RESEARCH

Local production of 17β-oestradiol in the endometrium during the implantation window: a pilot study

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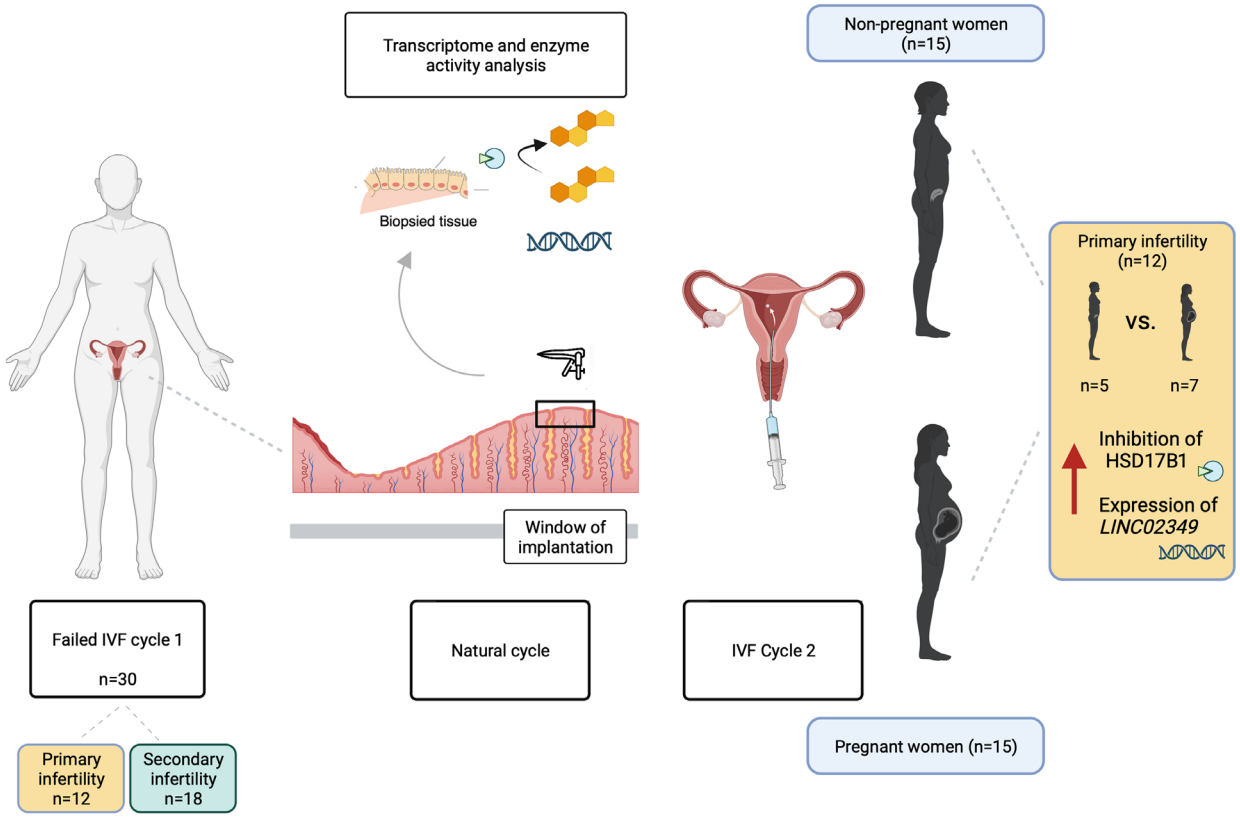
Graphical abstract



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Abstract

Sex steroids are converted to bioactive metabolites and vice versa by endometrial steroid-metabolising enzymes. Studies indicate that alterations in this metabolism might affect endometrial receptivity. This pilot study determined whether the endometrial formation and inactivation of 17β-oestradiol differed between the supposedly embryo-receptive endometrium and non-receptive endometrium of women undergoing IVF/intracytoplasmic sperm injection (ICSI). Endometrial biopsies were obtained from IVF/ICSI patients 5–8 days after ovulation in a natural cycle, prior to their second IVF/ICSI cycle with fresh embryo transfer (ET). Endometrial biopsies from patients who achieved clinical pregnancy after fresh ET ($n = 15$) were compared with endometrial biopsies from patients that did not conceive after fresh ET ($n = 15$). Formation of 17β-oestradiol (oxidative 17β-hydroxysteroid dehydrogenases (HSDs)), oestrone (reductive HSD17Bs) and inhibition of HSD17B1 activity were determined by high-performance liquid chromatography. The endometrial transcriptome was profiled using RNA sequencing followed by principal component analysis and differentially expressed gene analysis. The false discovery rate-adjusted $P < 0.05$ and log fold change >0.5 were selected as the screening threshold. Formation and inactivation of 17β-oestradiol resulted similar between groups. Inhibition of HSD17B1 activity was significantly higher in the non-pregnant group when only primary infertile women ($n = 12$) were considered (27.1%, $n = 5$ vs 16.2%, $n = 7$, $P = 0.04$). Gene expression analysis confirmed the presence of *HSD17B1* (encoding HSD17B1), *HSD17B2* (encoding HSD17B2) and 33 of 46 analysed steroid metabolising enzymes in the endometrium. In the primary infertile subgroup ($n = 10$) 12 DEGs were found including *LINC02349* which has been linked to implantation. However, the exact relationship between steroid-metabolising enzyme activity, expression and implantation outcome requires further investigation in larger, well-defined patient groups.

Lay summary

Sex hormones are produced and broken down by enzymes that can be found in the endometrium (the inner lining of the womb). This enzyme activity might influence the chances of becoming pregnant. We compared (i) enzyme activity in the endometrium of 15 women who did and 15 women who did not become pregnant in their second *in vitro* fertilisation attempt, (ii) how enzyme activity can be blocked by an inhibitor, and (iii) differences in gene expression (the process by which instructions in our DNA are converted into a product). Enzyme activity was similar between groups. We found that in women who have never been pregnant in the past, inhibition of enzyme activity was higher and found differences in a gene that has been linked to the implantation of the embryo, but future studies should be performed in larger, well-defined patient groups to confirm these findings.

Keywords: ▶ oestrogen ▶ implantation ▶ HSD17B1 ▶ endometrium ▶ receptivity ▶ IVF

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Introduction

In Europe, pregnancy rates after IVF have reached a plateau during the last years, with average pregnancy chances around 33% per embryo transfer for both IVF and intracytoplasmic sperm injection (ICSI) (European IVF Monitoring Consortium *et al.* 2022). For implantation to occur, the endometrium must undergo a series of molecular and cellular events that would lead to a receptive endometrial state. The period in which the endometrium is receptive to an embryo, or window of implantation (WOI), is limited to days 19–24 of a regular 28-day menstrual cycle and is modulated by steroid hormones.

In particular, 17β-oestradiol is one of the fundamental regulators of endometrial functions and receptivity. During the follicular phase, it induces endometrial cell proliferation and primes the endometrium to respond to progesterone during the luteal phase. During the luteal phase, serum 17β-oestradiol levels decline, and intracellular 17β-oestradiol signalling is blunted to allow for full activation of progesterone signalling, which in turn induces stroma cell decidualisation (DeMayo & Lydon 2020).

