrial tissue to peritoneal wall followed by treating with DMEM/F12 medium, ADSC-CM, ADSCs or ADSC-CM+ADSCs for 28 days. The area of endometriotic cysts and the degree of pelvic adhesion were measured. ICAM-1, VEGF and caspase 3 expression was assessed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and immunohistochemistry. Moreover, the mice were allowed to mate and deliver. The pregnancy outcomes were recorded. The ADSC-CM was subjected to proteomics analysis with further data mining with Ingenuity Pathway Analysis (IPA).

Results: Both ADSC-CM and ADSCs passed quality validation. ADSC-CM reduced the area of endometriotic cysts. The inhibition by ADSC-CM was

obliterated by adding ADSCs. The presence of ADSCs with or without ADSC-CM increased the peritoneal adhesion. ADSC-CM inhibited ICAM-1 and VEGF mRNA and protein expression, whereas the addition of ADSCs not only did not inhibit by itself, but also blocked the inhibition by ADSC-CM. The resorption rate was reduced by ADSC-CM. The number of live birth/dam and the survival rate of pup at 1 week-old were both increased by ADSC-CM in mice with endometriosis. IPA demonstrated that PTX3 was potentially critical for the inhibition of endometriosis by ADSC-CM due to its anti-inflammatory and antiangiogenic properties as well as its importance in implantation.

Conclusion: ADSC-CM inhibited endometriosis development and improved pregnancy outcomes in mice. Potential translation to clinical treatment for human endometriosis is expected.

KEYWORDS

endometriosis, adipose-derived stem cell, conditioned medium, proteomics, PTX3

Introduction

Endometriosis is initially a benign but frequently progressive disease that occurs in 10% of reproductive age women, 60% of women with pelvic pain, and 30% to 50% of infertile women. It is associated with a wide spectrum of clinical sequelae, including secondary dysmenorrhea and/or dyspareunia, infertility as well as symptoms associated with gastrointestinal and urinary tract involvement (1). Although coelomic metaplasia and direct transplantation *via* lymphatic or vascular metastasis may play a role, the etiology of endometriosis is frequently attributed to retrograde menstruation. Since only a small percentage of women with retrograde menstruation develop endometriosis, additional factors are likely to play roles in implantation, growth, and angiogenesis of ectopic endometrial tissue. Moreover, changes in the immune system have been recently suggested to play a causative role in the pathogenesis of endometriosis (2, 3). Despite evidence of abnormalities in cell-mediated and humoral immune response in both peripheral blood and peritoneal fluid of patients with endometriosis (4–7), the cause of these aberrant immune responses remains unclear.

Factors to be considered in the management of endometriosis include the age and reproductive desires of the patient, the stage of the disease as well as, most importantly, the symptoms. Surgical treatment is considered appropriate, especially for advanced stages of the disease. Specifically, conservative surgery preserves the reproductive organs and is an effective treatment for endometriosis-associated pain. Laparoscopic surgery is an effective approach with the goal of eradicating visible endometriotic lesions. Hysterectomy with bilateral salpingo-oophorectomy remains a main therapy for refractory endometriosis-associated pain in patients who have completed childbearing.

Endometriosis is also responsive to hormonal therapy. A hypoestrogenic and anovulatory state induced by various hormonal drugs leads to atrophic change of endometriotic tissue. The use of hormonal therapies, including oral contraceptives, progestins, danazol or such GnRH agonists as leuprolide acetate, goserelin acetate, selective progesterone receptor modulators, selective estrogen receptor modulators, and nafarelin acetate, is commonly accompanied with non-steroid anti-inflammatory drugs.(8) Moreover, RU486 (mifepristone), GnRH antagonists, pentoxifylline, tumor necrosis factor-alpha (TNF-α) inhibitors, matrix metalloproteinases, and angiogenesis inhibitors are still under investigation (8). However, clinical observations show that the outcomes of medical treatments are unpredictable. Although both surgical and medical treatments have been shown to be effective for treating complications associated with endometriosis, the recurrent rate is estimated to be more than 20% 2 years and 40-50% 5 years after treatment(s). Thus, the development of new therapeutic strategies is required to improve the efficacy of treatment for endometriosis.

Adipose-derived stem cells (ADSCs) isolated from adipose tissue are multipotent mesenchymal stem cells (MSCs) (9). Human adipose tissue is ubiquitous and easily obtainable in large quantity under local anesthesia with minimal patient discomfort. Thus, ADSCs can serve as an alternative source of stem cells for mesenchymal tissue-based regeneration and engineering. Recently, ADSC therapy has been utilized to repair various organs (10–12). For renal fibrosis, ADSCs were found to regulate inflammatory responses and reduce epithelial–mesenchymal transition both early and late after injury (13). Furthermore, ADSC-conditioned medium (ADSC-CM) exhibits anti-oxidative (14), anti-proliferative (15), immunoregulatory, and wound-healing effects on various organ systems (16).

MSC-CM contains adipokines, cytokines, growth factors, and extracellular vesicles (17–20). The complexity of ADSCs and ADSC-CM mandate further investigation of their potential therapeutic applications and their underlying mechanisms (21). Thus, this study aims to examine the effects of human ADSC-CM on the development of inflammation-associated endometriosis using a mouse model. The findings can potentially be translated to clinical application to combat endometriosis.

Materials and methods

Human ADSCs isolation and culture

Before processing, all chemical reagents, supplements, buffer solutions and culture media were checked for microbial contamination. The procedures of sterility test followed Chinese Pharmacopoeia 8th edition, general chapter <7001>. All isolation and cultivation procedures were accomplished in a clean laboratory at E-Da Cell Therapy Center and under Good Tissue Practice (GTP) and Good Manufacturing Practice (GMP) regulations.

The human adipose tissues were obtained from lipoaspiration under E-Da Hospital IRB (EMRP04109N) approval. As previously described, (22) after washing with DPBS x2, the tissues were minced and digested with 0.1% collagenase I (GMP-grade Clzyme AS, VitaCyte, Indianapolis, IN, USA) in DMEM for 1h at 37°C with gentle agitation. Then, the digestate was centrifuged for 10min at 1,000 rpm. The cell pellet was re-suspended in DMEM/F12 supplemented with 5% platelet-rich plasma (PRP, UltraGRO™-PURE GI, AventaCell BioMedical, USA) and filtered through a 100-μm mesh filter to remove debris. The filtrate was centrifuged at 400× g for 10min at room temperature followed by re-suspension in 10 mL of ammonium-chloride-potassium (ACK) lysis buffer. After adding 10 mL of 1× DPBS buffer containing 1% antibiotics (20 μg/mL penicillin/streptomycin), the mixture was centrifuged at 400 ×g for 5min followed by re-suspension in 5 mL of complete medium (DMEM/F12 containing 5% PRP and 1% antibiotics) and cultured at 37°C in 5% CO_2. After removal of unattached cells at the third day of cultivation, the complete medium was replaced every 3-4 days until the culture reached 85-90% confluence and passaged. Chromosomal stability of ADSCs was checked at passage three by karyotyping.

ADSC characterization

The expression of CD14 (for monocytes), CD19 (for B-cells), CD29 (for ADSCs), CD31 (for endothelial cells), CD34 (for hematopoietic cells), CD44 (for ADSCs), CD45 (pan-leukocyte marker), CD73 (for ADSCs), CD90 (for ADSCs) and CD105 (for ADSCs) in the third-passage cells were examined by flow cytometric analysis. After fixation with ice-cold methanol, 100 μL of cell suspension with density at 1×10^6 cells/mL were stained with each specific antibody for 30min at 37°C in dark. The samples were centrifuged for 5min at 400× g, washed with DPBS twice, and re-suspended in 0.4 mL of DPBS before analyzed by a BD Accuri flow cytometer (BD, Franklin Lakes, NJ, USA) and FlowJo v.10.6.1 software (BD Bioscience).

Conditioned medium production and quality control

Human ADSCs at the third passage were seeded to T75 flasks at a density of 2×10^4 cells/cm^2. At 80-90% confluence, ADSCs were washed with 1× DPBS and cultured with 10 mL of serum-free DMEM/F12 for 48h at 37°C in 5% CO_2. Then, CM was collected and filtered through a 0.22-μm filter to remove cell debris. CM was aliquoted and stored at -80°C for future experiments.

CM safety test

In support of pharmaceutical quality for media used in a GMP facility, culture medium for ADSCs and ADSC-CM were subjected to growth promotion test using such inoculum as Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Candida albicans or Aspergillus niger, prior to sterility test. Before releasing CM from Cell Therapy Center, its safety was confirmed by various tests for sterility, bacterial endotoxin and mycoplasma. All safety tests were performed under the guidance of Chinese Pharmacopoeia 8th edition, general chapters <7001>, <7008> and <7009>. Direct transfer sterility test in tryptic soy broth (TSB) and fluid thioglycollate medium (FTM) verified the sterility of CM by incubating for 14 days with daily examination for the presence of microbials. Mycoplasma was checked by the nucleic acid amplification test (NAAT) based on Chinese Pharmacopoeia 8th edition, general chapter <7009>. Limulus Amebocyte Lysate (LAL) test used chromogenic methods to measure the concentration of bacterial endotoxin in CM, which is expected to be less than 0.5 EU/mL.

Endometriosis mouse model

The animal studies were performed under E-Da Hospital Institutional Animal Care and Use Committee (IACUC-EDAH-108040) approval. Nine-week-old female C57BL/6JNarl mice purchased from BioLASCO Taiwan Co., Ltd. were kept under controlled conditions (24˚C, 12:12 light-dark cycle with lights on at 6:00 AM) and anesthetized with Zoteil (VIRBAC, Pukete, Hamilton, France) by intraperitoneal injection. The uterus was ligated at the internal cervical os followed by removal of uterus with both ovaries spared. Both uterine horns were longitudinally opened. Four pieces of uterine tissues with identical size generated by a disposable 2-mm dermal biopsy punch (Life Technologies, Carlsbad, CA, USA) were sutured to the peritoneal wall using a 6-0 monofilament nylon (UNIK, New Taipei City, Taiwan) with two pieces on each side (23). Subsequently, the mice were intraperitoneally treated with medium (control) (1 mL), ADSC-CM (1 mL), ADSC (1 x 10^6 cells) or ADSC-CM (1 mL) + ADSC (1 x 10^6 cells) only once before suturing the abdominal wound and sacrificed at day 28 to harvest the endometriotic tissues.

A total of 6 batches of ADSCs and ADSC-CM were used in 6 independent experiments with triplicate in each experiment. One half of the tissues were fixed in 4% paraformaldehyde for 24h at 4°C and the other half were snap-frozen and stored in −80°C for future use. The formation of adhesion bands between peritoneum and endometriotic tissues was graded based on a 0 to 3 scale: 0: no adhesion. 1: mild adhesions that can be easily separated. 2: moderate adhesions that can be separated by a pair of forceps. 3: dense adhesions that require to use scissors to separate. The area of the lesion was measured using Image-J v1.53 DIA software (IHC Image

Analysis Toolbox, National Institutes of Health, Washington, USA; https://imagej.nih.gov/ij/index.html). Since only ADSC-CM was effective in reducing the lesion size, the treatments of ADSC and ADSC-CM + ADSC were excluded in further studies of eutopic endometrial receptivity and pregnancy outcomes. For pregnancy outcomes, the female mice after 28-day treatment with either medium (control) (6 mice) or ADSC-CM (8 mice) were mated with an 8-week-old male C57BL/6JNarl and allowed to deliver at term. The endometrial receptivity of the eutopic endometrium were examined at gestational day (GD) 4. The pregnancy outcomes, including: 1) number of vaginal plug; 2) resorption rate; 3) live birth per dam; 4) pup survival rate at 1 week-old, were recorded (Figure 1).

H & E stain

Paraffin-embedded tissue sections (4 μm) prepared using an SM200R microtome (Leica, Wetzlar, Germany) were placed on slides pre-coated with poly-L-lysine followed by deparaffinization and rehydration. The sections were stained with hematoxylin and eosin followed by clearing with xylene for 5min x2 before mounting with DPX mounting medium and examination under an Olympus BX43 light microscope.

Quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from the endometriotic lesions using a total RNA purification plus kit. Reverse transcription used SuperScript™ III First-Strand Synthesis System. Specific primer sets for mouse ICAM-1 (forward: AAACCAGACCCTGGAA CTGCAC; reverse: GCCTGGCATTTCAGAGTCTGCT), VEGF (forward: CCTGAATCCTGGGAAATGTGCC; reverse: CGATTC GCACACGGTCTTCTGT), caspase-3 (forward: GGAG TCTGACTGGAAAGCCGAA; reverse: CTTCTGGCAAGCCATC TCCTCA), and GAPDH (forward: CATCACTGCCACCCAGAA GACTG; reverse: ATGCCAGTGAGCTTCCCGTTCAG) measured mRNA levels using PowerUp SYBR Green Master Mix (ThermoFisher Scientific) on a StepOneplus™ real-time PCR system. Relative gene expression was analyzed according to the $2^{-\Delta\Delta Ct}$ method. All samples were assayed in triplicate. Melting curve analysis determined the specificity of the amplified products and the absence of primer-dimer formation.

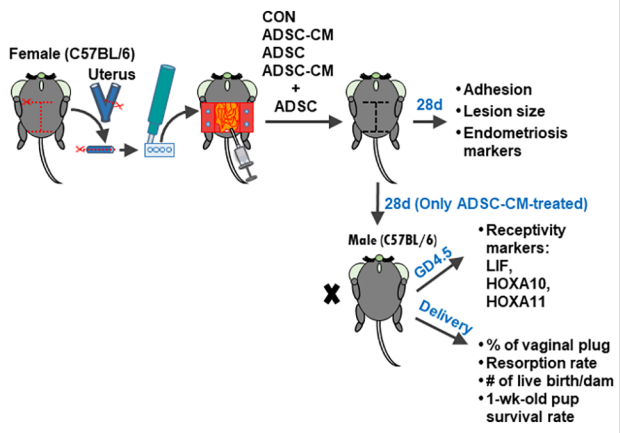

FIGURE 1
Experimental design Four pieces of uterine tissue were generated from the uterus of a female C57BL/6JNarl mice using a disposable 2-mm dermal biopsy punch and sutured to the peritoneal wall with two pieces on each side. Then, the culture medium, ADSC, ADSC-CM or ADSC + ADSC-CM was added into the peritoneal cavity. The endometriotic lesions were harvested at 28 days. To examine the pregnancy outcomes, the mouse was mated with a male C57BL/6JNarl at day 28 and allowed to deliver at term. The percentage of vaginal plug, resorption rate, live births per dam, and pup survival rate at 1 week-old were recorded.

Immunohistochemistry

The rehydrated paraffin sections were immersed in 3% H_2O_2 (in 100% methanol) for 10min to remove endogenous peroxidase activity followed by washing with PBS x3. Tissues were blocked with 0.5% BSA for 30min, then, incubated with monoclonal anti-mouse ICAM-1, VEGF, caspase-3 [1:2,000 (v/v)] or corresponding isotype IgG for 1h at room temperature. After washing, the slides were incubated with either anti-mouse or -rabbit secondary antibody for 40min at room temperature. The immunoreactivity was detected with 3,3'-diaminobenzidine (DAB) chromogen. Sections were lightly counterstained with hematoxylin for 1min and dehydrated in a gradient of alcohol and xylene. The slides were mounted with Permount mounting medium and examined with an Olympus BX43 light microscope. Immunoreactivity of different molecules were quantified using the Image-J v1.53 DIA software as previously described (24).

Isobaric tagging for relative and absolute quantification gel-free proteomics

Total protein was extracted from plain culture medium and 3 batches of ADSC-CM for iTRAQ labeling. The samples (100 μg) were digested for 16 h at 37°C (protein:trypsin=30:1) using Trypsin Gold (Promega, Madison, WI, USA). Digestate was processed with 4-plex iTRAQ (Applied Biosystems, Waltham, MA, USA) labelling following the manufacturer's instructions. Samples were labeled with the iTRAQ 114, 115, 116 and 117 (plain culture medium). Strong cation exchange (SCX) chromatography was performed using the LC-20AB HPLC Pump system (Shimadzu, Japan). SCX fractions were suspended in buffer A (2% ACN, 0.1% FA) followed by a centrifugation at 20,000x g for 10 min. Then, 10 μL of supernatant were loaded onto a 2-cm C18 trap column on a LC-20AD nanoHPLC (Shimadzu, Japan) by the auto sampler and eluted onto a 10-cm analytical C18 column (inner diameter 75 μm) packed in-house. Finally, the chromatographic conditions were restored in 1 min. Data acquisition was performed by a Q EXACTIVE (Thermo Fisher Scientific, CA) coupled to the HPLC. Compared to plain culture medium, proteins in ADSC-CM with 1.5-fold increase were selected for further analysis. All the differentially expressed proteins were first analyzed *via* UniProt (http://www.uniprot.org/). Further

data mining was done using Ingenuity Pathway Analysis (IPA) software (Qiagen, Hilden, Germany).

Results

Characterization of ADSCs

The cells isolated from lipoaspiration-derived adipose tissue were subjected to flow cytometric analysis of various markers. The expression of CD14, CD19, CD45, CD31, and CD34 was absent in 99.70 ± 0.21%, 97.37 ± 1.13%, 99.57 ± 0.17%, 98.63 ± 0.64%, and 99.83 ± 0.03% of cells, respectively. The cells expressing CD29, CD73, CD90 and CD105 were 99.13 ± 0.30%, 98.20 ± 0.62%, 99.33 ± 0.35%, and 95.50 ± 1.69%, respectively (Figure 2A). A representative karyotyping showed that the adipose tissues were obtained from patients with normal karyotype (Figure 2B).

ADSC-CM inhibits the development of endometriosis

Endometriotic lesions were stained with hematoxylin and eosin. Compared with control (Figure 3A), ADSC-CM decreased the area of endometriotic lesions by 27.64 ± 3.02% (Figures 3B, E). The addition of ADSCs alone did not show inhibition on endometriotic lesions (Figures 3C, E). In contrast, the inhibitory effect of ADSC-CM was obliterated by adding ADSCs (Figures 3D, E). Although ADSC-CM treatment did not show improvement in peritoneal adhesion, the addition of ADSCs alone increased the peritoneal adhesion (Figure 3F).

The expression of ICAM-1 and VEGF was suppressed by ADSC-CM

Quantitative RT-PCR and IHC were used to evaluate the expression of ICAM-1 and VEGF in endometriotic lesions. Quantitative RT-PCR demonstrated that ICAM-1 expression was inhibited by ADSC-CM by 39.15 ± 6.99%, whereas the addition of ADSCs not only did not exhibit inhibition by itself, but also blocked the ADSC-CM-induced inhibition by 75.02 ± 18.76% (Figure 4A). Compared to control, ADSC-CM significantly suppressed VEGF mRNA expression by 44.93 ± 4.46%. The addition of ADSCs did not exert any effects on VEGF expression, compared with control. The down-regulation of VEGF mRNA by ADSC-CM was inhibited by the addition of ADSCs by 75.32 ± 23.24% (Figure 4B). The expression of caspase-3 was not affected by any treatment (Figure 4C).

Consistently, immunostaining revealed that ADSC-CM reduced ICAM-1 and VEGF expression by 43.74 ± 11.36% and 44.59 ± 16.84%, respectively. The existence of ADSCs did not show effect on the expression of ICAM-1 (Figures 5A, B) and VEGF (Figures 5A, C), however, suppressed the inhibitory effect of ADSC-CM on the expression of ICAM-1 (Figures 5A, B) and VEGF (Figures 5A, C) by 68.52 ± 10.33% and 47.71 ± 12.81%, respectively.

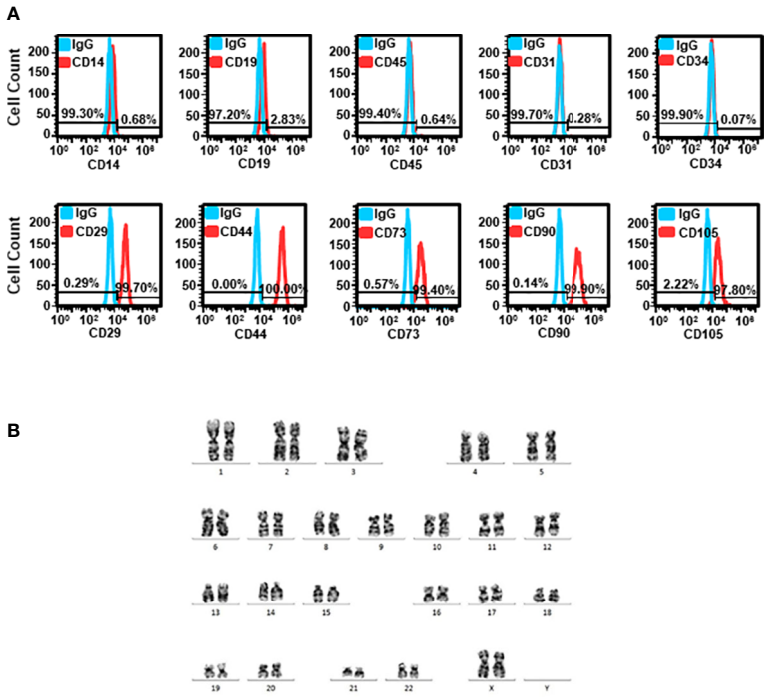

FIGURE 2
Characterization of ADSCs **(A)** Cells isolated from adipose tissue were stained with various markers, including CD29, CD44, CD73, CD90 and CD105 for MSC, CD14 for monocytes, CD19 for B-cells, CD31 for endothelial cells, CD34 for hematopoietic cells, and pan-leukocyte marker, CD45. Representative histograms of flow cytometric analysis are shown. **(B)** Representative karyotyping of cells isolated from adipose tissue.

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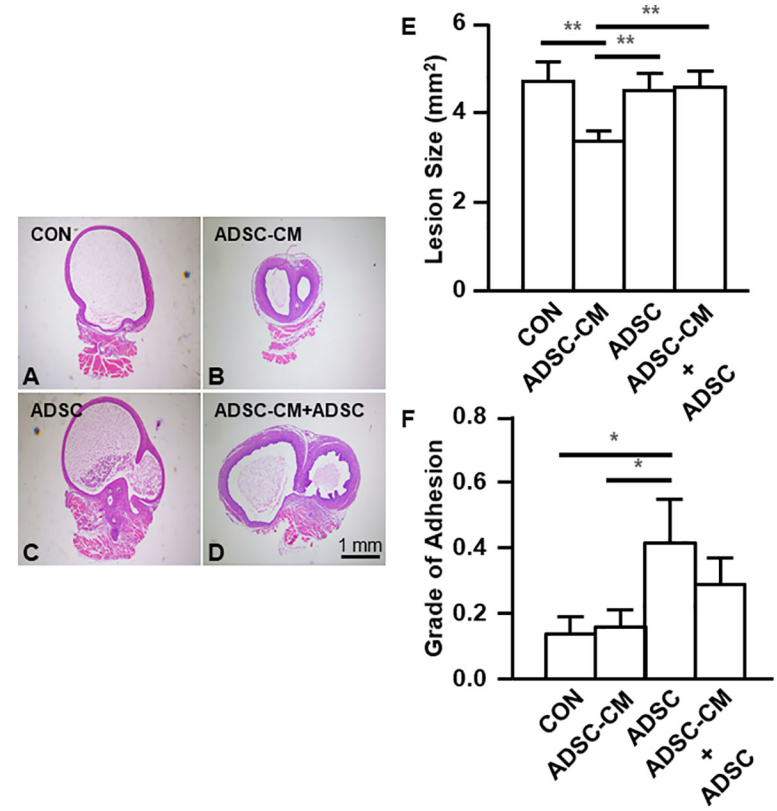

FIGURE 3
ADSC-CM inhibits the development of endometriosis Uterine tissue was sutured to the peritoneal wall of female C57B/6 mice and treated with **(A)** Culture medium, **(B)** ADSC-CM, **(C)** ADSC, **(D)** ADSC-CM + ADSC. **(E)** Lesion size and **(F)** grade of adhesion were measured. The data were reported as mean ± SEM. n=6; *p < 0.05.; **p < 0.01.

Caspase-3 expression in the endometriotic tissue was regulated by neither ADSC-CM nor ADSCs (Figures 5A, D).

ADSC-CM improved pregnancy outcomes in mice with endometriosis

Mice were mated with male mice at day 28 and allowed to proceed to delivery. The number of vaginal plug showed no different between control and ADSC-CM-treated groups (Figure 6A). The resorption rate was reduced by ADSC-CM from 18.33 ± 10.67% to 4.00 ± 4.00% (Figure 6B). The number of live birth/dam was increased by ADSC-CM treatment from 3.00 ± 0.41 to 5.40 ± 0.40 in mice with endometriosis (Figure 6C). The survival rate of pup at 1 week-old was improved from 47.92 ± 16.80% to 86.67 ± 6.24% (Figure 6D).

ADSC-CM improved endometrial receptivity in mice with endometriosis

To further study the mechanism associated with enhanced pregnancy outcomes, the endometrial receptivity of eutopic endometrium of mice with endometriosis was examined. Figure 7 demonstrated ADSC-CM up-regulated the mRNA expression of receptivity markers, including LIF, HOXA10 and HOXA11, by 1.72 ± 0.50- (Figure 7A), 3.25 ± 0.76- (Figure 7B), 4.67 ± 1.74-fold (Figure 7C), respectively. Compared to the control eutopic endometrium, ADSC-CM eliminated the immune cell infiltration, including lymphocytes and polymorphonuclear cells, in the sub-epithelial regions in eutopic endometrium of pregnant endometriotic mice at GD 4.5 (Figure 8A). IHC revealed that ADSC-CM consistently promoted the expression of LIF (Figures 8B, C), HOXA10 (Figures 8B, D) and HOXA11 (Figures 8B, E) by 90.17 ± 9.32%, 83.14 ± 5.95%, and 53.84 ± 9.46%, in epithelium, stroma and glands.

Proteomics analysis of ADSC-CM

ADSC-CM was subjected to iTRAQ analysis. A total of 1,348 proteins were detected in ADSC-CM (Supplementary Table 1). The results were further analyzed with Ingenuity Pathway Analysis (IPA) software. These proteins were shown to be involved in the signaling of 598 canonical pathways (Supplementary Table 2). Among which, acute phase response signaling, LXR/RXR activation, BAG2 signaling pathway, FAT10 signaling pathway, and clathrin-mediated endocytosis signaling were the most significant pathways associated with ADSC-secreted proteins. These ADSC-derived proteins also played a role in 500 diseases

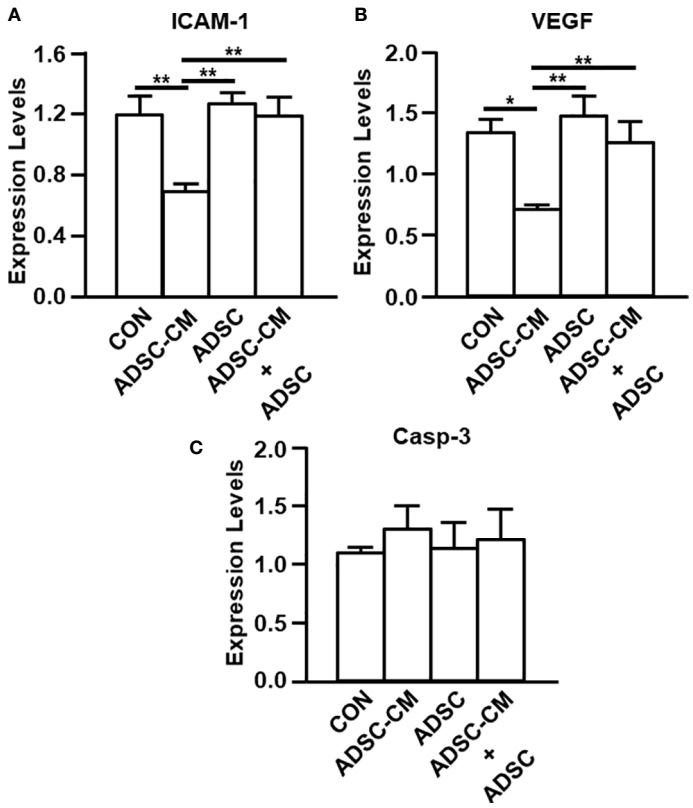

FIGURE 4
qRT-PCR of endometriosis-associated molecule expression The expression of **(A)** ICAM-1, **(B)** VEGF and **(C)** Caspase-3 in the endometriotic lesions were examined by qRT-PCR. The data were reported as mean ± SEM. n=6; *p < 0.05.; **p < 0.01.

and biofunctions (Supplementary Table 3) as well as 746 Tox functions (Supplementary Table 4). Specifically, 82 molecules were involved in the pathogenesis of endometriosis. A total of 4,033 upstream regulators were involved in the regulation of the expression of proteins in ADSC-CM (Supplementary Table 5). This regulation acts through 2,986 casual networks (Supplementary Table 6). The proteins in ADSC-CM form 25 interactive networks (Supplementary Table 7). Among 1,348 proteins, pentraxin 3 (PTX3) was found to interact with 114 molecules (Supplementary Table 8), particularly involved in regulating a variety of inflammatory cytokines and their receptors, including CCL2, CCL4, CCL5, CCL7, CCL8,CXCL8, CCR2, CCR5, and CX3CR1. In addition, IL-1α, IL-1β, TNF-α, IL-10, TLR2, TLR3, and TLR4 were shown to up-regulate PTX3, whereas PTX3 was down-regulated by IL-17A and TGF-β1.

Discussion

Endometriosis is known to be an inflammatory disease of pelvic cavity. The treatment of endometriosis aims to not only eradicate the ectopic endometrium, but also reduce inflammation and its associated complications, such as pain and adhesion-related sequelae. Due to high recurrence rate of endometriosis after medical treatment, patients suffering from endometriosis are usually destined for either infertility or surgical removal of uterus and adnexae after completing childbearing. Thus, an effective treatment to improve fertility and ameliorate symptoms is required to promote patients' quality of lives. ADSC-CM was used to treat endometriosis in the current study. After isolation, the purity of ADSCs was verified by the absence of CD14 (monocyte), CD19 (B-cell), CD45 (pan-leukocyte), CD31 (endothelial cell), and CD34 (hematopoietic cell) expression accompanied by expressing CD29, CD44, CD73, CD90 and CD105 with normal karyotype. The pharmaceutical quality of media used for ADSC culture was validated by growth promotion test. The preparation of ADSC and ADSC-CM was under the guidance of GTP and GMP regulations. The ADSC-CM was shown to be free of bacterial endotoxin and mycoplasma in ensuring the sterility and safety of the products.

The experimental mice were treated with or without ADSCs and/or ADSC-CM at the beginning of the experiments, the reduction of lesion size by ADSC-CM indicates its inhibitory effect on newly formed endometriotic lesions. Although MSCs acquired from other tissues were demonstrated to be anti-inflammatory (25), in the current study, the addition of ADSCs appears to offset the inhibition by ADSC-CM. Whether it's because of a tissue-specific effect or dosage-related issue needs to be further scrutinized. Also, treatment with ADSCs alone did not increase the growth of endometriotic tissue, suggesting that stem cells were not

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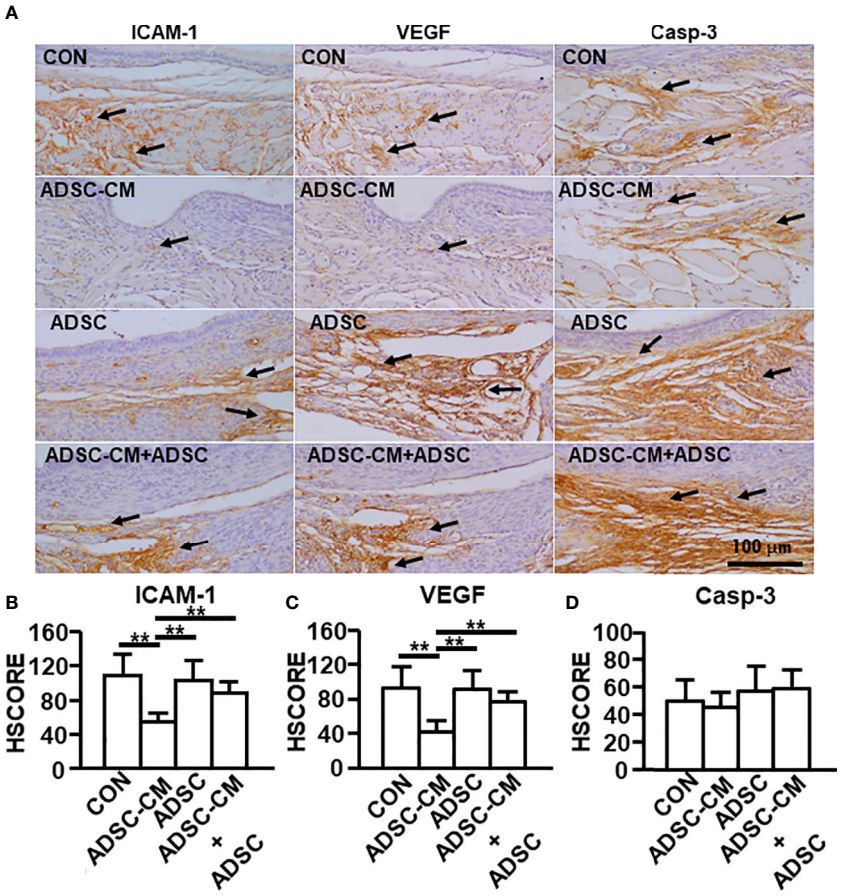

FIGURE 5
Immunohistochemical staining of endometriosis-associated molecules The expression of **(A)** ICAM-1, VEGF and caspase-3 in endometriotic lesions was demonstrated by immunohistochemistry. The immunoreactivity of **(B)** ICAM-1, **(C)** VEGF and **(D)** Caspase-3 in the tissue were semi-quantified by HSCORE. The data were reported as mean ± SEM. n=6; **p < 0.01.

involved in the growth of endometriosis under the pathogenic microenvironment. Furthermore, to test its therapeutic effect on the pre-existing endometriotic lesions in the future studies, the animals will not be treated until day 21 when the endometriotic lesions develop. Endometriosis is an inflammatory disease which usually leads to severe pelvic adhesion. In the current model, no apparently improvement in adhesion by ADSC-CM was observed. Although not statistically significant in comparison to non-treated group (p=0.574) and ADSC-CM-treated group (p=0.68), the addition of ADSCs seems to aggravate the adhesion. In addition to ADSC-CM, further studies are required to elucidate whether CM derived from stem cells isolated from mesenchyme of other tissues also exhibits similar inhibitory effects on the development of endometriosis.

