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Serum oestradiol concentrations during mid-secretory endometrial preparation and the position of the window of implantation determined by RNA-sequencing-based endometrial receptivity testing: a retrospective cohort study of 311 testing cycles

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Abstract

Background The window of implantation (WOI) is displaced later in 25% to 41% of women undergoing endometrial receptivity testing. Whether routine serum hormones reflect transcriptomically defined WOI position is unknown. We examined serum oestradiol (E_2), progesterone (P) and E_2/P ratios at three progesterone exposure timepoints in relation to WOI

position determined by RNA-sequencing-based endometrial receptivity testing (rsERT), and how regimens affect these hormones and window results.

Methods This single-centre retrospective cohort study analysed 311 consecutive hormone replacement therapy cycles undergoing rsERT (2022-2025). Serum E_2 and P were measured at progesterone conversion (P+0), P+3 and P+5 (biopsy day). Estrogen route was single-agent (n=216) or combined (n=95); progesterone was 40 mg/day (n=108), 60 mg/day (n=180) or step-up (n=23); 34 cycles included GnRH-a down-regulation. Outcomes were displaced windows and the rsERT-predicted implantation hour, analysed by non-parametric tests, logistic regression and ROC analysis, and repeated in the non-down-regulated subgroup.

Results Displaced windows occurred in 220 cycles (70.7%). Displaced cycles had higher P+3 and P+5 E_2 (P=0.003, P=0.002) and P+3 E_2/P (P=0.020); no P+0 variable and no P level differed. P+5 E_2 (rho=0.228) and P+3 E_2 (rho=0.214) correlated with predicted hour (both P<0.001), confined to displaced cycles. Across P+5 E_2 quartiles displacement rose from 63.6% to 81.5% (trend P=0.006). Per standard deviation, P+5 E2 gave an odds ratio (OR) for displacement of 1.96 (95% confidence interval [CI] 1.18-3.24), independent after regimen adjustment (OR=2.06, P=0.011), with area under the curve 0.626 (0.552-0.702). The association was stronger in the non-down-regulated subgroup (adjusted OR=2.10; AUC 0.652) without significant interaction (P=0.98). Down-regulation carried the strongest association (94.1% versus 67.9%, adjusted OR=8.58) despite lower serum E_2. Regimens markedly altered hormone levels (P+5 E_2 206.10 versus 134.87 pg/mL, P<0.001) without independently predicting WOI position.

Conclusions Mid-secretory serum E_2 is independently associated with rsERT-defined WOI position after adjustment for regimens, more strongly in non-down-regulated cycles, with effect size increasing with progesterone exposure duration. Discriminative ability is moderate: serum E_2 is an adjunctive indicator and cannot replace endometrial biopsy. Serum P reflects

dosing rather than window position. GnRH-a down-regulation associates with displacement independently of circulating E_2. Thresholds not standardised to regimen should not be applied across centres.

Keywords: Window of implantation; Endometrial receptivity; Estradiol; Progesterone; GnRH-a down-regulation; Hormone replacement cycle; rsERT

Background

Embryo implantation requires synchrony between a competent blastocyst and a receptive endometrium, and in natural cycles the window of implantation occupies only a few days of the mid-secretory phase [1, 2]. In hormone replacement therapy (HRT) frozen embryo transfer cycles the window is drug-induced, and transfer is conventionally performed on the fifth day of progesterone exposure [3]. Individual variation exists at this timepoint, and displacement of the window, most commonly to a later position, is considered an important cause of recurrent implantation failure (RIF) [4, 5, 6]. Histological dating lacks adequate accuracy [7], which has driven the development of transcriptomic tests: the Endometrial Receptivity Array (ERA) classifies endometrial phase from a 238-gene expression signature to guide personalized embryo transfer (pET) [8, 9, 10], and rsERT, an RNA-sequencing-based test combined with machine learning, was established in 2021 and predicts the optimal implantation point with hourly precision [11, 12]. Our centre has previously reported that hourly personalized embryo transfer improves pregnancy outcomes in patients with RIF [13].