Although 17β-oestradiol is mainly produced within the ovarian follicles, its production also occurs

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in peripheral tissues such as fat, breast, liver, brain and endometrium, a process known as local steroid metabolism or intracrinology (Labrie 1991). The endometrium expresses several enzymes responsible for the generation of steroids and 17β-oestradiol, and is therefore capable of regulating local 17β-oestradiol concentrations. As a consequence, 17β-oestradiol tissue levels may differ from serum levels, which may lead to altered intracellular signalling activation (Konings *et al.* 2018*a*). The enzymes 17β-hydroxysteroid dehydrogenases (HSD17Bs) are particularly relevant in such local steroid metabolism. The redox balance between reductive and oxidative HSD17Bs controls the balance between the active 17β-oestradiol and the less active oestrogen oestrone. Out of the 15 known HSD17Bs that have been characterised to date, the enzyme HSD17B1 is considered the most relevant for local 17β-oestradiol generation, as it has the highest affinity towards oestrogens, and catalyses the reduction of oestrone to 17β-oestradiol. The enzyme HSD17B2 catalyses the oxidation of 17β-oestradiol to oestrone. Besides HSD17B1 and 2, other reductive and oxidative HSD17Bs (i.e. HSD17B5, 6, 7, 8 and 14) might be engaged in the local oestrogen metabolism, but their role and impact *in vivo* are not fully elucidated (Konings *et al.* 2018*a*).

The role of 17β-oestradiol, oestrogen signalling and the influence of its metabolism during implantation is currently unclear. Previous studies on mice and human stroma cells *in vitro* indicate that local formation of 17β-oestradiol is fundamental for decidualisation of stroma cells (Das *et al.* 2009, Gibson *et al.* 2013). Altered regulation of local steroid metabolism by HSD17B enzymes has been linked to endometrial development throughout different stages of the menstrual cycle and to disorders such as endometriosis. Although other enzymes controlling local steroid metabolism have also been related with implantation in humans, there are no reports of *HSD17B1* expression being associated with it. However, one study on ectopic pregnancies detected *HSD17B1* expression in epithelial cells of the fallopian tube at the nidation site, suggesting a regulative role of this enzyme at the site of implantation (Li *et al.* 2003).

In the present study, we explored the redox balance of reductive and oxidative HSD17Bs in relation to endometrial receptivity during the window of implantation. We (i) compared the activity of reductive and oxidative HSD17B enzymes in mid-luteal endometrial biopsies in women who did or did not become pregnant in the IVF/ICSI cycle following the biopsy, (ii) assessed the engagement of HSD17B1 using a specific inhibitor and (iii) profiled the endometrial transcriptome to gain further insight into the molecular mechanisms underlying endometrial receptivity. We hypothesise that endometrial metabolism of oestrogen is altered in the supposed embryo-receptive endometria (women with clinical pregnancy) compared to endometria without implantation (women who did not conceive).

Materials and methods

Ethical approval

The Medical Ethics Committee of the University Medical Centre of Utrecht (UMCU) approved the study (registration number 15-495/D), and the study is registered at the Dutch Trial Registry (registration number NL5193/NTR5342; 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

In this prospective cohort study, endometrial tissue samples were obtained from the SCRaTCH study, a randomised controlled trial investigating the effect of endometrial scratching in women with implantation failure after a first unsuccessful IVF/ICSI cycle on subsequent live birth rate (van Hoogenhuijze *et al.* 2021). The full study protocol was published previously (van Hoogenhuijze *et al.* 2017). Briefly, women aged 18–44 years indicated for IVF/ICSI and who had undergone a previous first full IVF/ICSI cycle, in which all fresh and frozen embryos were transferred but which did not result in clinical pregnancy, were included. Women could 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, but are now unable to become pregnant.

Endometrial biopsies and follow-up

Patients allocated to the intervention ('endometrial scratching') underwent an endometrial biopsy during the luteal phase of the menstrual cycle prior to the

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second IVF/ICSI cycle. From cycle day 10 onwards, urine ovulation tests based on the detection of the luteinising hormone (LH) surge were performed twice a day at home. The biopsy was obtained 5–8 days after a positive urine ovulation test using a Pipelle catheter (CCD International, France). Tissue was stored at −80°C. After endometrial biopsy, patients continued their second IVF/ICSI cycle. During this second cycle, thus after study randomisation and biopsy, endometrial thickness at the time of planning the ovum pick-up, embryo quality and pregnancy results were recorded.

Sample selection and matching

A total of 141 endometrial biopsies were harvested for the SCRaTCH study in six Dutch centres. For the present study, to avoid potential biases, specific inclusion/exclusion criteria were applied to select subjects: patients had to be below 38 years and had to have a fresh single embryo transfer (ET) in the second IVF/ICSI cycle. Patients who did not have a fresh embryo transfer or who miscarried, were excluded. Women were further matched for age, embryo quality and fertility treatment indication. Following the matching procedure, 30 samples were selected to compare intra-tissue steroid levels and divided into two clinical groups based on the results of the second IVF/ICSI cycle: 15 women with a clinical pregnancy (which all resulted in a live birth) versus 15 non-pregnant women with a negative urine b-hCG (beta human chorionic gonadotrophin) 14 days after ET (the 'pregnant group' and 'non-pregnant group', respectively). In our primary analysis, we compared women who achieved a clinical pregnancy to those who did not. In a secondary analysis, we compared types of infertility: primary versus secondary infertility.

Synthesis of 2-(4-carboxy-phenyl)-5,6-dimethylbenzimidazole

The synthesis of 2-(4-carboxyphenyl)-5,6-dimethyl-benzimidazole (CDB) was adapted from the procedure reported by Katayama *et al.* (Katayama *et al.* 1991). Briefly, 4,5-dimethyl-*O*-phenylenediamine (5.1 g, 37.8 mmol, 1.00 equiv.) was dissolved in 160 mL ethanol in a 500 mL round-bottomed flask in an ice bath with a magnetic stir bar. A suspension of 4-formylbenzoic acid (5.8 g, 38.6 mmol, 1.02 equiv.) in 160 mL ethanol was added dropwise over the course of 1 h. After 1 h, the round-bottomed flask was the fitted with a condenser, transferred to an oil bath, and refluxed for 2 h. After cooling, an orange precipitate was allowed to develop in the fridge over 48 h, filtered, rinsed with 2× 50 mL cold ethanol, and then transferred to a conical flask. Subsequently, the washed precipitate was refluxed in a 1:1 water–methanol solution for 1 h, filtered, and then recrystallised from a minimum of boiling ethanol three times. Finally, the resulting solid was dried under vacuum for 48 h prior to NMR analysis. NMR spectra were acquired using a Bruker Avance III HD 700 MHz spectrometer equipped with a cryogenically cooled three-channel TCI probe at 299.7 K. Analysis of these spectra confirming the structure of CDB can be found in Supplementary file 1 (see section on supplementary materials given at the end of this article).