Endometriotic patients exert reduced cycle fecundity, suggesting a decrease in receptivity of the eutopic endometrium (26). Defective endometrial receptivity can lead to adverse pregnancy outcomes, such as miscarriage, preeclampsia, and fetal growth restriction, potentially due to suboptimal trophoblast invasion (27). Therefore, in addition to alleviating the lesions and their associated symptoms, improving eutopic endometrial receptivity is critical in treating endometriotic patients who desire reproduction. In the current study, the percentages of the existence of vaginal plug after mating are similar between ADSC-CM-treated and control endometriotic mice, indicating the mating behavior of mice was not changed by ADSC-CM. However, the reduction of resorption rate as well as the improvement of live birth number and postnatal pup survival rate by ADSC-CM suggest the enhancement of endometrial receptivity by ADSC-CM in endometriotic mice.

Although an array of secreted proteins, including an extensive range of cytokines, chemokines, adhesion molecules, proteases, shed receptors, and growth factors, have been shown in several studies, a complete secretome profile has not been demonstrated (28). In our preliminary analysis, PTX3 appears to be a potential candidate crucial for the inhibition of endometriosis by ADSC-CM (Figure 9). PTX3 functions as a soluble pattern recognition receptor (29). In human endometrial stromal cells, estradiol and progesterone induce PTX3 expression that plays an important role in implantation of blastocyst

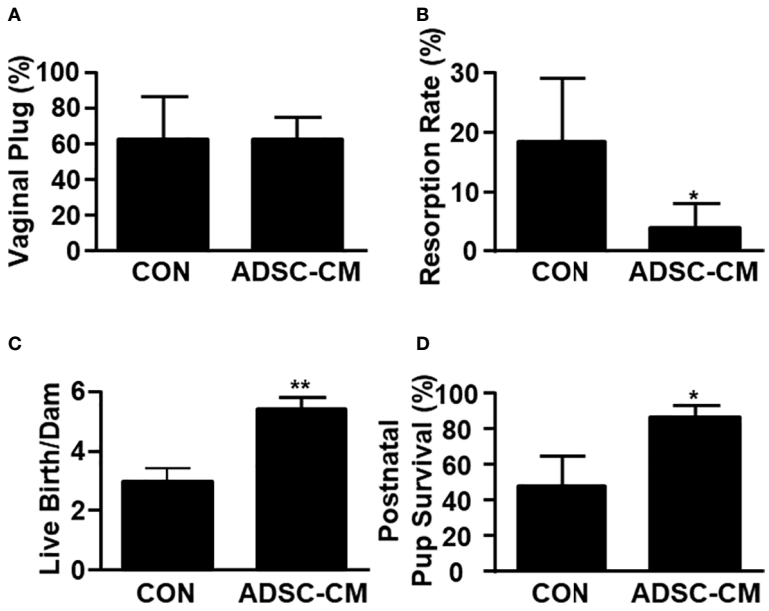

FIGURE 6
ADSC-CM Improves Pregnancy Outcomes in Mice with Endometriosis Fecundity and pregnancy outcomes represented by **(A)** numbers of vaginal plug, however, **(B)** resorption rate, **(C)** live births per dam, **(D)** pup survival rate at 1 week-old were recorded in mice complicated with endometriosis treated with or without ADSC-CM. The data were reported as mean ± SEM. n=4; *p < 0.05.; **p < 0.01.

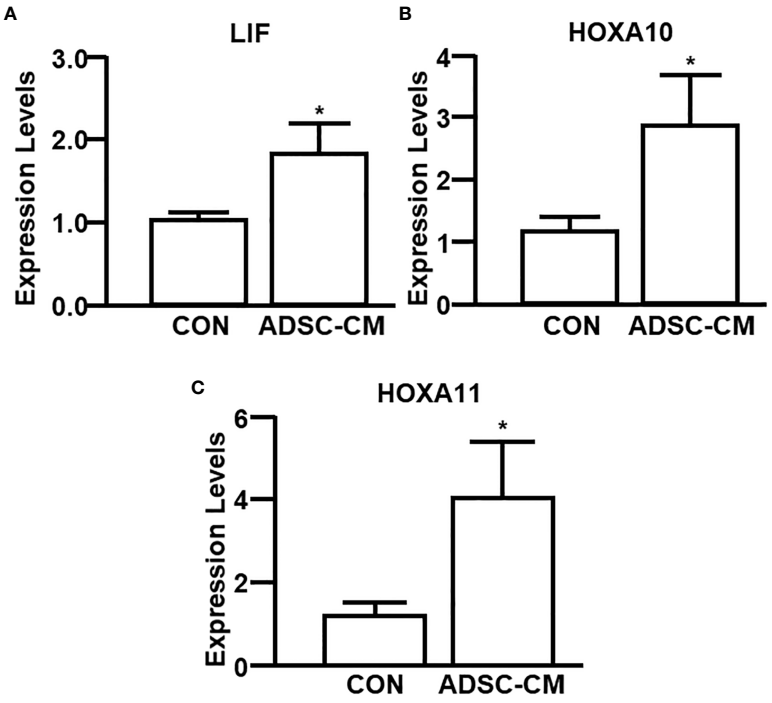

FIGURE 7
qRT-PCR of receptivity markers The expression of **(A)** LIF, **(B)** HOXA10 and **(C)** HOXA11 in the eutopic endometrium of mice with endometriosis at GD4 was examined by qRT-PCR. The data were reported as mean ± SEM. n=6; *p < 0.05.

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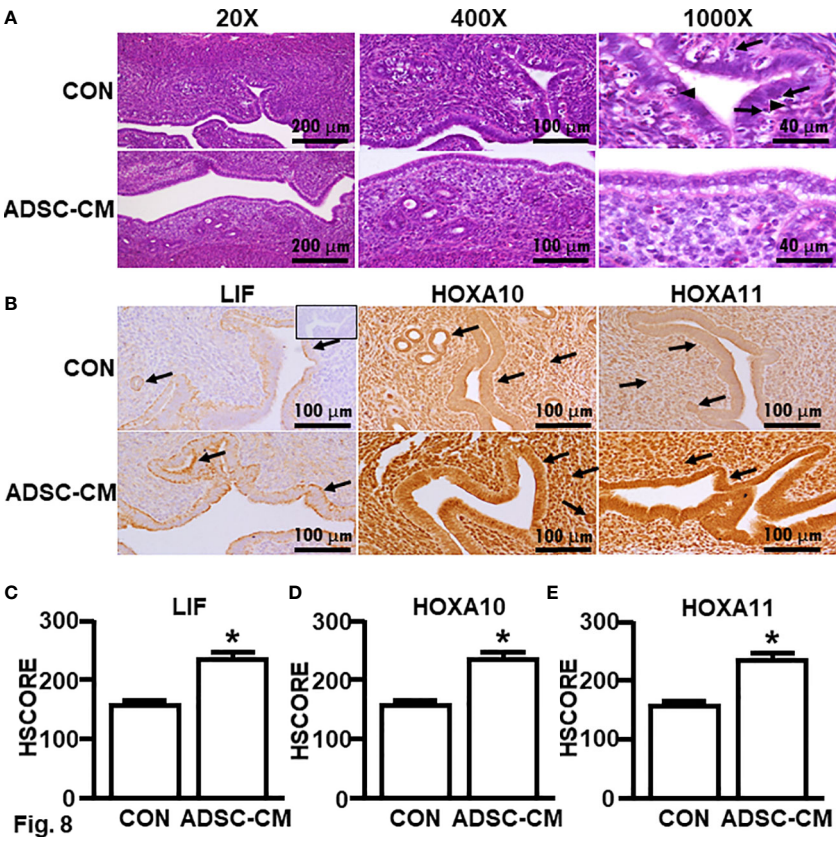

FIGURE 8
Histology and immunochemical staining of receptivity markers Eutopic endometria of mice with endometriosis at GD4 were subjected to **(A)** H & E stain. Arrows: lymphocytes; Arrow heads: polymorphonuclear cells. **(B)** Immunohistochemistry was used to assess the expression of **(C)** LIF, **(D)** HOXA10 and **(E)** HOXA11. The data were reported as mean ± SEM. n=6; *p < 0.05.

(30). Through binding with heavy chain-hyaluronan (HC-HA) (31), HC-HA PTX3 exerts anti-angiogenic (32), anti-inflammatory, anti-scarring (33) effects. Such binding also stabilizes HC-HA in the cumulus oophorous complex matrix (34). Macrophages are polarized to M2 subtype by HC-HA PTX3 (35). Nevertheless, the aforementioned proteins, DNA fragments, lipids, and RNA species (microRNA and long non-coding RNAs) can also be secreted *via* membrane-encapsulated particles, namely extracellular vesicles (EVs). Thus, the inhibition of the development of endometriosis by ADSC-CM is more likely to be attributed to a combinatory effect of these secreted molecules. The proteomic analysis in this study reveals the protein profile of ADSC-CM. Additional studies are required to demonstrate the distribution of various protein and non-protein molecules.

In conclusion, the current study showed for the first time that ADSC-CM effectively reduces the development of endometriosis and improves pregnancy outcomes. These findings open a new avenue in establishing a novel therapeutic strategy to combat endometriosis. These results can potentially be translated to clinical management of endometriosis.

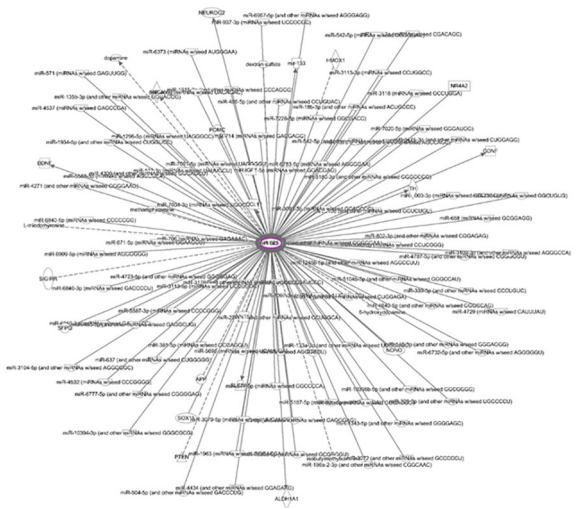

FIGURE 9
Network of molecules interacting with PTX3 One hundred and fourteen molecules interact with PTX3 in the ADSC-CM.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by E-Da Hospital Institutional Review Boards. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by E-Da Hospital Institutional Animal Care and Use Committee.

Author contributions

SJH and L-YS designed research and drafted the manuscript. Y-PH and C-CC provided the technical assistance and consultation for experimental design. C-YH and Y-CY performed animal studies, qRT-PCR, IHC, and data analyses. Y-HH provided the adipose tissue from lipoaspiration. L-YS and J-CL isolated the ADSCs and did the quality control tests. J-HC performed the proteomics analysis of ADSC-CM. SH did the IPA analysis of proteomics data. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1158527/full#supplementary-material

References

1. Frackiewicz EJ. Endometriosis: an overview of the disease and its treatment. *J Am Pharm Assoc (Wash)* (2000) 40:645–57. doi: 10.1016/S1086-5802(16)31105-6

2. Lebovic DI, Mueller MD, Taylor RN. Immunobiology of endometriosis. *Fertil Steril* (2001) 75:1–10. doi: 10.1016/S0015-0282(00)01630-7

3. Kayisli UA, Mahutte NG, Arici A. Uterine chemokines in reproductive physiology and pathology. *Am J Reprod Immunol* (2002) 47:213–21. doi: 10.1034/j.1600-0897.2002.01075.x

4. Haney AF, Muscato JJ, Weinberg JB. Peritoneal fluid cell populations in infertility patients. *Fertil Steril* (1981) 35:696–8. doi: 10.1016/S0015-0282(16)45567-6

5. Mathur S, Peress MR, Williamson HO, Youmans CD, Maney SA, Garvin AJ, et al. Autoimmunity to endometrium and ovary in endometriosis. *Clin Exp Immunol* (1982) 50:259–66.

6. Halme J, Becker S, Hammond MG, Raj MH, Raj S. Increased activation of pelvic macrophages in infertile women with mild endometriosis. *Am J Obstet Gynecol* (1983) 145:333–7. doi: 10.1016/0002-9378(83)90720-2

7. Kennedy SH, Starkey PM, Sargent IL, Hicks BR, Barlow DH. Antiendometrial antibodies in endometriosis measured by an enzyme-linked immunosorbent assay before and after treatment with danazol and nafarelin. *Obstet Gynecol* (1990) 75:914–8.

8. Kalaitzopoulos DR, Samartzis N, Kolovos GN, Mareti E, Samartzis EP, Eberhard M, et al. Treatment of endometriosis: a review with comparison of 8 guidelines. *BMC Womens Health* (2021) 21:397. doi: 10.1186/s12905-021-01545-5

9. Gimble JM, Katz AJ, Bunnell BA. Adipose-derived stem cells for regenerative medicine. *Circ Res* (2007) 100:1249–60. doi: 10.1161/01.RES.0000265074.83288.09

10. Kim WS, Park BS, Sung JH, Yang JM, Park SB, Kwak SJ, et al. Wound healing effect of adipose-derived stem cells: a critical role of secretory factors on human dermal fibroblasts. *J Dermatol Sci* (2007) 48:15–24. doi: 10.1016/j.jdermsci.2007.05.018

11. Pecanha R, Bagno LL, Ribeiro MB, Robottom Ferreira AB, Moraes MO, Zapata-Sudo G, et al. Adipose-derived stem-cell treatment of skeletal muscle injury. *J Bone Joint Surg Am* (2012) 94:609–17. doi: 10.2106/JBJS.K.00351

12. Chan TM, Chen JY, Ho LI, Lin HP, Hsueh KW, Liu DD, et al. ADSC therapy in neurodegenerative disorders. *Cell Transplant* (2014) 23:549–57. doi: 10.3727/096368914X678445

13. Donizetti-Oliveira C, Semedo P, Burgos-Silva M, Cenedeze MA, Malheiros DM, Reis MA, et al. Adipose tissue-derived stem cell treatment prevents renal disease progression. *Cell Transplant* (2012) 21:1727–41. doi: 10.3727/096368911X623925

14. Kim WS, Park BS, Kim HK, Park JS, Kim KJ, Choi JS, et al. Evidence supporting antioxidant action of adipose-derived stem cells: protection of human dermal fibroblasts from oxidative stress. *J Dermatol Sci* (2008) 49:133–42. doi: 10.1016/j.jdermsci.2007.08.004

15. Wang X, Wang Y, Zhou X, Liu F. Conditioned medium from adipose-derived stem cell inhibits jurkat cell proliferation through TGF-β1 and p38/MAPK pathway. *Analytical Cell Pathol* (2019) 2019:1-6. doi: 10.1155/2019/2107414

16. Kim WS, Park BS, Sung JH. The wound-healing and antioxidant effects of adipose-derived stem cells. *Expert Opin Biol Ther* (2009) 9:879–87. doi: 10.1517/14712590903039684

17. Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *JAMA* (2001) 286:327–34. doi: 10.1001/jama.286.3.327

18. Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, Bovenkerk JE, et al. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation* (2004) 109:1292–8. doi: 10.1161/01.CIR.0000121425.42966.F1

19. Kadowaki T, Yamauchi T, Kubota N, Hara K, Ueki K, Tobe K. Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. *J Clin Invest* (2006) 116:1784–92. doi: 10.1172/JCI29126

20. Deng ZB, Poliakov A, Hardy RW, Clements R, Liu C, Liu Y, et al. Adipose tissue exosome-like vesicles mediate activation of macrophage-induced insulin resistance. *Diabetes* (2009) 58:2498–505. doi: 10.2337/db09-0216

21. Dai M, Zhang Y, Yu M, Tian W. Therapeutic applications of conditioned medium from adipose tissue. *Cell Prolif* (2016) 49:561–7. doi: 10.1111/cpr.12281

22. Cheng JH, Hsu CC, Hsu SL, Chou WY, Wu YN, Kuo CA, et al. Adipose-derived mesenchymal stem cells-conditioned medium modulates the expression of inflammation induced bone morphogenetic protein-2, -5 and -6 as well as compared with shockwave therapy on rat knee osteoarthritis. *Biomedicines* (2021) 9:1-17. doi: 10.3390/biomedicines9101399

23. Fainaru O, Adini A, Benny O, Adini I, Short S, Bazinet L, et al. Dendritic cells support angiogenesis and promote lesion growth in a murine model of endometriosis. *FASEB J* (2008) 22:522–9. doi: 10.1096/fj.07-9034com

24. Skaland I, Janssen EA, Gudlaugsson E, Klos J, Kjellevold KH, Soiland H, et al. Phosphohistone H3 expression has much stronger prognostic value than classical prognosticators in invasive lymph node-negative breast cancer patients less than 55 years of age. *Mod Pathol* (2007) 20:1307–15. doi: 10.1038/modpathol.3800972

25. Mao F, Tu Q, Wang L, Chu F, Li X, Li HS, et al. Mesenchymal stem cells and their therapeutic applications in inflammatory bowel disease. *Oncotarget* (2017) 8:38008–21. doi: 10.18632/oncotarget.16682

26. Lessey BA, Kim JJ. Endometrial receptivity in the eutopic endometrium of women with endometriosis: it is affected, and let me show you why. *Fertil Steril* (2017) 108:19–27. doi: 10.1016/j.fertnstert.2017.05.031

27. Neykova K, Tosto V, Giardina I, Tsibizova V, Vakrilov G. Endometrial receptivity and pregnancy outcome. *J Matern Fetal Neonatal Med* (2022) 35:2591–605. doi: 10.1080/14767058.2020.1787977

28. Shukla L, Yuan Y, Shayan R, Greening DW, Karnezis T. Fat therapeutics: the clinical capacity of adipose-derived stem cells and exosomes for human disease and tissue regeneration. *Front Pharmacol* (2020) 11:158. doi: 10.3389/fphar.2020.00158

29. Bottazzi B, Doni A, Garlanda C, Mantovani A. An integrated view of humoral innate immunity: pentraxins as a paradigm. *Annu Rev Immunol* (2010) 28:157–83. doi: 10.1146/annurev-immunol-030409-101305

30. Tranguch S, Chakrabarty A, Guo Y, Wang H, Dey SK. Maternal pentraxin 3 deficiency compromises implantation in mice. *Biol Reprod* (2007) 77:425–32. doi: 10.1095/biolreprod.107.062414

31. Scarchilli L, Camaioni A, Bottazzi B, Negri V, Doni A, Deban L, et al. PTX3 interacts with inter-alpha-trypsin inhibitor: implications for hyaluronan organization and cumulus oophorus expansion. *J Biol Chem* (2007) 282:30161–70. doi: 10.1074/jbc.M703738200

32. Shay E, He H, Sakurai S, Tseng SC. Inhibition of angiogenesis by HC.HA, a complex of hyaluronan and the heavy chain of inter-alpha-inhibitor, purified from human amniotic membrane. *Invest Ophthalmol Vis Sci* (2011) 52:2669–78. doi: 10.1167/iovs.10-5888

33. He H, Li W, Tseng DY, Zhang S, Chen SY, Day AJ, et al. Biochemical characterization and function of complexes formed by hyaluronan and the heavy chains of inter-alpha-inhibitor (HC*HA) purified from extracts of human amniotic membrane. *J Biol Chem* (2009) 284:20136–46. doi: 10.1074/jbc.M109.021881

34. Salustri A, Garlanda C, Hirsch E, De Acetis M, Maccagno A, Bottazzi B, et al. PTX3 plays a key role in the organization of the cumulus oophorus extracellular matrix and in *in vivo* fertilization. *Development* (2004) 131:1577–86. doi: 10.1242/dev.01056

35. He H, Zhang S, Tighe S, Son J, Tseng SCG. Immobilized heavy chain-hyaluronic acid polarizes lipopolysaccharide-activated macrophages toward M2 phenotype. *J Biol Chem* (2013) 288:25792–803. doi: 10.1074/jbc.M113.479584

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Reproductive outcomes and risk factors of women with septate uterus after hysteroscopic metroplasty

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Background: Hysteroscopic metroplasty of the uterine septum has been the standard treatment strategy to improve reproductive outcomes, but there are still controversies about the appropriateness of metroplasty. In addition, there have been few studies of the factors related to reproductive outcomes of women after surgery. The study aimed to evaluate the reproductive outcomes and the associated risk factors that influence reproductive outcomes after hysteroscopic metroplasty of women with septate uterus and the desire to conceive.

Methods: This study was an observational study. Cases were screened by searching electronic patient files, and demographic factors were collected. We conducted telephone follow-ups to collect the postoperative reproductive outcomes. The primary outcome of this study was live birth, and secondary outcomes were ongoing pregnancy, clinical pregnancy, early miscarriage, and preterm birth. Demographic variables included patients' age, body mass index (BMI), the type of septum, infertility and miscarriage history, and complications including intrauterine adhesions, endometrial polyps, endometriosis, and adenomyosis were collected to perform univariate and multivariate analyses to predict the risk factors of reproductive outcomes after surgery treatment.

Results: In total, 348 women were evaluated and followed up. There were 95 cases (27.3%, 95/348) with combined infertility, 195 cases (56.0%, 195/348) with miscarriage history, and cases combined with intrauterine adhesions, endometrial polyps, endometriosis, and adenomyosis were 107 (30.7%, 107/348), 53 (15.2%, 53/348), 28 (8.0%, 28/348), and 5 (1.4%), respectively. Following surgery, the live birth rate and clinical pregnancy rate were significantly higher than prior to surgery (84.6% vs 3.7%, p= 0.000; and 78.2% vs 69.5%, p= 0.01, respectively), early miscarriage rate and preterm delivery rate were significantly lower (8.8% vs 80.6%, p= 0.000; and 7.0% vs 66.7%, p=0.000, respectively). After adjusting for body mass index, miscarriage history, and complications, multivariable logistic regression analysis revealed age ≥ 35 years and primary infertility as independent factors that affected postoperative clinical pregnancy (OR 4.025, 95% CI 2.063–7.851, p= 0.000; and OR 3.603, 95% CI 1.903–6.820,

p= 0.000; respectively) and ongoing pregnancy (OR 3.420, 95% CI 1.812–6.455, p= 0.000; and OR 2.586, 95% CI 1.419–4.712, p= 0.002; respectively).

Conclusions: Hysteroscopic metroplasty could lead to improved reproductive outcomes of women with septate uterus. Both age and primary infertility were independent factors for postoperative reproductive outcomes.

Trial registration: Chi ECRCT20210343

KEYWORDS

septate uterus, hysteroscopic metroplasty, reproductive outcomes, live birth, clinical pregnancy

Introduction

A septate uterus is the most common congenital uterine anomaly, characterized by a septum that divides the uterus into two cavities (1). It is thought to be associated with poor reproductive outcomes including reduced live birth (37.9% vs 84.8%) and increased miscarriage (36.2–77.1% vs 9.1–16.7%) (2, 3). In one prospective study, the risk of miscarriage of women with septate uterus increased with each loss from approximately 11% to 40% after three or more pregnancy losses (4). A meta-analysis suggested that prevalence of septate uterus is 18% after two miscarriages and 17% after three (5). In addition, there is possible involvement of septate uterus in infertility of women. Studies suggested that approximately 3.5–8% (6, 7) of subfertile women, and 15.4–24.5% (6) of those with a history of miscarriage and infertility, had a septate uterus; in comparison, this prevalence is 2–3% in the general female population (8). The effective uterine volume reduction of the septate uterus results in miscarriage. However, as most miscarriages occur in the first trimester of pregnancy in the women with septate uterus (9), there may be other mechanisms such as abnormal function of the endometrium including endometrial receptivity and decidual transformation. Hysteroscopic metroplasty or hysteroscopic transcervical division of the uterine septum has been the standard treatment strategy to improve reproductive outcomes (10). Following surgery treatment, the miscarriage rate decreased from 94.3% to 10.4%, and live birth rate increased from 2.4% to 81.3% (2). Recently, some studies dispute this. A large cohort study of 257 patients and an international, multicenter randomized controlled trial of 68 patients showed that surgery treatment did not improve chance of conception (11, 12). Therefore, there are still controversies about the appropriateness of metroplasty (13). In addition, patients' age, BMI, infertility and miscarriage history, and combinations may be the related demographic factors of fertility outcomes after surgery. A septate uterus does not always exist alone, and there are other possible uterine cavity disorders such as polyps, uterine adhesions, and endometriosis. However, there have been few studies of the factors related to reproductive outcomes of women after surgery.

This study aimed to 1) provide a general overview of reproductive outcomes after hysteroscopic metroplasty, 2) compare reproductive outcomes among women with a history of primary infertility and those without, and 3) analyze the demographic factors including patients' age, BMI, infertility and miscarriage history, and combinations such as endometrial polyps, uterine adhesions, endometriosis and adenomyosis associated with reproductive outcomes of septate uterus after surgery.

Abbreviations: BMI: body mass index.

Methods

Study population

Women with a septate uterus receiving hysteroscopic metroplasty in the Department of Minimally Invasive Gynecology at Beijing Obstetrics and Gynecology Hospital of Capital Medical University from February 2015 to February 2020 were retrospectively investigated and screened for inclusion in this study.

The inclusion criteria for women follow: (1) with septate uterus; (2) age 20–40 years; and (3) desiring pregnancy. The exclusion criteria for women follow: (1) with secondary surgery of residual uterine septum; (2) with submucosal uterine fibroids; and (3) with endometrial lesion (including endometrial intraepithelial neoplasia and endometrial tuberculosis).

Women with septate uterus were diagnosed by hysteroscopy or imaging examination. We conducted hysteroscopic metroplasty in patients with infertility, prior miscarriage, or adverse obstetrical outcome. In addition, because it was reasonable to consider surgical treatment following counseling regarding potential benefits, we also performed surgery on these women without previous poor reproductive outcomes. All patients signed informed consent for using their clinical data for this research.

This retrospective follow-up study was conducted according to the Declaration of Helsinki for Medical Research involving Human Subjects and was approved by the China Ethics Committee of Registering Clinical Trials (Chi ECRCT20210343).

Data collection

We identified women retrospectively by searching electronic patient files, and clinical data were collected and examined. The following information was extracted by trained researchers: demographic, medical and pregnancy history including clinical pregnancy, miscarriage, live birth, and other uterine findings including intrauterine adhesions, endometrial polyps, endometriosis, and adenomyosis. Meanwhile, the patients' further medical details and reproductive outcomes were obtained by telephone follow-up, and the follow-up timepoints were two years after hysteroscopic surgery.

Operative procedures

The procedure is preferably performed in the early follicular phase of the menstrual cycle. Hysteroscopic metroplasty was conducted under laparoscopic guidance on all patients. Before the operation, the type of septum was again confirmed. Then the septum was cut along the midline starting from the outer part to the base of the septum. In addition, patients with complications including intrauterine adhesions, endometrial polyps, or endometriosis underwent hysteroscopy with resection of polyps, adhesiolysis, and laparoscopic excision of endometriosis. We performed second-look hysteroscopy at 3 months after metroplasty to evaluate the intrauterine cavity.

Outcome measures and definitions

The primary outcome of the current study was live birth, defined as at least one live birth beyond 24 weeks of gestation. Secondary outcomes were ongoing pregnancy, clinical pregnancy, miscarriage, and preterm birth. Ongoing pregnancy was indicated by a viable intrauterine pregnancy of at least 12 weeks duration confirmed on an ultrasound scan (14). Clinical pregnancy referred to the presence of a gestational sac on ultrasound or by documentation of a birth, spontaneous abortion, or therapeutic abortion in the absence of ultrasound. Miscarriage was defined as intrauterine pregnancy loss before 24 weeks of gestation and early miscarriage was before 14 weeks of gestation. Preterm birth was defined as delivery of 24-37 weeks of gestation (15).

Statistical analyses

Data analysis was performed using SPSS software, version 23.0. Independent proportions were compared using chi-squared test or Fisher's exact probability method (when $n \leq 40$ or $T \leq 1$), whereas paired proportions were compared using McNemar chi-squared test. To evaluate factors of age, BMI, the type of septum, miscarriage history, infertility (primary and secondary), and complications (intrauterine adhesions, endometrial polyps, endometriosis, and adenomyosis) associated with reproductive outcomes after surgery treatment, we used a chi-square test for univariate analyses and multivariate logistic regression for multivariate analyses. Odds ratios (OR) with corresponding 95% confidence interval (95% CI) were calculated by adjusting for possible confounders.

Results

Patient characteristics

During the study period, 481 cases were searched; of these, eight cases were excluded and 125 were not followed. There were 348 cases ultimately included and completely followed (Figure 1). The median follow-up time after hysteroscopic metroplasty was 48 months (range 8–79 months). Mean age of included women was 30.1 ± 4.2 years and median age was 30 years (20–40 years). Mean BMI was 22.9 ± 3.6 kg/m^2. There were 257 women (73.9%, 257/348) with partial septate uterus, while 91 (26.1%, 91/348) had complete septate uterus. There were 95 cases (27.3%, 95/348) combined with infertility (57 cases of primary infertility, 16.4%; and 38 cases of secondary infertility, 10.9%). There were 195 cases of spontaneous abortion (56.0%, 195/348); 124 cases (35.6%, 124/348) had one miscarriage and 71 cases (20.4%, 71/348) had two or more miscarriages;189 cases (54.3%, 189/348) had combinations: 107 cases (30.7%, 107/348) were combined with intrauterine

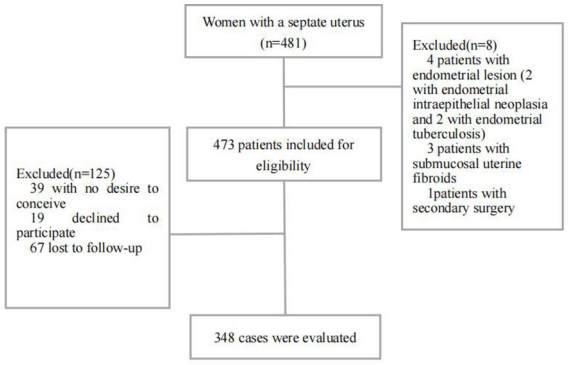

FIGURE 1
Flow chart of the screening of study population.

adhesions, and 53 cases (15.2%, 53/348) were combined with endometrial polyps. Endometriosis was combined in 28 cases (8.0%, 28/348) and adenomyosis in 5 cases (1.4%, 5/348). Characteristics of women are shown in Table 1.

Reproductive outcomes

Following surgery, the live birth rate was significantly higher than prior to surgery (84.6% vs 3.7%, $p = 0.000$), clinical pregnancy rate was significantly higher (78.2% vs 69.5%, $p = 0.010$), early miscarriage rate was significantly lower (8.8% vs 80.6%, $p = 0.000$), and preterm birth rate was significantly lower (7.0% vs 66.7%, p=0.000, Table 2). There was no statistical difference regarding live birth rate. In addition, of the 57 women with combined primary infertility, 28 (84.8%, 28/33) had live birth, 33 (57.9%, 33/57) achieved clinical pregnancy, 31 (54.4%, 31/57) had ongoing pregnancy, 3 (9.1%, 3/33) had early miscarriages, and 0 had preterm birth. The live birth rate between the two groups had no statistical significance. There was significant difference of clinical pregnancy and ongoing pregnancy between women with primary infertility and without (57.9% vs 82.1%, p=0.000; 54.4% vs 75.3%, p=0.001; respectively, Table 3).

When the complications (intrauterine adhesions, endometrial polyps, endometriosis, and adenomyosis) had been excluded, the live birth rate was significantly higher than prior to surgery (86.8% vs 5.4%, p=0.000), clinical pregnancy rate was significantly higher (76.1% vs 57.9%, p=0.001), early miscarriage rate was significantly lower (5.0%vs. 79.3%, p=0.000), and preterm birth rate was significantly lower (4.8% vs 60.0%, p=0.000) in the 159 women (Table 2). Of the 36 women with primary infertility, 16 (88.9%, 16/18) had live birth, 18 (50.0%,18/36) had clinical pregnancy, 18 (50.0%, 18/36) had ongoing pregnancy, 1 (5.6%, 1/18) had early miscarriage, and 0 had preterm live birth. No significant difference was found for the rate of live birth rate. There was significant difference of clinical pregnancy and ongoing pregnancy between women with primary infertility and without (50.0% vs 83.7%, p=0.000; 50.0% vs 75.6%, p=0.003; respectively). The reproductive outcomes are summarized in Table 3.

