An integrative analysis of endometrial steroid metabolism and transcriptome in relation to endometrial receptivity in in vitro fertilization patients

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Objective: To study the relationship between the steroid concentration in the endometrium, in serum, and the gene expression level of steroid-metabolizing enzymes in the context of endometrial receptivity in in vitro fertilization (IVF) patients.
Design: Case-control study of 40 IVF patients recruited in the SCRaTCH study (NTR5342), a randomized controlled trial investigating pregnancy outcome after "endometrial scratching." Endometrial biopsies and serum were obtained from patients with a first failed IVF cycle randomized to the endometrial scratch in the midluteal phase of the natural cycle before the next fresh embryo transfer during the second IVF cycle.
Setting: University hopsital.
Patients: Twenty women with clinical pregnancy were compared with 20 women who did not conceive after fresh embryo transfer. Cases and controls were matched for primary vs. secondary infertility, embryo quality, and age.
Intervention: None.
Main Outcome Measure(s): Steroid concentrations in endometrial tissue homogenates and serum were measured with liquid chromatography-mass spectrometry. The endometrial transcriptome was profiled by RNA-sequencing, followed by principal component analysis and differential expression analysis. False discovery rate-adjusted and log-fold change $>|0.5|$ were selected as the threshold for differentially expressed genes.

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Result(s): Estrogen levels were comparable in both serum (n = 16) and endometrium (n = 40). Androgens and 17-hydroxyprogesterone were higher in serum than that in endometrium. Although steroid levels did not vary between pregnant and nonpregnant groups, subgroup analysis of primary women with infertility showed a significantly lower estrone concentration and estrone:androstenedione ratio in serum of the pregnant group (n = 5) compared with the nonpregnant group (n = 2). Expression of 34 out of 46 genes encoding the enzymes controlling the local steroid metabolism was detected, and estrogen receptor β gene was differentially expressed between pregnant and nonpregnant women. When only the primary infertile group was considered, 28 genes were differentially expressed between pregnant and nonpregnant women, including *HSD11B2*, that catalyzes the conversion of cortisol into cortisone.
Conclusion(s): Steroidomic and transcriptomic analyses show that steroid concentrations are regulated by the local metabolism in the endometrium. Although no differences were found in endometrial steroid concentration in the pregnant and nonpregnant IVF patients, primary women with infertility showed deviations in steroid levels and gene expression, indicating that a more homogeneous patient group is required to uncover the exact role of steroid metabolism in endometrial receptivity.
Clinical Trial Registration Number: The study was registered in the Dutch trial registry (www.trialregister.nl), registration number NL5193/NTR5342, available at https://trialsearch.who.int/Trial2.aspx?TrialID=NTR6687. The date of registration is July 31, 2015. The first enrollment is on January 1, 2016. (Fertil Steril Sci® 2023;4:219–28. ©2023 by American Society for Reproductive Medicine.)
Key Words: Endometrium, Steroid, Transcriptome, Receptivity, IVF

Human embryo implantation is a relatively inefficient process, with approximately 30% success chance per cycle in natural conception (1), and implantation failure is one of the limiting factors for successful treatment during in vitro fertilization (IVF). Implantation requires a complex series of molecular and cellular events, which make the endometrium switch from an unreceptive to a receptive status, a 4/5-day period called window of implantation (WOI). During the WOI, the endometrium allows an embryo to appose, adhere, and invade (2).

Estradiol and progesterone are key regulators of endometrial function and receptivity. In mice, both the duration and dosage of estradiol exposure affect the length of the WOI (3). Also, in humans, high level of estradiol in the circulation (>2,500 pg/mL ≅ 9 pmol/mL) because of ovarian hyperstimulation in IVF can result in asynchrony of endometrial maturation and lower implantation rates (4–7), although this is not always confirmed (8, 9).

Progesterone is the master regulator of the decidualization of stromal cells and of the production of cytokines and growth factors responsible for communication with the blastocyst (10, 11). In IVF protocols, the early rise of progesterone during the follicular phase is detrimental to pregnancy chances (12).

The evidence outlined above indicates that a fine regulation of both estradiol and progesterone is crucial for WOI establishment and embryo implantation. Recently, it became evident that steroid hormones are not exclusively produced in the specialized glands but can be synthesized and metabolized locally, a process that is also referred to as "intracrinology" (13–17). Consequently, steroid tissue levels can differ from steroid blood levels. Deregulated local steroid metabolism is involved in several endometrial disorders, such as endometriosis, endometrial cancer, and infertility (18–20).

In this study, we explored the relationship between main steroids (estrogens, progestogens, androgens, and corticosteroids) and endometrial receptivity under the hypothesis that endometrial intracrinology (i.e., steroids and their metabolizing genes) is altered during the WOI in the embryo-receptive compared with nonreceptive endometria. Steroid levels (in serum and endometrium) and transcriptional profiles of the endometrium during WOI were compared between women who became or did not become pregnant.