Activity of HSD17B enzymes

Enzyme activity of reductive and oxidative HSD17Bs was determined in endometrial tissue extracts using high-performance liquid chromatography (HPLC) as previously described (Delvoux *et al.* 2007) with minor modifications. All measurements were performed in duplicate. Endometrial tissue was homogenised using a Potter homogenisation device in 1.5 mL Eppendorf tubes in RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA). Tissue homogenates were cleared by centrifugation. Protein concentration was determined by a BC Assay Protein Quantitation Kit (Uptima-Interchim, Montlucon, France).

The conversion of oestrone to 17β-oestradiol was carried out as follows: a 500 µL reaction mixture containing 3.2 mM NADP+, 9 mM glucose 6-phosphate, 1 U glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 10 nmol oestrone in 50 mM potassium phosphate buffer (pH 7.4) was incubated with 24 to 232 µg of the protein lysate at 37°C for 16 h. The conversion of 17β-oestradiol into oestrone was measured in a 500 µL reaction mixture containing 5 mM $NADP^+$, 10 nmol 17β-oestradiol in 50 mM sodium phosphate buffer (pH 7.4) and 24 to 232 µg of the protein lysate incubated at 37°C for 16 h.

To determine the oestrogen formation in the reaction mixture we used a HPLC method. After adding the internal standard (IS) butyl-4-hydroxybenzoate (300 ng), 2 mL of H_2O and 2.5 mL of chloroform to the reaction mixture, the oestrogens were extracted in the organic phase. The chloroform phase was evaporated under nitrogen at 45°C and oestrogens were derivatised with 250 µL CDB work and 250 µL

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1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) solution at 50°C for 20 min. The 500 µL reaction mix were diluted five times in acetonitrile and 10 µL were injected in the HPLC system.

The HPLC system (Shimadzu LC-10AD setup) consisted of a SIL-10ADvp autosampler, FCV-10ALvp gradient mixer, LC10Avp pump with high pressure gradient mixing extension, DGU-14A degasser and an RF-10Axl fluorescence detector (excitation wavelength 336 nm, emission wavelength 440 nm). Labsolutions software (Shimadzu) was used for instrument control, data acquisition and calculation of peak areas. The derivatised steroids were injected into a LiChroCART 250-4 RP 18 column (Merck) and eluted with a gradient of methanol–H_2O (80:20; v/v) at time 0 to methanol–water (90:10; v/v) at time 20 min. The flow rate was set to 0.8 mL/min.

HSD17B1 inhibition

Reactions were carried out with or without the presence of the specific HSD17B1 inhibitor 3-(15b-estronyl)-*N*-(5-methyl-thiazol-2-yl)-propionamide at 5 µM concentration, also described as compound 21 (Messinger *et al.* 2009). This HSD17B1 inhibitor is an oestrone substitute at the 15th carbon position and has previously been characterised as a potent and specific inhibitor of HSD17B1 with little affinity towards other HSD17Bs and oestrogen receptor alpha (ERa) (Saloniemi *et al.* 2010, Delvoux *et al.* 2014). The compound was gifted by Dr. Koskimies (Forendo Pharma Finland) and was previously used and tested by our team both *in vitro* and *in vivo* (Delvoux *et al.* 2014, Konings *et al.* 2018*b*, Xanthoulea *et al.* 2021, Poirier *et al.* 2022).

The inhibition of the HSD17B1 enzyme was computed as percentage of inhibition (or inhibition of oestrone to 17β-oestradiol conversion) by the formula: 100% – (formation of 17β-oestradiol with inhibitor/formation of 17β-oestradiol without inhibitor × 100%).

RNA extraction and sequencing

The RNA data are part of a larger data set (the SCRaTCH-2 study), and RNA extraction, sequencing and analysis of data (with a different sample selection) was previously described (Stevens Brentjens *et al.* 2023). The data described in the current article uses the same data set but has a different sample selection. Briefly, RNA was extracted using QIAsymphony SP with the QIAsymphony RNA kit (according to the Qiagen protocol) and the Illumina TruSeq Stranded mRNA kit was used according to the Illumina protocol. Trimming, alignment, counting and count matrix processing has been previously described (Stevens Brentjens *et al.* 2023). To identify the differentially expressed genes (DEGs), gene count matrix was analysed using DESeq2 (v1.34.0). The false discovery rate (FDR)-adjusted $P < 0.05$ and log fold change >0.5 were selected as the screening threshold for DEG comparison. The level of expression of 46 genes controlling the local steroid metabolism was analysed (Supplementary Table 1).

For three patients, data on LH timing were missing. Predictive mean matching (PMM) from M.I.C.E. (v. 3.16.0) was used for the imputation of the variable corresponding to LH timing using existing values from the complete cases matched with respect to Bayesian B and stochastic matching distance (van Buren & Groothuis-Oudshoorn 2011). Four samples were excluded due to low RNA integrity number or RNA concentration. Gene ontology analysis was performed on all genes ($P \leq 0.05$).

Statistical analysis

Enzyme activity data were analysed using the Wilcoxon–Mann–Whitney rank sum test. Statistical significance was defined as a $P < 0.05$. For transcriptome, statistical analyses were performed using R (version 4.2.2, R Foundation for Statistical Computing, Vienna, Austria). Statistical analyses of oestrogen metabolism related genes expression (see Supplementary Table 1) were performed using an unpaired two-sample Wilcoxon test adjusted using Bonferroni's correction. Correlations were considered significant with an FDR-adjusted $P < 0.05$, asymptotic two-tailed *P*-values estimated from Pearson's coefficients.

Results

Main characteristics of the patients and IVF/ICSI treatments

We explored whether endometrial oxidative and reductive HSD17B activity during the WOI differed in women who achieved a clinical pregnancy ($n = 15$) ('pregnant group') compared to women who did not ($n = 15$) ('non-pregnant group') after fresh single

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cleavage-stage embryo transfer. These women were enrolled in the SCRaTCH study and represent a selection of the larger study population ($n = 141$), as described in the 'Materials and Methods' section. There were no relevant statistically significant differences in patient characteristics and treatment characteristics between the study groups (see Supplementary Table 2A and B for the comprehensive data of all participants). Endometrial thickness on the day of the endometrial biopsy was unknown. The endometrial thickness in the subsequent cycle, on the day of ovulation triggering before the fresh embryo transfer, was significantly higher in the pregnant group (Table 1).

Oxidative and reductive *HSD17B* activity

Activity of the HSD17Bs responsible for the reduction of oestrone to 17β-oestradiol was detected in all samples via HPLC following the derivatisation of oestrogens with CDB (synthesis and characterisation are described in the 'Materials and Methods' section). Formation of 17β-oestradiol ranged from 55 to 1864 pmol/mg protein/h. Median 17β-oestradiol formation in pregnant women was 1053.68 pmol/mg protein/h (interquartile range (IQR): 591.84–1378.47) which was not significantly different compared to non-pregnant women (996.93 pmol/mg protein/h, IQR: 948.03–1302.64), $P = 0.97$ (Fig. 1A).