The proportion of displaced windows reported with these tests ranges from 25% to 41% [14, 15, 16], indicating that endometrial phase abnormalities are common. If routine serum markers could signal window displacement, they would have two clinical applications. First, because endometrial biopsy is invasive and testing is costly, a non-invasive marker could be used to identify who needs testing, or to detect inadequate endometrial preparation early.

Second, serum measurements are available within the testing cycle itself and could help explain why the window is displaced and guide regimen optimisation. Previous studies have shown that serum progesterone around transfer is associated with implantation and live birth [17, 18, 19, 20, 21], and that mid-luteal oestradiol is associated with live birth after frozen embryo transfer in HRT cycles [22], but these studies used pregnancy as the endpoint rather than the molecular window itself. The few studies that directly examined the relation between serum progesterone and endometrial receptivity status found only weak correlations [23]. Evidence on oestrogens is sparser: estrogen dose and route, GnRH-a down-regulation and progesterone dose all substantially alter serum E_2 and P, and are therefore important confounders for any hormonal inference; their distribution during the conversion period of receptivity testing cycles has rarely been systematically reported [3, 22].

Mechanistically, receptivity is established in two steps: estrogen initiates endometrial proliferation and glandular programmes via estrogen receptor alpha, after which progesterone drives stromal decidualisation via progesterone receptors A/B and suppresses estrogen-driven gene expression [1, 2]. Exogenous estradiol in HRT regimens exceeds mid-luteal levels of natural cycles, and interindividual differences in absorption, distribution and metabolism mean that tissue estrogen load differs between women on the same protocol day. Sustained estrogen signalling can antagonise progesterone-driven maturation through down-regulation of progesterone receptors and cross-talk with epithelial estrogen receptors [2], which in principle could delay the molecular phase. This hypothesis, however, lacks clinical data using hourly molecular testing as the endpoint.

We retrospectively analysed 311 endometrial preparation cycles in which rsERT was performed, to determine: whether serum E_2, P and the E_2/P ratio at P+0, P+3 and P+5 are associated with the rsERT-defined WOI position and predicted hour of implantation; whether such associations persist after adjustment for endometrial preparation regimens; how well

serum markers discriminate a displaced window; and how three classes of regimen (estrogen route, progesterone dose schedule and GnRH-a down-regulation) influence hormone levels and the window result.

Methods

Study population

We retrospectively analysed infertile women who underwent rsERT testing at the Reproductive Medicine Centre, Boai Hospital of Zhongshan, between January 2022 and December 2025. Testing indications included women meeting RIF criteria (failure to achieve clinical pregnancy after at least two transfers of good-quality embryos) and women who did not meet RIF criteria but requested testing for other clinical reasons, mainly valuable embryos in preimplantation genetic testing (PGT) cycles and a wish to optimise transfer timing after a single previous failed transfer.

Inclusion criteria were age below 45 years, normal karyotype and endometrial thickness of 7 mm or more. Exclusion criteria were untreated uterine pathology (endometrial polyps, endometritis, intrauterine adhesions, intramural fibroids compressing the endometrium, uterine malformations), natural or minimal stimulation cycles, vaginal progesterone supplementation, and oocyte or embryo donation cycles. Ages in this cohort ranged from 25 to 43 years with no participant aged 45 or above, so all 311 testing cycles were included.

The study was approved by the Ethics Committee of the Boai Hospital of Zhongshan (Application ID: KY-2022-010-14; date of approval: October 2022). Informed consent was waived because the analysis used retrospectively collected anonymised data.

Endometrial preparation regimens

Estrogen was started on cycle day 2 to 4: the single-agent group received oral (4-8mg/day, Progynova; Bayer) or transdermal (5-15 g/day, Oestrogel) oestradiol; the combined group received both preparations simultaneously with additive dosing. Estrogen doses were titrated according to endometrial thickness measured by transvaginal ultrasound every 3 to 4 days and serum E_2 levels, and endometrial thickness of 7 mm or more was confirmed after 10 to 14 days of treatment. In GnRH-a cycles, triptorelin 3.75 mg (Diphereline; Ipsen) was injected on cycle day 2 to 4, with estrogen started 28 days later.