MATERIALS AND METHODS

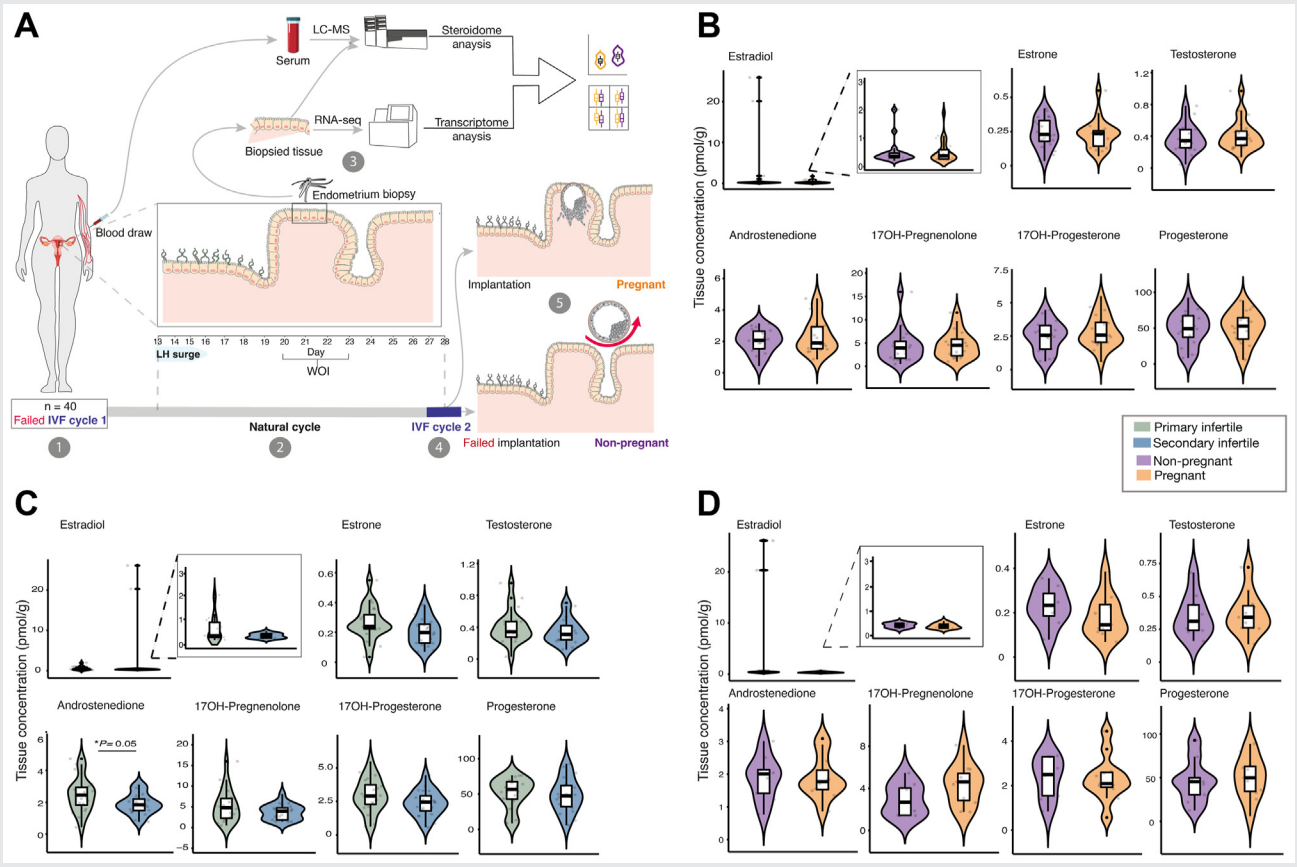
Ethical Approval

The study was approved by the Medical Ethics Committee of the University Medical Center of Utrecht, registration number 15-495/D, and registered in the Dutch trial registry (www.trialregister.nl), registration number NL5193/NTR5342, available at https://trialsearch.who.int/Trial2.aspx?TrialID=NTR6687. Written informed consent was obtained from all participating subjects in accordance with the Helsinki Declaration.

Study Design and Participants

This prospective cohort study was nested in a randomized controlled trial investigating the effect of endometrial scratching in women with implantation failure after a first unsuccessful IVF or intracytoplasmatic sperm injection (ICSI) cycle on subsequent live birth rate (the SCRaTCH study, NTR 5342 (21)). The full eligibility criteria, setting, and dates of the trial have been described elsewhere (22). Briefly, women aged 18–44 years planning a second full IVF/ICSI cycle with a regular indication for IVF/ICSI and a failed implantation after one full IVF/ICSI cycle, with all fresh and frozen embryo transfers (ETs) were included. Women could either have primary infertility, i.e., no pregnancy in the past, or secondary infertility, i.e., women who were pregnant (regardless of pregnancy outcome or method of conception) at least once in the past. Failed implantation was defined as the absence of a clinical pregnancy after one or more fresh and/or frozen ETs.

FIGURE 1



Study design and steroid concentration in tissue. (A) (1) Women were included after a first failed IVF cycle with at least one embryo transferred. Subsequently, (2) in a natural cycle, endometrial tissue was obtained (3) using a Pipelle catheter 5–8 days after a positive urine ovulation test based on the detection of the LH surge. At the same day of the biopsy, serum was isolated. After the biopsy, (4) patients continued with their second IVF cycle including stimulation, ovum pick up and fresh ET. (5) Pregnancy results were monitored regarding the result of the fresh ET. Steroidomic (LC-MS) and transcriptomic analyses were performed on both the endometrial tissue and serum. (B) Violin plot showing endometrial tissue concentration of estrogens (estradiol and estrone), androgens (testosterone and androstenedione) and progestogens (17OH-pregnenolone, 17OH-progesterone, and progesterone) in the pregnant and nonpregnant group, (C) Women with primary and secondary infertility, and (D) the pregnant and nonpregnant group considering women with primary infertility only. The boxplots show the interquartile range (box limits) and median (center line) of steroid levels. ET = embryo transfer; LC-MS = liquid chromatography - mass spectrometry; LH = luteinizing hormone; WOI = window of implantation.

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Endometrial Biopsies and Follow-up

Patients randomized to the intervention arm underwent an endometrial biopsy during the luteal phase of the natural cycle before the second IVF/ICSI cycle. The biopsy was performed 5–8 days after the luteinizing hormone (LH) surge, based on a positive urine ovulation LH test (Fig. 1A). The endometrial tissue was obtained with a Pipelle catheter (CCD International, France) and stored at -80 °C. On the same day of the endometrial biopsy, serum was isolated from peripheral blood (10 mL), which was left to clot for 30–60 minutes and centrifuged (2000× *g* for 10 minutes). After the endometrial biopsy, patients continued their IVF treatment. In the IVF/ICSI cycle after the biopsy, endometrial thickness at the time of ovum pick-up planning, number of embryos transferred, embryo quality, number of embryos frozen, pregnancy outcome in this cycle, and pregnancy in the 12-month follow-up period were recorded.

Sample Selection and Matching

Six Dutch centers biobanked 141 endometrial biopsies for the SCRaTCH study. For the present study, only biopsies of patients aged <38 years and who had a fresh single ET in the second IVF/ICSI cycle were included. Biopsies from patients with (i) a canceled cycle because of understimulation or overstimulation, (ii) with a freeze-all protocol, (iii) with no embryo available for transfer, or (iv) miscarriage were excluded. Subsequently, women were matched for age, embryo quality

TABLE 1

Patient and IVF/ICSI cycle characteristics of the participants.