TABLE 1 Baseline characteristics of the participants.

Characteristics	n (%)
Age (Mean, 30.1 ± 4.2; median, 30)	
<35	300 (86.2%)
≥35	48 (13.8%)
BMI (Mean, 22.9 ± 3.6)	
<30	271 (77.9%)
≥30	77 (22.1%)
classification of septum	
partial septate uterus	257 (73.9%)
complete septate uterus	91 (26.1%)
combined Infertility	95 (27.3%)
Primary	57 (16.4%)
Secondary	38 (10.9%)
Previous miscarriage	195 (56.0%)
one	124 (35.6%)
two or more	71 (20.4%)
combined intrauterine adhesion	107 (30.7%)
combined endometrial polyps	53 (15.2%)
combined endometriosis	28 (8.0%)
combined adenomyosis	5 (1.4%)
mode of conception	
natural pregnancy	308 (88.5%)
artificial assisted reproduction	40 (11.5%)
without primary infertility and miscarriage history	96 (27.6%)

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TABLE 2 Comparison of reproductive outcomes between before and post hysteroscopic metroplasty.

	live birth			clinical pregnancy			early miscarriage			preterm birth		
	before operation	postoperation	P	before operation	postoperation	P	before operation	postoperation	P	before operation	postoperation	P
All participants	9 (3.7%)	230 (84.6%)	0.000	242 (69.5%)	272 (78.2%)	0.010	195 (80.6%)	24 (8.8%)	0.000	6 (66.7%)	16 (7.0%)	0.000
Complications excluded	5 (5.4%)	105 (86.8%)	0.000	92 (57.9%)	121 (76.1%)	0.001	73 (79.3%)	6 (5.0%)	0.000	3 (60.0%)	5 (4.8%)	0.000

Factors associated with reproductive outcomes after surgery

The factors of age, BMI, the type of septum, miscarriage history, infertility (primary and secondary), and complications (intrauterine adhesions, endometrial polyps, endometriosis, and adenomyosis) were analyzed. Univariate analysis showed that age, combined primary infertility, and prior miscarriage history may be associated with clinical pregnancy after surgery. All these factors had no significant effect on live birth. The details are given in Table 4.

We further analyzed independent risk factors using a multivariate logistic regression analysis model; after adjusting for prior miscarriage history and complications, we found age ≥ 35 years and primary infertility had significant associations with lower clinical pregnancy (OR 4.025, 95% CI 2.063–7.851, $p = 0.000$; and OR 3.603, 95% CI 1.903–6.820, $p = 0.000$; respectively) and lower ongoing pregnancy (OR 3.420, 95% CI 1.812–6.455, $p = 0.000$; and OR 2.586, 95% CI 1.419–4.712, $p = 0.002$; respectively, Figure 2, Table 5).

Discussion

Our main findings are as follows: (1) live birth, clinical pregnancy, and early miscarriage rate were significantly improved following surgery treatment in patients with septate uterus with or without complications; (2) patients with primary infertility history had a lower clinical pregnancy rate compared to those who were not; and (3) of the possible factors associated with reproductive outcomes, age ≥ 35 years and primary infertility history may be independent risk factors for postoperative clinical pregnancy. Our study finally included 348 women, which was the largest sample size to date on this topic. Following surgery, live birth rate increased from 3.7% to 84.6%, clinical pregnancy rate increased from 69.5% to 78.2%, and early miscarriage decreased from 80.6% to 8.8%. In the previous studies, the reported live birth rate range was 76.2–81.3% after surgery compared to 2.4–4.3% prior to surgery (16). These other studies support that surgical treatment has a positive effect on the reproductive prognosis.

However, there is still controversy in regard to this topic. Some studies (11–13, 17) found no significant difference in reproductive outcomes between the two groups. A Cochrane review published in 2011 reported that there was no evidence for hysteroscopic metroplasty in women with recurrent miscarriage and a septate uterus, and advised against offering this intervention as routine practice. Recently, an international multicentre cohort study (11) was performed and included a total of 257 women with septate uterus: 151 underwent septum resection and 106 had expectant management. They suggested that surgical treatment did not improve reproductive outcomes. It is important that demographic factors including age, BMI, reproductive history, and comorbidity may be risk factors affecting the subsequent reproductive outcomes in women with septate uterus after surgical treatment. In our study, we performed univariate and multivariate analyses to find potential influencing factors. This is the first study to analyze risk factors

TABLE 3 Comparison of postoperative fertility outcomes of patients with and without primary infertility.

Outcomes	with primary infertility n=57	without primary infertility n=291	P	Complications excluded n=159		
				with primary infertility n=36	without primary infertility n=123	P
live birth, n (% of clinical pregnancy)	28 (84.8%)	202 (84.5%)	1.000	16 (88.9%)	91 (88.3%)	1.000
clinical pregnancy, n (%)	33 (57.9%)	239 (82.1%)	0.000	18 (50.0%)	103 (83.7%)	0.000
ongoing pregnancy, n (%)	31 (54.4%)	219 (75.3%)	0.001	18 (50.0%)	93 (75.6%)	0.003
early miscarriage, n (% of clinical pregnancy)	3 (9.1%)	21 (8.8%)	1.000	1 (5.6%)	5 (4.9%)	1.000
preterm birth, n (% of live birth)	0 (0%)	16 (7.9%)	0.230	0 (0%)	5 (5.5%)	1.000

associated with postoperative reproductive outcomes in women with septate uterus who desire to conceive. The results suggest that both age and primary infertility history were independent risk factors for postoperative reproductive outcome.

The surgery indication of septate uterus is not uniform, and current guidelines have different recommendations. The ASRM and NICE guidelines (18) recommend removing the intrauterine septum, whereas the ESHRE (19) and RCOG guidelines (20) recommend not performing surgery and suggest that the procedure should be evaluated in future studies. Some scholars suggest that women should not undergo extensive evaluation after a single first trimester or early second trimester miscarriage, given that these are relatively common with only a modestly increased risk of recurrence (21–23). The individualized treatment for patients of different ages and with comorbidities was ignored. Our findings provide some evidence for the clinical practice. For example, the indication may be relaxed for older women, and women with combined intrauterine adhesions, endometrial polyps, endometriosis, or adenomyosis. There may be potential benefits to consider septum resection after counseling regarding potential risks of the procedure in women. In addition, second-generation techniques have been used in hysteroscopy with good results. These new technological advances in the treatment of septate uterus have demonstrated sufficient efficacy in terms of reproductive outcomes (24–26).

However, the association between septate uterus and infertility is still unclear. Studies that evaluated women with primary infertility or unexplained infertility (2) showed an improved pregnancy rate followed surgery. In our study, we found the postoperative reproductive outcomes of women with primary infertility were improved but their clinical pregnancy was lower than women without it. We hypothesize that surgery could restore normal anatomy but not completely restore normal function. Initially, a uterine septum was believed to be predominantly fibrous tissue. However, magnetic resonance imaging and biopsy specimens suggest that the septum is primarily composed of muscle fibers and less

TABLE 4 Univariate analysis of reproductive outcomes after hysteroscopic metroplasty.

Variable	Live birth		Clinical pregnancy		Early miscarriage		Ongoing pregnancy		Preterm delivery	
	χ^2	P value	χ^2	P value	χ^2	P value	χ^2	P value	χ^2	P value
Age	——*	1.000	22.182	0.000	——*	0.471	——*	1.000	——*	1.000
BMI	0.315	0.574	1.710	0.191	0.679	0.410	0.000	0.987	0.256	0.613
Type of septum	0.248	0.618	6.864	0.009	0.247	0.619	0.557	0.455	1.683	0.194
Infertility	0.002	0.964	19.734	0.000	0.011	0.915	0.000	1.000	1.518	0.218
Primary	——*	1.000	16.401	0.000	0.000	1.000	——*	1.000	——*	0.232
secondary	——*	1.000	2.371	0.124	——*	1.000	——*	0.452	——*	1.000
miscarriage	0.158	0.924	6.279	0.043	1.008	0.604	0.923	0.630	2.962	0.227
combined intrauterine adhesion	2.325	0.127	0.032	0.859	1.544	0.214	0.386	0.534	0.004	0.948
combined endometrial polyps	0.390	0.533	0.024	0.878	0.000	1.000	0.000	1.000	0.431	0.511
combined endometriosis	——*	0.560	——*	0.159	——*	0.254	——*	0.130	——*	0.648
combined adenomyosis	——*	0.491	——*	1.000	——*	0.310	——*	1.000	——*	1.000
mode of conception	——*	0.596	0.012	0.914	——*	0.496	——*	0.293	——*	0.406

*Fisher's exact probability method.

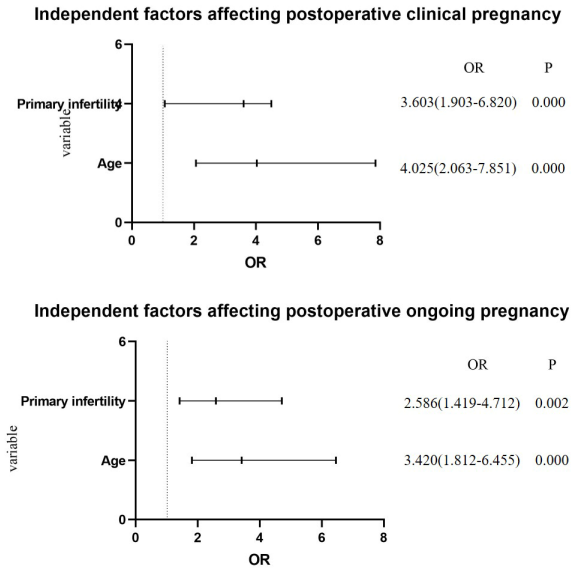

FIGURE 2
Independent factors.

TABLE 5 Multivariate analysis of reproductive outcomes after hysteroscopic metroplasty.

Variable	postoperative clinical pregnancy			postoperative ongoing pregnancy		
	n	OR (95%CI)	P value	n	OR (95%CI)	P value
age						
<35	247	4.025 (2.063–7.851)	0.000	227	3.603 (1.903–6.820)	0.000
≥35	25			23		
Primary infertility						
without	239	3.603 (1.903-6.820)	0.000	219	2.586 (1.419-4.712)	0.002
with	33			31		

connective tissue. One recent systematic literature review suggested that there was a lower number of glandular and ciliated cells and lower expression of HOXA10 genes and VEGF receptor genes in the endometrial lining of the septum, which possibly account for the poor reproductive outcomes (27). The vascularization, myometrium, and endometrium of septum are similar to the normal uterine wall. Further study should be performed to research the infertility mechanism and more effective treatment of septate uterus.

Our study has some limitations. The retrospective trial was at high risk of bias due to the before/after design. Although the factors of age, BMI, the type of septum, reproductive history, and comorbidities were analyzed to determine the influencing variables, there were other confounding factors such as women who were lost to follow-up, selection bias, and some unidentified factors. In addition, we found that early pregnancy accounted for 83.3% of all miscarriage in patients with septate uterus, so so we took early miscarriage as the outcome indicator of the study.

Conclusions

Hysteroscopic metroplasty could lead to improved reproductive outcomes of women with septate uterus, including those with miscarriage history and combined intrauterine adhesions, endometrial polyps, endometriosis, or adenomyosis. Both age and primary infertility were independent risk factors for postoperative reproductive outcomes. These provide some basis for the establishment of surgery treatment indication and personalized treatment strategy for women with septate uterus who desire to conceive.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the China Ethics Committee of Registering Clinical Trials. The patients/participants provided their written informed consent to participate in this study.

Author contributions

YC, MS, and HD conceived and designed the study. YC, MS, SW, and ZG searched and collected data. YC, MS, and ZG analyzed data. YC, MS, SW wrote the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Saridogan E, Salman M, Direk LS, Alchami A. Reproductive performance following hysteroscopic surgery for uterine septum: results from a single surgeon data. *J Clin Med* (2021) 10(1):130. doi: 10.3390/jcm10010130

2. Practice committee of the American society for reproductive medicine. Uterine septum: a guideline. *Fertil Steril* (2016) 106(3):530–40. doi: 10.1016/j.fertnstert.2016.05.014

3. Chan YY, Jayaprakasan K, Zamora J, Thornton JG, Raine-Fenning N, Coomarasamy A. The prevalence of congenital uterine anomalies in unselected and high-risk populations: a systematic review. *Hum Reprod Update* (2011) 17(6):761–71. doi: 10.1093/humupd/dmr028

4. Magnus MC, Wilcox AJ, Morken NH, Weinberg CR, Håberg SE. Role of maternal age and pregnancy history in risk of miscarriage: prospective register based study. *BMJ* (2019) 364:l869. doi: 10.1136/bmj.l869

5. Akhtar MA, Saravelos SH, Li TC, Jayaprakasan K. Reproductive implications and management of congenital uterine anomalies: scientific impact paper no. 62 November 2019. *BJOG* (2020) 127(5):e1–e13. doi: 10.1111/1471-0528.15968

6. Theodoridis TD, Pappas PD, Grimbizis GF. Surgical management of congenital uterine anomalies (including indications and surgical techniques). *Best Pract Res Clin Obstet Gynaecol* (2019) 59:66–76. doi: 10.1016/j.bpobgyn.2019.02.006

7. Rikken JFW, Kowalik CR, Emanuel MH, Bongers MY, Spinder T, de Kruif JH, et al. The randomised uterine septum transsection trial (TRUST): design and design and protocol. *BMC Womens Health* (2018) 18(1):163. doi: 10.1186/s12905-018-0637-6

8. Venetis CA, Papadopoulos SP, Campo R, Gordts S, Tarlatzis BC, Grimbizis GF. Clinical implications of congenital uterine anomalies: a meta-analysis of comparative studies. *Reprod BioMed Online* (2014) 29(6):665–83. doi: 10.1016/j.rbmo.2014.09.006

9. Valle RF, Ekpo GE. Hysteroscopic metroplasty for the septate uterus: review and meta-analysis. *J Minim Invasive Gynecol* (2013) 20(1):22–42. doi: 10.1016/j.jmig.2012.09.010

10. Saravelos SH, Cocksedge KA, Li TC. The pattern of pregnancy loss in women with congenital uterine anomalies and recurrent miscarriage. *Reprod BioMed Online.* (2010) 20(3):416–22. doi: 10.1016/j.rbmo.2009.11.021

11. Rikken JFW, Verhorstert KWJ, Emanuel MH, Bongers MY, Spinder T, Kuchenbecker W, et al. Septum resection in women with a septate uterus: a cohort study. *Hum Reprod* (2020) 35(7):1578–88. doi: 10.1093/humrep/dez284

12. Rikken JFW, Kowalik CR, Emanuel MH, Bongers MY, Spinder T, Jansen FW, et al. Septum resection versus expectant management in women with a septate uterus: an international multicentre open-label randomized controlled trial. *Hum Reprod* (2021) 36(5):1260–7. doi: 10.1093/humrep/deab037

13. Alvero R, Burney RO, Khorshid A, Saravanabavanandhan B, Pier BD, Rosalik KF, et al. Surgical treatment of uterine septum to improve reproductive outcomes - resect or not? *Fertil Steril* (2021) 116(2):298–305. doi: 10.1016/j.fertnstert.2021.06.009

14. Lawn JE, Kinney MV, Belizan JM, Mason EM, McDougall L, Larson J, et al. Born too soon: the global epidemiology of 15 million preterm births. *Reprod Health* (2013) 10(Suppl 1):S2. doi: 10.1186/1742-4755-10-S1-S6

15. Wang Z, An J, Su Y, Liu C, Lin S, Zhang J, et al. Reproductive outcome of a complete septate uterus after hysteroscopic metroplasty. *J Int Med Res* (2019) 48 (3):300060519893836. doi: 10.1177/0300060519893836

16. Tonguc EA, Var T, Batioglu S. Hysteroscopic metroplasty in patients with a uterine septum and otherwise unexplained infertility. *Int J Gynaecol Obstet* (2011) 113 (2):128–30. doi: 10.1016/j.ijgo.2010.11.023

17. Krishnan M, Narice BF, Ola B, Metwally M. Does hysteroscopic resection of uterine septum improve reproductive outcomes: a systematic review and meta-analysis. *Arch Gynecol Obstet* (2021) 303(5):1131–42. doi: 10.1007/s00404-021-05975-2

18. NICE. NICE: hysteroscopic metroplasty of a uterine septum for primary infertility. *Inf Dent* (2015), 1–8. Available at: https://www.nice.org.uk/guidance/ipg509/resources

19. ESHRE Guideline Group on RPL, Bender Atik R, Christiansen OB, Elson J, Kolte AM, Lewis S, et al. ESHRE guideline: recurrent pregnancy loss. *Hum Reprod Open* (2018) 2018(2):hoy004. doi: 10.1093/hropen/hoy004

20. RCOG. The investigation and treatment of couples with recurrent firsttrimester and second-trimester miscarriage. *Green-top Guideline No. 17* (2011), 1–18.

21. Knudsen UB, Hansen V, Juul S, Secher NJ. Prognosis of a new pregnancy following previous spontaneous abortions. *Eur J Obstet Gynecol Reprod Biol* (1991) 39 (1):31–6. doi: 10.1016/0028-2243(91)90138-B

22. Nybo Andersen AM, Wohlfahrt J, Christens P, Olsen J, Melbye M. Maternal age and fetal loss: population based register linkage study. *BMJ* (2000) 320(7251):1708–12. doi: 10.1136/bmj.320.7251.1708

23. Cohain JS, Buxbaum RE, Mankuta D. Spontaneous first trimester miscarriage rates per woman among parous women with 1 or more pregnancies of 24 weeks or more. *BMC Pregnancy Childbirth* (2017) 17(1):437. doi: 10.1186/s12884-017-1620-1

24. Nappi L, Pontis A, Sorrentino F, Greco P, Angioni S. Hysteroscopic metroplasty for the septate uterus with diode laser: a pilot study. *Eur J Obstet Gynecol Reprod Biol* (2016) 206:32–5. doi: 10.1016/j.ejogrb.2016.08.035

25. Esteban Manchado B, Lopez-Yarto M, Fernandez-Parra J, Rodriguez-Oliver A, Gonzalez-Paredes A, Laganà AS, et al. Office hysteroscopic metroplasty with diode laser for septate uterus: a multicenter cohort study. *Minim Invasive Ther Allied Technol* (2022) 31(3):441–7. doi: 10.1080/13645706.2020.1837181

26. Nappi L, Falagario M, Angioni S, De Feo V, Bollino M, Sorrentino F. The use of hysteroscopic metroplasty with diode laser to increase endometrial volume in women with septate uterus: preliminary results. *Gynecol Surg* (2021) 18:11. doi: 10.1186/s10397-021-01093-8

27. Rikken J, Leeuwis-Fedorovich NE, Letteboer S, Emanuel MH, Limpens J, van der Veen F, et al. The pathophysiology of the septate uterus: a systematic review. *BJOG* (2019) 126(10):1192–9. doi: 10.1111/1471-0528.15798

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The female upper reproductive tract harbors endogenous microbial profiles

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Introduction: The female reproductive tract harbours unique microbial communities (known as microbiota) which have been associated with reproductive functions in health and disease. While endometrial microbiome studies have shown that the uterus possesses higher bacterial diversity and richness compared to the vagina, the knowledge regarding the composition of the Fallopian tubes (FT) is lacking, especially in fertile women without any underlying conditions.

Methods: To address this gap, our study included 19 patients who underwent abdominal hysterectomy for benign uterine pathology, and 5 women who underwent tubal ligation as a permanent contraceptive method at Hospital Clínico Universitario Virgen de la Arrixaca (HCUVA). We analyzed the microbiome of samples collected from the FT and endometrium using 16S rRNA gene sequencing.

Results: Our findings revealed distinct microbiome profiles in the endometrial and FT samples, indicating that the upper reproductive tract harbors an endogenous microbiome. However, these two sites also shared some similarities, with 69% of the detected taxa Being common to both. Interestingly, we identified seventeen bacterial taxa exclusively present in the FT samples, including the genera *Enhydrobacter, Granulicatella, Haemophilus, Rhizobium, Alistipes*, and *Paracoccus*, among others. On the other hand, 10 bacterial taxa were only found in the endometrium, including the genera *Klebsiella, Olsenella, Oscillibacter* and *Veillonella* (FDR <0.05). Furthermore, our study highlighted the influence of the endometrial collection method on the findings. Samples obtained transcervically showed a dominance of the genus Lactobacillus, which may indicate potential vaginal contamination. In contrast, uterine samples obtained through hysterescopy revealed higher abundance of

the genera *Acinetobacter, Arthrobacter, Coprococcus, Methylobacterium, Prevotella, Roseburia, Staphylococcus*, and *Streptococcus*.

Discussion: Although the upper reproductive tract appears to have a low microbial biomass, our results suggest that the endometrial and FT microbiome is unique to each individual. In fact, samples obtained from the same individual showed more microbial similarity between the endometrium and FT compared to samples from different women. Understanding the composition of the female upper reproductive microbiome provides valuable insights into the natural microenvironment where processes such as oocyte fertilization, embryo development and implantation occur. This knowledge can improve *in vitro* fertilization and embryo culture conditions for the treatment of infertility.

KEYWORDS

fallopian tubes, endometrium, 16S rRNA gene, microbes, microbiome, microbiota, upper reproductive tract

Introduction

As our understanding of the human microbiota continues to expand, it becomes increasingly evident that it is ubiquitous and exerts significant influence on human physiology and pathophysiology (1–3). Within the female reproductive tract, a growing body of evidence is associating microbial composition to reproductive functions in both healthy and diseased states (4–7).

While numerous studies corroborate the important role of microbial communities in the female lower reproductive tract (vagina and cervix) in the defense against pathogens, the upper reproductive tract (endometrium, Fallopian tubes, ovaries) was traditionally considered a sterile environment, with the cervix acting as a barrier against bacterial passage (8). However, with the advent of microbiome studies focusing on the human upper reproductive tract and analysis of microbial genomes, it is now evident that this region possesses its own distinct microbial communities (7, 9, 10). Recent studies have consistently shown that the endometrium harbors greater bacterial diversity and richness compared to the lower reproductive tract. These microbial communities are mainly composed of bacteria belonging to the phyla *Firmicutes*, *Bacteroidetes* and *Proteobacteria*. The dominance of *Lactobacillus* in the uterus has been associated with a higher probability of live births, while the presence of *Gardnerella* or *Streptococcus* has been linked to early pregnancy loss or implantation failure in IVF treatment (1, 11). However, due to differences in study design and the absence of proper negative and positive controls, there is a lack of consensus among studies examining the upper reproductive tract microbiota (9, 12).

The microbial composition of the Fallopian tubes (FT) is less studied, primarily due to challenges associated with sample collection which may affect future fertility. The characterization of the endogenous microbiome of the FT is of particular interest because this microenvironment provides a stable temperature, optimal pH and dynamic fluid secretions that support oocyte fertilisation and the early stages of embryo development (13–15). The limited studies analysing samples from women with benign diseases or for prophylactic purposes suggest that the FT does indeed harbor an endogenous microbiome. Predominant bacterial taxa identified in these studies include *Firmicutes* (especially *Staphylococcus* sp., *Enterococcus* sp., and *Lactobacillus* sp.), *Pseudomonas* sp. *Burkholderia* sp., *Propionibacterium* sp. and *Prevotella* sp (15–18). However, there is ongoing debate regarding whether the FT truly harbors an endogenous microbiome and to what extent it impacts oocyte fertilisation and the initial stages of embryo development.

Given the anatomical connection between the uterus and the FT, with the intramural portion of the uterine tube preventing a complete physical separation between the two sites, it is reasonable to hypothesize that the microbiome of the FT may be similar to that of the uterus (19–21). Therefore, comparative studies analyzing uterine and FT samples collected simultaneously from the same donor are necessary to evaluate whether the organs comprising the female upper reproductive tract possess specific endogenous microbial profiles. In the current study, we aimed to analyse the 16S rRNA gene V2-4 and V6-9 regions of endometrial and FT samples obtained from fertile women, with the objective of identifying the microbiome of the female upper reproductive tract in disease-free individuals.

Materials and methods

Study population

This prospective studywas conducted at the Service of Obstetrics and Gynaecology of the HCUVA in Murcia, Spain.

Patients who underwent a planned laparoscopic hysterectomy with bilateral salpingo-oophorectomy or laparoscopic tubal ligation from January 2016 until June 2018 were recruited to participate in the study. Inclusion criteria were as follows: Caucasian women who had not received hormonal treatment for three months prior to surgery, regular menstrual cycles, and no history of fertility problems, endometriosis or other adnexal pathology detected by transvaginal ultrasound analysis and confirmed through histological examination. Nineteen participants underwent total laparoscopic hysterectomy with bilateral salpingo-oophorectomy to remove the uterus, cervix, ovaries, and FT due to the presence of uterine fibroids and associated abnormal bleeding (see Figure 1 for the study design). Additionally, five participants underwent tubal ligation to remove the FTs as a permanent contraception/sterilization measure. This study was approved by the Ethics Research Committee (CEIC) of HCUVA, Murcia, Spain (Approval No. EST: 04/16) and all participants provided written informed consent. Patient data and samples included in this study were registered, stored, and processed by the Biobanco en Red de la Región de Murcia, BIOBANC-MUR (registered on the Registro Nacional de Biobancos – ISCIII, no. B.0000859).

Collection of FT and endometrial samples

The collection method for the FT samples was standardizedfor all patients who underwent laparoscopic hysterectomy with bilateral salpingo-oophorectomy or laparoscopic tubal ligation. After the laparoscopic procedure, FTs were removed and transferred to ice-cold Petri dishes. Once dissected, FTs were clamped at both opposite ends to avoid sample waste. Manual mechanical pressure was applied between the extremities, the FT

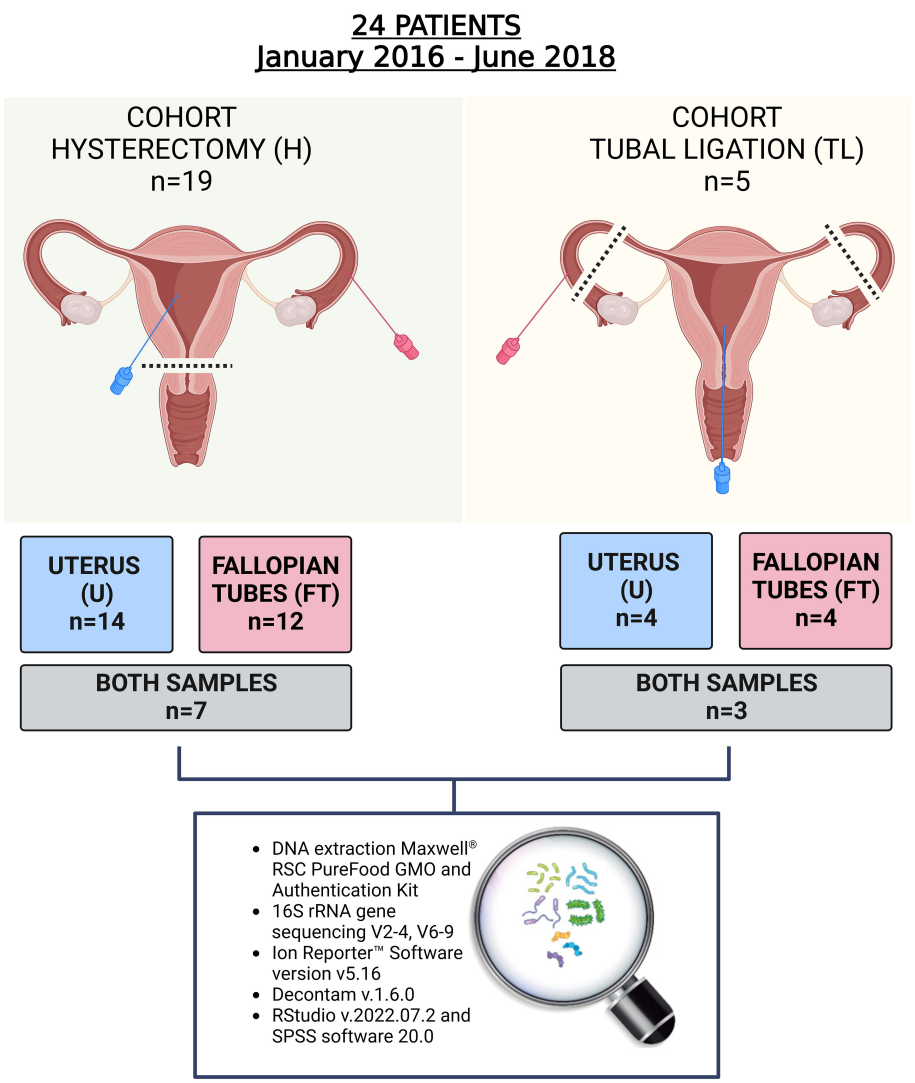

FIGURE 1
Study design. In total 24 women participated in the study and 34 samples from the upper reproductive tract were retrieved. In the hysterectomy cohort (H), seven women provided both endometrial and Fallopian tube (FT) samples, and in the tubal ligation cohort (TL), three women provided both samples. The rest of the participants provided only one of the samples due to the tissue damage during laparoscopic procedure, non-sterile condition, or blood contamination.

content that accumulated at the upper portion of the ampulla was aspirated through a sterile Mucat device (CDD Laboratorie, France). This class I medical device, complying with Directive 93/42/EEC, indicated for direct exocervical or endocervical aspiration and Hühner test, was adapted to be easily introduced into the tubes. Once introduced, aspiration of the content was performed with the integrated plunger, which slides up and down when pushed by a flexible acetal resin shaft, without a syringe. The content was immediately aliquoted in 1,5ml Eppendorf Safe-Lock® Tubes, frozen in liquid nitrogen until further analysis.

For the endometrial samples, the collection method avried depending on the type of surgery. During hysterectomy, when the entire upper reproductive tract was removed, direct access to the uterus was achieved using a sterile Mucat device (CDD Laboratorie, France). The device was carefully maneuvered to avoid sampling uterine fibroid tissue (clearly identified visually), as well as potential microbial contamination from the vagina or the cervix. In contrast, for patients undergoing tubal ligation without uterus removal, a speculum was inserted to gently separate the vagina, allowing visualization of the cervix. The cervix was cleaned with sterile saline solution and then the sterile Mucat device (CDD Laboratorie, France) was inserted into the cervix to reach the interior of the uterus. The aspiration of the uterine content was performed with the integrated plunger as previously described (22). The collected content was stored in 1,5ml Eppendorf Safe-Lock® Tubes, and frozen in liquid nitrogen until further analysis.

DNA extraction, amplification, library preparation, and sequencing

DNA extraction from the stored samples was performed using the Maxwell® RSC PureFood GMO and Authentication Kit and Maxwell® RSC Equipment (Promega, USA). A NanoDrop spectrophotometer was used to determine the DNA yield (A260) and purity (A260/A280 ratio) (Supplementary Table 1).

Bacterial identification was performed by Genomics Unit from Institute for Biomedical Research of Murcia IMIB-Arrixaca. The multiplex PCR using Ion Torrent 16S Metagenomics kit (Thermo Fisher Scientific Inc., **USA)** was used to amplify the 16S rRNA gene.Two sets of primers to target the regions V2, V4, V8, and V3, V6-7, V9 (Supplementary Table 2). Amplification was performed in a SimpliAmp thermal cycler (Applied Biosystems, USA) following the program: denaturation at 95°C for 10 min, followed by a cyclic 3-step stage consisting of 25 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 20 s; at the end of this stage, the program concluded with an additional extension period at 72°C for 7 min and the reaction was stopped by cooling at 4°C. The resulting amplicons were tested by electrophoresis using 2% agarose gel in tris-acetate-EDTA (TAE) buffer, purified with AMPure® XP Beads (Beckman Coulter Inc., USA), and quantified using QubitTM dsDNA HS Assay Kit in a Qubit 3 fluorometer (Invitrogen, Thermo Fisher Scientific Inc., **USA**). Afterwards, the Ion Plus Fragment Library Kit (Thermo Fisher Scientific Inc., **USA)** was used to generate a library from each sample. Each library was indexed by ligating Ion Xpress ™ Barcode Adapters (Thermo Fisher Scientific Inc. **USA)** to the amplicons. Libraries were purified with AMPure® XP Beads and quantified using the Ion Universal Library Quantitation Kit (Thermo Fisher Scientific Inc., **USA)** in a QuantStudio 5 Real-Time PCR Instrument (Applied Biosystems, USA).

Next, the libraries were pooled and clonally amplified onto Ion Sphere Particles (ISPs) by emulsion PCR in an Ion OneTouch™ 2 System (Thermo Fisher Scientific Inc., **USA)** according to the manufacturer´s instructions. Sequencing of the amplicon libraries was carried out on an Ion 530™ Kit (Thermo Fisher Scientific Inc. **USA**) on an Ion S5™ System (Thermo Fisher Scientific Inc., **USA)**.