Luteal transformation was initiated with intramuscular progesterone in oil using one of three regimens: fixed 40 mg/day, fixed 60 mg/day, or a step-up regimen consisting of 40 mg/day from P+0 to P+2 with escalation to 60 mg/day from P+3. Endometrial biopsy for rsERT was performed at 5 days following progesterone administration (P+5). The testing cycle was the unit of analysis; the endpoints were the rsERT-defined WOI position and the predicted hour of transfer. Subsequent frozen embryo transfer cycles and pregnancy outcomes after transfer were outside the scope of this study.

By estrogen route the cohort comprised 216 single-agent cycles and 95 combined cycles. Progesterone regimens were distributed as 180 fixed 60 mg, 108 fixed 40 mg and 23 step-up cycles. Thirty-four cycles (10.9%) included GnRH-a down-regulation.

Timepoint definitions and sample collection

Progesterone days were counted from the first day of progesterone administration as P+0. Blood was sampled at three timepoints in the testing cycle: conversion day (P+0), P+3 and P+5 (biopsy day). Serum E_2 (pg/mL) and P (ng/mL) were measured on the routine immunoassay platform of our laboratory. The E_2/P ratio was calculated by direct division of reported concentrations (pg/mL divided by ng/mL) as an index of estrogen exposure per unit of progesterone.

rsERT testing and window classification

P+5 biopsy specimens were subjected to RNA sequencing and classified by rsERT as pre-receptive, receptive or post-receptive, with a random forest regression model predicting the individual optimal implantation point, as previously described [11, 12, 13]. In this cohort samples were classified as receptive (normal window) or pre-receptive (displaced window, that is, an optimal implantation point later than P+5); no post-receptive results were obtained. The model also reports the predicted hour of transfer, expressed in hours of progesterone exposure with 120 h corresponding to P+5, together with an uncertainty interval.

Statistical analysis

Analyses were performed with Python 3.14 (pandas 3.1.0, SciPy 1.18.1, statsmodels 0.15.0) and figures were drawn with matplotlib 3.11.2. Distribution of continuous variables was assessed by the Shapiro-Wilk test; normally distributed data are presented as mean and standard deviation (SD) and non-normal data as median with interquartile range (IQR); categorical data are presented as number and percentage. Two groups were compared using the independent-samples t test or Welch t test for normally distributed variables and the Mann-Whitney U test otherwise; three or more groups were compared using one-way analysis of variance or the Kruskal-Wallis test; categorical variables were compared using the chi-square test with Yates continuity correction for 2x2 tables, and Fisher's exact test when the minimum expected frequency was below 5. Associations between hormone levels and predicted hour were assessed by Spearman rank correlation and repeated within window strata. Proportional trends across ordered groups were assessed by the Cochran-Armitage trend test.

Logistic regression was used to identify determinants of a displaced window. Continuous predictors are reported as odds ratios (OR) with 95% CI per SD increase; hormone markers were entered one at a time in univariable models, and the multivariable model simultaneously

adjusted for GnRH-a down-regulation, estrogen route (combined versus single) and progesterone regimen (60 mg and step-up versus fixed 40 mg as reference). E_2 at all three timepoints was also entered together to assess collinearity. The area under the ROC curve (AUC) for discriminating a displaced window was calculated non-parametrically with 95% CI from 1000 bootstrap resamples; P+5 E_2 was additionally analysed by quartiles to test for a gradient in displacement rates. Because serum E_2 was lower while displacement was highest in the GnRH-a down-regulated group, between-group comparisons, logistic regression, quartile gradients and AUC analysis were all repeated in the non-down-regulated subgroup as a sensitivity analysis, and a product term of E_2 and down-regulation was added to the overall model to test for interaction. Statistical power was calculated for two-sided tests comparing two independent proportions at alpha=0.05, using the pooled proportion for the critical value under the null and the unpooled standard error for power, and the minimum detectable effect (MDE) at 80% power is reported. A two-sided P value below 0.05 was considered statistically significant. No formal correction for multiple comparisons was applied in these exploratory analyses; the robustness of conclusions was judged by concordance across four types of analysis, namely between-group comparisons, continuous correlation, quartile gradients and adjusted models.