Variables	Pregnant (n = 20)	Nonpregnant (n = 20)	*P* value
Age at inclusion, y	33.4 (3.1)	33.4 (3.2)	.995[g]
BMI - kg/m^2[a]	24.0 (4.1)	23.1 (2.4)	.444[g]
Female smokers, n, (%)	2 (10)	2 (10)	1.000[h]
Primary infertility,[b] n, (%)	11 (55)	9 (45)	.527[h]
Age at inclusion, y	32.7 (3.6)	32.3 (4.1)	.795
BMI - kg/m^2[c]	23.7 (3.9)	22.9 (2.0)	.600
Duration of infertility - mo	33.8 (18.3)	31.6 (15.5)	.684[g]
Indication for IVF or ICSI:			.484[h]
Male factor, n, (%)	13 (65)	11 (55)	
Idiopathic, n, (%)	6 (30)	8 (40)	
Unilateral tubal pathology, n, (%)	0	1 (5)	
Combination n, (%)	1 (5)	0	
No. of embryos transferred before biopsy[d]			
Fresh	1	1	1.000[g]
Frozen	2.57 (0.9)	2.56 (1.4)	.984[g]
Endometrial biopsy available n, (%)	20 (100)	20 (100)	
Serum available n, (%)	10 (50)	6 (30)	
Days between LH surge and biopsy[e]	6.33 (0.8)	6.95 (1.0)	**.040**[g]
Endometrial thickness in cycle after biopsy, mm[f]	11.6 (3.1)	9.6 (2.2)	**.028**[g]
Embryo quality of fresh ET after biopsy:			
Days after ovum pick up	2.8 (0.41)	2.9 (0.31)	.389[g]
Number of cells	7.4 (2.0)	7.5 (1.4)	.927[g]
Fragmentation, n, (%)			.162[h]
0%–10%	17 (85)	12 (60)	
10%–20%	2 (10)	7 (35)	
20%–50%	1 (5)	1 (5)	

Note: Data are presented as mean (±SD) or number (%).
BMI = body mass index; LH = luteinizing hormone.
[a] Data were missing for 1 participant in the nonpregnant group.
[b] Primary infertility: female has never conceived before.
[c] Obesity was not associated with differences in steroid levels in this study population, see main text.
[d] The number of embryos before randomization for the SCRaTCH trial
[e] Data was missing for 1 participant in the nonpregnant and 2 participants in the pregnant group.
[f] Data was missing for 1 participant in the nonpregnant and 1 participant in the pregnant group.
[g] Independent samples *t*-test
[h] χ^2 test

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(number of cells), and fertility treatment indication. Following the matching procedure, 40 subjects were selected: 20 women with a clinical pregnancy (which all resulted in a live birth), hereafter called the "pregnant group" and 20 nonpregnant women with a negative urine human chorionic gonadotropin 14 days after ET, hereafter called the "nonpregnant group." Patient characteristics are summarized in Table 1. Endometrial tissue was available from all subjects, whereas serum was available from 16 patients (because of technical reasons).

Objectives

Our primary objective was to compare endometrial steroid levels (estrogens, androgens, progestogens, and corticosteroids) during the midluteal phase, WOI, between the pregnant and nonpregnant groups. The secondary objectives were (i) to compare steroid levels in endometrial tissue and serum and (ii) to profile the steroid-metabolizing genes and the whole transcriptome of WOI endometria in the pregnant and nonpregnant groups.

Whole-tissue and Serum Steroid analysis

The concentration of main steroids (i.e., estradiol, estrone, dehydroepiandrosterone [DHEA], testosterone, androstenedione, 17OH-pregnenolone, pregnenolone, 17OH-progesterone, progesterone, cortisol, cortisone, corticosterone, 11-deoxycortisol, and 21OH-progesterone) was measured in endometrium and serum samples using liquid chromatography-tandem mass spectrometry as previously described (23). To obtain comparable local and systemic estrogen concentrations, we considered that 1 g of endometrial tissue corresponds to 1 mL of serum, as previously published (24). Steroids are shown as median values with interquartile range (IQR). The DHEA and pregnenolone could not be determined in tissue and serum samples because sample impurities interfered with the measurement. Therefore, these results were not included in the analysis.

RNA Extraction and Sequencing

The RNA extraction, library preparation, sequencing, and RNA-sequencing analysis (trimming, alignment counting,

and count matrix processing) are reported in Supplemental Notes (available online).

Differential Gene Expression Analysis

To identify the differentially expressed genes (DEGs), the gene count matrix was analyzed using DESeq2 (version 1.34.0). The DEG comparison was performed at the false discovery rate criterion of <0.05 and $\log_2$FC (fold-change) of >0.5 or <-0.5 as the threshold for up-regulated and down-regulated genes. The level of expression of 46 genes controlling the local steroid metabolism was analyzed (Supplemental Table 1, available online).

Statistical Analysis

Steroid levels were tested for normality with the Shapiro-Wilk test and log transformed if necessary. Normal distributed steroid levels in tissue and serum were compared using a paired *t*-test or a nonparametric Wilcoxon's signed rank test for data that were not normally distributed. To compare steroid concentrations between the pregnant and nonpregnant group, the independent samples Student's *t*-test was performed for normally distributed data and the Mann-Whitney *U* test for data that were not normally distributed. Multivariate logistic regression analysis was used to assess the influence of confounding determinants, body mass index (BMI, kg/m^2), and age, and confounder-adjusted *P* values were given. Statistically significant differences in the expression of steroid metabolism-related genes were determined using an unpaired 2-sample Wilcoxon test adjusted using Bonferroni's correction. Missing data were excluded from the analysis and is reported in the tables. In the batch correction for LH timing, the respective measurements were missed for 3 patients, and they were omitted from the further analysis.

RESULTS

Main Characteristics of the Patients and IVF Treatments

In the present study, we explored whether the levels of the major steroids in the endometrium during the WOI differed in women who achieved a clinical pregnancy (n = 20) ("pregnant group") compared with those who did not (n = 20) ("nonpregnant group") after fresh single cleavage-stage ET (Fig. 1A). Subjects consisted of a well-defined study group of women selected from a larger study population (n = 141) enrolled in the SCRaTCH-2 study. According to the SCRatCH-2 study protocol, all subjects had undergone a previous unsuccessful IVF cycle (see **Materials and Methods**).

The endometrial biopsy was taken between 5 and 8 days after the LH surge, and embryos were transferred during the next cycle. The interval after LH surge resulted 0.5 days longer in the nonpregnant group compared with the pregnant group (6.95 ± 1.0 vs. 6.33 ± 0.8 days, $P=.04$). Also, endometrial thickness, which was measured before the fresh ET in the cycle after the biopsy, was higher in the pregnant group than that in the nonpregnant group (11.6 ± 3.1 vs. 9.6 ± 2.2 mm, $P=.03$) (Table 1). Neither patient characteristics nor the number of previous fresh or frozen embryos transferred nor other treatment characteristics differed between subjects in the first failed IVF cycle (the cycle before the biopsy).