The activity of the enzymes responsible for the oxidation of 17β-oestradiol into oestrone ranged from 32 to 1731 oestrone formed/mg protein/h. Median oestrone formation in pregnant women was not significantly different compared to non-pregnant women (737.06 pmol/mg protein/h, IQR: 334.05–1034.22 vs 623.63 pmol/mg protein/h, IQR: 462.26–876.04, respectively, $P = 0.90$) (Fig. 1B). The ratio of reductive versus oxidative HSD17B had a median of 1.63 (IQR: 1.12–2.00) in the pregnant woman compared to 1.95 (IQR: 1.38–2.44) in the group of non-pregnant woman, which was not significantly different ($P = 0.57$) (Fig. 1C).

Inhibition of HSD17B1

Since HSD17B1 is the most relevant enzyme in the conversion of oestrone to 17β-oestradiol *in vivo*, and it was specifically shown to be involved human embryo implantation (Li *et al.* 2003), we further explored the engagement of HSD17B1 in the measured reductive

Table 1 Patient and IVF/ICSI cycle characteristics of the participants. Data are presented as mean (±S.D.) or number (%).

	Pregnant ($n = 15$)	**Non-pregnant** ($n = 15$)	***P***
Age at inclusion – years	33.5 (3.5)	33.8 (3.1)	0.821[f]
BMI – kg/m^2 [c]	24.43 (4.45)	23.15 (2.41)	0.346[f]
Female smokers	2 (13.3%)	2 (13.3%)	1.000[g]
Primary infertility[a]	7 (46.7%)	5 (33.3%)	0.445[g]
Duration of infertility (months)	38.7 (17.4)	33.6 (16.7)	0.422[f]
Indication for IVF or ICSI			0.394[g]
Male	10 (66.7%)	7 (46.7%)	
Idiopathic	5 (33.3%)	7 (46.7%)	
Unilateral tubal pathology	0 (0%)	1 (6.7%)	
Number of embryos transferred before biopsy[b]			
Fresh	1	1	
Frozen	2.5 (0.9)	2.91 (1.4)	0.421[f]
Days between LH surge and biopsy[d]	6.5 (0.8)	6.9 (1.0)	0.189[f]
Endometrial thickness at last ultrasound before ovulation triggering – mm[c]	12.0 (3.1)	9.5 (2.1)	**0.018**[f]
Embryo quality of fresh ET[e] after biopsy			
Days after ovum pick-up	2.7 (0.5)	2.9 (0.4)	0.379[f]
Number of cells	7.27 (2.3)	7.13 (1.5)	0.849[f]
Fragmentation			0.183[g]
0–10%	13 (86.7%)	9 (60%)	
10–20%	1 (6.7%)	5 (33.3%)	
20–50%	1 (6.7%)	1 (6.7%)	

[a]Primary infertility: female has never conceived before; [b]The number of embryos before randomisation for the SCRaTCH trial; [c]Data were missing for one participant in the pregnant group; [d]Data were missing for two participants in the pregnant group and one participant in non-pregnant group; [e]ET, embryo transfer; [f]By Independent samples *t*-test; [g]By chi-square test.

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activity by using a specific HSD17B1 inhibitor. A trend was shown when comparing inhibition of HSD17B1 in the pregnant and non-pregnant group albeit non-significant (17.8% vs 27.5%, $P=0.059$) (Fig. 2A).

As the endometrial contribution to implantation failure might be different in primary infertile women (who never had a pregnancy before) compared to secondary infertile women (where the endometrium showed to be recipient in previous pregnancies), and previous studies with these samples found more pronounced differences between these groups (Bui *et al.* 2023, Stevens Brentjens *et al.* 2023), we performed a subgroup analysis with respect to fertility status (primary vs secondary infertility). When only primary infertile women ($n=12$) were considered, inhibition was significantly decreased in the pregnant group ($n=7$) compared to the non-pregnant group ($n=5$) (16.2% vs 27.1%, $P=0.042$) (Fig. 2B). The activity of HSD17B1 was not significantly different between the pregnant primary infertile and non-pregnant primary infertile group (1029 pmol 17β-oestradiol/mg protein/h vs 953 pmol 17β-oestradiol/mg protein/h, $P=0.23$).

Transcriptome analysis in pregnant and non-pregnant groups

RNA-sequencing data were available for 26 of the 30 patients (pregnant, $n=14$ and non-pregnant, $n=12$). Four samples were excluded due to low RNA quality or concentration. We first analysed the expression levels of the 46 genes encoding for the enzymes controlling the local steroid metabolism (Supplementary Table 1) (Konings *et al.* 2018*a*), and 11 genes out of the 46 (i.e. *AKR1C4, AKR1D1, ARO, CYP11B1, CYP11B2, CYP17A1, HSD11B1, HSD3B1, HSD3B2, SULT1B1* and *SULT2A1*) were not detected. In the 35 detectable transcripts, genes encoding for steroid hormone receptors (*ESR1* and *PGR*), for the androgen-metabolising enzyme *SRD5A3* and for hydroxysteroid-dehydrogenases *HSD11B2* and *HSD17B4* showed the highest expression levels. No genes were differentially expressed between the study groups, although a trend was observed for HSD17B13 (adjusted $P=0.075$) when comparing pregnant ($n=14$) and non-pregnant ($n=12$) women (Fig. 3A).