Data processing

After sequencing, the individual sequence reads were filtered by the Torrent Suite ™ Software v5.12.1 to remove the low quality and polyclonal sequences. The quality filtered data were analyzed using Ion Reporter™ Software version v5.16. Clustering into operational taxonomic units (OTUs) and taxonomic assignment were performed based on the Basic Local Alignment Search Tool (BLAST) using two reference libraries, MicroSEQ® 16S Reference Library v2013.1 and the Greengenes v13.5 database (Life Technologies Corporation, USA). For an OTU to be accepted as valid, at least ten reads with an alignment coverage ≥ 90% between the hit and query were required. Identifications were accepted at the genus level with sequence identity > 97%.

Given that characterization of the low microbial biomass site like the upper reproductive tract requires meticulous contamination control, *in-silico* decontamination approach using Decontam v.1.6.0 (23, 24) was applied to discern between the true bacterial sequences and potential contaminants. To use this method, a table of the relative abundances of OTUs (columns) in each sample (rows) was created from the raw data. Next, we included DNA concentration of each sample in the model (from Supplementary Table 1). The Decontam score threshold was set to 0.1 as a default setting to define contaminating phylotypes (23). The relative abundance of the considered contaminant phylotypes was set to zero as described previously (24). Furthermore, for diversity and abundance analyses we additionally filtered out those taxa that were detected in less than 30% of the remaining samples, as previously described (25).

Statistical analyses

Statistical analyses were performed using the R statistical software v.4.2.1 under RStudio v.2022.07.2 and SPSS software 20.0 (SPSS, USA). Microbiome data were aggregated to genus level for diversity and abundance comparisons. All relative abundances were expressed as median and first and third quartiles (q1, q3). Normal distribution of the variables was tested by using the Shapiro-Wilk test. Relative abundances of identified genera did not meet normality and were analyzed using the nonparametric Mann-Whitney *U* test. Furthermore, the Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) was performed to validate our results. Benjamini-Hochberg

method (false discovery rate [FDR]) was used to obtain adjusted p-values in multiple comparisons. Differences were considered statistically significant between groups when $p < 0.05$. Alpha-diversity indices (Shannon diversity index and OTUs number [i.e., richness]) were calculated using the diversity function of the *vegan* R package, both in FT and endometrial samples. Differences among the groups of samples' diversity indices were tested using Mann-Whitney *U* test. Additionally, alpha-diversity was compared between women with both types of samples using a Wilcoxon signed-rank test for paired data. Bray-Curtis dissimilarity was calculated using *vegdist* R function and Permutational Analysis of Variance (PERMANOVA) was performed to analyze beta-diversity using *adonis* R function.

Results

Samples

A total of 34 samples were collected from 24 enrolled patients. The patients' characteristics are presented in Table 1 and Supplementary Table 3. As indicated in Figure 1, from the group that underwent laparoscopic tubal ligation, four FT samples and four transcervical endometrial samples were collected. In the hysterectomy group, which involved the extraction of the upper reproductive tract, 12 FT samples and 14 endometrial samples were obtained from the uterus, avoiding uterine fibroid tissue. It was not always possible to collect both types of samples from each patient because some anatomical pieces were damaged after being removed by laparoscopic techniques, and due to the impossibility of collecting some samples with the required sterile conditions and without blood contamination. Both FT and endometrial samples were successfully collected from seven out of 19 patients of the hysterectomy cohort, while three out of the five patients in the tubal ligation cohort provided both samples (Figure 1).

Data processing

A total of 245 and 252 bacterial genera were identified in the endometrial and FT samples, respectively. The average number of reads per FT sample was 25241,44 ± 10845,46 (mean ± SD). For the endometrial samples, the average number of reads per sample was 30845 ± 18702,56 (mean ± SD). Applying the decontamination method using Decontam, two genera, *Aerococcus* in FT samples and *Acidovorax* in the endometrial samples, were identified as contaminant phylotypes and removed from the analysis. Furthermore, to ensure the identification of the "core" microbiome of both sites, an additional filtering step was applied, eliminating bacterial taxa present in less than 30% of the participants, as previously described (25). As a result, a total of 77 bacterial genera were identified in the FT samples (Supplementary Table 4), and 70 bacterial genera were identified in the endometrial samples (Supplementary Table 5).

Microbial profiles of FT samples

The microbial composition at the genus level in FT samples exhibited variability across different samples (Figure 2; Supplementary Figure 1 at family taxonomic level). The most abundant genera among all samples were *Lactobacillus* (relative abundance =14.3 [3.48;24.4]), *Prevotella* (relative abundance = 9.29 [0.31;12.7]), *Acinetobacter* (relative abundance =3.20 [1.36;11.7]), *Propionibacterium* (relative abundance =3.09 [2.45;5.86]) and *Faecalibacterium* (relative abundance =3.09 [0.68;4.97]) (Supplementary Table 4).

Since the fertile women undergoing tubal ligation had no associated pathology, while women undergoing hysterectomy were diagnosed with benign uterine fibroids, a comparative microbiome analysis was performed to investigate any potential influence of uterine fibroids on the microbial microenvironment in the tubes. No significant differences were revealed in microbial diversity, or in the differential abundance analysis between the two groups (Supplementary Table 6).

Microbial profiles of endometrial samples

The microbiome composition revealed heterogeneity among the endometrial samples. The genus *Lactobacillus* showed the highest average abundance (relative abundance =23.0 [6.89;49.8]),

TABLE 1 Demographic characteristics (age, body mass index- BMI and parity) of the study population and collected samples from two groups of patients: patients who underwent a total laparoscopic hysterectomy with bilateral salpingo-oophorectomy and patients who submitted to a laparoscopic tubal ligation.

Study Population Groups	Hysterectomy n=19	Tubal ligation n=5
Age (years)	45 ± 3	37 ± 4
BMI	28,5 ± 4,7	28,3 ± 4
Parity	1,8 ± 0,9	2,2 ± 0,5
Fallopian tube samples	12	4
Endometrial samples	14	4
Both tissue samples	7	3

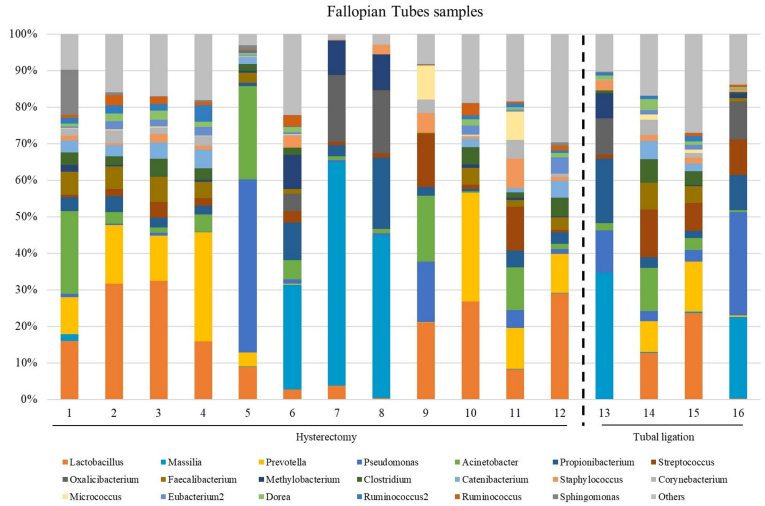

FIGURE 2
The most abundant genera detected in the Fallopian tubes (FT) samples from patients underwent atotal laparoscopic hysterectomy with bilateral salpingo-oophorectomy (patients 1, 2, 3, 5, 9, 10, 11, 12, 13, 15, 16 and 17) or laparoscopic tubal ligation (patients 20, 21, 23 and 24). Percent-stacked barchart of those genera whose mean relative abundances were higher than 1% are represented.

followed by *Prevotella* (relative abundance =4.13 [0.85;13.7]), *Faecalibacterium* (relative abundance =2.18 [0.24;4.12]), and *Clostridum* (relative abundance =2.08 [0.32;5.06]) (Figure 3; Supplementary Table 5, and Supplementary Figure 2 indicating family taxonomic level).

Unlike FT samples, the collection method for endometrial samples varied depending on the surgical procedure. In patients undergoing hysterectomy for benign uterine conditions, the entire upper reproductive tract was extracted, allowing direct access to the uterine cavity without passing through the vaginal and cervical canal. The fibroid tissue was visually identified and biopsied, focusing on tissue that presented unaltered morphological characteristics. On the other hand, in women undergoing to tubal ligation for contraceptive purposes and without underlying disease, endometrial biopsy was obtained transcervically. Therefore, we aimed to compare whether the uterine microenvironment could be influenced by the fibroids and whether the sampling method *via* cervix (high bacterial contamination risk) could have an impact on the microbial composition in the endometrial samples. When comparing the microbiome of the two sampling techniques, 20 genera presented significantly different abundance (Supplementary Table 7). When applying the multiple testing correction, nine genera remained as marginally different between the groups, where *Lactobacillus* was more abundant while *Acinetobacter, Arthrobacter, Coprococcus, Methylobacterium, Prevotella, Roseburia, Staphylococcus, Streptococcus* were less abundant in samples obtained transcervically (Figure 4; Supplementary Table 7).

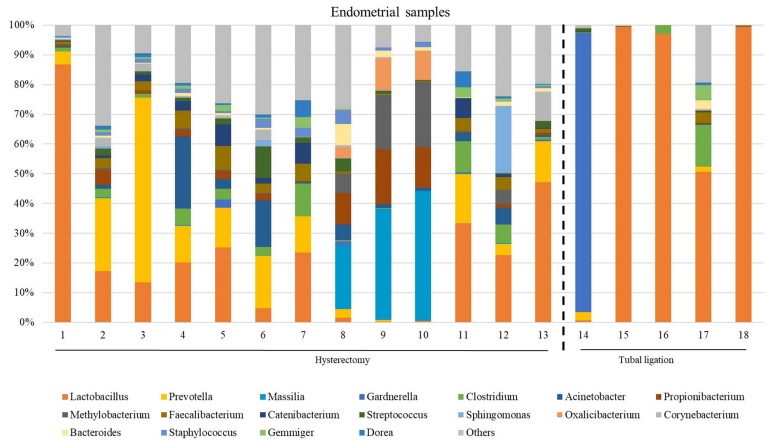

FIGURE 3
The most abundant genera detected in the endometrial samples from patients undergoing a total laparoscopic hysterectomy with bilateral salpingo-oophorectomy (patients 1, 2, 3, 4, 6, 7, 8, 10, 12, 13, 14, 15, 18 and 19) or laparoscopic tubal ligation (patients 20, 22, 23 and 24). Percent-stacked barchart of those genera whose mean relative abundances were higher than 1% are represented.

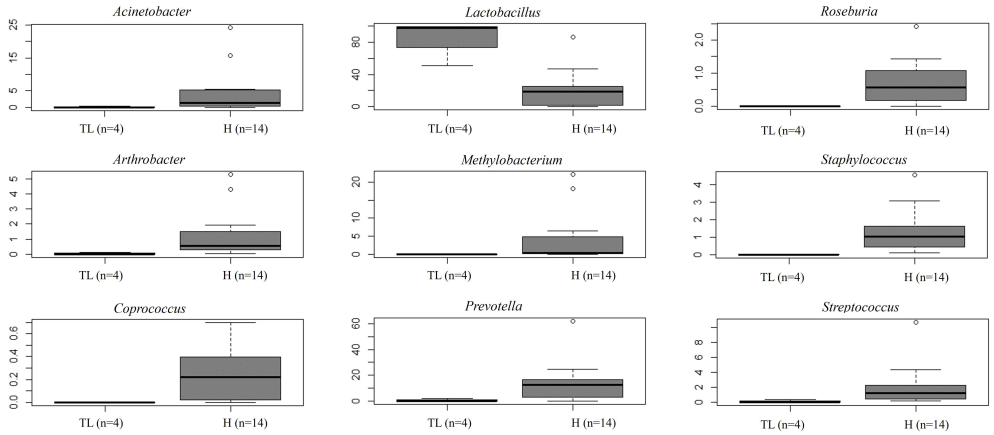

FIGURE 4
Relative abundance of nine bacterial genera between samples obtained directly from the uterus (hysterectomy, H) (fertile women with fibroids) and transcervically when undergoing tubal ligation (TL) (fertile women without the disease). After multiple testing correction adjustment, the difference remained marginal (FDR=0.083 for all plots).

Microbiome composition between endometrial and FT samples

When comparing microbial composition between the endometrium and FT, the endometrial samples from the tubal ligation group were excluded f4rom the analysis. This decision was made due to significant microbiome differences, potentially indicating vaginal orcervical contamination characterized by a high abundance of *Lactobacillus*). Thus, 16 FT samples and 14 endometrial samples were compared. A considerable portion of the detected taxa (60 genera) was found in both sites, indicating shared microbial composition. Additionally, 17 bacterial genera were exclusively detected in the FT samples, while 10 genera were considered endometrial-specific (Figure 5; Table 2). Out of these detected genera (Supplementary Table 8), the relative abundance of 11 genera was significantly between uterine and FT samples, as confirmed by both the Mann-Whitney U test and ANCOM-BC analysis. Specifically, *Gardnerella* (p=0.002; FDR=0.042), *Klebsiella* (p=0.004; FDR=0.042), *Olsenella* (p=0.004; FDR=0.042), *Oscillibacter* (p=0.004; FDR=0.042) and *Veillonella* (p=0.004; FDR=0.042) were found to be more prevalent in the endometrium.Conversely, *Enhydrobacter* (p=0.001; FDR=0.042), *Granulicatella* (p=0.001; FDR=0.042), *Haemophilus* (p=0.003; FDR=0.042), *Rhizobium* (p=0.003; FDR=0.042), *Alistipes* (p=0.006; FDR=0.048) and *Paracoccus* (p=0.006; FDR=0.048) were more abundant in FT samples (p values obtained from the strict Mann-Whitney U test analysis).

No significant differences were detected between the endometrial and FT samples in alpha-diversity metrics when comparing the microbiome diversity of endometrial and FT samples (i.e., Shannon, OTUs number [richness]) (Figure 6A). Also, beta-diversity represented by PCoA blot based on Bray-Curtis distances did not show any significant dissimilarities between the microbiome composition between the two sample types (Figure 6B).

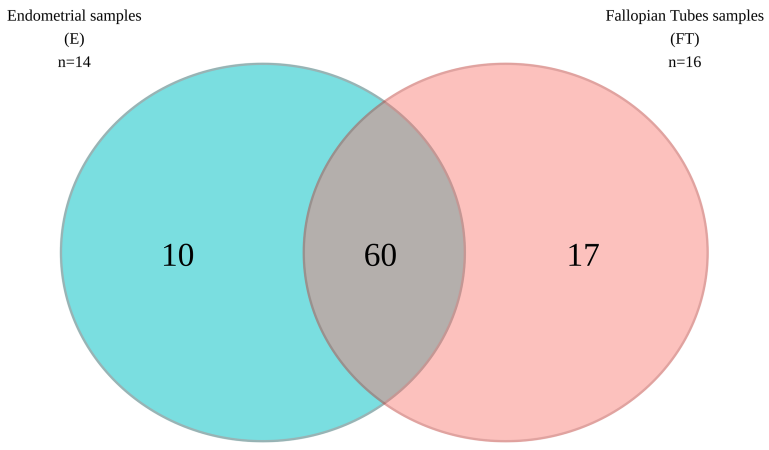

FIGURE 5
Venn diagram illustrating the bacterial genera present in the upper reproductive tract.

TABLE 2 Microbial composition of the endometrial and Fallopian tube (FT) samples.

Fallopian tubes	Fallopian tubes and Endometrium			Endomet
Aeromonas †	*Acinetobacter*	*Actinomyces*	*Anaerococcus*	*Barnesiella* †
*Alistipes**†	*Arthrobacter*	*Bacillus*	*Bacteroides*	*Brachymonas*†
Bifidobacterium †	*Bilophila*	*Blautia*	*Butyricimonas*	*Chryseobacterium*†
Brachyspira†	*Campylobacter*	*Catenibacterium*	*Cloacibacterium*	*Gardnerella**†
Brevundimonas†	*Clostridium*	*Collinsella*	*Coprococcus*	*Klebsiella**†
Burkholderia †	*Corynebacterium*	*Desulfovibrio*	*Dialister*	*Olsenella**†
Comamonas †	*Dolosigranulum*	*Dorea*	*Enterococcus*	*Oscillibacter**†
*Enhydrobacter**†	*Eubacterium*	*Eubacterium2*	*Faecalibacterium*	*Serratia* †
Flavonifractor †	*Finegoldia*	*Gemella*	*Gemmiger*	*Veillonella**†
Fusobacterium †	*Helicobacter*	*Herbaspirillum*	*Kocuria*	*Vibrio*†
*Granulicatella** †	*Lachnoclostridium*	*Lactobacillus*	*Lactococcus*	
*Haemophilus**†	*Massilia*	*Megasphaera*	*Methylobacterium*	
*Paracoccus**†	*Microbacterium*	*Micrococcus*	*Mitsuokella*	
Parasutterella †	*Moraxella*	*Neisseria*	*Oxalicibacterium*	
*Rhizobium**†	*Parabacteroides*	*Pelomonas*	*Phascolarctobacterium*†	
Shewanella †	*Porphyromonas*	*Prevotella*	*Propionibacterium*	
Sutterella †	*Pseudoflavonifractor*	*Pseudomonas*	*Ralstonia*	
	Roseburia	*Rothia*	*Ruminiclostridium*	
	Ruminococcus	*Ruminococcus2*	*Sphingomonas*	
	Staphylococcus	*Streptococcus*	*Subdoligranulum*	

The asterisks (*) represent the differentially abundant microbial taxa between uterine and FT samples analyzed by the non-parametric Mann-Whitney U test ($p<0.05$). The crosses (†) represent the differentially abundant microbial taxa between the endometrial and FT samples analyzed by the Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) ($p<0.05$). P-values were adjusted for the multiple testing correction (False Discovery Rate, FDR).

Sensitivity analysis in paired endometrial and FT samples

A sensitivity analysis was performed using samples exclusively from patients who underwent hysterectomy (n=7) and had valid samples from both tissues (endometrium and FT) (Figure 1; Supplementary Table 3). This approach aimed to avoid the possible contamination effect from cervical bacteria.

The comparison of microbial diversity between endometrial and FT samples revealed no significant differences in alpha- (Figure 7A) and beta- (Figure 7B) diversity metrics. (p >0,05).

In this more restricted subset of samples, the previously observed statistical differences in the relative abundances of the 11 genera (*Gardnerella, Klebsiella, Olsenella, Oscillibacter, Veillonella, Enhydrobacter, Granulicatella, Haemophilus, Rhizobium, Alistipes and Paracoccus*) between endometrial and

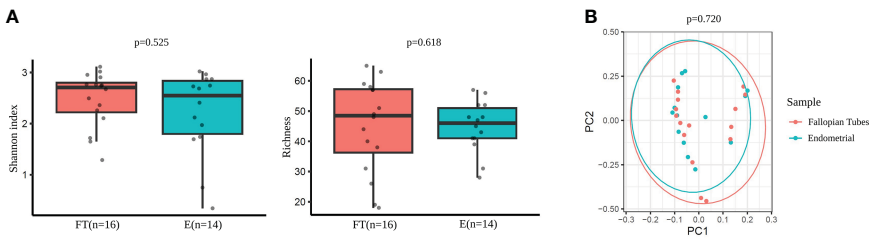

FIGURE 6
Diversity indices in Fallopian tubes (FT) and endometrial (E) samples. **(A)** Alpha-diversity metrics (i.e., Shannon, OTUs number [richness]) of endometrial and FT samples. **(B)** Beta-diversity represented by principal coordinate analysis (PCoA) based on Bray-Curtis distances (PERMANOVA, R2=0.024, p=0.720) between endometrial and FT samples.

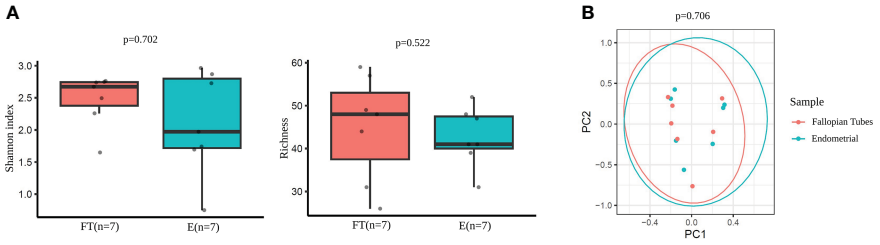

FIGURE 7
Diversity indices in paired endometrial and FT samples. **(A)** Alpha-diversity metrics (i.e., Shannon, OTUs number [richness]) of endometrial and FT samples when the restricted group of patients with paired samples was selected. **(B)** Beta-diversity represented by principal coordinate analysis (PCoA) based on Bray-Curtis distances of patients with paired samples (PERMANOVA, R2 = 0.048, p=0.706).

FT samples did not remain statistically significant after adjusting for the multiple testing correction (FDR) (Supplementary Table 9).

As a next step, we performed an additional comparison considering each pair of samples from the same patient. Alpha-diversity analysis did not detect any statistically significant differences when comparing the paired tissue samples of each patient (Shannon diversity index and OTUs number with p >0.05; Supplementary Tables 10, 11, respectively) (Figure 8A). However, beta-diversity analysis revealed a significant dissimilarity when comparing the paired samples from the same woman (PERMANOVA, p=0.044) (Figure 8B). This finding suggests that the microbiome within an individual, even from two different tissue types (endometrium and FT), is more similar than the same tissue type (e.g. endometrium) between different individuals.

Discussion

The female upper reproductive tract plays a critical role in oocyte fertilisation, early embryo development, and embryo implantation. Understanding the detailed microenvironment in the FT and endometrium is essential for manipulating and improving conditions in assisted reproduction technologies. Over 20% of couples at reproductive age suffer infertility, and with the socioeconomic situation where couples delay family planning and have children later in life, the demand for infertility treatment continues to rise worldwide (26).

There is a growing awareness that the microbes colonizing our body are involved in various pathological processes. Therefore, studying the microbiome of female reproductive tract has become a hot topic in order to understand its role in crucial events such as embryo development and pregnancy establishment (6). Imbalances in the uterine cavity microbiome have been associated with implantation failure, decreased success of assisted reproductive technologies, as well as conditions like endometriosis, endometritis, polyps, and endometrial cancer (10, 27, 28). However, very few studies have analysed FT microbiome due to ethical and technical challenges associated with obtaining FT sample without compromising future fertility. As a result, there is currently no consensus on the core microbial composition of the upper reproductive tract, whether in healthy or pathological conditions (10, 12, 29–31), and further research is needed.

The current study analysed the microbial composition of the upper reproductive tract in women with confirmed fertility. We examined FT and endometrial samples from patients diagnosed with benign uterine pathology or without the disease. Our findings revealed a shared (~70%) endogenous microbial community present in both sites of the upper reproductive tract, whith *Lactobacillus*, *Prevotella*, and *Faecalibacterium* being the most prevalent taxa. Considering that the intramural portion of the uterine tube in humans does not allow for physical separation between the FT and uterine environments, it is reasonable to assume that there is smooth communication between these anatomical regions, resulting in similar microbiomes. We detected 60 bacterial genera common to both tissues, while 17 bacterial genera were FT-specific and 10 were uniquely present in the endometrium. *Gardnerella*, *Klebsiella*, *Olsenella*, *Oscillibacter*, and *Veillonella* were significantly associated

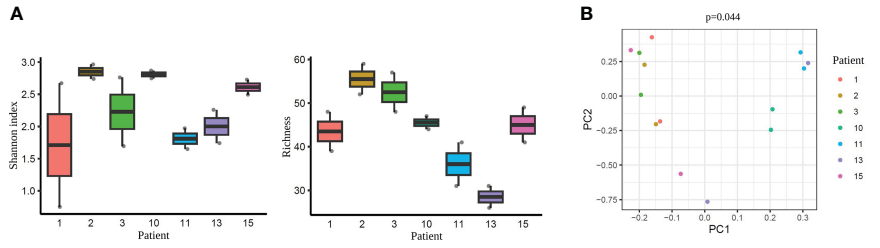

FIGURE 8
Diversity indices each pair of the tissue samples corresponding to their respective patient. **(A)** Alpha-diversity metrics (i.e., Shannon, OTUs number) of paired endometrium and Fallopian tube (FT) samples from the same women (n=7), all values p >0.05. Each label indicates a patient (e.g. 1). **(B)** Beta-diversity represented by principal coordinate analysis (PCoA) based on Bray-Curtis distances of patients with paired samples (PERMANOVA, R^2 = 0.622, p=0.044). Each patient is indicated with one colour, where the two dots of the same colour represent individuals' endometrial and FT samples.

with the endometrial samples, while *Enhydrobacter*, *Granulicatella*, *Haemophilus*, *Rhizobium*, *Alistipes*, and *Paracoccus* were more abundant in FT samples. Although the presence of these genera in the upper reproductive tract has been previously described (8, 16, 32), the site specificity demonstrated in our results has not been reported before.

When comparing the FT and endometrial samples obtained from the same women, although the sample size was limited, it seems that the two distinct tissue microbiomes were more similar within an individual than the same tissue sample between different individuals. These data support the hypothesis that each person has their own "microbial fingerprint", with microbial residents tailored to their environmental conditions – namely their genetics, diet, and developmental history. These residents persist over time and help to defend against invaders (33). So, it is expected that there would be more microbial similarities between different body sites within an individual compared to specific body sites between different individuals. Similar results have been described previously, although with more heterogenous cohorts (18). Thus, establishing a 'core' microbiome becomes challenging, as what might be considered healthy in one person may differ from another, adding complexity to the investigation of the human microbiome.

Our study included fertile women with benign uterine conditions (fibroids) and women without the disease who underwent tubal ligation as a terminal contraceptive method. This led to two different methods for obtaining study material: hysterectomy and tubal ligation. The study evaluated the effect of fibroids-related uterine microenvironment on the FT microbiome. FT samples were obtained in both cohorts using the same method, allowing us to study this effect. Our findings showed no association between the fibroids-free endometrial microbiome from women with uterine fibroids and the microbiome of FT. This suggests that fibroids-related uterine environment does not seem to affect the FT microenvironment.

In contrast, the sampling method for obtaining endometrial samples differed considerably between the two cohorts.: In the hysterectomy cases, the reproductive organs were removed, and the endometrial samples were obtained directly by opening the uterus under sterile conditions. However, in the tubal ligation cases, the endometrial samples were obtained transcervically, posing a higher risk of bacterial contamination from the lower reproductive tract (vagina/cervix). Thus, when analysing the endometrial samples from these two cohorts, we cannot determine whether the significant differences observed in the endometrial microbial composition are due to the fibroids-associated uterine microenvironment or the sampling method itself. After applying multiple testing correction, nine genera remained marginally different between the groups. *Lactobacillus* was more abundant in samples obtained transcervically, while *Acinetobacter, Arthrobacter, Coprococcus, Methylobacterium, Prevotella, Roseburia, Staphylococcus, Streptococcus* were more abundant in hysterectomy samples. The difference in *Lactobacillus* abundance depending on the sampling method has been previously reported, with lower dominance linked to surgeries carrying a lower contamination risk from the vagina and cervix, such as hysterectomy (30), laparoscopy (8) and/or cesarean section (34) (10). In line with these studies, the uterine samples collected transcervically in our study showed a clear dominance of *Lactobacillus* (abundance of 98,2%), while samples obtained during hysterectomy showed higher diversity and lower prevalence of *Lactobacillus* (abundance of 18,7%). Based on these findings, we believe that the sampling method had a stronger effect on the endometrial microbiome than the fibroids-free uterine sample. A previous study by Winters et al. reported that the endometria of women with a median age of 45, who underwent hysterectomy for fibroids were dominated by *Acinetobacter* (abundance of 60%) (30). Other studies have suggested that *Acinetobacter* may be associated with a normal (or benign) endometrium, while *Methylobacterium* has been associated with endometrial cancer (35). In our study, disease-free endometrial samples from women with uterine fibroids showed a small relative abundance of *Acinetobacter* and *Methylobacterium*. These two genera, however, along with *Arthrobacter, Coprococcus, Prevotella, Roseburia, Staphylococcus*, and *Streptococcus*, which showed differential presence in endometrial samples, are considered common contaminant genera (9). Therefore, further research is required to determine which genera are contaminant and which have a role in uterine health. This could involve enrichment analysis of metabolic pathways using RNAseq analysis or whole metagenomics analysis, as well as investigating the impact of factors like uterine fibroids and other pathologies on the microbial composition. Interestingly, a recent study has associated *Clostridium, Ruminococcus, Blautia* and *Lactobacillus* (which were found in both tissues in our study) with Tryptophan metabolism (12). This suggests a potential host–microbiota crosstalk in the biosynthesis of serotonin and melatonin, as well as serotonin degradation, where Tryptophan acts as a precursor. Specifically, dysregulation of melatonin has been linked to altered uterine functions, including endometrial receptivity and recurrent spontaneous abortion (36).

Our study is the first to analyze the endometrial and FT samples together from women with confirmed fertility. Nevertheless, some limitations should be acknowledged. Firstly, the relatively small sample size makes the study results preliminary and highlight the need for confirmation in a larger sample size. Secondly, the analysis focused on older reproductive-aged women, and therefore the results should not be generalized for younger women, as age might influence the microbial composition. Thirdly, the endometrial samples were obtained at different cycle phases, which restricts our ability to examine endometrial receptivity. Fourthly, despite taking utmost care to obtain fibroid-free tissue when sampling endometrial biopsies, the effect of fibroids on uterine microenvironment cannot be ruled out. Lastly, the study design lacked negative controls in the sampling process and separate validation, thus, stringent decontamination tools and strict data processing methods were applied.

In conclusion, our study results corroborate that the female upper reproductive tract harbours an endogenous microbiome, although with low microbial biomass. We observed that a significant portion of the microbial profile is shared between the FT and the endometrium, with approximately ~70% of the detected taxa being shared. Interestingly, women have unique microbial profiles, wherein two distinct tissues (FT and endometrium) displayed greater bacterial similarities than the same tissue sample (e.g. endometrium) between two individuals. Unravelling the female upper reproductive microbiome, helps understanding the

natural microenvironment where crucial processes of oocyte fertilisation and embryo development occur. This knowledge can be used to improve *in vitro* fertilisation and embryo culture conditions for the treatment of infertility.

Data availability statement

The data presented in the study are deposited in the NCBI SRA Database, accession number PRJNA915312 (Raw Data in Biom format).

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Research Committee (CEIC) of HCUVA, Murcia, Spain (Approval No. EST: 04/16). The patients/participants provided their written informed consent to participate in this study.