Results

Cohort characteristics and distribution of the window of implantation

Among 311 testing cycles, 91 (29.3%) were receptive and 220 (70.7%) were displaced, with no advanced windows. The overall median predicted hour of optimal implantation was 144.0 (135.0-153.0) h, being 129.0 (123.0-133.0) h in receptive cycles and 149.5 (144.0-156.0)

h in displaced cycles (P<0.001); the median model uncertainty interval was 3 h in both groups (P=0.284) (Figure 1A).

Median age of the cohort was 34.0 (31.0-38.0) years and median BMI was 22.0 (20.2-24.4) kg/m^2. Age (P=0.920), BMI (P=0.472), number of previous failed transfers of good-quality embryos (P=0.341), proportion of PGT cycles (P=0.454) and distribution of progesterone regimens (P=0.512) did not differ between the two groups, whereas displaced cycles included a higher proportion of GnRH-a down-regulated cycles (14.5% versus 2.2%, P=0.003) and of combined estrogen regimens (34.5% versus 20.9%, P=0.025) (Table 1).

Stratifying by the number of previous failed transfers of good-quality embryos (0 in 28 cycles, 1 in 47, 2 in 142, 3 or more in 90), displacement rates were 82.1%, 72.3%, 69.0% and 70.0% respectively (chi-square P=0.563) and median predicted hours were 151, 150, 144 and 144 h, indicating that window displacement is not confined to women with RIF. The number of previous failures correlated weakly and inversely with predicted hour (rho=-0.162, P=0.004).

Missing data were as follows: P+5 hormones missing in 50 cycles (16.1%), P+3 hormones in 2 (0.6%), no missing P+0 hormones, and BMI missing in 1 cycle (0.3%).

Serum hormones during the testing cycle in relation to WOI position

Displaced cycles had higher P+3 E_2 than receptive cycles [160.62 (116.67-222.37) versus 134.10 (106.66-177.53) pg/mL, P=0.003] and higher P+5 E_2 [163.54 (117.49-220.61) versus 133.42 (106.99-172.61) pg/mL, P=0.002]. The P+3 E_2/P ratio was 15.12 (9.88-23.30) in displaced versus 12.50 (8.24-19.71) in receptive cycles (P=0.020). P+0 E_2 [149.66 (120.37-208.40) versus 140.88 (112.58-185.79) pg/mL, P=0.086] and the P+0 and P+5 E_2/P ratios (P=0.106 and P=0.114) did not differ significantly. Progesterone levels did not differ between groups at any timepoint (P+0: 0.33 versus 0.35 ng/mL, P=0.415; P+3: 10.95 versus 11.08 ng/mL, P=0.604; P+5: 12.36 versus 11.35 ng/mL, P=0.131) (Table 2, Figure 2).

P+5 E_2 (rho=0.228, P<0.001), P+3 E_2 (rho=0.214, P<0.001) and P+5 P (rho=0.206, P=0.001) all correlated positively with predicted hour, as did the P+3 E_2/P ratio (rho=0.129, P=0.023), whereas P+0 markers showed weak or no correlation (|rho| at most 0.104, all P>0.06) (Table 2). On stratified analysis, correlations between E_2 and predicted hour were confined to displaced cycles (P+5 E_2: rho=0.157, P=0.031; P+3 E_2: rho=0.166, P=0.014) and absent in receptive cycles (P+5 E_2: rho=-0.053, P=0.661); the correlation for P+5 P was likewise restricted to displaced cycles (rho=0.248, P<0.001) (Figure 3).