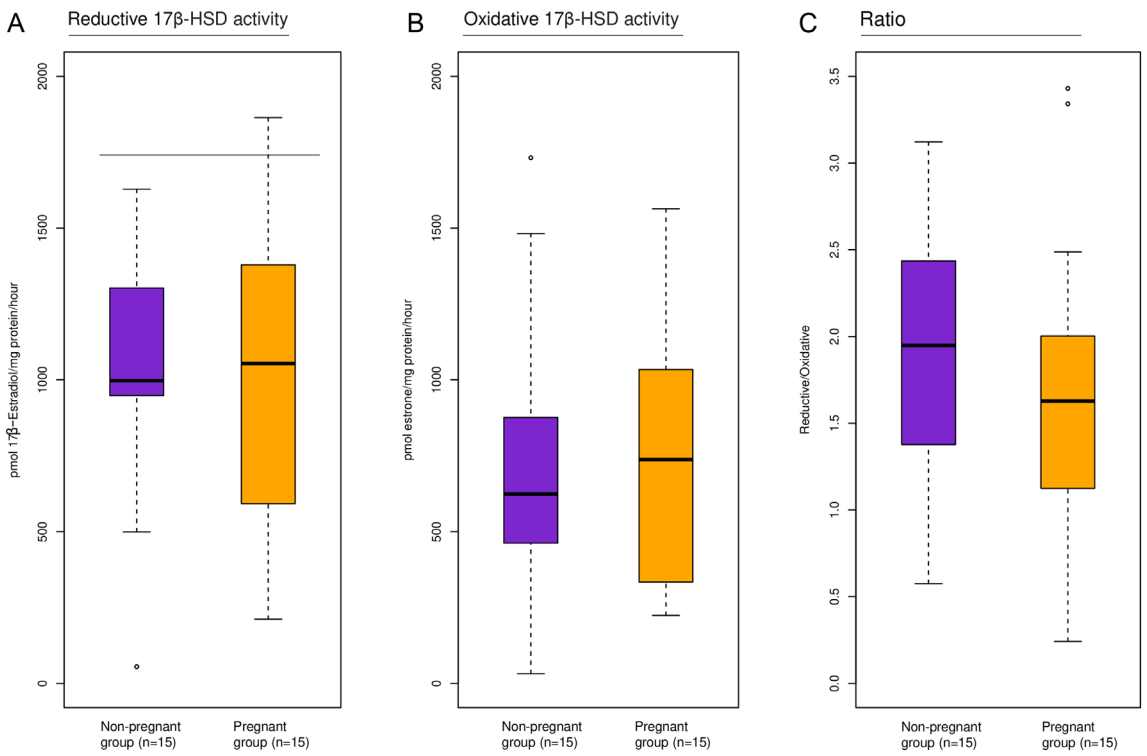

Figure 1 Activity of (A) reductive and (B) oxidative HSD17Bs and (C) activity ratio of the reductive and oxidative HSD17Bs. The (A) conversion of oestrone to 17β-oestradiol, (B) conversion of 17β-oestradiol to oestrone and (C) the activity ratio of reductive and oxidative HSD17Bs was similar in the pregnant ($n=15$) and non-pregnant ($n=15$) group. Box plots show the 25th and 75th percentile within the median, and whiskers indicate 1.5 times the interquartile range. Statistical significance was determined using a Wilcoxon–Mann–Whitney rank sum test.

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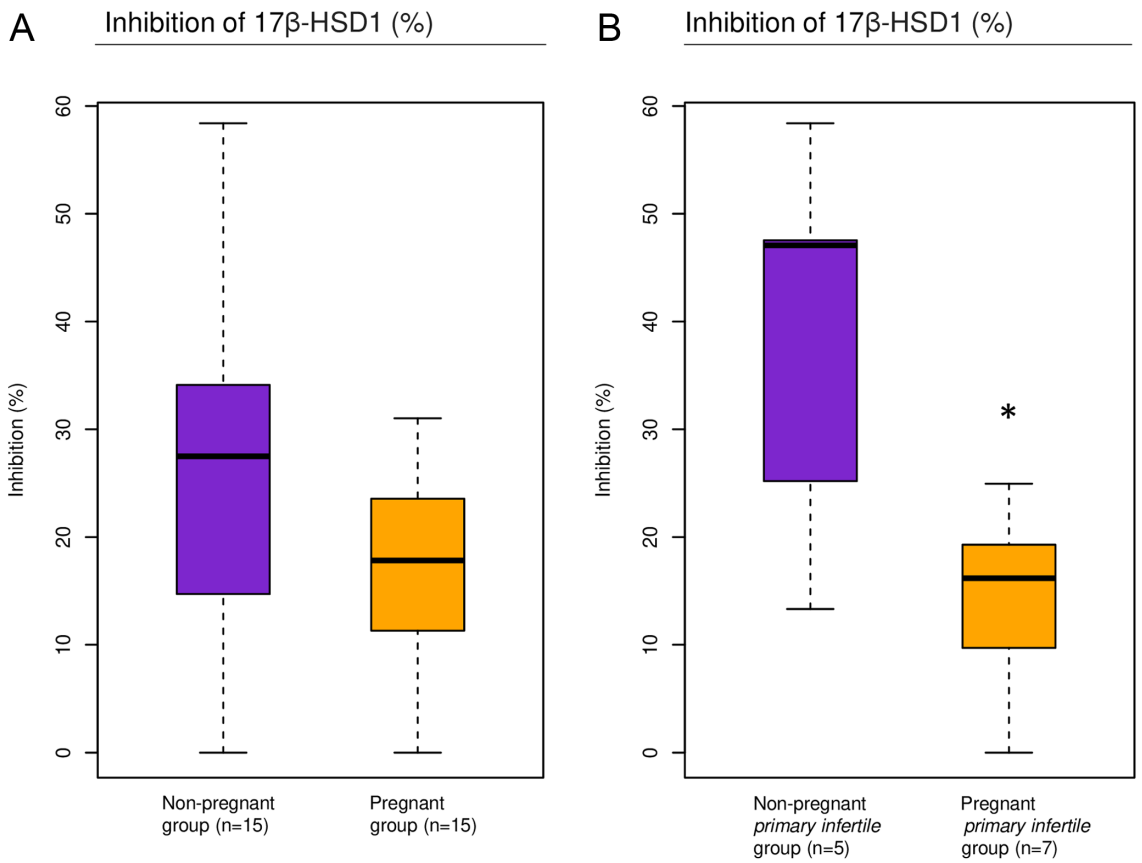

Figure 2 Inhibition of HSD17B1 in the complete study group (A) and subgroup of primary infertile women (B). The inhibition of HSD17B1 was similar in the pregnant ($n = 15$) and non-pregnant ($n = 15$) group (A). However, when only women with primary infertility were considered, inhibition of HSD17B1 was significantly lower in the pregnant group ($n = 7$) compared to the non-pregnant group ($n = 5$, $P = 0.042$) (B). Box plots show the 25th and 75th percentile within the median, and whiskers indicate 1.5 times the interquartile range. Statistical significance was determined using a Wilcoxon–Mann–Whitney rank sum test; *$P < 0.05$.

Next, we analysed the complete transcriptome and four DEGs were identified: *HBA1*, *HBB*, *GDF15* and *CDKL1* (adjusted $P < 0.05$) (Table 2 and 3). Characteristics of the expression profiles inspected through a principal component analysis (PCA) did not separate the pregnant ($n = 14$) and non-pregnant ($n = 12$) groups (Supplementary Fig. 1A). Likewise, the PCA plot from the expression matrix of steroid-metabolising genes only did not distinguish between the two groups (Supplementary Fig. 2). Neither the activity nor the inhibition of HSD17B1 was correlated with *HSD17B1* expression (Supplementary Fig. 3A and B). Correspondingly, activity of HSD17B2 was not correlated with *HSD17B2* expression (Supplementary Fig. 3C). Although the sample size is small, the expression of *HSD17B1* was correlated with the expression of *HSD17B5,* and the expression of *HSD17B7* was correlated with the expression of *HSD17B1,* 2 and 5 in the non-pregnant group, but no correlation could be seen in the pregnant group (Supplementary Fig. 3A and B).

Transcriptome analysis in relation to infertility type

As the differences in 17BHSD1 inhibition were more pronounced in primary infertile women ($n = 12$) we performed a subgroup analysis with respect to infertility type (primary infertility, $n = 10$ vs secondary infertility, $n = 16$, irrespective of the outcome of the second cycle). Data from four RNA samples were missing due to low RNA quality or concentration (primary infertility, $n = 2$, secondary infertility, $n = 2$). When analysing genes encoding for steroid-metabolising enzymes irrespective of pregnancy status, no significantly different expression was found (Fig. 4). PCA did not separate the primary and secondary infertile groups (Supplementary Fig. 1B).