Steroid Levels in Serum and Endometrium

Steroid concentrations were measured in the 40 endometrial biopsies and 16 matched serum samples isolated from peripheral blood. First, we analyzed whether steroid levels differed between endometrial tissue and serum (Supplemental Table 2, available online). Estrone and estradiol resulted within the same range in serum and tissue (estrone with median 0.29 pmol/g [IQR = 0.19–0.37] in serum vs. 0.23 [0.14–0.30] pmol/g in tissue and estradiol 0.45 [0.28–0.69] pmol/g in serum vs. 0.37 [0.28–0.62] pmol/g in tissue). Contrary to estrogens, androstenedione, and testosterone showed a 2- to 3-fold higher level in serum compared with tissue (androstenedione with median 6.06 [4.64–7.82] pmol/g vs. 2.02 [1.51–2.82] pmol/g respectively, $P<.001$, and testosterone with median 0.86 [0.67–1.02] pmol/g vs. 0.34 [0.26–0.49] pmol/g respectively, $P<.001$). The 17OH-progesterone levels were almost doubled in serum as compared with tissue (median 5.92 [4.13–7.52] pmol/g vs. 2.57 [2.00–3.36] pmol/g, $P<.001$), whereas 17OH-Pregnenolone levels did not vary between serum and tissue (median 4.52 [2.50–7.76] pmol/g vs. 4.20 [1.86–5.72] pmol/g).

Estrone:androstenedione and estradiol:testosterone ratios were decreased in serum compared with tissue (estrone:androstenedione ratio 0.04 [0.03–0.05] vs. 0.11 [0.08–0.12] respectively, $P<.001$ and estradiol:testosterone ratio median 0.51 [0.30–0.91] vs. 1.10 [0.70–2.32] respectively, $P=.01$). Testosterone:androstenedione ratio resulted decreased in serum compared with tissue (0.14 [0.12–0.18] vs. 0.18 [0.14–0.21] respectively, $P=.04$) whereas the estradiol:estrone ratio did not vary (1.86 [1.55–2.09] vs. 1.81 [1.34–3.58] respectively).

Progesterone levels were not compared between tissue and serum because this steroid resulted above the upper limit of quantification (33.31 pmol/mL) in 12 out of 16 serum samples. As described in the methods, DHEA and pregnenolone could not be determined because of technical issues.

Steroid Levels In Pregnant vs. Nonpregnant Women

Next, mean steroid concentrations in endometrial tissue and serum were compared between pregnant and nonpregnant groups. None of the steroids analyzed, or their ratios differed between the 2 groups (Table 2 and Fig. 1B).

Steroid Levels in Primary Infertile Women

As the endometrial contribution to implantation failure might be different in women with primary infertility, i.e., women who never had a pregnancy before, in contrast to women with secondary infertility, subgroup analyses were performed to compare these groups. Serum concentrations of estradiol and 17OH-progesterone were lower in women with primary infertility (n =7) compared with women with secondary infertility (n = 9), irrespective of pregnancy status. For estradiol, the median concentration was 0.27 (IQR = 0.25–0.38) pmol/g vs. 0.59

TABLE 2

Median, lower, and upper quartile of the concentrations of steroids and steroid ratios in serum and endometrium of pregnant and nonpregnant women.

			Estradiol			Estrone			Testosterone		
Origin	Group	n	median (pmol/g)	0.25/0.75	P	median (pmol/g)	0.25/0.75	P	median (pmol/g)	0.25/0.75	P
Serum	Nonpregnant	6	0.49	0.31/0.71	.73[a]	0.29	0.19/0.38	.6[a]	0.96	0.48/0.99	.43[a,b]
	pregnant	10	0.45	0.25/0.72		0.27	0.18/0.36		0.84	0.74/1.35	
Endometrium	Nonpregnant	20	0.37	0.31/0.61	.57[c]	0.23	0.16/0.35	.74[a]	0.34	0.24/0.49	.53[c]
	Pregnant	20	0.38	0.26/0.68		0.23	0.13/0.27		0.37	0.27/0.49	

			Androstenedione			17OH-Pregnenolone			17OH-Progesterone		
			median (pmol/g)	0.25/0.75	P	median (pmol/g)	0.25/0.75	P	median (pmol/g)	0.25/0.75	P
Serum	Nonpregnant	6	6.53	4.56/9.02	.81[a,b]	3.69	1.80/8.03	.26[a,b]	5.5	4.08/8.44	.92[a]
	Pregnant	10	6.06	4.38/9.52		4.99	3.72/8.56		6.55	3.92/7.28	
Endometrium	Nonpregnant	20	2.07	1.45/2.68	.29[a]	3.95	1.55/5.43	.57[a,b]	2.55	1.49/3.30	.57[a]
	Pregnant	20	1.9	1.51/2.98		4.5	2.00/5.85		2.57	2.02/3.60	

			Progesterone			E_2:E_1			T:A4		
			median (pmol/g)	0.25/0.75	P	Median	0.25/0.75	P	Median	0.25/0.75	P
Serum	Nonpregnant	6	ULOQ	ND	ND	1.90	1.51/2.10	.85[a]	0.15	0.11/0.18	.92[a]
	Pregnant	10	ULOQ	ND		1.80	1.50/2.07		0.13	0.11/0.17	
Endometrium	Nonpregnant	20	49.38	32.74/67.51	.84[a]	1.86	1.34/5.00	.74[c]	0.17	0.13/0.23	.92[a,b]
	Pregnant	20	52.92	33.41/65.44		1.77	1.34/3.57		0.18	0.16/0.21	

			E_2:T			E1:A4		
			Median	0.25/0.75	P	Median	0.25/0.75	P
Serum	Nonpregnant	6	0.65	0.32/1.10	.41[a,b]	0.05	0.03/0.06	.45[a]
	Pregnant	10	0.40	0.29/0.91		0.03	0.02/0.05	
Endometrium	Nonpregnant	20	1.21	0.66/2.43	.48[c]	0.11	0.08/0.16	.29[a,b]
	Pregnant	20	1.05	0.71/1.66		0.09	0.06/0.16	

ULOQ = upper limit of quantification; ND = not determined; E_2:E_1 = estradiol to estrone ratio; T:A4 = testosterone to androstenedione ration; E_2:T = estradiol to testosterone ratio; E_1:A4 = estrone to androstenedione ratio.
[a] Independent samples t-test.
[b] Log 10 transformed.
[c] Mann-Whitney *U* test.