Across quartiles of P+5 E_2 (cut-offs 114.7, 153.5 and 215.5 pg/mL), displacement rates were 63.6%, 66.2%, 80.0% and 81.5% (chi-square=6.685, P=0.038; trend z=2.74, P=0.006), with median predicted hours of 142, 143, 148 and 147 h (Figure 5A).

In univariable logistic regression, each SD increase in P+5 E_2 (151.66 pg/mL) was associated with an OR for displacement of 1.956 (95% CI 1.183-3.236, P=0.009); P+3 E_2 gave an OR of 1.742 (95% CI 1.182-2.566, P=0.005) and the P+3 E_2/P ratio an OR of 1.684 (95% CI 1.142-2.482, P=0.009). The OR for P+0 E_2 was 1.330 (P=0.068), and P+0 and P+5 E_2/P ratios and P levels at all three timepoints were not significant (OR 0.915 to 1.395, all P>0.07) (Table 3, Figure 5B). When E_2 at the three timepoints was entered simultaneously, no timepoint remained significant owing to collinearity (P+0 OR=0.932, P=0.781; P+3 OR=1.591, P=0.207; P+5 OR=1.452, P=0.277, n=259).

ROC analysis gave an AUC of 0.626 (95% CI 0.552-0.702) for P+5 E_2 in discriminating a displaced window, 0.606 (0.544-0.677) for P+3 E_2, 0.584 (0.519-0.653) for the P+3 E_2/P ratio, 0.564 (0.491-0.637) for the P+5 E_2/P ratio, 0.562 (0.492-0.639) for P+0 E_2, 0.558 (0.488-0.622) for the P+0 E_2/P ratio, 0.561 (0.480-0.640) for P+5 P and 0.481 (0.410-0.549) for P+3 P (Table 4, Figure 4).

Influence of endometrial preparation regimens on hormone levels

Estrogen route had the largest effect on E_2 concentrations. Median P+5 E_2 was 206.10 (152.80-325.41) pg/mL in combined cycles versus 134.87 (109.12-179.02) pg/mL in single-agent cycles (P<0.001, rho=0.408), with the same pattern at P+3 and P+0 (198.71 versus 139.01 pg/mL, P<0.001; 164.89 versus 143.75 pg/mL, P=0.004). The median P+5 E_2/P ratio was 19.43 (12.84-33.23) in combined versus 10.57 (7.51-16.44) in single-agent cycles (P<0.001, rho=0.421). P+5 P was lower in combined than in single-agent cycles [11.09 (8.22-14.42) versus 12.93 (10.03-16.59) ng/mL, P=0.007], as was P+0 P (0.29 versus 0.35 ng/mL, P=0.014), while P+3 P did not differ (P=0.226) (Table 5, Figure 6A).

In GnRH-a down-regulated cycles, E_2 was lower at all three timepoints (P+0: 119.71 versus 150.61 pg/mL, P<0.001; P+3: 122.54 versus 152.31, P=0.014; P+5: 127.17 versus 157.38, P=0.013), whereas P+5 P was higher (14.21 versus 11.55 ng/mL, P=0.012); P+0 and P+3 P did not differ (P=0.117 and P=0.151). The P+5 and P+3 E_2/P ratios were both lower with down-regulation (9.06 versus 13.79, P=0.004; 10.33 versus 14.39, P=0.012) (Table 5, Figure 6C).

Progesterone regimen determined P concentrations. Median P+5 P was 14.11 (11.27-17.43) ng/mL with fixed 60 mg, 11.35 (9.66-12.26) ng/mL with the step-up regimen and 9.44 (7.20-12.11) ng/mL with fixed 40 mg (P<0.001). At P+3 the step-up group was still receiving 40 mg/day and its P level was 6.90 (6.05-8.10) ng/mL, lower than 9.50 (7.53-11.82) ng/mL with fixed 40 mg and 12.70 (10.10-16.36) ng/mL with fixed 60 mg (P<0.001). The P+5 E_2/P ratio showed the inverse gradient across progesterone regimens (18.41 with 40 mg, 14.85 with step-up and 10.44 with 60 mg, P<0.001), while E_2 levels did not differ among regimens at any timepoint (P=0.101 to 0.575) (Table 5, Figure 6B).