Author contributions

AC-G, PC and SA conceived the idea and designed the study. PC, MP-S, MS-F provided all the required documentation to the Ethics Research Committee (CEIC) of Clinical University Hospital "Virgen de la Arrixaca" (HCUVA). MP-S, MS-F and AC-G recruited the patients. AC-G performed the sample and data collection. TE was the responsible of the registration, storage and processing of the samples at the Biobanco en Red de la Región de Murcia, BIOBANC-MUR. CR performed the amplification, library preparation and sequencing of the samples. IP-P, ES-E, IL-B analyzed the data. SA, AC-G and IP-P wrote the first manuscript draft. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1096050/full#supplementary-material

References

1. Franasiak JM, Scott RT. Reproductive tract microbiome in assisted reproductive technologies. *Fertil Steril* (2015) 104(6):1364–71. doi: 10.1016/j.fertnstert.2015.10.012

2. NIH HMP Working Group, Peterson J, Garges S, Giovanni M, McInnes P, Wang L, et al. The NIH human microbiome project. *Genome Res* (2009) 19(12):2317–23. doi: 10.1101/gr.096651.109

3. Altmäe S, Franasiak JM, Mändar R. The seminal microbiome in health and disease. *Nat Rev Urol* (2019) 16(12):703–21. doi: 10.1038/s41585-019-0250-y

4. Moreno I, Simon C. Deciphering the effect of reproductive tract microbiota on human reproduction. *Reprod Med Biol* (2019) 18(1):40–50. doi: 10.1002/rmb2.12249

5. Benner M, Ferwerda G, Joosten I, van der Molen RG. How uterine microbiota might be responsible for a receptive, fertile endometrium. *Hum Reprod Update* (2018) 24(4):393–415. doi: 10.1093/humupd/dmy012

6. Baker JM, Chase DM, Herbst-Kralovetz MM. Uterine microbiota: residents, tourists, or invaders? *Front Immunol* (2018) 9(MAR). doi: 10.3389/fimmu.2018.00208

7. Koedooder R, Singer M, Schoenmakers S, Savelkoul PHM, Morré SA, de Jonge JD, et al. The vaginal microbiome as a predictor for outcome of *in vitro* fertilization with or without intracytoplasmic sperm injection: a prospective study. *Hum Reprod* (2019) 34(6):1042–54. doi: 10.1093/humrep/dez065

8. Chen C, Song X, Wei W, Zhong H, Dai J, Lan Z, et al. The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. *Nat Commun* (2017) 8(1):875. doi: 10.1038/s41467-017-00901-0

9. Molina NM, Sola-Leyva A, Haahr T, Aghajanova L, Laudanski P, Castilla JA, et al. Analysing endometrial microbiome: methodological considerations and recommendations for good practice. *Hum Reprod* (2021) 36(4):859–79. doi: 10.1093/humrep/deab009

10. Molina NM, Sola-Leyva A, Saez-Lara MJ, Plaza-Diaz J, Tubić-Pavlović A, Romero B, et al. New opportunities for endometrial health by modifying uterine microbial composition: present or future? *Biomolecules* (2020) 10(4):593. doi: 10.3390/biom10040593

11. Moreno I, Franasiak JM. Endometrial microbiota–new player in town. *Fertil Steril* (2017) 108(1):32–9. doi: 10.1016/j.fertnstert.2017.05.034

12. Sola-Leyva A, Andrés-León E, Molina NM, Terron-Camero LC, Plaza-Díaz J, Sáez-Lara MJ, et al. Mapping the entire functionally active endometrial microbiota. *Hum Reprod* (2021) 36(4):1021–31. doi: 10.1093/humrep/deaa372

13. Ng KYB, Mingels R, Morgan H, Macklon N, Cheong Y. *In vivo* oxygen, temperature and pH dynamics in the female reproductive tract and their importance in human conception: a systematic review. *Hum Reprod Update* (2017) 24(1):15–34. doi: 10.1093/humupd/dmx028

14. Leese HJ. The formation and function of oviduct fluid. *J Reprod Fert* (1988) 82:843–56. doi: 10.1530/jrf.0.0820843

15. Li S, Winuthayanon W. Oviduct: roles in fertilization and early embryo development. *J Endocrinol* (2017) 232(1):R1–26. doi: 10.1530/JOE-16-0302

16. Pelzer ES, Willner D, Buttini M, Hafner LM, Theodoropoulos C, Huygens F. The fallopian tube microbiome: implications for reproductive health. *Oncotarget* (2018) 9(30):21541–51. doi: 10.18632/oncotarget.25059

17. Pelzer ES, Willner D, Huygens F, Hafner LM, Lourie R, Buttini M. Fallopian tube microbiota: evidence beyond DNA. *Future Microbiol* (2018) 13(12):1355–61. doi: 10.2217/fmb-2018-0118

18. Miles SM, Hardy BL, Merrell DS. Investigation of the microbiota of the reproductive tract in women undergoing a total hysterectomy and bilateral salpingo-oopherectomy. *Fertil Steril* (2017) 107(3):813–820.e1. doi: 10.1016/j.fertnstert.2016.11.028

19. Strandell A, Lindhard A. Why does hydrosalpinx reduce fertility? the importance of hydrosalpinx fluid. *Hum Reprod* (2002) 17(5):1141–5. doi: 10.1093/humrep/17.5.1141

20. Ng EH, Ajonuma LC, Lau EY, Yeung WS, Ho PC. Adverse effects of hydrosalpinx fluid on sperm motility and survival. *Hum Reprod* (2000) 15(4):772–7. doi: 10.1093/humrep/15.4.772

21. Meyer WR, Castelbaum AJ, Somkuti S, Sagoskin AW, Doyle M, Harris JE, et al. Hydrosalpinges adversely affect markers of endometrial receptivity. *Hum Reprod* (1997) 12(7):1393–8. doi: 10.1093/humrep/12.7.1393

22. Canha-Gouveia A, Paradela A, Ramos-Fernández A, Prieto-Sánchez MT, Sánchez-Ferrer ML, Corrales F, et al. Which low-abundance proteins are present in the human milieu of Gamete/Embryo maternal interaction? *Int J Mol Sci* (2019) 20 (21):5305. doi: 10.3390/ijms20215305

23. Davis NM, Proctor DM, Holmes SP, Relman DA, Callahan BJ. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome* (2018) 6(1):226. doi: 10.1186/s40168-018-0605-2

24. O'Callaghan JL, Turner R, Dekker Nitert M, Barrett HL, Clifton V, Pelzer ES, et al. Re-assessing microbiomes in the low-biomass reproductive niche. *BJOG Int J Obstet Gynaecol.* (2020) 127(2):147–58. doi: 10.1111/1471-0528.15974

25. Lüll K, Arffman RK, Sola-Leyva A, Molina NM, Aasmets O, Herzig KH, et al. The gut microbiome in polycystic ovary syndrome and its association with metabolic traits. *J Clin Endocrinol Metab* (2021) 106(3):858–71. doi: 10.1210/clinem/dgaa848

26. Sunde A, Brison D, Dumoulin J, Harper J, Lundin K, Magli MC, et al. Time to take human embryo culture seriously. *Hum Reprod* (2016) 31(10):2174–82. doi: 10.1093/humrep/dew157

27. Moreno I, Garcia-Grau I, Perez-Villaroya D, Gonzalez-Monfort M, Bahçeci M, Barrionuevo MJ, et al. Endometrial microbiota composition is associated with reproductive outcome in infertile patients. *Microbiome* (2022) 10(1):1. doi: 10.1186/s40168-021-01184-w

28. Peric A, Weiss J, Vulliemoz N, Baud D, Stojanov M. Bacterial colonization of the female upper genital tract. *Int J Mol Sci* (2019) 20(14):3405. doi: 10.3390/ijms20143405

29. Jiang I, Yong PJ, Allaire C, Bedaiwy MA. Intricate connections between the microbiota and endometriosis. *Int J Mol Sci* (2021) 22(11):5644. doi: 10.3390/ijms22115644

30. Winters AD, Romero R, Gervasi MT, Gomez-Lopez N, Tran MR, Garcia-Flores V, et al. Does the endometrial cavity have a molecular microbial signature? *Sci Rep* (2019) 9(1):9905. doi: 10.1038/s41598-019-46173-0

31. Altmäe S, Rienzi L. Endometrial microbiome: new hope, or hype? *Reprod BioMed Online* (2021) 42(6):1051–2. doi: 10.1016/j.rbmo.2021.05.001

32. Moreno I, Codoñer FM, Vilella F, Valbuena D, Martinez-Blanch JF, Jimenez-Almazán J, et al. Evidence that the endometrial microbiota has an effect on implantation success or failure. *Am J Obstet Gynecol.* (2016) 215(6):684–703. doi: 10.1016/j.ajog.2016.09.075

33. Franzosa EA, Huang K, Meadow JF, Gevers D, Lemon KP, Bohannan BJM, et al. Identifying personal microbiomes using metagenomic codes. *Proc Natl Acad Sci* (2015) 11:201423854. doi: 10.1073/pnas.1423854112

34. Leoni C, Ceci O, Manzari C, Fosso B, Volpicella M, Ferrari A, et al. Human endometrial microbiota at term of normal pregnancies. *Genes (Basel)* (2019) 10 (12):971. doi: 10.3390/genes10120971

35. Kaakoush NO, Olzomer EM, Kosasih M, Martin AR, Fargah F, Lambie N, et al. Differences in the active endometrial microbiota across body weight and cancer in humans and mice. *Cancers (Basel)* (2022) 14(9):2141. doi: 10.3390/cancers14092141

36. Chuffa LG de A, Lupi LA, Cucielo MS, Silveira HS, Reiter RJ, Seiva FRF. Melatonin promotes uterine and placental health: potential molecular mechanisms. *Int J Mol Sci* (2019) 21(1):300. doi: 10.3390/ijms21010300

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Transcriptomic analyses of ovarian clear-cell carcinoma with concurrent endometriosis

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Introduction: Endometriosis, a benign inflammatory disease whereby endometrial-like tissue grows outside the uterus, is a risk factor for endometriosis-associated ovarian cancers. In particular, ovarian endometriomas, cystic lesions of deeply invasive endometriosis, are considered the precursor lesion for ovarian clear-cell carcinoma (OCCC).

Methods: To explore this transcriptomic landscape, OCCC from women with pathology-proven concurrent endometriosis (n = 4) were compared to benign endometriomas (n = 4) by bulk RNA and small-RNA sequencing.

Results: Analysis of protein-coding genes identified 2449 upregulated and 3131 downregulated protein-coding genes (DESeq2, P< 0.05, log2 fold-change > |1|) in OCCC with concurrent endometriosis compared to endometriomas. Gene set enrichment analysis showed upregulation of pathways involved in cell cycle regulation and DNA replication and downregulation of pathways involved in cytokine receptor signaling and matrisome. Comparison of pathway activation scores between the clinical samples and publicly-available datasets for OCCC cell lines revealed significant molecular similarities between OCCC with concurrent endometriosis and OVTOKO, OVISE, RMG1, OVMANA, TOV21G, IGROV1, and JHOC5 cell lines. Analysis of miRNAs revealed 64 upregulated and 61 downregulated mature miRNA molecules (DESeq2, P< 0.05, log2 fold-change > |1|). MiR-10a-5p represented over 21% of the miRNA molecules in OCCC with endometriosis and was significantly upregulated (NGS: log2fold change = 4.37, P = 2.43e-18; QPCR: 8.1-fold change, P< 0.05). Correlation between miR-10a expression level in OCCC cell lines and IC50 (50% inhibitory concentration) of carboplatin *in vitro* revealed a positive correlation (R^2 = 0.93). MiR-10a overexpression *in vitro* resulted in a significant decrease in proliferation (n = 6; P< 0.05) compared to transfection with a non-targeting control miRNA.

Similarly, the cell-cycle analysis revealed a significant shift in cells from S and G_2 to G_1 (n = 6; P< 0.0001). Bioinformatic analysis predicted that miR-10a-5p target genes that were downregulated in OCCC with endometriosis were involved in receptor signaling pathways, proliferation, and cell cycle progression. MiR-10a overexpression *in vitro* was correlated with decreased expression of predicted miR-10a target genes critical for proliferation, cell-cycle regulation, and cell survival including [*SERPINE1* (3-fold downregulated; P< 0.05), *CDK6* (2.4-fold downregulated; P< 0.05), and *RAP2A* (2-3-fold downregulated; P< 0.05)].

Discussion: These studies in OCCC suggest that miR-10a-5p is an impactful, potentially oncogenic molecule, which warrants further studies.

KEYWORDS

endometriosis, ovarian endometrioma, ovarian clear-cell carcinoma, transcriptomic profiling, miRNA

Introduction

Previous studies have shown that each histotype of epithelial ovarian carcinoma, including high-grade serous, endometrioid, and clear-cell carcinomas, are transcriptomically distinct (1, 2). Large-scale molecular analyses of high-grade serous ovarian carcinomas showed unique classifications of tumors based on integrating multi-platform profiling (3). Molecular profiling of the endometriosis-associated ovarian carcinomas, including ovarian endometrioid and clear-cell carcinomas, showed frequent loss-of-function mutations in *ARID1A* (4–6). Previous work showed a unique transcriptomic profile in endometrioid ovarian carcinoma from women with concurrent endometriosis compared to those without concurrent endometriosis, supporting the role of the endometriotic microenvironment in ovarian cancer development (7, 8).

Endometriosis, a benign, chronic inflammatory condition where endometrial-like tissue grows outside the uterus, is a significant and potentially modifiable risk factor for ovarian cancer development (9, 10). Women with any amount or anatomical location of endometriosis have an increased risk of developing ovarian endometrioid (3.1-fold) or clear-cell (5.1-fold) carcinoma (11, 12). Specifically, women with ovarian endometriomas or deep infiltrating endometriotic lesions of the ovary are at even higher risk for developing ovarian endometrioid (4.7-fold) or clear-cell (10.1-fold) carcinoma (11). OCCC is a rare histological subtype composing 5-25% of ovarian malignancies, with the wide variation thought to be due to the subjective evaluation of histologic features and country-specific differences in prevalence (13–15).

Unlike the large sample size high-grade serous transcriptomic profiling studies focusing only on high-grade serous histology, transcriptomic profiling of OCCC is more limited in sample size or primarily used to show differences between transcriptomic profiles of different histological types of epithelial ovarian cancers (3, 16–21). Significantly, up to 50% of OCCCs are associated with endometriosis (22, 23). However, most transcriptomic studies of primary tumors classified as OCCC do not characterize samples as coming from women with concurrent endometriosis, pathology-proven endometriosis, or even a history of endometriosis. Only recently was a large sample size, OCCC-focused, multi-platform study performed that characterized samples as from women with a history of endometriosis (16). In this study, samples from women with a history of endometriosis were more likely to have loss-of-function mutations in *ARID1A*. In contrast, those with p53 mutations were likely to have poorer outcomes (16). While the tumors transcriptomically clustered into two groups – a traditional OCCC group and an aggressive p53-mutant high-grade serous-like group (16), the study did not discern particular transcriptomic contributions in samples from women with a history of endometriosis. To fill this gap, we focused our transcriptomic profiles on OCCC with pathology-proven concurrent endometriosis using both bulk RNA and miRNA sequencing.

MicroRNAs (miRNAs) are single-stranded, 22-24 nucleotide RNA molecules that function through an eight nucleotide seed sequence to modulate gene networks (24). MiRNAs are dysregulated in malignant and benign gynecological diseases and play impactful, functional roles in endometriomas and OCCC cell lines (24–27). For example, previous work has shown that endometriomas have distinct miRNA profiles and specific miRNAs, including miR-29c, play critical roles in uterine dysfunction (26). As another example, next-generation sequencing of OCCC cell lines showed that miR-100 played a critical role in rapamycin resistance *in vitro* (27). These limited studies suggest that miRNA molecules play essential roles. However, miRNA profiles in OCCC with concurrent endometriosis have not been examined. As a multi-platform approach, we integrated small RNA sequencing for miRNAs on matched samples of OCCC from women with pathology-confirmed concurrent endometriosis. From our list of dysregulated miRNAs, miR-10a was chosen to explore potential cellular and molecular associations in OCCC cell lines.

Materials and methods

Institutional review board approval for collection of human tissues and metadata

The expedited protocol to obtain and use tissues for this study was reviewed and approved by the Institutional Review Board (IRB) at Indiana University (#1812764043). The participants provided written informed consent to participate in this study. De-identified flash-frozen specimens, surgical pathology reports, and demographic data were obtained from tissue banks or previous studies (26). Tissue banks included the NRG Oncology Biospecimen Bank (NRG BB) and the Biospecimen Collection and Banking Core (BC^2) at the Indiana University Melvin and Bren Simon Comprehensive Cancer Center (IUSCCC). Supplementary Table S1 lists the metadata and associated experiments for each de-identified human tissue sample.

OCCC with concurrent endometriosis and OCCC without endometriosis were pure clear-cell histology samples collected from adnexal masses. Tumors with mixed histology (*i.e.*, clear-cell with endometrioid or clear-cell with serous) were not included in these studies. Inclusion criteria for OCCC with concurrent endometriosis samples were defined as the explicit mention of endometriosis on the surgical pathology report. Per banking protocols, ovarian cancer samples were taken away from obvious pathologies such as necrotic tissue or endometriosis. The malignant samples were 50-90% malignant cells (Supplementary Table S1). Endometrioma cyst wall tissues were collected as described previously (26).

Next-generation sequencing studies

Total RNA was isolated from 50-100 mg of fresh frozen tissue using the *mir*Vana miRNA Isolation Kit with phenol (Thermo Fisher Scientific, Waltham, MA). RNA was treated with the Turbo DNA-*free* Kit (Thermo Fisher Scientific). RNA quality control was assessed using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) at the Center for Medical Genomics at Indiana University School of Medicine (Indianapolis, IN). High-quality RNA samples were sent to the Center for Genomics and Bioinformatics at Indiana University (Bloomington, IN). Poly-A RNA libraries were constructed using mRNA Stranded TruSeq protocol (Illumina, San Diego, CA). Small RNA library construction was performed using the TruSeq Small RNA kit (Illumina). Purified libraries were visualized and quantified using a TapeStation HSD1000 (Agilent Technologies).

For poly-A bulk RNA sequencing analysis, NextSeq reads were trimmed using fastp (version 0.23.2) with parameters "-l 17 -g -p" (28). The resulting reads were mapped against GRCh38 using HISAT2 version 2.2.1 with default parameters (29). HISAT uses Bowtie2, based on the Burrows-Wheeler transform algorithm, for sequence alignment and allows for mapping across exon junctions (30). Read counts for each gene were created using featureCounts from the Subread package version 2.0.3 with the parameters "-O -M –primary –largestOverlap -s 2" and Gencode v42 as the annotation (31–33). For small RNA sequencing analysis, NextSeq reads were trimmed using fastp (version 0.23.2) with parameters "-l 17 -g -p" (28). MiRDeep2 version 2.0.0.8 was used to map the resulting reads against GRCh38 and miRBase version 22 as a reference to detect known, mature miRNA sequences (34–36). MiRDeep2 uses Bowtie to perform mapping of the reads and includes tools for the identification and quantification of miRNAs (37). Bowtie version 1.3.0 was the version of Bowtie installed. Differential expression analysis for bulk RNA and miRNA was performed using the DESeq2 package (version 1.36.0) in R/Bioconductor (R version 4.2.0) (38). Transcriptomic data have been deposited into the Gene Expression Omnibus (GSE230956). Figures from poly-A bulk RNA and small RNA sequencing analysis were created using R (version 4.2.0) and R libraries: ggplot2, complex heatmap, and ggrepel.

Quantitative PCR for mRNA and miRNA expression

Total RNA was extracted from 50-100 mg of fresh frozen tissue or cultured cells using the miRNeasy Mini Kit (Qiagen, Hilden Germany) following the manufacturer's protocol with on-column RNase-Free DNase Set (Qiagen) or previously extracted DNase-treated RNA using the *mir*Vana kit described above. A NanoDrop ND-1000 (Thermo Fisher Scientific) was used for the determination of RNA quantity and purity. For mRNA expression experiments, 1000 ng of DNase-treated RNA was reverse transcribed in a 20 µL reaction using 50 units MultiScribe Reverse Transcriptase (Thermo Fisher Scientific), 1X reverse transcriptase Buffer (Thermo Fisher Scientific), 0.5 mM deoxynucleotide triphosphate (Thermo Fisher Scientific), 6 units RNase Inhibitor (Thermo Fisher Scientific), and 2.5 µM random hexamers (Thermo Fisher Scientific) on a 2720 Thermo Cycler (Thermo Fisher Scientific): 10 minutes at 25°C, 30 minutes at 48°C, and 5 minutes at 95°C. Samples were diluted 1:5 for qPCR. QPCR was performed using 2 µL of diluted cDNA using either SYBR Green PCR Master Mix (Thermo Fisher Scientific) with previously published primers (39) or custom-designed primers (Supplementary Table S2) in a reaction volume of 10 µl. Only custom-designed primer pairs specific for the gene of interest, intron-spanning, with a primer efficiency of 80-110%, lacking primer-dimers, and R^2 >0.95 were used (40, 41). mRNA experiments were normalized to the human β-actin (*ACTB*) (39). For miRNA expression experiments, total RNA (25 ng) was reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) in a reaction volume of 15 µl. Mature miRNA expression was performed using TaqMan mature microRNA assays on undiluted cDNA. U6 snRNA was used for normalization (26, 42). Supplementary Table S2 lists the TaqMan assays used.

Both mRNA and miRNA assays were run on a QuantStudio 3 Real-Time PCR Instrument (Thermo Fisher Scientific) with reaction conditions as follows: 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C (denaturation), and 1 minute at 60°C (annealing/extension). All SYBR Green assays ran dissociation curves to detect primer dimers. Each sample was analyzed in duplicate. Expression fold change calculations utilized

the $2^{-\Delta\Delta CT}$ method (43). Data were plotted as mean ± SEM, and statistical analyses were performed with GraphPad Prism (Dotmatics, Boston, MA). $P< 0.05$ was considered statistically significant. Power analyses were performed using G*Power (version 3.1.9.7) (44, 45). *Post-hoc* analysis of A2780 and A2780CR5 miR-10a expression, with a type I error set at 0.05, found that we had greater than 95% power to detect a three-fold change with effect size d= 9.6 with a sample size of two in each group using a two-tailed t-test. For tissue miR-10a expression, due to greater variability, a total sample size of 24 was calculated to achieve greater than 80% power to detect a 0.7 effect size f and a type I error set at 0.05 using a one-way ANOVA.

Tissue-cell line transcriptomics data comparison analyses

To compare tissue and cell line collected from different studies, we computed the pathway activity scores (PAS) of an extensive collection of canonical biological pathways for each sample and utilized the PAS to assess the similarity between samples. We assume that the cell lines and tissue samples of high similarity should have a similar profile in more similarly activated cellular pathways. Noting that cell line samples do not have the biological characteristics of tumor microenvironments, we excluded stromal genes and related pathways from the PAS analysis. Specifically, canonical gene sets were downloaded from MSigDB version 6 c2, containing 1329 gene sets (46–48). Cancer Cell Line Encyclopedia (CCLE) cell line RNA-seq gene expression data were downloaded from the Broad Institute (49). The housekeeping genes and immune and stromal cell marker genes derived from our previous analysis were excluded (50, 51). Pathway activity scores (PAS) were assigned using the following function for each sample and pathway. For a given gene expression profile $x^{1\times N}$ of N genes and a pathway P as a set of genes, denote $y^{1\times N}$ as the sorted $x^{1\times N}$ in the decreasing order and i_g as the rank of gene g in y, the pathway activity score of P on x, denoted as $PAS(x,P,K)$ is computed by

$$PAS(x,P,K)=\frac{\sum_{g\in P}\max\left\{\frac{K-i_g}{|P|},0\right\}}{K}$$

where K is the hyperparameter in this study. Here, K is set as 3000. Here, the *PAS* can be viewed as a normalized and weighted sum of the rank of the pathway genes whose expression is within the top K=3000 rank. PAS is computed for each pathway and each sample. Then Pearson Correlation Coefficients of the PAS of all pathways were computed between samples and used as their molecular similarity measure.

Ovarian cancer cell lines

ES-2 (52, 53), TOV-21G (54), and IGROV-1 (55) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). SKOV3ip1 (56) was obtained from the Cytogenetics and Cell Authentication Core at the University of Texas M.D. Anderson Cancer Center (Houston, TX, USA). OVISE (57), OVAS (58), and OVTOKO (57) were generously obtained from Dr. Hiroshi Minaguchi (Yokohama City University, Yokohama, Japan). KK (59) was generously obtained from Dr. Yoshihiro Kikuchi (National Defense Medical College, Tokorozawa, Japan). SMOV-2 (60) was generously obtained from Dr. Tomohiro Iida (St. Marianna University, Kawasaki, Japan). A2780 (61) and A2780CR5 (62) were provided by Dr. Kenneth P. Nephew (Indiana University, Bloomington, IN, USA). SKOV3 (63) was generously obtained from Dr. Salvatore Condello (Indiana University School of Medicine, Indianapolis, IN, USA). RMG-I (64) was generously obtained from Dr. Samuel C. Mok (The University of Texas MD Anderson Cancer Center, Houston, TX, USA). Cell line authentication was confirmed using a short tandem repeat (STR) marker profile (IDEXX BioAnalytics, Westbrook, ME) within six months of experiments and tested for mycoplasma contamination monthly (MycoAlert Plus Mycoplasma Detection Kit, Lonza, Switzerland).

KK, OVISE, OVTOKO, IGROV-1, RMG-I, A2780, A2780CR5, and SKOV3 were maintained in RPMI 1640 (Thermo Fisher Scientific). OVAS was maintained in DMEM/F12 (Thermo Fisher Scientific). TOV-21G was maintained in a 1:1 ratio of Medium 199 to MCBD 105 (Sigma-Aldrich, St. Louis, MO). ES-2 was grown in McCoy's (Thermo Fisher Scientific). All cell lines were supplemented with 1% penicillin and streptomycin (Thermo Fisher Scientific) and 10% fetal bovine serum (Atlanta Biologicals, Minneapolis, MN) except for TOV-21G, which was supplemented with 15%. All cells were cultured in a humidified incubator at 37°C with 5% carbon dioxide. Supplementary Table S3 lists the published common genetic mutations, drug responses, and the experimental uses for each of the cell lines used in this manuscript.

Carboplatin cytotoxicity assays

Carboplatin cytotoxicity assays were performed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) (Promega, Madison, WI). Cells were plated $1x10^3$ cells per 96-well. After 24 hours, cells were treated with ten increasing (5-200 μM) doses of carboplatin [cis-diammine (1,1-cyclobutane-dicarboxylate) platinum, (C2358, Sigma)] diluted in tissue culture grade water (Thermo Fisher Scientific) in triplicate. Following 72-hours of carboplatin treatment, absorbance was read on a Synergy H1 Hybrid Reader (BioTek, Winooski, VT), background absorbance was subtracted, and data were presented as normalized to vehicle control. GraphPad Prism version 9.3.0 (Dotmatics) was used to calculate an IC50 (50% inhibitory concentration). GraphPad Prism (Dotmatics) was used to calculate the correlation between IC50 and miR-10a-5p expression. With a type I error set at 0.05, we will have 90% power to detect a correlation of 0.85 with a total sample size of 8. Figures were created using GraphPad Prism (Dotmatics).

miRNA target prediction

Putative miRNA:mRNA pairs were facilitated using SigTerms (65) with input from TargetScan (66–68), miRDB (69, 70), and miRTarBase (71). Putative target genes were further curated for potential as impactful miR-10a-5p targets using hand annotation.

MiR-10a-5p mimic transfection of human OCCC cell lines

Each cell line underwent optimization of transfection conditions using the siGLO Red Transfection Indicator (Horizon Discovery, Cambridge, United Kingdom) to determine the optimum amount of lipid transfection reagent, miRNA mimic concentration, and initial cell density. Cells were seeded at a density of 2-3x10^5 cells per well of a 6-well plate. After 24 hours, cells were transfected using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific) with 100 nM hsa-miR-10a-5p mimic (*mir*Vana miRNA mimic, Assay ID MC10787) or 100 nM negative control (*mir*Vana miRNA mimic, Negative Control #1, catalog #4464058).

Transfected cells were used simultaneously for four different endpoints: confirmation of miR-10a overexpression, cellular proliferation, cell cycle analysis, and associated putative target gene expression by qPCR. To confirm miR-10a overexpression and associated putative target gene expression, cells were lysed at 24 hours after transfection for RNA isolation. To evaluate the effects of miR-10a overexpression on proliferation, 24 hours after transfection, cells were seeded into a 96-well plate at a density of 1000 cells/well. Cellular proliferation was measured using CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) (Promega) in triplicate at 24-hour intervals. Absorbance was read with the Synergy H1 Hybrid Reader (BioTek). Proliferation was plotted as the percent of viable cells as a function of time using GraphPad Prism (Dotmatics). To assess the effects of miR-10a overexpression on the cell cycle, 24 hours post-transfection cells were fixed using 66% ethanol (Decon Laboratories Inc., King of Prussia, PA) and stained with Propidium Iodide (PI) according to the manufacturer's protocol (Thermo Fisher Scientific, F#10797). Stained cells were analyzed using BD LSRFortessa (BD Biosciences, Franklin Lakes, NJ), and cell cycle analysis was performed with ModFit LT4.1 (Verity Software House, Topsham, ME). A two-tailed Student's t-test was performed using GraphPad Prism (Dotmatics). Figures were created using GraphPad Prism (Dotmatics).

Results

OCCC samples with concurrent endometriosis had unique molecular characteristics

While nearly half of all women with OCCC have endometriosis (22, 23), transcriptomic profiling studies have not examined OCCC samples from women with pathology-proven endometriosis. Only one transcriptomic study contained OCCC samples from women with a history of endometriosis (16), but the samples were not defined as pathology-proven nor were their transcriptomic profiles analyzed independently. Here, we focused on OCCC samples from women with pathologically-confirmed endometriosis. Table 1 summarizes the clinical and pathological characteristics. OCCC with concurrent endometriosis was defined as having endometriosis at the time of staging surgery, listed on the pathology report. Women with OCCC were significantly older (median 53 years; range 39-79 years, $P< 0.0001$) than women with benign endometriomas (median 30.5 years; range 21-48 years). Women with OCCC and concurrent endometriosis did not differ in age (median 51 years; range 45-72 years, $P = 0.15$) from those without concurrent endometriosis (median 56.5 years; range 39-79). Using the Federation Internationale de Gynécologie et d'Obstétrique (FIGO) ovarian cancer staging system implemented in 2014 (72), there was no difference in the stage between the women with and without concurrent endometriosis (Fisher's exact test = 1, $P > 0.1$). Thus, the OCCC samples were clinically similar except for concurrent endometriosis.

TABLE 1 Clinical characteristics of patient samples.

	Benign (n = 16)	Malignant (n = 19)		*P*-value
Median age, y (range)	30.5 (21-48)	53 (39-79)		*$P< 0.0001$
		With Endometriosis (n = 9)	Without Endometriosis (n = 10)	
Median age, y (range)		51 (45-72)	56.5 (39-79)	*$P = 0.15$
Stage				^$P >0.10$
I		4 (44%)	5 (50%)	
II		3 (33%)	2 (20%)	
III		2 (22%)	1 (10%)	
IV		0	2 (20%)	

*, Student's t-test, one-sided, unpaired; ^Fisher's exact test, comparing stage I to stage II+.

Poly-A bulk RNA and small RNA sequencing were performed on RNA isolated from specimens (n = 8), including endometrioma (n = 4) and OCCC with concurrent endometriosis (n = 4). Endometrioma samples were full cyst wall thickness samples taken from areas without gross pathology such as necrosis or abscess, with pathology-proven endometriosis without atypia and no evidence of ovarian cancer. OCCC samples were taken from adnexal masses, and samples were taken from areas free from gross endometriosis, necrosis, or abscess.

Poly-A bulk sequencing revealed over 437 million mapped reads (mean: 54,646,311 ± 6,117,236 mapped reads per clinical sample). There was no difference between endometrioma and OCCC with concurrent endometriosis samples regarding overall alignment rate, as all eight bulk RNA samples had greater than 97% of mapped reads aligned. To categorize mapped reads into RNA categories or feature counts, HISAT analysis was used (Supplementary Table S4). More reads were assigned in endometrioma (145,847,035 reads) than OCCC with concurrent endometriosis (124,361,541 reads, Student's t-test, P< 0.01). OCCC with concurrent endometriosis had more reads assigned to mitochondrial RNA species (33,929,857 to 22,296,699; endometrioma, Student's t-test, P< 0.01). More reads were assigned to protein-coding genes in benign endometrioma (98,430,773 reads) than OCCC with concurrent endometriosis (70,984,332 reads, Student's t-test, P< 0.01).

Transcriptomic profiles of the endometrioma and OCCC with concurrent endometriosis samples were first evaluated using multidimensional scaling (MDS) analysis (73). The MDS plot shows a significant differential clustering of the OCCC samples with concurrent endometriosis from endometriomas (Figure 1A). This difference is most apparent at the MDS1 level. Similar clustering was noted on uniform manifold approximation and projection (UMAP) and principal component analysis (PCA) for dimension reduction plots (Supplementary Figures S1A, B). At a global level, malignant OCCC with concurrent endometriosis is molecularly distinct from benign endometriomas.

We directly compared transcriptomic profiles of OCCC with concurrent endometriosis (n = 4) to endometriomas (n = 4). Endometrioma was used as a comparison tissue due to its strong increase in risk for the development of OCCC, studies supporting increased molecular mutations in atypical endometriosis and concurrent OCCC, strong genomic correlation and causal relationship between endometriosis and OCCC, and the high incidence of concurrent endometriosis seen in women with OCCC

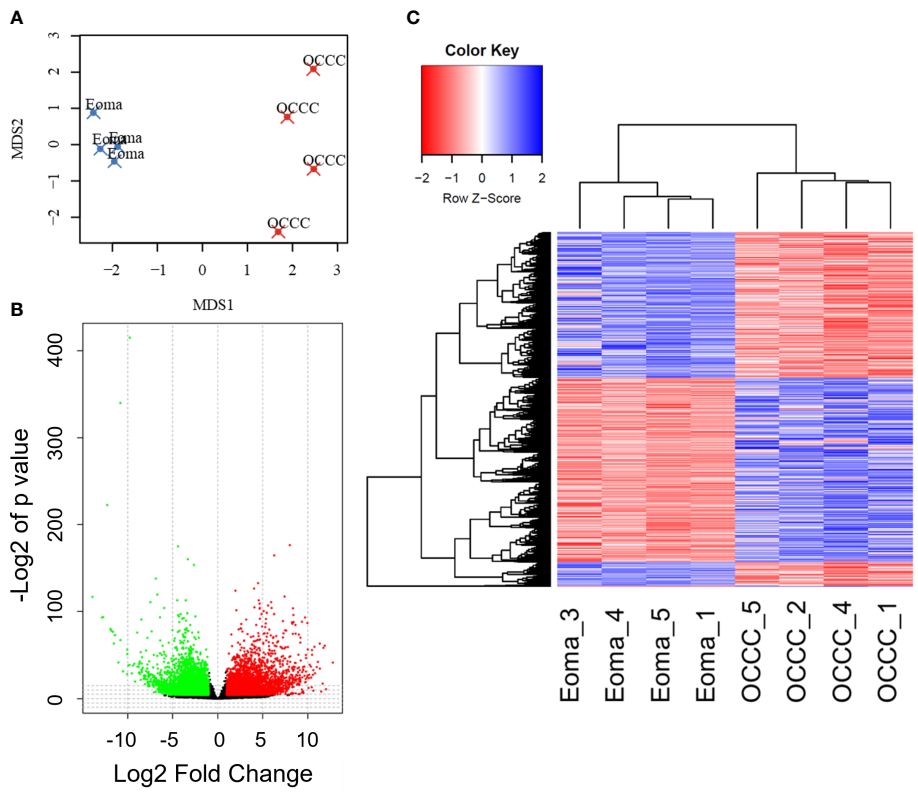

FIGURE 1
The OCCC with concurrent endometriosis transcriptomic profile is molecularly distinct from benign endometrioma. **(A)** Multidimensional scaling (MDS) plot of transcriptomic profiles for endometrioma (Eoma, blue X's) and ovarian clear cell carcinoma (OCCC, red X's) with concurrent endometriosis. **(B)** Volcano plot representation of protein-coding transcripts overexpressed (red dots), similarly expressed (black dots), and under-expressed (green dots) in OCCC with concurrent endometriosis versus endometriomas (P_{adj}<0.05; Log2-Fold change<|1|. **(C)** Heat map representation of 5575 differentially expressed unique protein-coding gene transcripts overexpressed (red) and under-expressed (blue). Dendrogram of hierarchical clustering. Rows, protein-coding gene transcripts; columns, profiled samples.