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(0.45–0.77) pmol/g, $P=.01$. After adjusting for confounders BMI and age, this difference remained significant (adjusted-$P=.03$). For 17OH-progesterone the median concentration was 4.16 (3.95–6.39) pmol/g vs. 7.17 (5.50–8.56) pmol/g respectively, $P=.04$ (adjusted-$P=.01$).

In tissue, androstenedione concentration was lower when comparing women with primary infertility (n = 20) with those with secondary infertility (n = 20). Androstenedione had a median concentration of 1.84 (1.44–2.33) pmol/g vs. 2.45 (1.65–2.99) pmol/g, respectively, $P=.047$ (adjusted-$P=.02$) (Fig. 1C). These differences were present irrespective of nonsignificant mean age differences in the group (32.53 ± 3.73 vs. 34.22 ± 2.10 years, $P=.09$).

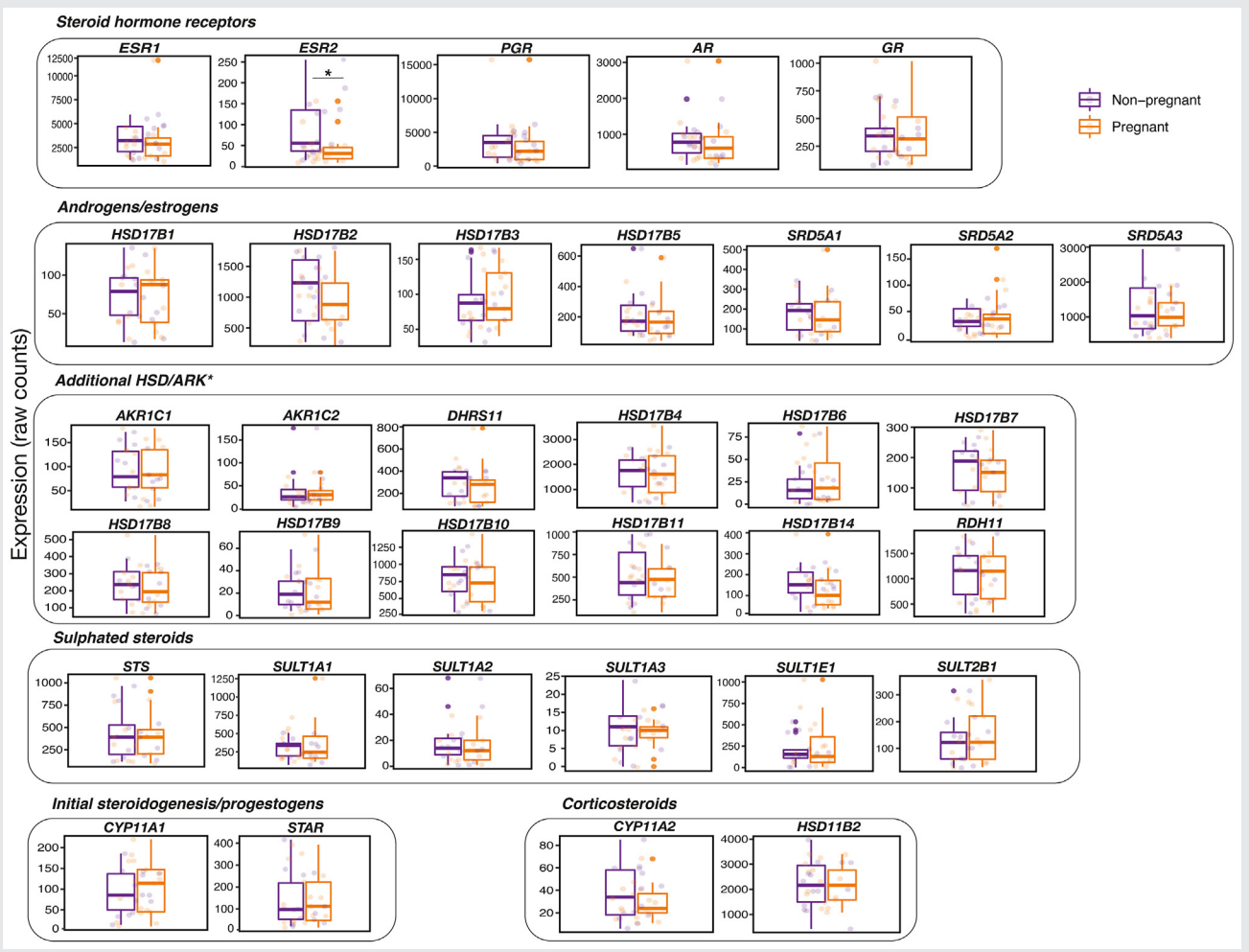
When women with primary infertility were considered only, serum estrone concentration was significantly lower in the pregnant group (n = 5) as compared with the nonpregnant (n = 2) group (estrone 0.19 [0.12–0.21] pmol/g vs. 0.34 [0.3–ND] pmol/g respectively, $P=.01$, adjusted-$P=.03$). The estrone:androstenedione ratio in serum was also lower in the pregnant compared with the nonpregnant group, but this difference did not remain significant after correction for confounders BMI and age (0.03 [0.03–0.05] vs. 0.05 [0.05–ND], respectively; $P=.049$ (adjusted-$P=.29$) (Supplemental Table 3, available online). There were no differences in steroid concentrations in endometrial tissue (Fig. 1D).

To assess whether obesity could be a confounding factor for steroid levels, we compared steroid levels in serum and tissues in obese (BMI ≥ 30) and nonobese women (BMI < 30), and no difference was found (it should be, however, noted that because BMI >35 was part of the exclusion criteria of the SCRaTCH-2 study, only one obese subject could be analyzed in the primary infertile group and 2 in the complete study population).