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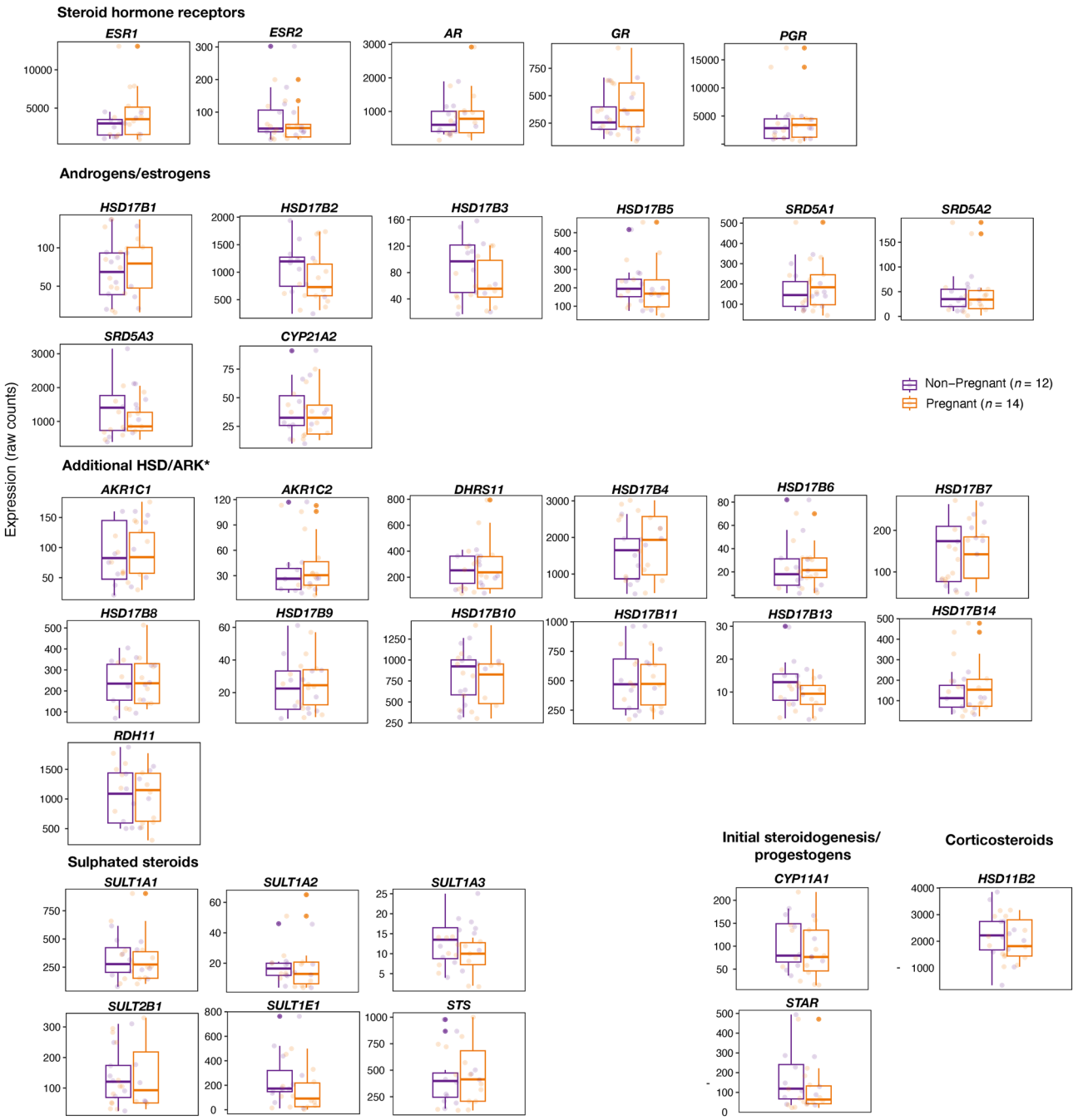

Figure 3 Expression of steroid metabolism related genes by RNA sequencing. Expression of 34 steroid metabolism related genes in the pregnant ($n = 12$) and non-pregnant ($n = 14$) group. Box plots show the 25th and 75th percentile within the median, and whiskers indicate 1.5 times the interquartile range. Statistical significance was determined using an unpaired two-sample Wilcoxon test adjusted using Bonferroni's correction; *Putative steroid-metabolising action.

When considering only primary infertile women ($n = 10$) transcriptome analysis revealed 12 DEGs between pregnant ($n = 6$) and non-pregnant ($n = 4$) primary infertile women, although PCA analysis did not show separation of the groups (Supplementary Fig. 1C). The 12 DEGs were *FLVCR1-DT, RASAL2-AS1, FAM225B, IGKC, JCHAIN, IGHGP, USP6, ALOX15B, IGHM, IGHG1, LINC02349*, and *IGHG3* (Table 2 and 3). Gene ontology analysis indicated two enriched gene ontology biological processes categories: humoral immune response and defence response to bacterium. No steroid-metabolising enzyme was present in the DEGs (Fig. 5).

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Table 2 Differentially expressed gene (DEG) analysis between the pregnant ($n = 14$) and non-pregnant ($n = 12$) group[a].

Gene name	Log2fold change	Log fold change SE	*P*	Adjusted *P*
HBA1	1.975	0.433	5.23×10^{-06}	0.026
HBB	1.960	0.425	4.03×10^{-06}	0.026
GDF15	1.127	0.241	3.02×10^{-06}	0.026
CDKL1	−0.567	0.115	8.81×10^{-07}	0.017

[a]Data were missing from four participants due to low RNA quality or concentration.

Discussion

In this study, we compared the activity of oxidative and reductive HSD17B enzymes in combination with the endometrial transcriptome between pregnant and non-pregnant women after a fresh ET. The activity of the oxidative and reductive HSD17Bs did not differ between the two patient groups, but in primary infertile patients, inhibition of HSD17B1 was significantly decreased in pregnant compared to non-pregnant women. In addition, among primary infertile patients, 12 DEGs were found between pregnant and non-pregnant women.

To our knowledge, this is the first study that explores oestrogen-metabolising enzyme activity in the endometrium of pregnant and non-pregnant IVF/ICSI patients. Previous studies have explored enzyme activity and HSD17B1 inhibition in both healthy endometrial tissue and women with endometriosis (Delvoux *et al.* 2009, 2014). Here, the activity of HSD17Bs converting oestrone into 17β-oestradiol was found to be higher in endometriosis tissue compared to the endometrium inside the uterus. Similar to our results, formation of 17β-oestradiol could not always be completely blocked using an HSD17B1-specific inhibitor, indicating that other reducing HSD17Bs may be involved in the generation of 17β-oestradiol in the endometrium (Konings *et al.* 2018*a*).

Table 3 Differentially expressed gene (DEG) analysis between the pregnant ($n = 6$) and non-pregnant ($n = 4$) group for primary infertile women only[a].