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(4, 11, 17, 18, 22, 23). Differential gene expression analysis was conducted with DESeq2. Significant differential expression was determined using a false discovery rate<0.05, giving 6865 protein-coding transcripts significantly differentially expressed. Limiting to log2 fold-change >|1| identified 2449 upregulated and 3131 downregulated unique protein-coding genes (Figures 1B, C, Supplementary Tables S5, S6). Hierarchical clustering shows that endometriomas cluster separately from OCCC with concurrent endometriosis (Figure 1C).

Matrisome, secreted factors, cell cycle, and DNA replication pathways are dysregulated in OCCC with concurrent endometriosis

To explore potentially impactful molecular processes, we performed pathway enrichment analysis of the upregulated and downregulated genes using a hypergeometric test and Gene Set Enrichment Analysis (GSEA) against MSigDB v6 canonical pathway set, with a significant cutoff determined by $P< 0.005$ (46–48). Complete lists of the pathways significantly enriched by upregulated and downregulated genes are given in Supplementary Tables S7, S8. We observed a limited list of pathways from upregulated genes in OCCC with concurrent endometriosis (Supplementary Table S7). The upregulated genes showed significant enrichment in cell cycle and DNA replication pathways, including cyclin A B1-mediated G2-M transition, G1-S transcription, and E2F-mediated DNA replication (Supplementary Figures S2A-C). Previous work has shown that OCCC exhibited dysregulation of p27-related cell cycle effects (74). Important drivers of p27-related cell cycle dysregulation that were upregulated in OCCC with concurrent endometriosis include *SKP2* (S-phase kinase-associated protein 2, log2 fold-change = 1.3, P = 1.1e-2), *CKS2* (CDC28 protein kinase regulatory subunit 2, log2 fold-change = 2.2, P = 6.6e-9), *CCNA2* (Cyclin A2, log2 fold-change = 1.7, P = 2.9e-3), and *CCNE1* (Cyclin E1, log2 fold-change = 5.2, P = 8.5e-10). GSEA plots of the cyclin A-mediated G2-M transition (P = 8.95e-6) and E2F-mediated DNA replication (P = 8.61e-4) top enriched pathways from upregulated genes are shown in Supplementary Figures S2B, C. Supplementary Table S9 lists the upregulated genes involved in the cell cycle with their fold change.

There were many more significantly downregulated pathways in OCCC with concurrent endometriosis. Significantly downregulated genes in OCCC with concurrent endometriosis showed significant enrichment in the pathways of matrisome, secreted factors, GPCR signaling, and cytokine-cytokine-receptor interaction (Figure 2A). GSEA plots of matrisome (P = 2.64e-69) and cytokine-cytokine receptor interaction (P = 2.43e-20) pathways from downregulated genes are shown in Figures 2B, C. Key genes involved in the cytokine-cytokine receptor interaction pathway are largely upregulated in endometriomas (7, 26, 75–78). Supplementary Table S10 shows the downregulated genes in OCCC with concurrent endometriosis compared to endometrioma in the cytokine-cytokine receptor interaction pathway.

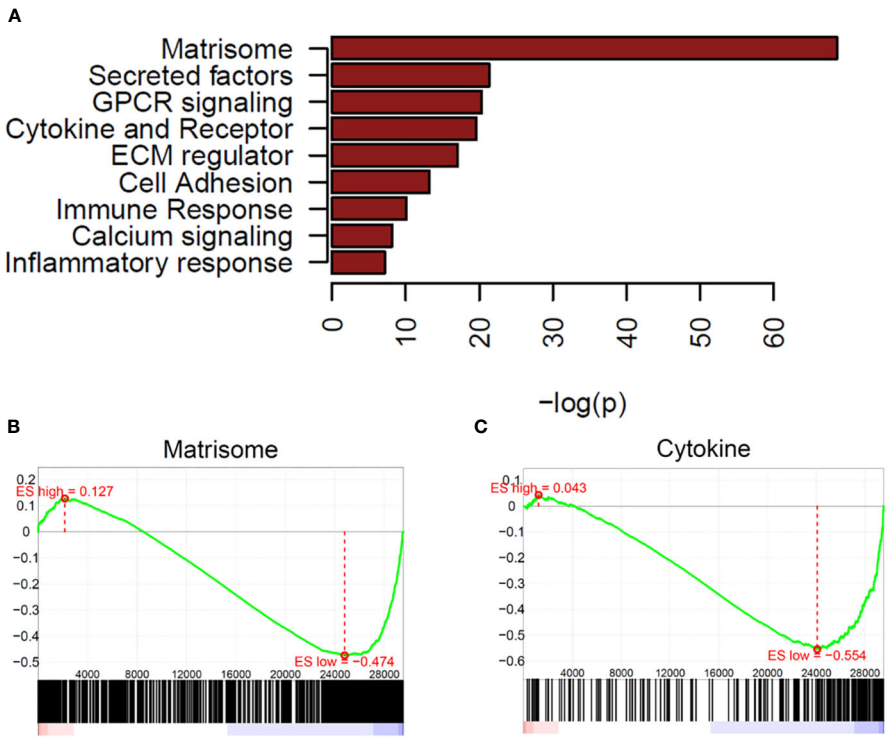

FIGURE 2
Matrisome and cytokine pathways are enriched in downregulated genes from OCCC with concurrent endometriosis. **(A)** Waterfall plot of significantly downregulated pathways in OCCC with concurrent endometriosis. Gene set enrichment plots for **(B)** NABA_MATRISOME and **(C)** KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION.

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Determining a model cell line of OCCC with concurrent endometriosis based on human gene expression by RNA sequencing

Genomically, OCCC cell lines frequently classify as OCCC rather than other histological subtypes of epithelial ovarian cancer (79–84). However, none of the widely shared or commercially available OCCC cell lines are characterized as being derived from OCCC with concurrent endometriosis. To determine which available cell lines most closely recapitulate our transcriptomic profiling data from OCCC with concurrent endometriosis, we used bioinformatic analysis of datasets from the Cancer Cell Line Encyclopedia (CCLE) (49). Gene expression datasets for ovarian carcinoma cell lines [endometrioid/clear-cell (IGROV-1, SKOV3, A2780), clear cell (TOV-21G, OVTOKO, OVISE, OVMANA, JHOC_5), p53-altered clear-cell (RMG-I, ES-2), endometrioid (TOV112D), high-grade serous (OVCAR8), and low-grade serous (HEYA8)], endometrial cancer cell lines (AN3CA, HEC1A, ISHIKAWA, HEC1B), and a leukemia cell line (JURKAT) were downloaded and used. For the RNA-seq gene expression data from the OCCC with concurrent endometriosis tissue samples and endometrioma samples, we utilized multiclass logistic regression with variable selection by L1 penalty. We selected pathways whose pathway activity scores (PAS) are most predictive of cancer types. PAS of the 394 cancer-types predictive pathways were computed for OCCC with concurrent endometriosis tissue, endometrioma, and the selected cell line samples. We computed a Pearson correlation between the PAS of 394 pathways in tissue and cell line data to measure the similarity.

Examination of the PAS results (Figure 3) showed that endometrioma samples (Eoma1, Eoma4, Eoma5) clustered most closely to endometrioma sample 3 (Eoma3), ES2, and HEYA8. ES-2 is considered a p53-altered OCCC cell line (81, 85), and HEYA8 is considered a KRAS-mutant low-grade serous line (85). Studies suggest that both HEYA8 and ES-2 most closely represent low-grade serous (83). The dendrogram suggests that ES2 and HEYA8 cluster more closely to each other than Eoma3. OCCC samples with concurrent endometriosis (OCCC4, OCCC2) clustered together along with OCCC5, OVTOKO, OVISE, RMG-I, and OVMANA. OVTOKO, OVISE, and OVMANA are considered clear-cell ovarian cancer cell lines as they were derived from metastatic lesions of OCCC and contain a mutant ARID1A (57, 80, 83). RMG-I may be a p53-mutant clear cell type rather than a mutant ARID1A type (16, 83, 84). OCCC sample with concurrent endometriosis 1 (OCCC1) clustered with IGROV-1, JHOC5, SKOV3, and TOV-21G. IGROV-1 is derived from a mixed histology tumor and contains mutant ARID1A and PIK3CA and could be considered a clear cell-like line (81–83, 85). SKOV3 was derived from ascites of ovarian adenocarcinoma and contains mutant ARID1A and PIK3CA, and

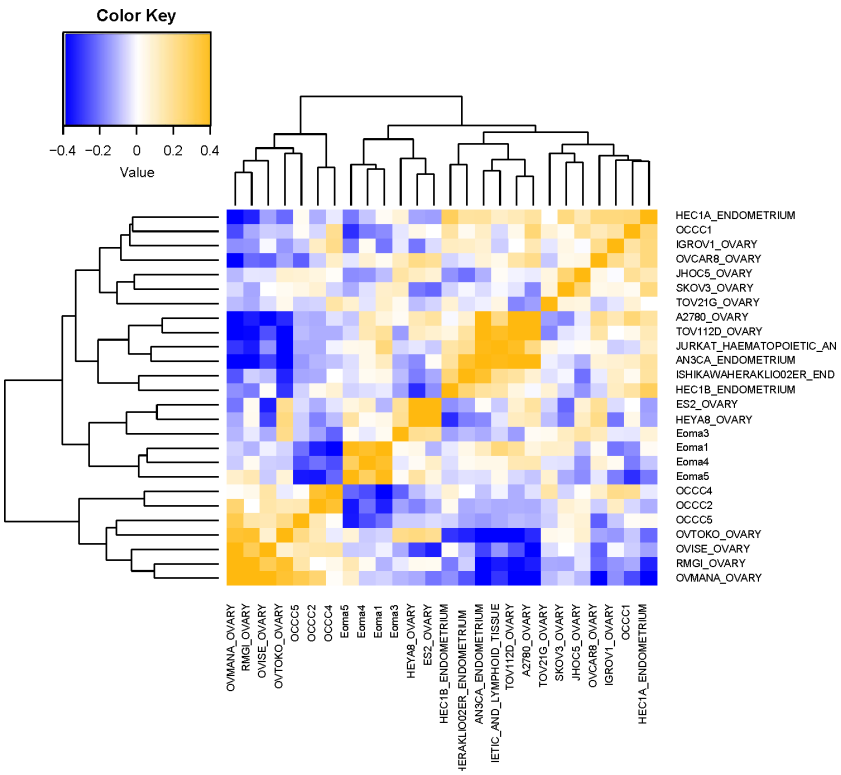

FIGURE 3
Molecular comparison of cell line transcriptomic profiles with clinical samples. Computational analysis of the molecular similarity of the clinical samples (Eoma, benign endometrioma or OCCC, OCCC with concurrent endometriosis) and cell lines from CCLE. A pathway activation score (PAS) was first computed for each pathway and each sample. Then the similarity between the samples was assessed by Pearson Correlation Coefficients (PCC) of the PAS. Cell line nomenclature is the CCLE name of the line_tissue type. A yellow color represents a higher PCC; blue, a lower PCC.

is frequently more closely associated with clear-cell tumors (63, 81–83, 85). TOV-21G is considered a clear cell line as it was derived from OCCC and contains mutations in ARID1A and PIK3CA (54, 81–83, 85) (Supplementary Table S3).

OCCC with concurrent endometriosis samples have dysregulated miRNA expression

MiRNAs are impactful for their potential as disease biomarkers and role as upstream regulators of multiple signaling pathways in diseases of the female reproductive tract (24, 25). However, large-scale profiling of miRNAs has focused broadly on epithelial ovarian cancers without a direct analysis of OCCC, included only a small number of OCCC samples, or did not describe any samples with a history of or pathology-proven endometriosis (86–90). To complement our protein-coding transcriptomic studies, we profiled miRNAs on RNA isolated from clinical samples (n = 8), including endometrioma (n = 4) and OCCC with concurrent endometriosis (n = 4).

Small RNA sequencing gave over 43 million reads (mean: 5,382,728 ± 644,063 mapped reads per clinical sample). There were no significant differences in the mirDeep2 total mapped count percentage between endometrioma and OCCC with concurrent endometriosis (Supplementary Table S11). Of the 2588 human mature miRNA molecules, 446 were expressed in at least one clinical sample. Principal component analysis (Figure 4A) with miRNA expression profiles showed PC1 and PC2 differential clustering of the OCCC with concurrent endometriosis from the benign endometrioma. Differential miRNA expression analysis was conducted with DESeq2. A comparison of dysregulated miRNAs between endometrioma and OCCC with endometriosis is shown on the volcano plot (Figure 4B). Significant differential expression was determined using a false discovery rate< 0.05, giving 128 significantly differentially expressed mature miRNA molecules. Limiting to log2 fold change > |1| identified 64 upregulated and 61 downregulated mature miRNA molecules (Supplementary Tables S12, S13).

Because fold change up- or downregulated is a relative number, we examined the most abundant miRNA molecules in endometriomas with statistically significant lower expression in OCCC with concurrent endometriosis. The most abundant miRNA in endometriomas was hsa-miR-143-3p, representing 14.5%. Hsa-miR-146b-5p represented nearly 5% (or 4.47%) of endometrioma miRNAs. Table 2 shows the top ten downregulated mature miRNA molecules in OCCC with concurrent endometriosis. Table 3 shows the top ten upregulated mature miRNA molecules in OCCC with concurrent endometriosis. MiR-10a-5p was the most abundant miRNA in OCCC with concurrent endometriosis, representing nearly a quarter of all miRNAs (21.5%). Other significantly abundant and upregulated miRNAs included hsa-miR-30a-5p (6.1%), two other miR-30 family members [hsa-miR30d-5p (0.71%) and hsa-miR-30c-5p (0.12%)], and hsa-miR-141-3p (1.35%). Three mature miRNA molecules had a log2 fold change >4 and were in the top ten in terms of abundance in OCCC with concurrent endometriosis: hsa-miR-10a-5p (log2 fold change = 4.37, P = 2.43e-18), hsa-miR-141-3p (log2 fold change = 4.67, P = 1.31e-15), and hsa-miR-183-5p (log2 fold change = 4.62, P = 4.90e-6).

Overexpression of miR-10a has been found in breast, cervical, acute myeloid leukemia, and pancreatic ductal adenocarcinomas (91–98). Further, miR-10a overexpression was correlated with an increased risk of recurrent breast cancer and decreased response to platinum agents *in vitro* (92, 96, 97, 99). Disease progression on first-line platinum therapy is a hallmark of OCCC, with response rates to chemotherapy ranging as low as 11% (100–105). While platinum resistance is the most common reason for death from

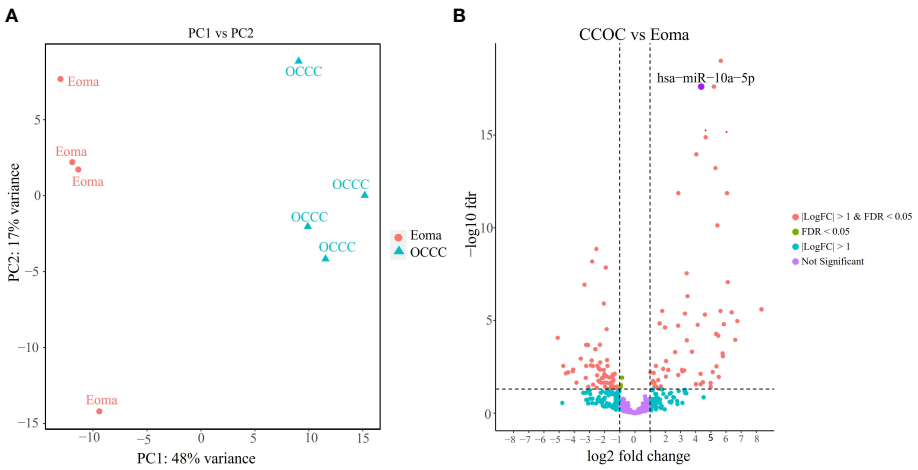

FIGURE 4
OCCC with concurrent endometriosis is molecularly distinct from benign endometrioma using miRNA expression. **(A)** Principal component (PC) analysis of malignant OCCC with concurrent endometriosis (triangles, OCCC) clusters separately from benign endometrioma samples (circles, Eoma), using PC1 and PC2. **(B)** Volcano plot of the significantly dysregulated miRNAs. Red represents miRNAs with P< 0.05, log2 fold-change > |1|. Blue dots represent miRNAs log2 fold-change > |1|. Green represents miRNAs with P< 0.05. Purple dots represent non-statistically significant miRNAs. The labeled dot represents hsa-miR-10a-5p.

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TABLE 2 Top ten downregulated mature miRNAs in OCCC with concurrent endometriosis.

Mature miRNA	log2FoldChange	P_{adj}
hsa-miR-143-3p	-2.81	6.61E-09
hsa-miR-127-3p	-1.91	1.41E-08
hsa-let-7c-5p	-3.09	2.11E-04
hsa-miR-99a-5p	-3.57	1.16E-03
hsa-miR-27b-3p	-1.80	2.87E-03
hsa-miR-199a-3p	-2.38	3.86E-03
hsa-miR-199b-3p	-2.38	3.86E-03
hsa-miR-146b-5p	-2.12	1.18E-02
hsa-miR-199a-5p	-2.18	1.31E-02
hsa-miR-23b-3p	-1.60	4.23E-02

recurrence across all ovarian cancers, progression on platinum therapy is more prevalent in OCCC (103, 104, 106). Thus, we explored the cellular and molecular effects of miR-10a in OCCC. QPCR expression showed that benign endometrioma exhibited a significantly lower expression of miR-10a-5p than OCCC with or without endometriosis (one-way ANOVA, $P< 0.05$, Figure 5). OCCC with concurrent endometriosis exhibited an 8-fold overexpression of miR-10a-5p compared to benign endometrioma (Student's t-test, $P = 0.01$). However, there was no statistically significant difference in miR-10a-5p expression between OCCC with concurrent endometriosis and OCCC without endometriosis (Student's t-test, $P = 0.90$).

MiR-10a-5p expression in human OCCC cell lines correlates with carboplatin IC50

To explore the role of miR-10a in OCCC, the expression of miR-10a-5p was examined in a panel of human ovarian cancer cell lines (Figure 6A). There appeared to be two groups of cell lines – those with low miR-10a expression (A2780, OVISE, TOV-21G, OVTOKO, KK, and SMOV-2) and those with high miR-10a expression (A2780CR5, SKOV3ip1, RMG-I, SKOV3, OVAS, ES-2). To confirm similar results, we analyzed the next-generation small RNA sequencing data from Nagaraja et al. (27). The relative expression of miR-10a in each cell line was similar to our expression. There was a low miR-10a expression in OVISE, TOV-21G, SMOV-2, and KK, and those with high relative miR-10a expression included RMG-I, ES-2, and OVAS (Supplementary Figure S3). ES-2 transcriptomically clustered with endometriomas (Figure 3) and exhibited relatively high expression of miR-10a in both our qPCR analyses (Figure 6A) and next-generation sequencing data from Nagaraja et al. (27). RMG-I and SKOV3 transcriptomically clustered with OCCC with concurrent endometriosis (Figure 3) and showed high expression of miR-10a (Figure 6A). On the other hand, OVISE, TOV-21G, and OVTOKO transcriptomically clustered with OCCC with concurrent endometriosis (Figure 3) but exhibited low expression of miR-10a (Figure 6A).

TABLE 3 Top ten upregulated mature miRNA in OCCC with concurrent endometriosis.

Mature miRNA	log2FoldChange	P_{adj}
hsa-miR-10a-5p	4.37	2.43E-18
hsa-miR-141-3p	4.67	1.31E-15
hsa-miR-30a-5p	2.87	1.37E-12
hsa-miR-183-5p	4.62	4.90E-06
hsa-miR-30d-5p	1.63	1.44E-05
hsa-miR-30c-5p	1.98	2.39E-05
hsa-miR-182-5p	3.77	4.94E-04
hsa-miR-98-5p	1.39	2.87E-03
hsa-miR-148b-3p	1.02	6.38E-03
hsa-miR-191-5p	1.24	6.87E-03

A2780CR5 cells are an isogenic line of A2780 that is resistant to platinum (62). Interestingly, the miR-10a-5p expression was 3.3-fold higher in the platinum-resistant line, A2780CR5 (Mann-Whitney, $P< 0.01$) than in A2780. Increased miR-10a expression has previously been correlated with platinum resistance in lung cancer (99, 107). As a result of this increased miR-10a-5p expression in the platinum-resistant line, carboplatin response was compared across OCCC cell lines. Carboplatin response was expressed as the half maximum inhibitory capacity (IC50) and correlated with miR-10a-5p expression. A positive correlation ($R^2 = 0.93$) was found between miR-10a expression and carboplatin IC50 (Figure 6B).

MiR-10a-5p overexpression decreases cellular proliferation.

SKOV3ip1cells are a xenograft-derived line of SKOV3. Previous work showed increased cellular proliferation of SKOV3ip1 cells compared to SKOV3 (56). The miR-10a-5p expression was almost 2-fold higher in SKOV3ip1 (un-paired t-test, $P<0.05$) than SKOV3. *In vitro* studies overexpressing miR-10a-5p showed potentially cancer type specific effects on cellular proliferation. For example, overexpression of miR-10a in melanoma, acute myeloid leukemia, and laryngeal squamous cell carcinoma cells decreased cellular proliferation in these cancers (108–110).

To study the effects of miR-10a overexpression on proliferation in OCCC cell lines, SMOV-2 and KK were transiently transfected with a mature miR-10a-5p mimic. After optimization of transfection conditions (data not shown), overexpression of miR-10a was confirmed (Supplementary Figure S4). SMOV-2 and KK miR-10a overexpressing cells (SMOV2-10a and KK-10a) had a statistically significant and sustained decrease in cellular proliferation compared to the non-targeting control transfected cells (SMOV2-10actl and KK-10actl) (Figure 7). For SMOV-2 cells, there was a statistically significant decrease in proliferation

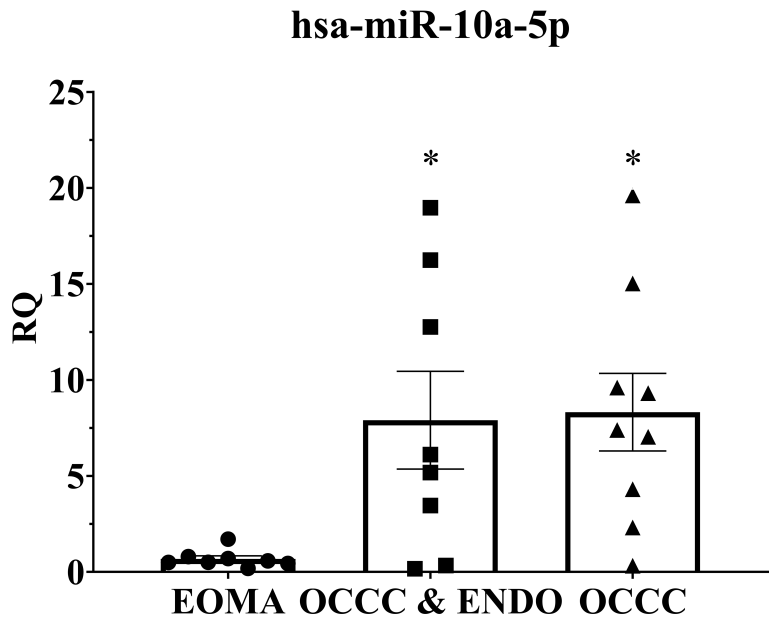

FIGURE 5
MiR-10a-5p is significantly upregulated in human tissue samples of ovarian clear cell carcinoma. Examination of miR-10a-5p expression in endometrioma (EOMA, *n* = 8), ovarian clear cell carcinoma from women with concurrent endometriosis (OCCC & ENDO, *n* = 8), and ovarian clear cell carcinoma from women without pathologically confirmed endometriosis (OCCC, *n* = 9). RQ, the relative quantity of hsa-miR-10a-5p to U6 snRNA, normalized to EOMA. Error bars represent ± SEM. **P*< 0.05, one-way ANOVA.

beginning at 96 hours. Overexpression of miR-10a in SMOV-2 cells showed an almost 2-fold increase in doubling time, from 42.8 hours to 84.9 hours. A statistically significant decrease in cell density was noted beginning at 72 hours in KK cells overexpressing miR-10a compared to a non-targeting control and continuing through 120 hours, there was a statistically significant decrease in proliferation. Overexpression of miR-10a in KK cells lengthened the double time from 40.9 hours to 47.2 hours.

MiR-10a-5p overexpression shifts cells from S and G_2 to G1 phase.

Cell cycle distribution was analyzed in SMOV-2 and KK cells overexpressing miR-10a-5p compared to non-targeting control transfected cells. SMOV-2 and KK cells overexpressing miR-10a-5p had a statistically significant increase in the G_1 population and a decrease in S and G_2 populations (*P*< 0.001) (Figure 8). SMOV2-10a

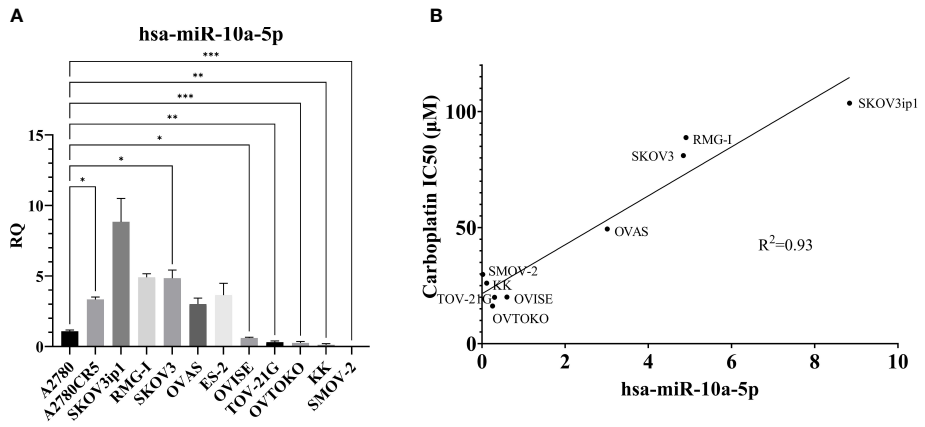

FIGURE 6
MiR-10a-5p expression across a panel of ovarian cancer cell lines. **(A)** RQ, the relative quantity of hsa-miR-10a-5p to U6 snRNA, normalized to A2780. ****P*<0.001, ***P*<0.001, **P*< 0.05, Brown-Forsythe and Welch ANOVA. A2780 (*n* = 6), A2780CR5 (*n* = 2), SKOV3ip1 (*n* = 3), RMG-I (*n* = 2), SKOV3 (*n* = 4), OVAS (*n* = 4), ES-2 (*n* = 4), OVISE (*n* = 9), TOV-21G (*n* = 4), OVTOKO (*n* = 7), KK (*n* = 3), and SMOV-2 (*n* = 6). **(B)** Correlation of miR-10a-5p expression to carboplatin IC50 in OCCC cell lines. RMG-I (*n* = 2), OVAS (*n* = 4), ES-2 OVISE (*n* = 9), TOV-21G (*n* = 2), OVTOKO (*n* = 7), KK (*n* = 2), and SMOV-2 (*n* = 6). Carboplatin IC50 is *n* ≥ 5 for each cell line.

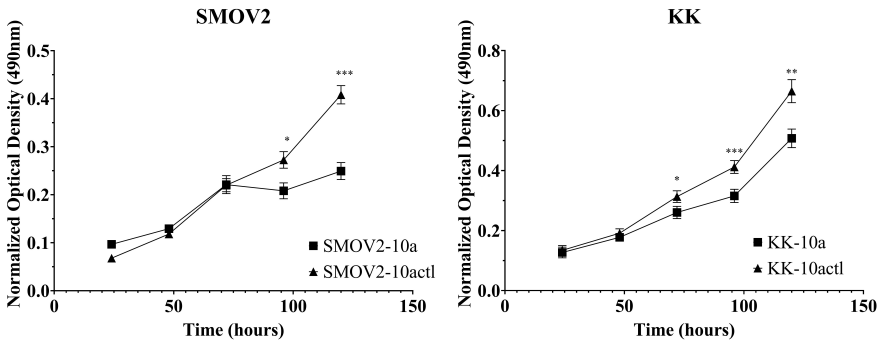

FIGURE 7
MiR-10a overexpression resulted in a significant decrease in cellular proliferation in SMOV2 and KK cells. MTS was normalized to the growth medium for each measurement. Cells transfected with mature miRNA mimic for miR-10a (SMOV2-10a and KK-10a) were compared to cells transfected with negative control #1 (SMOV2-10actl and KK-10actl) for each cell line and each time point. ***P< 0.0001, **P< 0.001, *P< 0.05, Student's two-tailed t-test at each time point. n =6 for each cell line, timepoint, and transfection condition.

had more than a 6% increase in the G_1 population with a 4.5% decreased percent in S phase (P< 0.0001). KK-10a had a similar 7% increase in cells in the G_1 phase, but KK-10a cells had a more distributed decrease in S phase (4%, P = 0.01) and G_2 (3%, P = 0.002). The amount of cellular debris was not significantly changed in either SMOV-2 or KK samples (Figure 8).

Predicted miR-10a-5p target genes dysregulated in OCCC with endometriosis play a role in signaling receptor binding.

MiRNA molecules are considered epigenetic regulators of gene expression (111). Overexpression of miRNA molecules leads to downregulation largely by destabilization of mRNA transcripts. Importantly, most mRNA molecules are targets of miRNAs (112). Each miRNA molecule has relative specificity of gene targets based on nucleotide sequence in the 3'UTR of the target gene. *In silico* prediction of genes that could be targeted by individual miRNA families is available in several databases. Target Scan predicts miRNA binding through complementary binding of the seed region of the mature miRNA molecule to the mRNA molecule, typically within the 3'UTR (66–68). As a slightly different algorithm for miRNA target gene predictions, miRDB uses *in silico* predicted miRNA binding to mRNA targets and downregulation of target gene expression from high-throughput sequencing data to identify putative targets. Additional predictions are added to miRDB from computational modeling and literature curation (69, 70). As another resource, miRTarBase uses natural language processing (NLP) to extract miRNA-predicted target gene data across the literature, to give miRNA-target interactions (MTIs). Examples of MTIs from miRTarBase include direct interaction studies of

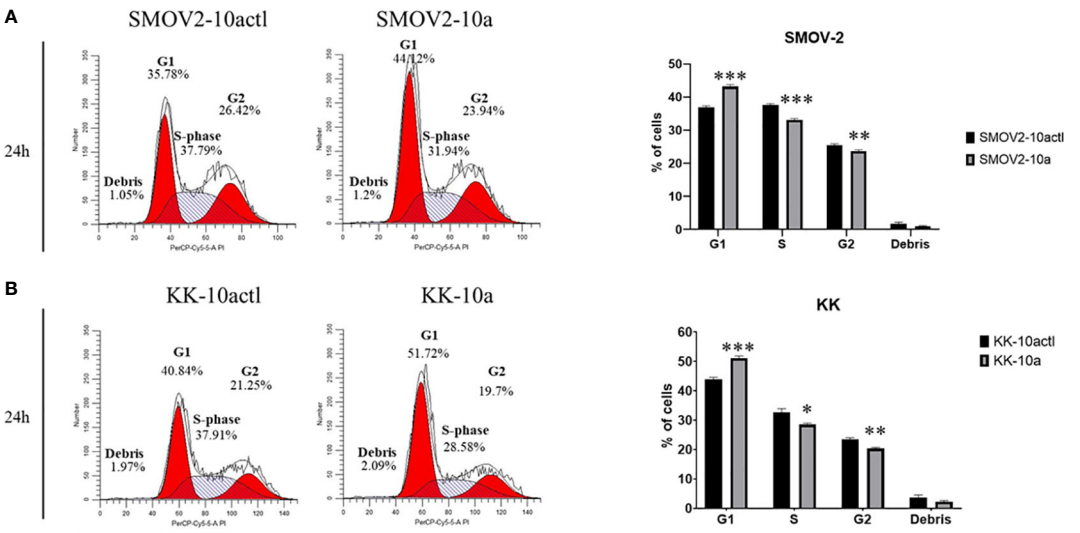

FIGURE 8
Overexpression of hsa-miR-10a-5p shifts cell cycle from S and G2 phase to G1. Cells transfected with mature miRNA mimic for miR-10a (SMOV2-10a and KK-10a) were compared to cells transfected with negative control #1 (SMOV2-10actl and KK-10actl) for each cell line. The flow histograms depict a representative biological replicate for **(A)** SMOV-2 and **(B)** KK. Graphical depictions represent n = 6 for each cell line and transfection condition. Statistical analysis was conducted using a 2-tailed Student's t-test: ***P< 0.0001, **P< 0.001, *P< 0.05.