Corticosteroid Levels

Corticosterone and 11-deoxycortisol showed higher serum vs. tissue level (median 9.58 pmol/g [IQR = 5.80–11.40] vs. 0.68 [0.45–1.34] pmol/g, respectively; $P<.001$ for corticosterone; and median serum value 0.42 [0.29–0.73] pmol/g vs. tissue

FIGURE 2



Expression of steroid metabolism-related genes by RNA-sequencing. Expression of 34 steroid metabolism-related genes in the pregnant and nonpregnant group. Box plots show the 25th and 75th percentile, and whiskers indicate 1.5 times the interquartile range. Statistical significance was determined using an unpaired 2-samples Wilcoxon test adjusted using Bonferroni's correction. *$P<.05$.

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0.30 [0.18–0.45] pmol/g; $P<.001$ for 11-deoxycortisol) whereas cortisone was lower in serum compared with tissue (69.51 [62.90–71.73] pmol/g vs. 190.18 103.59–235.92] pmol/g, respectively, $P<.001$) (n=16). When considering pregnancy status, 11-deoxycortisol in serum was significantly higher in the pregnant group as compared with the nonpregnant group (0.95 [0.4–2.55] pmol/g vs. 0.44 [0.24–0.68] pmol/g, $P=.04$) (Supplemental Table 4, available online). Cortisol in serum was above upper limit of quantification (133.24 pmol/mL). In addition, the time during the day at which sampling took place was not recorded.

Steroid-Metabolizing Genes are Expressed in Human Endometrium

To gather information on the expression levels of the genes encoding for the enzymes controlling the local steroid metabolism, global RNA expression profiling was performed. Six out of 40 samples were excluded because of low RNA integrity number or RNA concentration.

Out of 46 steroid-metabolizing genes (16), the expression of 12 genes could not be detected (*AKR1C4, AKR1D1, ARO, CYP11B1, CYP11B2, CYP17A1, HSD11B1, HSD17B13, HSD3B1, HSD3B2, SULT1B1,* and *SULT2A1*; see Supplemental Table 1 for the comprehensive list of these genes). Among the 34 detectable transcripts, the highest expression levels were seen in genes encoding for the steroid hormone receptors (*AR, ERS1, GR,* and *PGR*), for known endometrial luteal phase markers (*HSD17B2* and *SRD5A*s), and for some hydroxysteroid-dehydrogenases, including the *HSD11B1/2* involved in glucocorticoid activation. Only the expression level of the *ESR2* gene was lower in the pregnant group (n = 19) compared with the nonpregnant group (n = 15; adjusted-$P=.02$, Fig. 2), whereas the expression levels of the remaining transcripts did not vary between study groups.

Next, we performed the principal component analysis (PCA) on the whole transcriptome profiles of our study groups, but no clear separation between the pregnant and nonpregnant groups was shown (Supplemental Fig. 1A, available online). This was confirmed by subsequent differential expressed gene (DEG) analysis using a mixed linear model where no DEGs were detected. Possible bias because of differences in menstrual cycle progression was controlled for by correcting for the time between the positive ovulation test and the biopsy (LH timing). No differences were seen in the PCA plot with uncorrected samples vs. samples corrected for LH timing (Supplemental Fig. 1B, available online).

Because the largest differences in steroid concentrations were seen in the subgroup of primary infertile women, RNA expression was analyzed in the groups with respect to infertility status. Among genes encoding for steroid-metabolizing enzymes *AR, ESR2, HSD17B8,* and *HSD17B9* had significantly lower expression in primary (n = 14) vs. secondary infertile women (n = 17) (adjusted-P<.05), irrespective of pregnancy status (Supplemental Fig. 2A, available online). The DEG analysis and PCA on the whole transcriptome did not show differences between groups (Supplemental Fig. 3, available online). However, among women with primary infertility, 28 DEGs were found between pregnant (n = 9) and nonpregnant (n = 5) women, of which 17 were up-regulated and 11 down-regulated. Among significantly enriched biologic processes, the regulation of microtubule depolymerization, as well as mitotic spindle organization, were present. Enriched molecular functions included protein kinase activator activity and microtubule binding. Among the detected genes, only *HSD11B2* from the steroid-metabolizing genes, catalyzing the conversion of cortisol into inactive cortisone, was increased in pregnant compared with nonpregnant women with primary infertility (Supplemental Fig. 2B, available online). The PCA showed the separation of expression profiles of pregnant primary infertile and nonpregnant primary infertile women, but the number of included women was small (Supplemental Fig. 4, available online).

DISCUSSION

In this study, we explored the influence of the circulating/tissue steroid hormones and endometrial transcriptome on endometrial receptivity in patients who did and did not become pregnant after a second IVF attempt. Genes encoding for steroid-metabolizing enzymes were found to be expressed in the endometrium, and differences in estrone level and the ratio of estrone:androstenedione were detected in the serum of a subgroup of patients with primary infertility but not in the complete study group.

Three previous studies have explored steroid levels in endometrial tissue (24–26). Huhtinen et al (24, 25) focused on the normal endometrium and endometriosis. In line with our results, androgen concentrations were higher in serum than that in tissue. Although binding to sex-hormone binding globulin in serum, these data may also suggest the presence of active conversion of androgens to other compounds in endometrial tissue. In this context, aromatase (*CYP19A1*), converting androgens to estrogens, could not be detected in our study, in contrast to previous literature (16). However, 5α-reductases (*SDR5As*), converting testosterone to DHT, were expressed at high levels in our study, in line with a previous study (25).

Although Huhtinen et al (24) described a two-fold higher estradiol concentration in serum compared with the endometrium, this was not reproduced in our study. Labarta et al. (26) explored the serum and endometrial progesterone levels in relation to implantation. The investigators used the endometrial receptivity array as an outcome. Progesterone levels in serum were comparable with the levels we found in the present study, but tissue levels of progesterone were significantly higher because of the use of a vaginal progesterone supplementation as part of the hormone replacement therapy. No association between serum progesterone and endometrial receptivity was found (26). In line with our study, endometrial concentrations of estradiol and estrone were not associated with receptivity (as determined by endometrial receptivity array). However, high endometrial progesterone was associated with a higher proportion of receptive endometria, and a similar association was found with low levels of 17OH-progesterone (26). The association between 17OH-progesterone and endometrial receptivity was not reproduced in our study.

Previous studies have shown a correlation between obesity and estrogen metabolism, the possibility of adipose tissue to convert estrogens, and the deleterious effect of obesity on human reproduction (27–29). In our study, no major differences were found in steroid levels between obese and nonobese women, but it should be noted that only 2 obese women were included.