Gene name	Log2fold change	Log fold change SE	*P*	Adjusted *P*
FLVCR1-DT	1.912	0.450	2.17×10^{-05}	0.047
RASAL2-AS1	−1.439	0.330	1.30×10^{-05}	0.034
FAM225B	−1.653	0.342	1.34×10^{-06}	0.007
IGKC	−2.690	0.556	1.29×10^{-06}	0.007
JCHAIN	−2.810	0.615	4.85×10^{-06}	0.021
IGHGP	−2.903	0.673	$1{,}60 \times 10^{-05}$	0.038
USP6	−3.026	0.693	$1{,}25 \times 10^{-05}$	0.034
ALOX15B	−3.177	0.719	$1{,}00 \times 10^{-05}$	0.033
IGHM	−3.419	0.708	$1{,}37 \times 10^{-06}$	0.007
IGHG1	−3.790	0.6675	1.34×10^{-08}	<0.000
LINC02349	−4.100	0.922	8.81×10^{-06}	0.033
IGHG3	−4.919	0.965	3.42×10^{-07}	0.004

[a]Data were missing from four participants due to low RNA quality or concentration.

Four genes were differentially expressed between pregnant and non-pregnant women. *GDF15*, which encodes for a secreted ligand of the transforming growth factor beta superfamily of proteins, is involved in decidualisation, placentation and pregnancy. In a study with pregnant rats, *GDF15* expression was increased at the implantation site, suggesting a role in embryo implantation (Zhao *et al.* 2016). Furthermore, in addition to secretion of hCG, the pregnancy-specific glycoprotein GDF15 was detected in the secretome of human trophoblast organoids (Turco *et al.* 2018). In our analysis, the expression of *GDF15* was higher in the pregnant group.

More pronounced differences in the transcriptome were found in primary infertile women. Among the DEGs specifically identified in this subgroup analysis, *LINC02349* has been linked to endometrial receptivity associated pathways before. This long non-coding RNA (lncRNA) may play crucial roles in a series of endometrial events before or during implantation by targeting the Wnt pathways (Tepekoy *et al.* 2015). In a study using transcriptome data of endometrial tissues from recurrent implantation failure (RIF) patients and fertile controls (data accession number GSE26787) to determine biomarkers for RIF, *LINC02349* was identified as one of the eight differentially expressed lncRNAs that distinguish RIF from fertile women. In line with our results, the expression of *LINC02349* was downregulated in RIF women compared to fertile controls (Lin & Lin 2022).

One study explored the endometrial intracrinology during IVF/ICSI treatment. Oocyte donors were treated with a standard IVF/ICSI antagonist protocol including ovarian hyperstimulation with follicle-stimulating hormone, and an endometrial biopsy was conducted in the putative WOI. Among other genes, the expression of *HSD17B2* was lower compared to control subjects in a natural menstrual cycle, indicating that in stimulated cycles, where the endometrium is exposed to supraphysiological steroid hormone levels, alterations in steroid-metabolising enzymes are induced (Vani *et al.* 2007). In our study, biopsies were taken in the cycle preceding the IVF/ICSI cycle, and no differences

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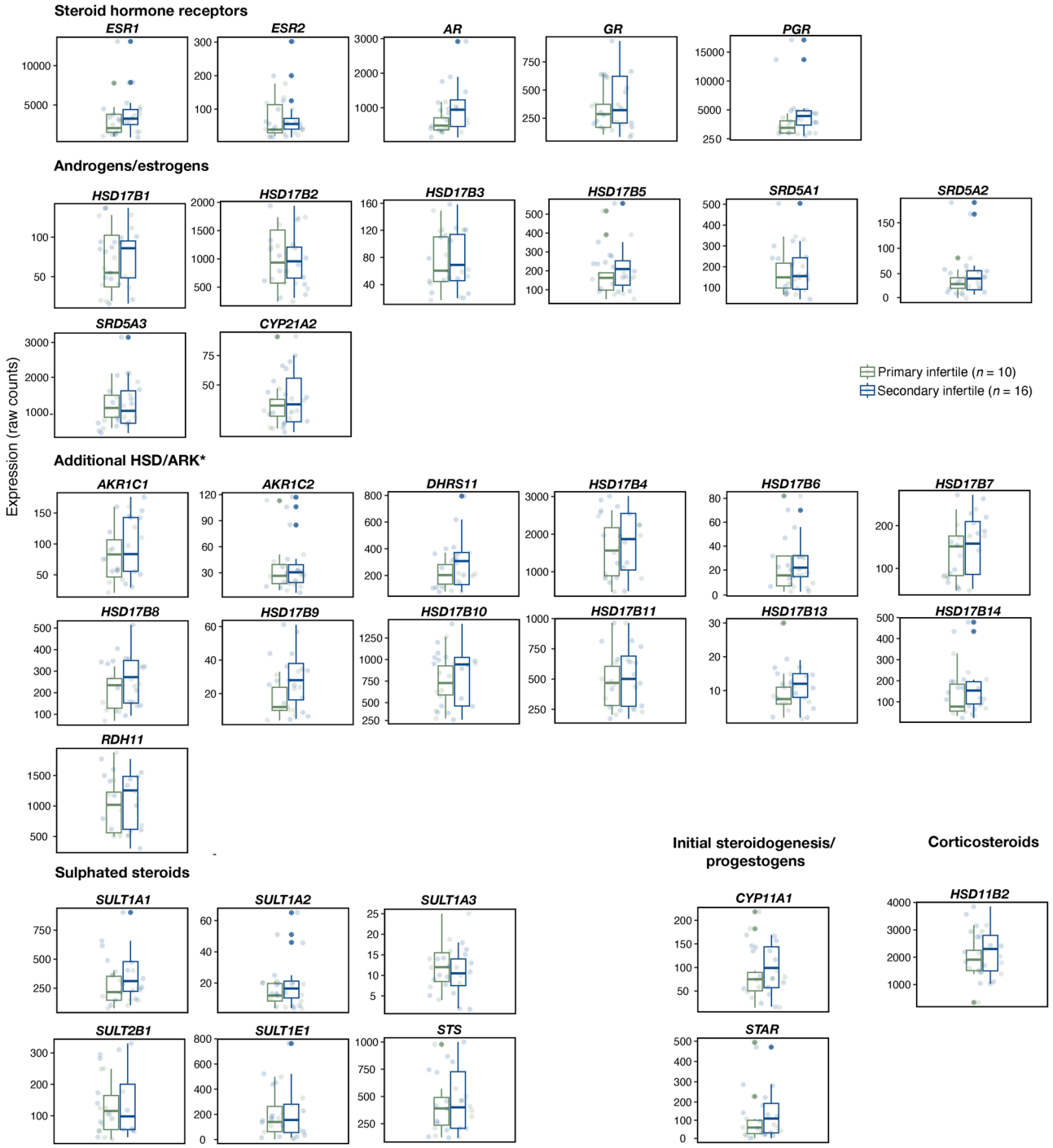

Figure 4 Expression of steroid metabolism related genes by RNA sequencing. Expression of 34 steroid metabolism related genes in primary infertile ($n = 10$) and secondary infertile ($n = 16$) women. Box plots show the 25th and 75th percentile within the median, and whiskers indicate 1.5 times the interquartile range. Statistical significance was determined using an unpaired two-sample Wilcoxon test adjusted using Bonferroni's correction; *Putative steroid-metabolising action.

in *HSD17B2* expression were found between pregnant and non-pregnant IVF/ICSI patients.

One strength of our study is its prospective design, where biopsies were obtained as part of a randomised controlled trial in well-defined patient groups. The biopsy was precisely timed between 5 and 8 days after the LH surge and the transcriptomic data was corrected for differences in LH timing accordingly.