Regimens in relation to window results

Displacement occurred in 94.1% (32/34) of GnRH-a down-regulated cycles versus 67.9% (188/277) of cycles without down-regulation (chi-square P=0.003), and predicted hour was later with down-regulation [157.0 (150.3-161.0) versus 144.0 (133.0-151.0) h, P<0.001]. Displacement occurred in 80.0% (76/95) of combined estrogen cycles versus 66.7% (144/216) of single-agent cycles (P=0.025), with a slightly later predicted hour [145.0 (140.0-155.0) versus 144.0 (134.0-151.3) h, P=0.042]. Displacement rates with the three progesterone regimens were 66.7% (40 mg), 72.8% (60 mg) and 73.9% (step-up) without significant difference (P=0.512), although predicted hours did differ among them (Kruskal-Wallis P=0.043) (Table 6, Figure 1B).

In multivariable logistic regression adjusting simultaneously for GnRH-a down-regulation, estrogen route and progesterone regimen, P+5 E_2 remained independently associated with a displaced window (OR 2.060 per SD, 95% CI 1.178-3.605, P=0.011), as did GnRH-a down-regulation (OR 8.575, 95% CI 1.948-37.753, P=0.005), whereas estrogen route (combined versus single OR 1.156, 95% CI 0.574-2.327, P=0.685) and progesterone regimen (60 versus 40 mg OR 0.743, P=0.359; step-up versus 40 mg OR 0.842, P=0.776) were not significant (n=261, 190 events, model converged) (Table 3, Figure 5B). A reduced model containing only P+5 E_2, down-regulation and estrogen route gave consistent results (E_2 OR=2.070, P=0.011; down-regulation OR=7.907, P=0.006).

Sensitivity analysis in the non-down-regulated subgroup

Serum E_2 was lower in the GnRH-a down-regulated group, yet that group had the highest displacement rate (94.1%), the opposite direction to the main finding that higher E_2 accompanies displacement. To exclude interference from down-regulated cycles, all analyses were repeated in the non-down-regulated subgroup of 277 cycles, with results consistent with and stronger than those in the overall cohort (Table 8, Figure 7).

Between-group comparisons: displaced cycles had higher P+5 E_2 (170.06 versus 133.42 pg/mL, P=0.0003), higher P+3 E_2 (165.46 versus 134.10 pg/mL, P=0.0002) and higher P+0 E2 (158.00 versus 142.00 pg/mL, P=0.012). The P+3 E_2/P ratio was 15.74 versus 12.50 (P=0.003) and the P+5 E_2/P ratio 14.91 versus 11.53 (P=0.036). P levels did not differ at any timepoint (P=0.209 to 0.495).

Effect estimates: per SD increase in P+5 E_2 the unadjusted OR was 2.188 (95% CI 1.281-3.740, P=0.004) and the OR adjusted for estrogen route and progesterone regimen was 2.101 (95% CI 1.193-3.697, P=0.010); adjusted ORs were 1.852 for P+3 E_2 (P=0.006), 1.403 for P+0 E_2 (P=0.043) and 1.905 for the P+3 E_2/P ratio (P=0.006). P+5 P was not significant (adjusted OR=1.332, P=0.099).

Gradient: across quartiles of P+5 E_2 (cut-offs 117.0, 157.4 and 219.7 pg/mL), displacement rates were 55.2%, 64.9%, 77.6% and 82.1% (chi-square=12.264, P=0.007; trend z=3.45, P=0.0006), a steeper gradient than in the overall cohort (63.6% to 81.5%).

Discrimination: the AUC for P+5 E_2 was 0.652 (95% CI 0.569-0.726), for P+3 E_2 0.637 (0.568-0.705), for the P+3 E_2/P ratio 0.613 (0.541-0.679) and for P+0 E_2 0.594 (0.525-0.665), all exceeding the corresponding overall values (0.626, 0.606, 0.584 and 0.562).