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miRNA and target genes from CLIP-seq data, *in silico* seed sequence binding to mRNA from miRanda and miRBase, and experimental validation through reporter assays, western blots, or qPCR (71). To determine which dysregulated genes in OCCC with concurrent endometriosis were predicted targets of miR-10a-5p, miRNA:mRNA functional interaction prediction was undertaken using datasets from Target Scan v7.2, miRDB, and miRTarBase 2022 (65). Target Scan predicted 61 (Supplementary Table S14), miRDB predicted 62 (Supplementary Table S15), and miRTarBase predicted 67 (Supplementary Table S16) unique protein-coding genes downregulated in OCCC with concurrent endometriosis to be putative miR-10a-5p targets.

While 151 unique protein-coding genes were predicted to be miR-10a-5p target genes in at least one of the three algorithms, *BDNF* (brain-derived neurotrophic factor, log2 fold-change -2.42, P = 6.8e-5), *RORA* (RAR related orphan receptor A, log2 fold-change -1.98, P = 1.48e-7), *CSRNP3* (cysteine and serine-rich nuclear protein3, log2 fold-change -1.93, P = 1.57e-3), *CHL1* (cell adhesion molecule L1 like, log2 fold-change -3.08, P = 2.09e-5), *LIX1L* (limb and CNS expressed 1 like, log2 fold-change -1.13, P = 7.11e-5), and *RAP2A* (RAP2A, member of RAS oncogene family, log2 fold-change -1.52, P = 2.63e-5) were genes predicted to be miR-10a-5p targets in each of the three datasets. Using the 151 genes as input, the WEB-based Gene SeT AnaLysis Toolkit [WebGestalt (113)] revealed that the top network for miRNA targeting was the miR-10 family as expected (enrichment ratio = 13.67, Supplementary Table S17). Gene ontology molecular function analysis (Supplementary Table S18) showed enrichment in signaling receptor binding genes (enrichment ratio = 1.92, P = 1.5e-3). Pathway analysis (Supplementary Table S18) showed enrichment in cellular senescence genes (enrichment ratio = 4.71, P = 4.24e-3) and TGFβ signaling pathway (enrichment ratio = 7.17, P = 2.34e-3). A listing of the downregulated predicted miR-10a-5p target genes from the signaling receptor binding molecular function is listed in Table 4. Signaling receptor binding, cellular senescence, and TGFβ-signaling all involve the cell cycle.

MiR-10a-5p overexpression downregulating genes involved in proliferation and cell cycle progression.

The 151 genes that were downregulated in OCCC with concurrent endometriosis and were putative miR-10a-5p target

TABLE 4 Putative miR-10a-5p target genes downregulated in OCCC with concurrent endometriosis within the receptor signaling pathway.

Gene Name	description	log2FoldChange	P_{adj}
ACVR2A	activin A receptor type 2A	-1.52	5.14E-05
ARRDC3	arrestin domain containing 3	-2.11	1.38E-05
BAMBI	BMP and activin membrane bound inhibitor	-2.57	1.03E-10
BDNF	brain derived neurotrophic factor	-2.42	4.93E-04
DLG4	discs large MAGUK scaffold protein 4	-1.55	7.17E-06
EPHA4	EPH receptor A4	-3.29	2.75E-13
FEM1B	fem-1 homolog B	-1.56	3.87E-09
FHL2	four and a half LIM domains 2	-2.70	1.60E-08
FLRT2	fibronectin leucine rich transmembrane protein 2	-3.15	5.94E-13
GNAL	G protein subunit alpha L	-3.60	3.56E-06
HLA-E	major histocompatibility complex, class I, E	-1.32	4.39E-03
IL12A	interleukin 12A	-1.82	4.61E-02
IRS1	insulin receptor substrate 1	-1.54	1.10E-02
MMP14	matrix metallopeptidase 14	-2.98	9.40E-06
NEDD4	NEDD4 E3 ubiquitin protein ligase	-1.48	6.79E-05
PANX1	pannexin 1	-2.03	2.86E-06
PIK3CG	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma	-2.49	2.24E-05
PLSCR1	phospholipid scramblase 1	-1.37	4.91E-02
SERPINE1	serpin family E member 1	-4.78	1.10E-06
TGFB3	transforming growth factor beta 3	-3.11	2.05E-12
TNFRSF8	TNF receptor superfamily member 8	-2.93	4.28E-03
VDR	vitamin D receptor	-2.12	4.67E-02

genes were hand-annotated for functional roles in proliferation or cell cycle. To examine the association of miR-10a-5p expression on these hand-selected putative target genes, mature miR-10a-5p was overexpressed in OCCC cell lines and target gene expression was examined by qPCR. Overexpression of miR-10a resulted in a nearly 2-fold decrease in *PALM2-AKAP2* in SMOV2 cells ($P< 0.01$, Figure 9A). A smaller, non-statistically significant effect was demonstrated in KK cells overexpressing miR-10a ($P = 0.12$, Figure 9B). *PALM2-AKAP2* is a newly named fusion gene with a yet unknown function, but it has been correlated with functions similar to the previously distinct *PALM2* and *AKAP2* genes, such as proliferation in colorectal cancer cell lines (114). Similarly, decreased *AKAP2* was shown to decrease cellular proliferation in ovarian cancer and decreased proliferation through regulation of *ERK1/2* (115, 116). Overexpression of miR-10a-5p was associated with a 2.4-fold decrease in Cyclin dependent kinase 6 (*CDK6*) gene expression in both SMOV2 and KK cells ($P< 0.05$, Figures 9A, B). CDK6 is a critical molecule for cellular proliferation and cell cycle progression from G1 to S phase (117, 118). Dysregulation of CDK6 is common in cancers and has been previously been implicated in dysfunctional proliferation and disease progression in ovarian carcinomas (119, 120). Overexpression of miR-10a-5p was associated with a 2-fold decrease in RAP2A, member of RAS oncogene family (*RAP2A*) gene expression in SMOV2 ($P< 0.01$, Figure 9A) and 3-fold decrease in KK cells ($P< 0.001$, Figure 9B). *RAP2A* is involved in cellular proliferation, has been positively correlated with increased platinum resistance in gastric cancer cells, and is a downstream target of *TP53* in cell cycle regulation (121–123). Overexpression of miR-10a-5p was associated with a more than 3-fold decrease in Serpin Family E Member 1 (*SERPINE1*) gene expression in KK cells ($P = 0.01$, Figure 9B). *SERPINE1* has been found to increase cancer cell proliferation through its regulation by miR-10a in clear cell renal carcinoma (124). Overexpression of miR-10a-5p was associated with a non-statistically significant decrease in Ephrin type A receptor 4 (*EPHA4*) gene expression in SMOV2 cells ($P< 0.07$, Figure 9A). *EPHA4* is a receptor involved in cancer cell proliferation in breast cancer cells through *AKT* signaling, where downregulation of *EPHA4* decreased proliferation and increasing *EPHA4* increased proliferation (125, 126). *EPHA4* is not expressed in KK cells (data not shown).

Discussion

Ovarian carcinomas are the fifth leading cause of cancer-related death for women in the United States, accounting for over 13,000 deaths annually (127). While multi-platform analyses are attempting to categorize epithelial ovarian cancers beyond histology to discover molecular features that will modulate therapeutic benefit (1–3), current first-line therapy for women with ovarian carcinomas remains similar for all histological subtypes and includes surgical debulking to remove maximum tumor tissue and six cycles of carboplatin and paclitaxel or neoadjuvant chemotherapy (128). Fortunately, 70% of women with high-grade serous ovarian carcinomas show a complete response to these standard regimens (129). Unfortunately, up to 89% of women with OCCC show progression of disease with this standard protocol (100, 130, 131). These epidemiological data highlight a critical need for further understanding of the molecular features of OCCC to improve treatment options and discoveries.

Towards this need for understanding the molecular underpinnings of ovarian cancer, large sample size, multi-platform epigenetic (*i.e.*, DNA methylation, histone binding), genomic (*i.e.*, whole genome sequencing, exome sequencing, targeted gene sequencing, copy number variant), transcriptomic (*i.e.*, bulk RNA, small RNA, target gene expression), and proteomic (*i.e.*, targeted immunohistochemistry, reverse phase protein array) studies have been undertaken. Many of the multi-platform studies utilize the much more abundant sample numbers from high-grade serous tumors (3). Publicly-available transcriptomic datasets for OCCC are available within the Gene Expression Omnibus (Supplementary Table S19), and many

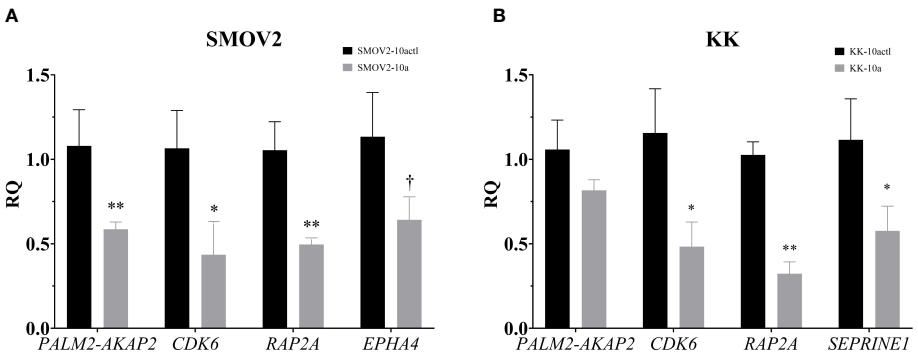

FIGURE 9
Putative miR-10a target genes, involved in cellular proliferation and the G1/S checkpoint, are downregulated with miR-10a-5p overexpression. Cells transfected with mature miRNA mimic for miR-10a (SMOV2-10a and KK-10a) were compared to cells transfected with negative control #1 (SMOV2-10actl and KK-10actl) for each cell line. **(A)** SMOV2 and **(B)** KK gene expression panels. RQ, the relative quantity of gene of interest to *ACTB*, normalized to negative control #1. Expression is plotted as mean ± SEM. Each gene was run with $n = 6$ for each cell line and treatment group. $^{**}P< 0.001$, $^{*}P< 0.05$, $^{\dagger}P< 0.07$ using unpaired Mann-Whitney or Welch's one-tailed t-test within cell lines.

of these datasets are published (16–21). Some studies on OCCC utilize transcriptomic profiles from OCCC cell lines (20, 27). For example, Yamaguchi et al. (20) created an OCCC signature from OCCC cell lines and compared it to multiple published or publicly available OCCC datasets. Nagaraja et al. (27) integrated transcriptomic microarray data with small RNA data from next-generation sequencing of a panel of OCCC cell lines compared to primary cultures of normal ovarian surface epithelium.

While up to 50% of OCCCs are associated with endometriosis (22, 23), most transcriptomic studies of primary tumors classified as OCCC do not characterize samples as coming from women with concurrent endometriosis, pathology-proven endometriosis or even a history of endometriosis (16–21). One of the studies that did delineate endometriosis was Bolton et al. (16) that performed the most extensive multi-platform sequencing of OCCC. They used both genomic (n = 421 samples) and transcriptomic (n = 211 samples) profiling. While more than 10% of their samples of OCCC were classified as coming from women with endometriosis (16), they did not analyze data from OCCC with concurrent endometriosis independently from those without endometriosis. This lack of concurrent endometriosis analysis could have been due to the endometriosis being based on patient-reported history and only confirmed by histology on samples from one institution. Shih et al. (18) included endometriosis from women without OCCC, endometriosis adjacent to OCCC, atypical endometriosis, and OCCC without endometriosis. The results showed that transcriptomic profiles from endometriosis adjacent to OCCC were most similar to atypical endometriosis. Similar to our results, they showed that endometriosis from women without OCCC was distinct from OCCC (18). Although they used laser capture microdissection, Shih et al. (18) did not examine OCCC samples from women with endometriosis. They focused more on the endometriosis samples and the transcriptomic transformation from endometriosis to atypical endometriosis to OCCC. Therefore, our study is unique for its inclusion and analyses of OCCC samples with pathologically confirmed endometriosis. Similar to Shih et al. (18), we used endometrioma samples without OCCC as a comparison tissue. All OCCC with concurrent endometriosis samples were primary tumor tissue with concurrent endometriosis confirmed by pathology reports with concurrent endometriosis. Unfortunately, these strict inclusion criteria and the limited availability of samples due to OCCC's rarity restricted the sample size in our study. While we had OCCC samples without endometriosis available for study, we did not compare OCCC transcriptomic profiles with and without endometriosis. Previously published transcriptomic studies of OCCC without endometriosis (16–21) had a significant number of samples already profiled (>200) and publicly available within GEO. Thus, we focused on well-characterized samples of OCCC with pathology-proven concurrent endometriosis compared to ovarian endometriomas, as this analysis had not been undertaken previously. Zhang et al. (7) showed unique molecular profiling in ovarian endometrioid carcinomas with and without endometriosis. Future studies will focus on analyses of publicly available OCCC datasets from women with and without endometriosis. Additionally, future studies will focus on obtaining matched endometrioma and adjacent OCCC samples from the same patient. Given the likelihood of a contribution of the endometriotic tumor microenvironment, evaluation using spatial transcriptomics would provide considerable insight.

On our well-characterized samples, we performed poly-A bulk RNA. Like previous studies in ovarian endometrioid carcinomas with concurrent endometriosis (7), we identified signaling pathways dysregulated in OCCC with concurrent endometriosis, including cytokine-cytokine receptor interaction, GPCR signaling, matrisome, and cell cycle and DNA repair pathways. Ovarian cancer cell lines are widely used as a model for epithelial ovarian carcinomas, and multiple studies have shown representative mutational, transcriptomic, and histological similarities between primary OCCC and OCCC cell lines (54, 57, 79–85). Many of these cell lines, including those derived from endometriosis-associated ovarian carcinomas (OCCC and ovarian endometrioid carcinomas), are not characterized by endometriosis status. In order to identify cell lines as the closest model of OCCC with concurrent endometriosis, our study utilized publicly available transcriptomic data for cancer cell lines, differentially expressed genes, and pathway activation scores. From this analysis we found that the OVTOKO, OVISE, RMG1, OVMANA, TOV21G, IGROV1, and JHOC5 cell lines are the cell lines with the most similar molecular profile to our OCCC with concurrent endometriosis dataset. Future studies with larger sample sizes of clinical samples would allow for a more comprehensive study of the subtle molecular nuances of the cell lines.

As a multi-platform transcriptomic approach, we also profiled small RNA molecules. To our knowledge, this represents the first small RNA sequencing from OCCC with concurrent endometriosis. Small RNA sequencing identified miR-141-3p, miR-183-5p, and miR-10a-5p as the top three most upregulated miRNAs in OCCC with concurrent endometriosis. MiR-141-3p overexpression has been demonstrated in a panel of platinum-resistant cell ovarian cancer lines (132). Further, increased expression of miR-141-3p was associated with increased cellular proliferation in esophageal cancer (133). Like miR-141-3p, studies of miR-183-5p in ovarian carcinoma are limited, but bioinformatic analysis in high-grade serous ovarian carcinoma correlated miR-183-5p with platinum-resistance (134). The specific role of these miRNAs in OCCC is currently unknown and will be crucial components in future studies.

MiR-10a-5p was the most abundant miRNA in OCCC with concurrent endometriosis, comprising 21% of the miRNA molecules. MiR-10a has been found to be upregulated in primary ductal breast carcinomas, squamous cell cervical carcinomas, acute myeloid leukemia, and pancreatic ductal adenocarcinomas and correlated with disease progression and platinum-resistance (91–98, 135, 136). Similar to other cancers, our study observed a strong, positive relationship between miR-10a-5p expression and platinum response in a panel of OCCC cell lines (R^2 = 0.93). Focusing on benign disease and ovarian function, previous studies have shown that miR-10a-5p expression is significantly lower in endometriomas compared to matched and unmatched eutopic endometrium (26,

137). Further, increased expression of miR-10a-5p in granulosa cells resulted in decreased proliferation (138), consistent with our results in OCCC cell lines. Moreover, increased expression of miR-10a-5p in granulosa cells led to cell cycle deficiencies, mediated through indirect regulation of cyclin-dependent kinase 2 (138). Dysregulated genes in the cell cycle and DNA repair pathways have been implicated in OCCC and associated with its disease progression and platinum-resistant phenotype: *RAP2A* (139–141), *CDK6* (119, 120, 142), *SERPINE1* (143, 144), and *EPHA4* (145–148) are involved in disease progression, drug response, and markers of progression. Overexpression of miR-10a-5p in OCCC cell lines showed an associated decrease in the expression of these putative miR-10a-5p target genes. These studies do not prove a direct effect of miR-10a-5p on this target gene expression. However, several studies have shown direct effects or associated effects of miR-10a on *SERPINE1* (124) and *EPHA4* (148, 149) gene expression. Figure 10 shows our working hypothesis of the role of miR-10a-5p effects on cell cycle progression in OCCC. In summary, we found a significant

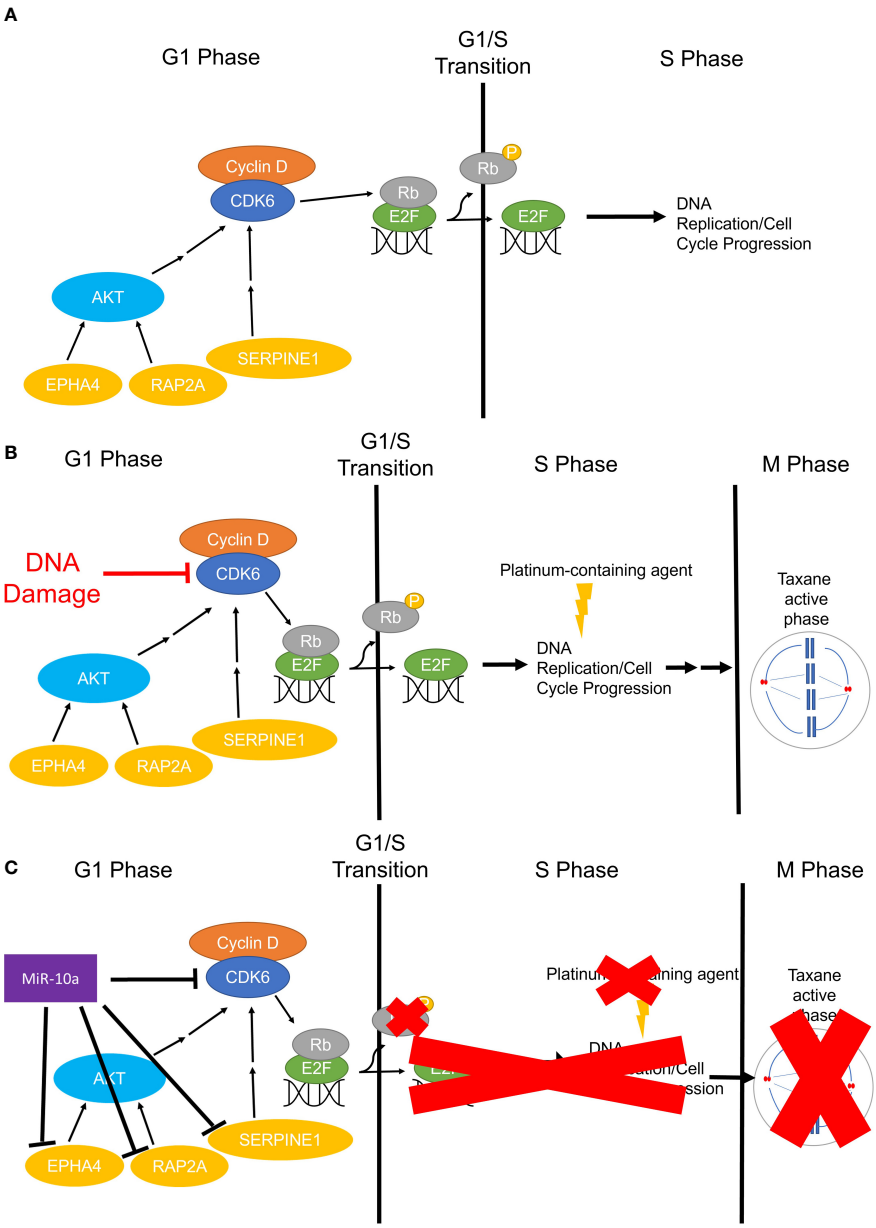

FIGURE 10
The working hypothesis for the mechanism of miR-10a-5p overexpression in OCCC G_0 or G_1/S Checkpoint. **(A)** Simplified representation of cell cycle progression function in non-cancerous cells, whereby CDK6 phosphorylates Rb freeing E2F for DNA replication in the S phase. **(B)** Infographic representation of cell cycle progression in non-malignant cells treated with platinum and/or taxane-containing agents. Cells will sustain DNA and/or microtubule damage resulting in no continued progression through the cell cycle and subsequent cell death. **(C)** Represents the working hypothesis for cell cycle progression in miR-10a-5p overexpressing OCCC cells. MiR-10a-5p downregulates *CDK6* and other important regulators of the cell cycle slowing or halting phosphorylation of Rb leading to inactive or prolonged inactivation of E2F and transition to S Phase and DNA replication. Cells slowed in G_1 or senescing in G_0 miss the critical chemotherapeutic effects in the S and M phases (red "X"s).

decrease in cellular proliferation with overexpression of miR-10a-5p. This decrease in proliferation may be due to a deficit in the G1/S checkpoint as a significant increase in cell population in G_1 was seen in cell cycle analysis while also having a significant decrease in cells in S and G_2 phases. Upon further evaluation of miR-10a target genes involved in proliferation, genes involved in regulating the G_1/S checkpoint were downregulated in SMOV-2 and KK cells transfected to overexpress miR-10a. More specifically, the miR-10a-5p target gene, *CDK6*, is well known for its regulation of a cell's progression to the S phase through its dimer with Cyclin-dependent kinase 4 (*CDK4*) (150). Other genes, including *EPHA4* (126), *RAP2A* (123), and *SERPINE1* (151) have been implicated in cell cycle progression. Of particular interest to the increased number of cells in G_1 are *CDK6* and *EPHA4* with downregulation of both being linked to cells remaining in G_0/G_1 (126, 152). The increase in this population of cells is clinically significant for the phases of the cell cycle in which platinum and taxanes show efficacy (153, 154). These drugs are commonly effective during S and M phases causing DNA and mitotic spindle damage leading to cell death. However, if OCCC cells are overexpressing miR-10a and stuck in a senescent or earlier stage of the cell cycle for an extended period, they will be less likely to undergo damage and death from cytotoxic platinum and taxane agents, leading to resistant tumors. Future studies, including the evaluation of phosphorylated proteins, are needed to explore this hypothesis further.

Data availability statement

The data presented in the study are deposited in the Gene Expression Omnibus (GEO), accession number GSE230956.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board (IRB) at Indiana University (#1812764043). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

KC, XW, AB, DR, CZ, and SH contributed to conception and design of the study. AB performed RNA sequencing analysis. KC, XW, YK, ND, MM, and SH performed bench experiments. KC, AB, DR, CZ, KS, CC, and SH performed bioinformatic and computational analysis. KC and SH wrote the first draft of the manuscript. KC, XW, YK, ND, MM, CZ, KS, AB, DR, and SH wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1162786/full#supplementary-material

References

1. Fridley BL, Dai J, Raghavan R, Li Q, Winham SJ, Hou X, et al. Transcriptomic characterization of endometrioid, clear cell, and high-grade serous epithelial ovarian carcinoma. *Cancer Epidemiol Biomarkers Prev* (2018) 27(9):1101–9. doi: 10.1158/1055-9965.Epi-17-0728

2. Bast RCJr., Matulonis UA, Sood AK, Ahmed AA, Amobi AE, Balkwill FR, et al. Critical questions in ovarian cancer research and treatment: report of an American association for cancer research special conference. *Cancer* (2019) 125(12):1963–72. doi: 10.1002/cncr.32004

3. Cancer Genome Atlas Research N. Integrated genomic analyses of ovarian carcinoma. *Nature* (2011) 474(7353):609–15. doi: 10.1038/nature10166

4. Wiegand KC, Shah SP, Al-Agha OM, Zhao Y, Tse K, Zeng T, et al. Arid1a mutations in endometriosis-associated ovarian carcinomas. *N Engl J Med* (2010) 363 (16):1532–43. doi: 10.1056/NEJMoa1008433

5. Jones S, Wang TL, Shih Ie M, Mao TL, Nakayama K, Roden R, et al. Frequent mutations of chromatin remodeling gene arid1a in ovarian clear cell carcinoma. *Science* (2010) 330(6001):228–31. doi: 10.1126/science.1196333

6. Ayhan A, Mao TL, Seckin T, Wu CH, Guan B, Ogawa H, et al. Loss of arid1a expression is an early molecular event in tumor progression from ovarian endometriotic cyst to clear cell and endometrioid carcinoma. *Int J Gynecol Cancer* (2012) 22(8):1310–5. doi: 10.1097/IGC.0b013e31826b5dcc

7. Zhang C, Wang X, Anaya Y, Parodi L, Cheng L, Anderson ML, et al. Distinct molecular pathways in ovarian endometrioid adenocarcinoma with concurrent endometriosis. *Int J Cancer* (2018) 143(10):2505–15. doi: 10.1002/ijc.31768

8. Wendel JRH, Wang X, Hawkins SM. The endometriotic tumor microenvironment in ovarian cancer. *Cancers (Basel)* (2018) 10(8):E261. doi: 10.3390/cancers10080261

9. Practice bulletin no. 114: management of endometriosis. *Obstet Gynecol* (2010) 116(1):223–36. doi: 10.1097/AOG.0b013e3181e8b073

10. Zondervan KT, Becker CM, Missmer SA. Endometriosis. *N Engl J Med* (2020) 382(13):1244–56. doi: 10.1056/NEJMra1810764

11. Saavalainen L, Lassus H, But A, Tiitinen A, Harkki P, Gissler M, et al. Risk of gynecologic cancer according to the type of endometriosis. *Obstet Gynecol* (2018) 131 (6):1095–102. doi: 10.1097/AOG.0000000000002624

12. Pearce CL, Templeman C, Rossing MA, Lee A, Near AM, Webb PM, et al. Association between endometriosis and risk of histological subtypes of ovarian cancer: A pooled analysis of case-control studies. *Lancet Oncol* (2012) 13(4):385–94. doi: 10.1016/S1470-2045(11)70404-1

13. Seidman JD, Horkayne-Szakaly I, Haiba M, Boice CR, Kurman RJ, Ronnett BM. The histologic type and stage distribution of ovarian carcinomas of surface epithelial origin. *Int J Gynecol Pathol* (2004) 23(1):41–4. doi: 10.1097/01.pgp.0000101080.35393.16

14. Machida H, Matsuo K, Yamagami W, Ebina Y, Kobayashi Y, Tabata T, et al. Trends and characteristics of epithelial ovarian cancer in Japan between 2002 and 2015: A jsgo-jsog joint study. *Gynecol Oncol* (2019) 153(3):589–96. doi: 10.1016/j.ygyno.2019.03.243

15. Kobel M, Kalloger SE, Huntsman DG, Santos JL, Swenerton KD, Seidman JD, et al. Differences in tumor type in low-stage versus high-stage ovarian carcinomas. *Int J Gynecol Pathol* (2010) 29(3):203–11. doi: 10.1097/PGP.0b013e3181c042b6

16. Bolton KL, Chen D, Corona de la Fuente R, Fu Z, Murali R, Kobel M, et al. Molecular subclasses of clear cell ovarian carcinoma and their impact on disease behavior and outcomes. *Clin Cancer Res* (2022) 28(22):4947–56. doi: 10.1158/1078-0432.CCR-21-3817

17. Mortlock S, Corona RI, Kho PF, Pharoah P, Seo JH, Freedman ML, et al. A multi-level investigation of the genetic relationship between endometriosis and ovarian cancer histotypes. *Cell Rep Med* (2022) 3(3):100542. doi: 10.1016/j.xcrm.2022.100542

18. Shin HY, Yang W, Chay DB, Lee EJ, Chung JY, Kim HS, et al. Tetraspanin 1 promotes endometriosis leading to ovarian clear cell carcinoma. *Mol Oncol* (2021) 15 (4):987–1004. doi: 10.1002/1878-0261.12884

19. Suryawanshi S, Huang X, Elishaev E, Budiu RA, Zhang L, Kim S, et al. Complement pathway is frequently altered in endometriosis and endometriosis-associated ovarian cancer. *Clin Cancer Res* (2014) 20(23):6163–74. doi: 10.1158/1078-0432.CCR-14-1338

20. Yamaguchi K, Mandai M, Oura T, Matsumura N, Hamanishi J, Baba T, et al. Identification of an ovarian clear cell carcinoma gene signature that reflects inherent disease biology and the carcinogenic processes. *Oncogene* (2010) 29(12):1741–52. doi: 10.1038/onc.2009.470

21. Marquez RT, Baggerly KA, Patterson AP, Liu J, Broaddus R, Frumovitz M, et al. Patterns of gene expression in different histotypes of epithelial ovarian cancer correlate with those in normal fallopian tube, endometrium, and colon. *Clin Cancer Res* (2005) 11(17):6116–26. doi: 10.1158/1078-0432.CCR-04-2509

22. Jimbo H, Yoshikawa H, Onda T, Yasugi T, Sakamoto A, Taketani Y. Prevalence of ovarian endometriosis in epithelial ovarian cancer. *Int J Gynaecol Obstet* (1997) 59 (3):245–50. doi: 10.1016/s0020-7292(97)00238-5

23. Stamp JP, Gilks CB, Wesseling M, Eshragh S, Ceballos K, Anglesio MS, et al. Baf250a expression in atypical endometriosis and endometriosis-associated ovarian cancer. *Int J Gynecol Cancer* (2016) 26(5):825–32. doi: 10.1097/IGC.0000000000000698

24. Logan M, Hawkins SM. Role of micrornas in cancers of the female reproductive tract: insights from recent clinical and experimental discovery studies. *Clin Sci (Lond)* (2015) 128(3):153–80. doi: 10.1042/CS20140087

25. Wang X, Ivan M, Hawkins SM. The role of microrna molecules and microrna-regulating machinery in the pathogenesis and progression of epithelial ovarian cancer. *Gynecol Oncol* (2017) 147(2):481–7. doi: 10.1016/j.ygyno.2017.08.027

26. Hawkins SM, Creighton CJ, Han DY, Zariff A, Anderson ML, Gunaratne PH, et al. Functional microrna involved in endometriosis. *Mol Endocrinol* (2011) 25 (5):821–32. doi: 10.1210/me.2010-0371

27. Nagaraja AK, Creighton CJ, Yu Z, Zhu H, Gunaratne PH, Reid JG, et al. A link between mir-100 and frap1/mtor in clear cell ovarian cancer. *Mol Endocrinol* (2010) 24 (2):447–63. doi: 10.1210/me.2009-0295

28. Chen S, Zhou Y, Chen Y, Gu J. Fastp: an ultra-fast all-in-one fastq preprocessor. *Bioinformatics* (2018) 34(17):i884–i90. doi: 10.1093/bioinformatics/bty560

29. Kim D, Langmead B, Salzberg SL. Hisat: A fast spliced aligner with low memory requirements. *Nat Methods* (2015) 12(4):357–60. doi: 10.1038/nmeth.3317

30. Langmead B, Salzberg SL. Fast gapped-read alignment with bowtie 2. *Nat Methods* (2012) 9(4):357–9. doi: 10.1038/nmeth.1923

31. Liao Y, Smyth GK, Shi W. Featurecounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* (2014) 30(7):923–30. doi: 10.1093/bioinformatics/btt656

32. Frankish A, Diekhans M, Ferreira AM, Johnson R, Jungreis I, Loveland J, et al. Gencode reference annotation for the human and mouse genomes. *Nucleic Acids Res* (2019) 47(D1):D766–D73. doi: 10.1093/nar/gky955

33. Frankish A, Diekhans M, Jungreis I, Lagarde J, Loveland JE, Mudge JM, et al. Gencode 2021. *Nucleic Acids Res* (2021) 49(D1):D916–D23. doi: 10.1093/nar/gkaa1087

34. Friedlander MR, Chen W, Adamidi C, Maaskola J, Einspanier R, Knespel S, et al. Discovering micrornas from deep sequencing data using mirdeep. *Nat Biotechnol* (2008) 26(4):407–15. doi: 10.1038/nbt1394

35. Kozomara A, Griffiths-Jones S. Mirbase: annotating high confidence micrornas using deep sequencing data. *Nucleic Acids Res* (2014) 42(Database issue):D68–73. doi: 10.1093/nar/gkt1181

36. Kozomara A, Birgaoanu M, Griffiths-Jones S. Mirbase: from microrna sequences to function. *Nucleic Acids Res* (2019) 47(D1):D155–D62. doi: 10.1093/nar/gky1141

37. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol* (2009) 10(3): R25. doi: 10.1186/gb-2009-10-3-r25

38. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for rna-seq data with deseq2. *Genome Biol* (2014) 15(12):550. doi: 10.1186/s13059-014-0550-8

39. Banu SK, Lee J, Starzinski-Powitz A, Arosh JA. Gene expression profiles and functional characterization of human immortalized endometriotic epithelial and stromal cells. *Fertil Steril* (2008) 90(4):972–87. doi: 10.1016/j.fertnstert.2007.07.1358

40. *QPCR standard curve* . Available at: https://www.sigmaaldrich.com/US/en/technical-documents/protocol/genomics/qpcr/qpcr-efficiency-determination.

41. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. Primer sequence disclosure: A clarification of the miqe guidelines. *Clin Chem* (2011) 57 (6):919–21. doi: 10.1373/clinchem.2011.162958

42. Yu Z, Kim J, He L, Creighton CJ, Gunaratne PH, Hawkins SM, et al. Functional analysis of mir-34c as a putative tumor suppressor in high-grade serous ovarian cancer. *Biol Reprod* (2014) 91(5):113. doi: 10.1095/biolreprod.114.121988

43. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative pcr and the 2(-delta delta C(T)) method. *Methods* (2001) 25(4):402–8. doi: 10.1006/meth.2001.1262

44. Faul F, Erdfelder E, Lang AG, Buchner A. G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav Res Methods* (2007) 39(2):175–91. doi: 10.3758/bf03193146

45. Faul F, Erdfelder E, Buchner A, Lang AG. Statistical power analyses using G*Power 3.1: tests for correlation and regression analyses. *Behav Res Methods* (2009) 41 (4):1149–60. doi: 10.3758/BRM.41.4.1149

46. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U.S.A.* (2005) 102(43):15545–50. doi: 10.1073/pnas.0506580102

47. Liberzon A, Birger C, Thorvaldsdóttir H, Ghandi M, Mesirov JP, Tamayo P. The molecular signatures database (Msigdb) hallmark gene set collection. *Cell Syst* (2015) 1 (6):417–25. doi: 10.1016/j.cels.2015.12.004

48. Liberzon A, Subramanian A, Pinchback R, Thorvaldsdottir H, Tamayo P, Mesirov JP. Molecular signatures database (Msigdb) 3.0. *Bioinformatics* (2011) 27 (12):1739–40. doi: 10.1093/bioinformatics/btr260

49. Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, et al. The cancer cell line encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* (2012) 483(7391):603–7. doi: 10.1038/nature11003

50. Lu X, Tu SW, Chang W, Wan C, Wang J, Zang Y, et al. Ssmd: A semi-supervised approach for a robust cell type identification and deconvolution of mouse transcriptomics data. *Brief Bioinform* (2021) 22(4):1–13. doi: 10.1093/bib/bbaa307

51. Wan C, Chang W, Zhang Y, Shah F, Lu X, Zang Y, et al. Ltmg: A novel statistical modeling of transcriptional expression states in single-cell rna-seq data. *Nucleic Acids Res* (2019) 47(18):e111. doi: 10.1093/nar/gkz655

52. Lau DH, Lewis AD, Ehsan MN, Sikic BI. Multifactorial mechanisms associated with broad cross-resistance of ovarian carcinoma cells selected by cyanomorpholino doxorubicin. *Cancer Res* (1991) 51(19):5181–7.

53. Lau DH, Ross KL, Sikic BI. Paradoxical increase in DNA cross-linking in a human ovarian carcinoma cell line resistant to cyanomorpholino doxorubicin. *Cancer Res* (1990) 50(13):4056–60.

54. Provencher DM, Lounis H, Champoux L, Tetrault M, Manderson EN, Wang JC, et al. Characterization of four novel epithelial ovarian cancer cell lines. *In Vitro Cell Dev Biol Anim* (2000) 36(6):357–61. doi: 10.1290/1071-2690(2000)036<0357:COFNEO>2.0.CO;2

55. Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, et al. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res* (1988) 48(3):589–601.

56. Yu D, Wolf JK, Scanlon M, Price JE, Hung MC. Enhanced C-erbb-2/neu expression in human ovarian cancer cells correlates with more severe Malignancy that can be suppressed by E1a. *Cancer Res* (1993) 53(4):891–8.

57. Gorai I, Nakazawa T, Miyagi E, Hirahara F, Nagashima Y, Minaguchi H. Establishment and characterization of two human ovarian clear cell adenocarcinoma lines from metastatic lesions with different properties. *Gynecol Oncol* (1995) 57(1):33–46. doi: 10.1006/gyno.1995.1097

58. Morisawa T, Kuramoto H, Shimoda T, Sakamoto I, Kato Y, Hamano M. [Establishment and characterization of a new scc antigen producing cell line (Hcs-2) from a carcinoma of the uterine cervix]. *Hum Cell* (1988) 1(3):308–14.

59. Sasa H, Ishii K, Hirata J, Kikuchi Y, Nagata I, Kawai T, et al. Establishment and characterization of a ca125-producing human ovarian clear cell carcinoma cell line. *Hum Cell* (1993) 6(4):279–86.

60. Yonamine K, Hayashi K, Iida T. Establishment and characterization of human ovarian clear cell adenocarcinoma cell line (Smov-2), and its cytotoxity by anticancer agents. *Hum Cell* (1999) 12(3):139–48.

61. Eva A, Robbins KC, Andersen PR, Srinivasan A, Tronick SR, Reddy EP, et al. Cellular genes analogous to retroviral onc genes are transcribed in human tumour cells. *Nature* (1982) 295(5845):116–9. doi: 10.1038/295116a0

62. Li M, Balch C, Montgomery JS, Jeong M, Chung JH, Yan P, et al. Integrated analysis of DNA methylation and gene expression reveals specific signaling pathways associated with platinum resistance in ovarian cancer. *BMC Med Genomics* (2009) 2:34. doi: 10.1186/1755-8794-2-34

63. Fogh J, Fogh JM, Orfeo T. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J Natl Cancer Inst* (1977) 59(1):221–6. doi: 10.1093/jnci/59.1.221

64. Nozawa S, Tsukazaki K, Sakayori M, Jeng CH, Iizuka R. Establishment of a human ovarian clear cell carcinoma cell line (Rmg-I) and its single cell cloning–with special reference to the stem cell of the tumor. *Hum Cell* (1988) 1(4):426–35.

65. Creighton CJ, Nagaraja AK, Hanash SM, Matzuk MM, Gunaratne PH. A bioinformatics tool for linking gene expression profiling results with public databases of microrna target predictions. *RNA* (2008) 14(11):2290–6. doi: 10.1261/rna.1188208

66. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microrna targets. *Cell* (2005) 120(1):15–20. doi: 10.1016/j.cell.2004.12.035

67. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mamMalian microrna targets. *Cell* (2003) 115(7):787–98. doi: 10.1016/s0092-8674(03)01018-3

68. Agarwal V, Bell GW, Nam JW, Bartel DP. Predicting effective microrna target sites in mamMalian mrnas. *Elife* (2015) 4:e05005. doi: 10.7554/eLife.05005

69. Liu W, Wang X. Prediction of functional microrna targets by integrative modeling of microrna binding and target expression data. *Genome Biol* (2019) 20(1):18. doi: 10.1186/s13059-019-1629-z

70. Chen Y, Wang X. Mirdb: an online database for prediction of functional microrna targets. *Nucleic Acids Res* (2020) 48(D1):D127–D31. doi: 10.1093/nar/gkz757

71. Huang HY, Lin YC, Cui S, Huang Y, Tang Y, Xu J, et al. Mirtarbase update 2022: an informative resource for experimentally validated mirna-target interactions. *Nucleic Acids Res* (2022) 50(D1):D222–D30. doi: 10.1093/nar/gkab1079

72. Prat J, Belhadj H, Berek J, Bermudez A, Bhatla N, Cain J, et al. Abridged republication of figo's staging classification for cancer of the ovary, fallopian tube, and peritoneum. *Eur J Gynaecol Oncol* (2015) 36(4):367–9.

73. Chen Y, Meltzer PS. Gene expression analysis via multidimensional scaling. *Curr Protoc Bioinf* (2005) 10:7.11.1–7.11.9. doi: 10.1002/0471250953.bi0711s10

74. Yamamoto S, Tsuda H, Miyai K, Takano M, Tamai S, Matsubara O. Cumulative alterations of P27-related cell-cycle regulators in the development of endometriosis-associated ovarian clear cell adenocarcinoma. *Histopathology* (2010) 56(6):740–9. doi: 10.1111/j.1365-2559.2010.03551.x

75. Hever A, Roth RB, Hevezi P, Marin ME, Acosta JA, Acosta H, et al. Human endometriosis is associated with plasma cells and overexpression of B lymphocyte stimulator. *Proc Natl Acad Sci U.S.A.* (2007) 104(30):12451–6. doi: 10.1073/pnas.0703451104

76. Chand AL, Murray AS, Jones RL, Hannan NJ, Salamonsen LA, Rombauts L. Laser capture microdissection and cdna array analysis of endometrium identify ccl16 and ccl21 as epithelial-derived inflammatory mediators associated with endometriosis. *Reprod Biol Endocrinol* (2007) 5:18. doi: 10.1186/1477-7827-5-18

77. Lawson C, Bourcier N, Al-Akoum M, Maheux R, Naud F, Akoum A. Abnormal interleukin 1 receptor types I and II gene expression in eutopic and ectopic endometrial tissues of women with endometriosis. *J Reprod Immunol* (2008) 77(1):75–84. doi: 10.1016/j.jri.2007.04.002

78. Zhao H, Wang Q, Bai C, He K, Pan Y. A cross-study gene set enrichment analysis identifies critical pathways in endometriosis. *Reprod Biol Endocrinol* (2009) 7:94. doi: 10.1186/1477-7827-7-94

79. Beaufort CM, Helmijr JC, Piskorz AM, Hoogstraat M, Ruigrok-Ritstier K, Besselink N, et al. Ovarian cancer cell line panel (Occp): clinical importance of in vitro morphological subtypes. *PloS One* (2014) 9(9):e103988. doi: 10.1371/journal.pone.0103988

80. Anglesio MS, Wiegand KC, Melnyk N, Chow C, Salamanca C, Prentice LM, et al. Type-specific cell line models for type-specific ovarian cancer research. *PloS One* (2013) 8(9):e72162. doi: 10.1371/journal.pone.0072162

81. Papp E, Hallberg D, Konecny GE, Bruhm DC, Adleff V, Noe M, et al. Integrated genomic, epigenomic, and expression analyses of ovarian cancer cell lines. *Cell Rep* (2018) 25(9):2617–33. doi: 10.1016/j.celrep.2018.10.096

82. Barnes BM, Nelson L, Tighe A, Burghel GJ, Lin IH, Desai S, et al. Distinct transcriptional programs stratify ovarian cancer cell lines into the five major histological subtypes. *Genome Med* (2021) 13(1):140. doi: 10.1186/s13073-021-00952-5

83. McCabe A, Zaheed O, McDade SS, Dean K. Investigating the suitability of in vitro cell lines as models for the major subtypes of epithelial ovarian cancer. *Front Cell Dev Biol* (2023) 11:1104514. doi: 10.3389/fcell.2023.1104514

84. Tan TZ, Ye J, Yee CV, Lim D, Ngoi NYL, Tan DSP, et al. Analysis of gene expression signatures identifies prognostic and functionally distinct ovarian clear cell carcinoma subtypes. *EBioMedicine* (2019) 50:203–10. doi: 10.1016/j.ebiom.2019.11.017

85. Domcke S, Sinha R, Levine DA, Sander C, Schultz N. Evaluating cell lines as tumour models by comparison of genomic profiles. *Nat Commun* (2013) 4:2126. doi: 10.1038/ncomms3126

86. Ditto A, De Cecco L, Paolini B, Alberti P, Martinelli F, Leone Roberti Maggiore U, et al. Validation of mirovar, a microrna-based predictor of early relapse in early stage epithelial ovarian cancer as a new strategy to optimise patients' Prognostic assessment. *Eur J Cancer* (2022) 161:55–63. doi: 10.1016/j.ejca.2021.11.003

87. Bagnoli M, Canevari S, Califano D, Losito S, Maio MD, Raspagliesi F, et al. Development and validation of a microrna-based signature (Mirovar) to predict early relapse or progression of epithelial ovarian cancer: A cohort study. *Lancet Oncol* (2016) 17(8):1137–46. doi: 10.1016/S1470-2045(16)30108-5

88. Vilming Elgaaen B, Olstad OK, Haug KB, Brusletto B, Sandvik L, Staff AC, et al. Global mirna expression analysis of serous and clear cell ovarian carcinomas identifies differentially expressed mirnas including mir-200c-3p as a prognostic marker. *BMC Cancer* (2014) 14:80. doi: 10.1186/1471-2407-14-80

89. Wyman SK, Parkin RK, Mitchell PS, Fritz BR, O'Briant K, Godwin AK, et al. Repertoire of micrornas in epithelial ovarian cancer as determined by next generation sequencing of small rna cdna libraries. *PloS One* (2009) 4(4):e5311. doi: 10.1371/journal.pone.0005311

90. Dahiya N, Sherman-Baust CA, Wang TL, Davidson B, Shih Ie M, Zhang Y, et al. Microrna expression and identification of putative mirna targets in ovarian cancer. *PloS One* (2008) 3(6):e2436. doi: 10.1371/journal.pone.0002436

91. Yu T, Liu L, Li J, Yan M, Lin H, Liu Y, et al. Mirna-10a is upregulated in nsclc and may promote cancer by targeting pten. *Oncotarget* (2015) 6(30):30239–50. doi: 10.18632/oncotarget.4972

92. Chang CH, Fan TC, Yu JC, Liao GS, Lin YC, Shih AC, et al. The prognostic significance of runx2 and mir-10a/10b and their inter-relationship in breast cancer. *J Transl Med* (2014) 12:257. doi: 10.1186/s12967-014-0257-3

93. Xiong G, Huang H, Feng M, Yang G, Zheng S, You L, et al. Mir-10a-5p targets tfap2c to promote gemcitabine resistance in pancreatic ductal adenocarcinoma. *J Exp Clin Cancer Res* (2018) 37(1):76. doi: 10.1186/s13046-018-0739-x

94. Zeng T, Li G. Microrna−10a enhances the metastatic potential of cervical cancer cells by targeting phosphatase and tensin homologue. *Mol Med Rep* (2014) 10(3):1377–82. doi: 10.3892/mmr.2014.2370

95. Long MJ, Wu FX, Li P, Liu M, Li X, Tang H. Microrna-10a targets chl1 and promotes cell growth, migration and invasion in human cervical cancer cells. *Cancer Lett* (2012) 324(2):186–96. doi: 10.1016/j.canlet.2012.05.022

96. Sun W, Ma Y, Chen P, Wang D. Microrna-10a silencing reverses cisplatin resistance in the A549/cisplatin human lung cancer cell line *via* the transforming growth factor-beta/smad2/stat3/stat5 pathway. *Mol Med Rep* (2015) 11(5):3854–9. doi: 10.3892/mmr.2015.3181

97. Pogribny IP, Filkowski JN, Tryndyak VP, Golubov A, Shpyleva SI, Kovalchuk O. Alterations of micrornas and their targets are associated with acquired resistance of mcf-7 breast cancer cells to cisplatin. *Int J Cancer* (2010) 127(8):1785–94. doi: 10.1002/ijc.25191

98. Lee JW, Choi CH, Choi JJ, Park YA, Kim SJ, Hwang SY, et al. Altered microrna expression in cervical carcinomas. *Clin Cancer Res* (2008) 14(9):2535–42. doi: 10.1158/1078-0432.CCR-07-1231

99. Huang T, Ren K, Ding G, Yang L, Wen Y, Peng B, et al. Mir10a increases the cisplatin resistance of lung adenocarcinoma circulating tumor cells *via* targeting pik3ca in the pi3k/akt pathway. *Oncol Rep* (2020) 43(6):1906–14. doi: 10.3892/or.2020.7547

100. Sugiyama T, Okamoto A, Enomoto T, Hamano T, Aotani E, Terao Y, et al. Randomized phase iii trial of irinotecan plus cisplatin compared with paclitaxel plus carboplatin as first-line chemotherapy for ovarian clear cell carcinoma: jgog3017/gcig trial. *J Clin Oncol* (2016) 34(24):2881–7. doi: 10.1200/JCO.2016.66.9010

101. Utsunomiya H, Akahira J, Tanno S, Moriya T, Toyoshima M, Niikura H, et al. Paclitaxel-platinum combination chemotherapy for advanced or recurrent ovarian clear cell adenocarcinoma: A multicenter trial. *Int J Gynecol Cancer* (2006) 16(1):52–6. doi: 10.1111/j.1525-1438.2006.00289.x

102. Goff BA, Sainz de la Cuesta R, Muntz HG, Fleischhacker D, Ek M, Rice LW, et al. Clear cell carcinoma of the ovary: A distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy in stage III disease. *Gynecol Oncol* (1996) 60(3):412–7. doi: 10.1006/gyno.1996.0065

103. Sugiyama T, Kamura T, Kigawa J, Terakawa N, Kikuchi Y, Kita T, et al. Clinical characteristics of clear cell carcinoma of the ovary: A distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy. *Cancer* (2000) 88(11):2584–9. doi: 10.1002/1097-0142(20000601)88:11<2584::AID-CNCR22>3.0.CO;2-5

104. Takano M, Kikuchi Y, Yaegashi N, Kuzuya K, Ueki M, Tsuda H, et al. Clear cell carcinoma of the ovary: A retrospective multicentre experience of 254 patients with complete surgical staging. *Br J Cancer* (2006) 94(10):1369–74. doi: 10.1038/sj.bjc.6603116

105. Ho CM, Huang YJ, Chen TC, Huang SH, Liu FS, Chang Chien CC, et al. Pure-type clear cell carcinoma of the ovary as a distinct histological type and improved survival in patients treated with paclitaxel-platinum-based chemotherapy in pure-type advanced disease. *Gynecol Oncol* (2004) 94(1):197–203. doi: 10.1016/j.ygyno.2004.04.004

106. Pectasides D, Fountzilas G, Aravantinos G, Kalofonos C, Efstathiou H, Farmakis D, et al. Advanced stage clear-cell epithelial ovarian cancer: the hellenic cooperative oncology group experience. *Gynecol Oncol* (2006) 102(2):285–91. doi: 10.1016/j.ygyno.2005.12.038

107. Sun W, Ma Y, Chen P, Wang D. Microrna-10a silencing reverses cisplatin resistance in the A549/cisplatin human lung cancer cell line *via* the transforming growth factor-β/smad2/stat3/stat5 pathway. *Mol Med Rep* (2015) 11(5):3854–9. doi: 10.3892/mmr.2015.3181

108. Zhu H, Kang M, Bai X. Tcf21 regulates mir-10a-5p/lin28b signaling to block the proliferation and invasion of melanoma cells. *PloS One* (2021) 16(8):e0255971. doi: 10.1371/journal.pone.0255971

109. Yuan Z, Wang W. Lncrna snhg4 regulates mir-10a/pten to inhibit the proliferation of acute myeloid leukemia cells. *Hematology* (2020) 25(1):160–4. doi: 10.1080/16078454.2020.1754636

110. Zhu M, Liu C, Chen S, Zhang C, Zhou P, Sun J, et al. Circrna circsept9 downregulates mir-10a through methylation to promote cell proliferation in laryngeal squamous cell carcinoma. *Crit Rev Eukaryot Gene Expr* (2021) 31(6):17–22. doi: 10.1615/CritRevEukaryotGeneExpr.2021039092

111. He L, Hannon GJ. Micrornas: small rnas with a big role in gene regulation. *Nat Rev Genet* (2004) 5(7):522–31. doi: 10.1038/nrg1379

112. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mamMalian mrnas are conserved targets of micrornas. *Genome Res* (2009) 19(1):92–105. doi: 10.1101/gr.082701.108

113. Wang J, Vasaikar S, Shi Z, Greer M, Zhang B. Webgestalt 2017: A more comprehensive, powerful, flexible and interactive gene set enrichment analysis toolkit. *Nucleic Acids Res* (2017) 45(W1):W130–W7. doi: 10.1093/nar/gkx356

114. Bracker TU, Sommer A, Fichtner I, Faus H, Haendler B, Hess-Stumpp H. Efficacy of ms-275, a selective inhibitor of class I histone deacetylases, in human colon cancer models. *Int J Oncol* (2009) 35(4):909–20. doi: 10.3892/ijo_00000406

115. Li X, Wang C, Zhang G, Liang M, Zhang B. Akap2 is upregulated in ovarian cancer, and promotes growth and migration of cancer cells. *Mol Med Rep* (2017) 16(4):5151–6. doi: 10.3892/mmr.2017.7286

116. Wang B, Jiang B, Li Y, Dai Y, Li P, Li L, et al. Akap2 overexpression modulates growth plate chondrocyte functions through erk1/2 signaling. *Bone* (2021) 146:115875. doi: 10.1016/j.bone.2021.115875

117. Meyerson M, Harlow E. Identification of G1 kinase activity for cdk6, a novel cyclin D partner. *Mol Cell Biol* (1994) 14(3):2077–86. doi: 10.1128/mcb.14.3.2077-2086.1994

118. Romero-Pozuelo J, Figlia G, Kaya O, Martin-Villalba A, Teleman AA. Cdk4 and cdk6 couple the cell-cycle machinery to cell growth *via* mtorc1. *Cell Rep* (2020) 31(2):107504. doi: 10.1016/j.celrep.2020.03.068

119. Cao J, Zhang Y, Mu J, Yang D, Gu X, Zhang J. Exosomal mir-21-5p contributes to ovarian cancer progression by regulating cdk6. *Hum Cell* (2021) 34(4):1185–96. doi: 10.1007/s13577-021-00522-2

120. Duan L, Yan Y, Wang G, Xing YL, Sun J, Wang LL. Muir-182-5p functions as a tumor suppressor to sensitize human ovarian cancer cells to cisplatin through direct targeting the cyclin dependent kinase 6 (Cdk6). *J BUON* (2020) 25(5):2279–86.

121. Wang T, Wang J, Liang R, He J, Liao Q, Jiang Y. Rap2a/P-erk1/2 is involved in glioma proliferation and migration. *Curr Mol Med* (2022). doi: 10.2174/1566524022666220804152049

122. Zhang J, Wei Y, Min J, Wang Y, Yin L, Cao G, et al. Knockdown of rap2a gene expression suppresses cisplatin resistance in gastric cancer cells. *Oncol Lett* (2020) 19(1):350–8. doi: 10.3892/ol.2019.11086

123. Wu JX, Zhang DG, Zheng JN, Pei DS. Rap2a is a novel target gene of P53 and regulates cancer cell migration and invasion. *Cell Signal* (2015) 27(6):1198–207. doi: 10.1016/j.cellsig.2015.02.026

124. Liu RJ, Xu ZP, Li SY, Yu JJ, Feng NH, Xu B, et al. Bap1-related cerna (Neat1/mir-10a-5p/serpine1) promotes proliferation and migration of kidney cancer cells. *Front Oncol* (2022) 12:852515. doi: 10.3389/fonc.2022.852515

125. Dong Y, Liu Y, Jiang A, Li R, Yin M, Wang Y. Microrna-335 suppresses the proliferation, migration, and invasion of breast cancer cells by targeting epha4. *Mol Cell Biochem* (2018) 439(1-2):95–104. doi: 10.1007/s11010-017-3139-1

126. Ding L, Shen Y, Ni J, Ou Y, Ou Y, Liu H. Epha4 promotes cell proliferation and cell adhesion-mediated drug resistance *via* the akt pathway in multiple myeloma. *Tumour Biol* (2017) 39(3):1010428317694298. doi: 10.1177/1010428317694298

127. Siegel RL, Miller KD, Wagle NS, Jemal A. Cancer statistics, 2023. *CA Cancer J Clin* (2023) 73(1):17–48. doi: 10.3322/caac.21763

128. Armstrong DK, Alvarez RD, Bakkum-Gamez JN, Barroilhet L, Behbakht K, Berchuck A, et al. Nccn guidelines insights: ovarian cancer, version 1.2019. *J Natl Compr Canc Netw* (2019) 17(8):896–909. doi: 10.6004/jnccn.2019.0039

129. Bast RCJr., Hennessy B, Mills GB. The biology of ovarian cancer: new opportunities for translation. *Nat Rev Cancer* (2009) 9(6):415–28. doi: 10.1038/nrc2644

130. Itamochi H, Kigawa J, Sugiyama T, Kikuchi Y, Suzuki M, Terakawa N. Low proliferation activity may be associated with chemoresistance in clear cell carcinoma of the ovary. *Obstet Gynecol* (2002) 100(2):281–7. doi: 10.1016/s0029-7844(02)02040-9

131. Itamochi H, Kigawa J, Terakawa N. Mechanisms of chemoresistance and poor prognosis in ovarian clear cell carcinoma. *Cancer Sci* (2008) 99(4):653–8. doi: 10.1111/j.1349-7006.2008.00747.x

132. Ying HC, Xu HY, Lv J, Ying TS, Yang Q. Microrna signatures of platinum-resistance in ovarian cancer. *Eur J Gynaecol Oncol* (2015) 36(1):16–20.

133. Phatak P, Noe M, Asrani K, Chesnick IE, Greenwald BD, Donahue JM. Microrna-141-3p regulates cellular proliferation, migration, and invasion in esophageal cancer by targeting tuberous sclerosis complex 1. *Mol Carcinog* (2021) 60(2):125–37. doi: 10.1002/mc.23274

134. Qi X, Yu C, Wang Y, Lin Y, Shen B. Network vulnerability-based and knowledge-guided identification of microrna biomarkers indicating platinum resistance in high-grade serous ovarian cancer. *Clin Transl Med* (2019) 8(1):28. doi: 10.1186/s40169-019-0245-6

135. Ujifuku K, Mitsutake N, Takakura S, Matsuse M, Saenko V, Suzuki K, et al. Mir-195, mir-455-3p and mir-10a(*) are implicated in acquired temozolomide resistance in glioblastoma multiforme cells. *Cancer Lett* (2010) 296(2):241–8. doi: 10.1016/j.canlet.2010.04.013

136. Slaby O, Svoboda M, Michalek J, Vyzula R. Micrornas in colorectal cancer: translation of molecular biology into clinical application. *Mol Cancer* (2009) 8:102. doi: 10.1186/1476-4598-8-102

137. Haikalis ME, Wessels JM, Leyland NA, Agarwal SK, Foster WG. Microrna expression pattern differs depending on endometriosis lesion type. *Biol Reprod* (2018) 98(5):623–33. doi: 10.1093/biolre/ioy019

138. Li D, Li X, He H, Zhang Y, He H, Sun C, et al. Mir-10a-5p inhibits chicken granulosa cells proliferation and progesterone(P4) synthesis by targeting mapre1 to suppress cdk2. *Theriogenology* (2022) 192:97–108. doi: 10.1016/j.theriogenology.2022.08.019

139. Han Y, Wang X, Mao E, Shen B, Huang L. Lncrna flvcr1−As1 drives colorectal cancer progression via modulation of the mir−381/rap2a axis. *Mol Med Rep* (2021) 23(2):139. doi: 10.3892/mmr.2020.11778

140. Wang X, Meng R, Hu QM. Linc00319-mediated mir-3127 repression enhances bladder cancer progression through upregulation of rap2a. *Front Genet* (2020) 11:180. doi: 10.3389/fgene.2020.00180

141. Wu F, Lin X, Shan SK, Li F, Xu F, Zhong JY, et al. The suppression of mir-199a-3p by promoter methylation contributes to papillary thyroid carcinoma aggressiveness by targeting rap2a and dnmt3a. *Front Cell Dev Biol* (2020) 8:594528. doi: 10.3389/fcell.2020.594528

142. Weiner-Gorzel K, Dempsey E, Milewska M, McGoldrick A, Toh V, Walsh A, et al. Overexpression of the microrna mir-433 promotes resistance to paclitaxel through the induction of cellular senescence in ovarian cancer cells. *Cancer Med* (2015) 4(5):745–58. doi: 10.1002/cam4.409

143. Pan JX, Qu F, Wang FF, Xu J, Mu LS, Ye LY, et al. Aberrant serpine1 DNA methylation is involved in carboplatin induced epithelial-mesenchymal transition in epithelial ovarian cancer. *Arch Gynecol Obstet* (2017) 296(6):1145–52. doi: 10.1007/s00404-017-4547-x

144. Zhao C, Liu Z. Microrna 617 targeting serpine1 inhibited the progression of oral squamous cell carcinoma. *Mol Cell Biol* (2021) 41(6):e0056520. doi: 10.1128/MCB.00565-20

145. Giaginis C, Tsoukalas N, Bournakis E, Alexandrou P, Kavantzas N, Patsouris E, et al. Ephrin (Eph) receptor A1, A4, A5 and A7 expression in human non-small cell lung carcinoma: associations with clinicopathological parameters, tumor proliferative

capacity and patients' Survival. *BMC Clin Pathol* (2014) 14(1):8. doi: 10.1186/1472-6890-14-8

146. Kori M, Yalcin Arga K. Potential biomarkers and therapeutic targets in cervical cancer: insights from the meta-analysis of transcriptomics data within network biomedicine perspective. *PloS One* (2018) 13(7):e0200717. doi: 10.1371/journal.pone.0200717

147. Saintigny P, Peng S, Zhang L, Sen B, Wistuba II, Lippman SM, et al. Global evaluation of eph receptors and ephrins in lung adenocarcinomas identifies epha4 as an inhibitor of cell migration and invasion. *Mol Cancer Ther* (2012) 11(9):2021–32. doi: 10.1158/1535-7163.MCT-12-0030

148. Yan Y, Luo YC, Wan HY, Wang J, Zhang PP, Liu M, et al. Microrna-10a is involved in the metastatic process by regulating eph tyrosine kinase receptor A4-mediated epithelial-mesenchymal transition and adhesion in hepatoma cells. *Hepatology* (2013) 57(2):667–77. doi: 10.1002/hep.26071

149. Luo L, Yu ZP, Qin H, Zhu ZX, Liao MH, Liao HT, et al. Exosomal microrna-10a is associated with liver regeneration in rats through downregulation of epha4. *Chin Med J (Engl)* (2018) 131(4):454–60. doi: 10.4103/0366-6999.225057

150. Goel S, Bergholz JS, Zhao JJ. Targeting cdk4 and cdk6 in cancer. *Nat Rev Cancer* (2022) 22(6):356–72. doi: 10.1038/s41568-022-00456-3

151. Szoltysek K, Janus P, Zajac G, Stokowy T, Walaszczyk A, Widlak W, et al. Rrad, il4i1, cdkn1a, and serpine1 genes are potentially co-regulated by nf-kappab and P53 transcription factors in cells exposed to high doses of ionizing radiation. *BMC Genomics* (2018) 19(1):813. doi: 10.1186/s12864-018-5211-y

152. Goel S, DeCristo MJ, Watt AC, BrinJones H, Sceneay J, Li BB, et al. Cdk4/6 inhibition triggers anti-tumour immunity. *Nature* (2017) 548(7668):471–5. doi: 10.1038/nature23465

153. Horwitz SB, Lothstein L, Manfredi JJ, Mellado W, Parness J, Roy SN, et al. Taxol: mechanisms of action and resistance. *Ann N Y Acad Sci* (1986) 466:733–44. doi: 10.1111/j.1749-6632.1986.tb38455.x

154. Basourakos SP, Li L, Aparicio AM, Corn PG, Kim J, Thompson TC. Combination platinum-based and DNA damage response-targeting cancer therapy: evolution and future directions. *Curr Med Chem* (2017) 24(15):1586–606. doi: 10.2174/0929867323666161214114948

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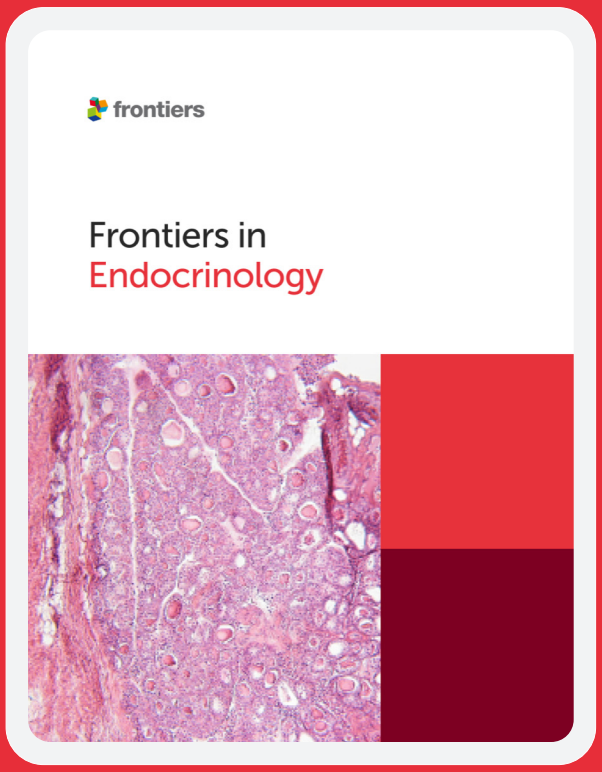

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