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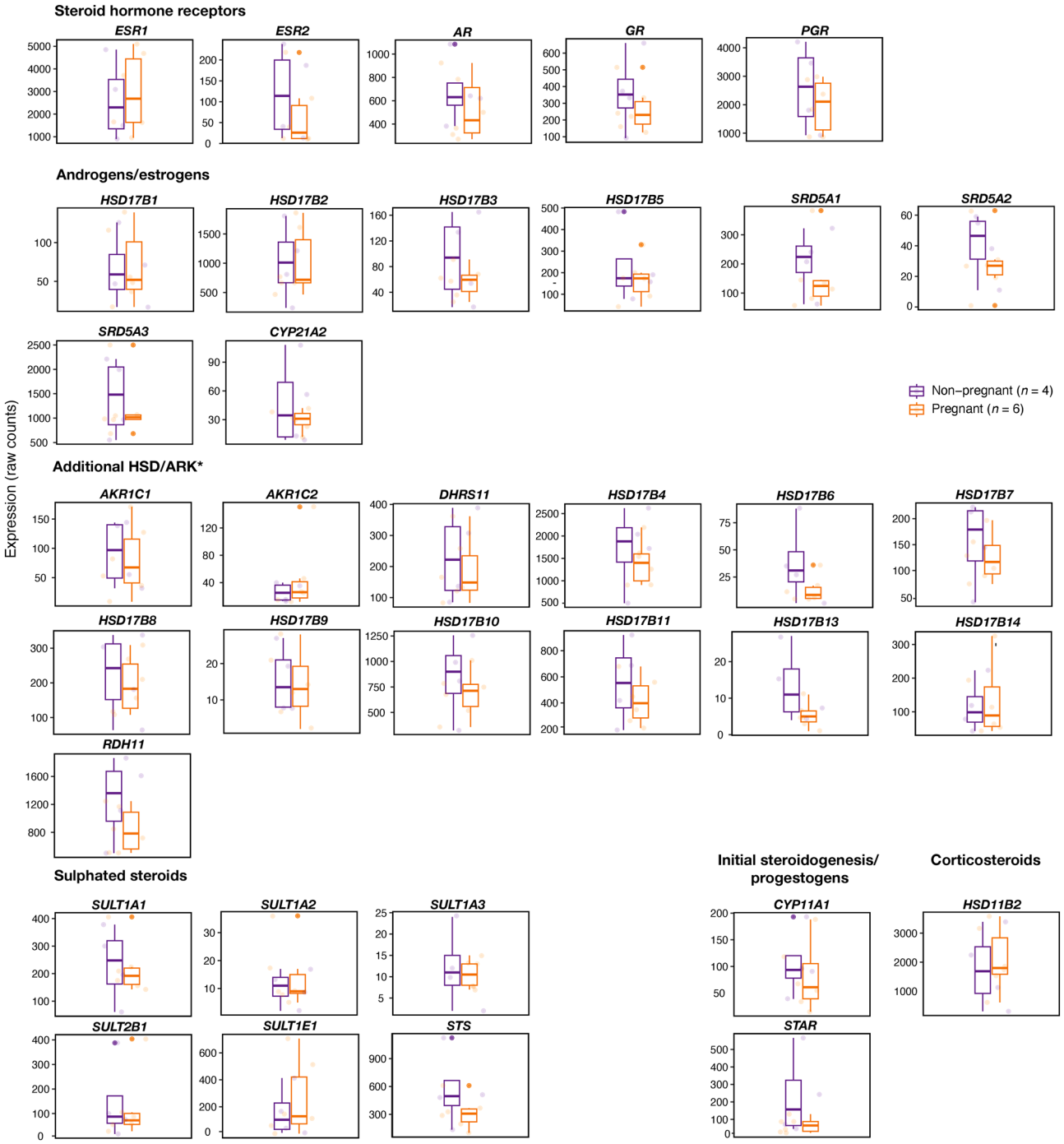

Figure 5 Expression of steroid metabolism related genes by RNA sequencing. Expression of 34 steroid metabolism related genes in the pregnant ($n = 4$) and non-pregnant ($n = 6$) group considering women with primary infertility only. Box plots show the 25th and 75th percentile within the median, and whiskers indicate 1.5 times the interquartile range. Statistical significance was determined using an unpaired two-sample Wilcoxon test adjusted using Bonferroni's correction; *Putative steroid-metabolising action.

It should be noted, however, that ovulation was not confirmed by serial ovarian ultrasound monitoring. Two limitations arise from the invasive nature of endometrial tissue sampling and the corresponding detrimental effect of sampling in the cycle of the ET: (i) the tissue is scarce, which has led to a smaller study sample size. This could be improved by larger future studies with better differentiation between the causes of infertility, and (ii) potential intercyclic variation bias, since the biopsy has to be taken in the cycle preceding

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the embryo transfer cycle. Consequently, a pregnancy in the cycle after the biopsy might not always reflect a receptive endometrium during the cycle of the biopsy. Finally, embryo quality has been taken into account based on morphological embryo criteria. However, aneuploidy status of the embryo was unknown as pre-implantation genetic testing for aneuploidy is currently prohibited in the Netherlands. The discovery of 12 DEGs between pregnant and non-pregnant primary infertile patients is interesting, but a recent publication by Walker *et al.* has pointed out high inconsistencies in reported DEGs between studies on the endometrial transcriptome (Walker *et al.* 2023). The current study is limited by the fact that the findings could not be validated with another technique such as real-time PCR, as no surplus samples were available. For a possible translation to relevant biomarkers or druggable pathways, future research with validation in independent larger sample sets or studies with stricter selection criteria for reproductive failure are warranted. For instance, only women with primary infertility or women with RIF could be considered instead of the currently included women with a first failed IVF/ICSI cycle. Besides, non-invasive methods for endometrial assessment in the cycle of the transfer such as a liquid biopsy (i.e. sampling endometrial fluid) could eliminate intercyclic variation in the future (Schobers *et al.* 2021, Stevens Brentjens *et al.* 2022).

Conclusions

In conclusion, endometrial intracrinology involves intricate and highly complex pathways that generate steroid metabolites with various activities. The participation of other steroid-metabolising enzymes and the various steroid metabolites including progestogens, androgens and corticosteroids was outside of the scope of this study, but their role in receptivity and implantation in the same study population is described elsewhere (Stevens Brentjens *et al.* 2023). To the best of our knowledge, this pilot study is the first to explore the endometrial oestrogen balance in humans during implantation by using an unique study population. The study results, especially the role of other HSD17Bs (such as 5, 7 and 12) in pregnancy, need to be reconfirmed in future studies, as this study was limited by a small sample size given its exploratory and pilot nature.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/RAF-23-0065.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the study reported.

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Author contribution statement

Study design: LSB, AR, BD, FB. Tissue collection: LSB, JdH, RvG, Fe.M, JdB, GT. Data collection: DO, BD, MZE, Fr.M, MB, BB. Data analysis: LSB, BD, DO, MZE, AR. Manuscript writing: LSB, AR. Manuscript review and approval: all authors.

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