Correlation: P+5 E_2 correlated with predicted hour at rho=0.301 (P<0.001), P+3 E_2 at rho=0.306 (P<0.001) and P+5 P at rho=0.157 (P=0.017), all stronger than in the overall cohort (rho=0.228, 0.214 and 0.206).

Adding a product term of E_2 and down-regulation to the overall model gave no significant interaction (OR=0.953, P=0.983), indicating that down-regulation does not modify the effect of E_2 and that the two associations are independent.

Statistical power

Power to detect the difference in displacement between estrogen routes (66.7% versus 80.0%, n=216/95) was 0.674 and between GnRH-a down-regulation status (67.9% versus 94.1%, n=277/34) was 0.980, whereas power for the comparison between progesterone 60 mg and 40 mg (72.8% versus 66.7%, n=180/108) was only 0.198 (Table 7).

Discussion

In 311 rsERT testing cycles we observed that mid-secretory serum E_2 concentrations are associated with the transcriptomically defined position of the WOI, and that this association remains independent after adjustment for the entire endometrial preparation regimen. E_2 at P+3 and P+5 gave concordant results across four types of analysis: between-group comparisons, continuous correlation, quartile gradient (63.6% to 81.5%, trend P=0.006) and multivariable models (OR=2.06, P=0.011). The effect size increased with duration of progesterone exposure (OR 1.33 at P+0, P=0.068; 1.74 at P+3, P=0.005; 1.96 at P+5, P=0.009), whereas P levels at every timepoint were unrelated to window position. This progressive increase with timepoint accords with the biological sequence of receptivity establishment. Notably, P+0 E_2 was not significant in the overall cohort but reached significance in the non-down-regulated subgroup (adjusted OR=1.40, P=0.043), suggesting that down-regulated cycles diluted the estrogen signal at earlier timepoints and further supporting an independent pathway for down-regulation.

Receptivity is established in two steps: estrogen initiates endometrial proliferation and glandular programmes via ERalpha, after which progesterone drives stromal decidualisation via PGR-A/B and suppresses estrogen-driven gene expression [1, 2]. In our cohort the association between E_2 and displacement strengthened with duration of progesterone exposure (OR rising from 1.33 at P+0 to 1.96 at P+5), indicating that the closer the endometrium is to receiving progesterone signalling and initiating decidualisation, the more prominent the influence of estrogen load on the molecular phase. This is consistent with the hypothesis that

326 persistently elevated estrogen signalling antagonises progesterone-driven maturation [2].
327 Exogenous estradiol in HRT regimens exceeds mid-luteal levels of natural cycles, and
328 interindividual differences in absorption and metabolism mean that tissue estrogen load differs
329 between women on the same protocol day, which may be one cause of window displacement.
330 The P+3 E_2/P ratio was likewise associated with displacement (OR=1.68); because this ratio
331 integrates information on both estrogen exposure and progesterone response, it further suggests
332 that estrogen load relative to progesterone is more informative than either hormone
333 concentration alone. It should be emphasised that serum E_2 does not directly reflect local
334 endometrial estrogenic activity: discriminative ability in this study was moderate (AUC 0.626),
335 consistent with previously reported weak concordance between serum and endometrial
336 progesterone [23] and with only moderate correlations between hormones and predicted hour
337 in our cohort (rho=0.13 to 0.23), indicating that peripheral markers only partially reflect local
338 tissue status.

339 GnRH-a down-regulation carried the strongest association with displacement (94.1%,
340 adjusted OR=8.58) and predicted hours were later (157.0 h). Of note, serum E_2 at all three
341 timepoints was lower with down-regulation and the E_2/P ratio was also lower, the opposite
342 direction to the finding that higher E_2 accompanies displacement. This apparent contradiction
343 can be resolved by distinguishing two pathways. First, serum E_2 reflects exogenous estrogen
344 exposure, and sensitivity analysis in the non-down-regulated subgroup showed that after
345 excluding down-regulated cycles the association between E_2 and displacement was not only
346 retained but stronger (P+5 E_2 adjusted OR=2.10; quartile displacement 55.2% to 82.1%, trend
347 P=0.0006; AUC 0.652), while the E_2-by-down-regulation interaction was not significant
348 (P=0.98), indicating that down-regulation does not modify the effect of E_2 and that the two are
349 independent. Second, the displacement rate of 94.1% in the down-regulated group is close to a
350 ceiling, so displacement there is evidently not driven by serum E_2; the more plausible