When exploring the expression profile, it was found that *ESR2* expression, encoding for estrogen receptor β, was lower in pregnant women compared with nonpregnant women. Although estrogen receptor α appears to be the dominant receptor during implantation, the precise role of estrogen receptor β remains unclear, and some studies indicate that it might regulate estrogen receptor α expression (30). Similarly to what was observed with steroid levels, most differences in the transcriptome were found specifically for women with primary infertility. Comparing pregnant and nonpregnant women with primary infertility revealed increased expression of the gene *HSD11B2* in pregnant women, which is responsible for the conversion of active cortisol into inactive cortisone. Additionally, pregnant women had a higher serum concentration of the metabolite of cortisol (11-deoxycortisol). Although the role of corticoids in embryo implantation is elusive, one study described that *HSD11B2* is expressed in mice endometrial stromal cells during early pregnancy and is mainly up-regulated by progesterone (31). Possibly, the endometrium needs to inactivate cortisol to achieve a receptive state. The role of corticoid signaling, inflammation, and implantation remains to be explored in future studies.

In addition, gene ontology analyses revealed alterations in biologic processes related to microtubule and mitotic spindle organization. Specifically, *KIF2C* (Kinesin Family Member

2C) and *NAV3* (Neuron Navigator 3) were up-regulated, whereas *TPX2* (TPX2 Microtubule Nucleation Factor) was down-regulated in the pregnant compared with the nonpregnant group. Although these genes have not been reported as associated with endometrial receptivity, they are involved in fertility and endometria-related processes. The *KIF2C* depolymerizes microtubules, promotes mitotic chromosome segregation, and has been implicated in female infertility in recent animal studies, along with a larger set of deregulated transcripts (including epithelial splicing regulatory protein 1; (32)). The *NAV3* is involved in axon guidance and was found hypermethylated in placentas associated with gestational diabetes mellitus (33). Similar to *KIF2C*, *TPX2* is involved in mitotic spindle assembly and is an important mediator of *AURKA* function. The *TPX2* is also involved in spindle microtubule regulation. Interestingly, both *KIF2C* and *AURKA* were suggested to play a role in the process of stroma cell decidualization and its impairment in women with polycystic ovary syndrome (34). All genes were also associated with various endometrial pathologies (35–37).

Compared with previous literature, our study presents several novel aspects: (i) it is prospective; (ii) it integrates endometrial steroid metabolism with transcriptome; (iii) the precise timing of the endometrial biopsy based on the LH surge. Although there was a difference of 0.5 days in the timing of the biopsy between both groups, correction for this confounder did not change our results.

The major limitation of our work is that the study population was heterogeneous with respect to various IVF indications and included both patients with primary and secondary infertility. Additionally, women were included after a first failed IVF cycle, and deviations in steroid profiles are more likely to be found between women that represent the opposite ends of the implantation spectrum, such as patients with recurrent implantation failure or recurrent miscarriages, compared with healthy fertile women. Our power calculation, derived from previous investigations (24–26) because no data were available to compute a rigorous one, was based on our primary objective, i.e., to compare endometrial steroid levels during the WOI in pregnant and nonpregnant women. However, the most significant outcomes were obtained in subgroup analyses among primary infertile patients. Results here are encouraging but should be further explored in larger study populations. Additional limitations of our study are: (i) we analyzed endometrial biopsies taken during the cycle before the ET; hence, the potential bias of cycle-to-cycle variations cannot be excluded (technically, it is not feasible to obtain a biopsy during the same cycle of the ET); (ii) embryo quality was evaluated based on morphology, but no preimplantation genetic testing for aneuploidy was performed.

CONCLUSIONS

Endometrial intracrinology was deviated in a subgroup of women with primary infertility, which may represent the cause of impaired endometrial receptivity. Because the cause of implantation failure is highly varied, future studies should focus on study groups with more homogeneous clinical phenotypes, such as primary infertility or recurrent implantation failure, in which the cause of implantation failure may be less diverse. More pronounced differences in steroid levels are expected to be found in study groups where the endometrial factor is the predominant cause of implantation failure.

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REFERENCES

1. Zinaman MJ, Clegg ED, Brown CC, O'Connor J, Selevan SG. Estimates of human fertility and pregnancy loss. Fertil Steril 1996;65:503–9.
2. Cha J, Sun X, Dey SK. Mechanisms of implantation: strategies for successful pregnancy. Nat Med 2012;18:1754–67.
3. Ma WG, Song H, Das SK, Paria BC, Dey SK. Estrogen is a critical determinant that specifies the duration of the window of uterine receptivity for implantation. Proc Natl Acad Sci U S A 2003;100:2963–8.
4. Noci I, Borri P, Coccia ME, Criscuoli L, Scarselli G, Messeri G, et al. Hormonal patterns, steroid receptors and morphological pictures of endometrium in hyperstimulated IVF cycles. Eur J Obstet Gynecol Reprod Biol 1997;75:215–20.
5. Pellicer A, Valbuena D, Cano F, Remohi J, Simon C. Lower implantation rates in high responders: evidence for an altered endocrine milieu during the preimplantation period. Fertil Steril 1996;65:1190–5.
6. Simon C, Cano F, Valbuena D, Remohi J, Pellicer A. Clinical evidence for a detrimental effect on uterine receptivity of high serum oestradiol concentrations in high and normal responder patients. Hum Reprod 1995;10:2432–7.
7. Devroey P, Bourgain C, Macklon NS, Fauser BC. Reproductive biology and IVF: ovarian stimulation and endometrial receptivity. Trends Endocrinol Metab 2004;15:84–90.
8. Mackens S, Santos-Ribeiro S, Orinx E, De Munck N, Racca A, Roelens C, et al. Impact of serum estradiol levels prior to progesterone administration in artificially prepared frozen embryo transfer cycles. Front Endocrinol (Lausanne) 2020;11:255.
9. Bocca S, Real EB, Lynch S, Stadtmauer L, Beydoun H, Mayer J, et al. Impact of serum estradiol levels on the implantation rate of cleavage stage cryopreserved-thawed embryos transferred in programmed cycles with exogenous hormonal replacement. J Assist Reprod Genet 2015;32:395–400.
10. DeMayo FJ, Lydon JP. 90 years of progesterone: new insights into progesterone receptor signaling in the endometrium required for embryo implantation. J Mol Endocrinol 2020;65:T1–4.
11. Halasz M, Szekeres-Bartho J. The role of progesterone in implantation and trophoblast invasion. J Reprod Immunol 2013;97:43–50.
12. Lawrenz B, Melado L, Fatemi H. Premature progesterone rise in ART-cycles. Reprod Biol 2018;18:1–4.
13. Labrie F. All sex steroids are made intracellularly in peripheral tissues by the mechanisms of intracrinology after menopause. J Steroid Biochem Mol Biol 2015;145:133–8.
14. Dassen H, Punyadeera C, Kamps R, Delvoux B, Van Langendonckt A, Donnez J, et al. Estrogen metabolizing enzymes in endometrium and endometriosis. Hum Reprod 2007;22:3148–58.
15. Gibson DA, Simitsidellis I, Collins F, Saunders PTK. Endometrial intracrinology: oestrogens, androgens and endometrial disorders. Int J Mol Sci 2018;19:3276.
16. Konings G, Brentjens L, Delvoux B, Linnanen T, Cornel K, Koskimies P, et al. Intracrine regulation of estrogen and other sex steroid levels in endometrium and non-gynecological tissues; pathology, physiology, and drug discovery. Front Pharmacol 2018;9:940.
17. Labrie F. Intracrinology. Mol Cell Endocrinol 1991;78:C113–8.
18. Cornel KM, Kruitwagen RF, Delvoux B, Visconti L, Van de Vijver KK, Day JM, et al. Overexpression of 17beta-hydroxysteroid dehydrogenase type 1 increases the exposure of endometrial cancer to 17beta-estradiol. J Clin Endocrinol Metab 2012;97:E591–601.
19. Delvoux B, D'Hooghe T, Kyama C, Koskimies P, Hermans RJ, Dunselman GA, et al. Inhibition of type 1 17beta-hydroxysteroid dehydrogenase impairs the

synthesis of 17beta-estradiol in endometriosis lesions. J Clin Endocrinol Metab 2014;99:276–84.
20. Brosens J, Verhoeven H, Campo R, Gianaroli L, Gordts S, Hazekamp J, et al. High endometrial aromatase P450 mRNA expression is associated with poor IVF outcome. Hum Reprod 2004;19:352–6.
21. van Hoogenhuijze NE, Torrance HL, Mol F, Laven JSE, Scheenjes E, Traas MAF, et al. Endometrial scratching in women with implantation failure after a first IVF/ICSI cycle; does it lead to a higher live birth rate? The SCRaTCH study: a randomized controlled trial (NTR 5342). BMC Womens Health 2017;17:47.
22. van Hoogenhuijze NE, Mol F, Laven JSE, Groenewoud ER, Traas MAF, Janssen CAH, et al. Endometrial scratching in women with one failed IVF/ICSI cycle-outcomes of a randomised controlled trial (SCRaTCH). Hum Reprod 2021;36:87–98.
23. Hakkinen MR, Heinosalo T, Saarinen N, Linnanen T, Voutilainen R, Lakka T, et al. Analysis by LC-MS/MS of endogenous steroids from human serum, plasma, endometrium and endometriotic tissue. J Pharm Biomed Anal 2018;152:165–72.
24. Huhtinen K, Desai R, Stahle M, Salminen A, Handelsman DJ, Perheentupa A, et al. Endometrial and endometriotic concentrations of estrone and estradiol are determined by local metabolism rather than circulating levels. J Clin Endocrinol Metab 2012;97:4228–35.
25. Huhtinen K, Saloniemi-Heinonen T, Keski-Rahkonen P, Desai R, Laajala D, Stahle M, et al. Intra-tissue steroid profiling indicates differential progesterone and testosterone metabolism in the endometrium and endometriosis lesions. J Clin Endocrinol Metab 2014;99:E2188–97.
26. Labarta E, Sebastian-Leon P, Devesa-Peiro A, Celada P, Vidal C, Giles J, et al. Analysis of serum and endometrial progesterone in determining endometrial receptivity. Hum Reprod 2021;36:2861–70.
27. Edman CD, MacDonald PC. Effect of obesity on conversion of plasma androstenedione to estrone in ovulatory and anovulator young women. Am J Obstet Gynecol 1978;130:456–61.
28. Hetemaki N, Mikkola TS, Tikkanen MJ, Wang F, Hamalainen E, Turpeinen U, et al. Adipose tissue estrogen production and metabolism in premenopausal women. J Steroid Biochem Mol Biol 2021;209:105849.
29. Yang T, Zhao J, Liu F, Li Y. Lipid metabolism and endometrial receptivity. Hum Reprod Update 2022;28:858–89.
30. Large MJ, DeMayo FJ. The regulation of embryo implantation and endometrial decidualization by progesterone receptor signaling. Mol Cell Endocrinol 2012;358:155–65.
31. Zheng HT, Fu T, Zhang HY, Yang ZS, Zheng ZH, Yang ZM. Progesterone-regulated Hsd11b2 as a barrier to balance mouse uterine corticosterone. J Endocrinol 2020;244:177–87.
32. Yu L, Zhang H, Guan X, Qin D, Zhou J, Wu X. Loss of ESRP1 blocks mouse oocyte development and leads to female infertility. Development 2021;148:dev196931.
33. Wang WJ, Huang R, Zheng T, Du Q, Yang MN, Xu YJ, et al. Genome-wide placental gene methylations in gestational diabetes mellitus, fetal growth and metabolic health biomarkers in cord blood. Front Endocrinol (Lausanne) 2022;13:875180.
34. Sutaji Z, Elias MH, Ahmad MF, Karim AKA, Abu MA. A systematic review and integrated bioinformatic analysis of candidate genes and pathways in the endometrium of patients with polycystic ovary syndrome during the implantation window. Front Endocrinol (Lausanne) 2022;13:900767.
35. Adams CL, Dimitrova I, Post MD, Gibson L, Spillman MA, Behbakht K, et al. Identification of a novel diagnostic gene expression signature to discriminate uterine leiomyoma from leiomyosarcoma. Exp Mol Pathol 2019;110:104284.
36. Zhang J, An L, Zhao R, Shi R, Zhou X, Wei S, et al. KIF4A promotes genomic stability and progression of endometrial cancer through regulation of TPX2 protein degradation. Mol Carcinog 2023;62:303–18.
37. Aly JM, Lewis TD, Parikh T, Britten J, Malik M, Catherino WH. NAV3, a tumor suppressor gene, is decreased in uterine leiomyoma tissue and cells. Reprod Sci 2020;27:925–34.