explanation is receptivity abnormality inherent in the indications for down-regulation (such as adenomyosis or a predisposition to endometriosis), together with direct effects of profound pituitary suppression on the local endometrial hormonal microenvironment, neither of which can be captured by circulating E_2 concentrations. In other words, low E_2 is the expected pharmacological consequence of down-regulation rather than the cause of displacement, and including down-regulated cycles in the overall analysis dilutes the E_2 effect size through their low-E_2/high-displacement profile, which explains why the E_2 effect was stronger in the non-down-regulated subgroup. We could not further separate confounding by indication from direct effects of pituitary suppression, and with only 34 down-regulated cycles the confidence interval was wide (1.95 to 37.75). These findings indicate that window timing deserves particular attention in down-regulated women, who may stand to gain most from personalized transfer, in keeping with the direction of our centre's previous report of improved pregnancy rates after personalized transfer in non-receptive women [13], although prospective confirmation is required.

Regimens exerted large effects on hormone levels, with direct implications for the clinical use of serum markers. P+5 E_2 was about 1.5-fold higher in combined than in single-agent estrogen cycles (206.10 versus 134.87 pg/mL), P+5 P about 1.5-fold higher with 60 mg than with 40 mg progesterone (14.11 versus 9.44 ng/mL), and E_2 about 20% lower with down-regulation. Importantly, although dosing regimens markedly altered hormone levels, they did not independently predict window position in the multivariable model (combined estrogen OR=1.16, P=0.685); that is, the association between hormone levels and window position cannot be explained by differences in drug administration. Hormone changes in the step-up group matched its day-by-day dose escalation design exactly (P lowest at P+3 while still on 40 mg, at 6.90 ng/mL, and rising to 11.35 ng/mL at P+5 after two days of 60 mg, between the two fixed-dose groups), providing internal validation of the consistency between hormone

measurement and medication records in this cohort. These findings indicate that hormone thresholds not standardised to regimen should not be applied directly across centres; if serum E_2 is to be used in future to select women for testing, thresholds must be set stratified by estrogen route or provided in the form of adjusted models.

The clinical relevance of this study lies in providing a basis for non-invasive prediction of window position. Previous work has shown that deviations in window position affect not only implantation but also pregnancy loss: when transfer is performed more than 12 h away from the predicted window, pregnancy loss more than doubles compared with transfer within the window [14], and hourly-precise personalized transfer improves clinical outcomes in women with RIF [13]. If serum E_2 can flag a later window in advance, its primary value would be in selecting women who should undergo endometrial biopsy testing; with a displacement rate of 70.7% in our cohort, women with higher P+3 or P+5 E_2 could be prioritised for testing where resources are limited. A second value is providing background information for interpreting test results, allowing clinicians to anticipate the likely need for personalized transfer before biopsy. It must be emphasised that the AUC in this study was only 0.626 (95% CI 0.552 to 0.702), so discriminative ability is moderate and serum E_2 cannot replace endometrial biopsy; its appropriate role is as an adjunctive indicator, and thresholds must be set according to estrogen route (see above). We did not assess pregnancy outcomes after transfer, so whether the association between serum E_2 and window position carries direct prognostic value requires prospective study.

Strengths of this study include the relatively large sample of 311 consecutive testing cycles sampled at three fixed timepoints; the unit of analysis strictly confined to the testing cycle, focusing on the hormones-window relationship itself and avoiding interference from outcome information; explicit treatment of regimens as covariates with systematic quantification of their hormonal effects, including additive-dose combined estrogen and day-

by-day step-up progesterone; four mutually corroborating types of analysis (between-group comparisons, continuous correlation, quartile gradients and adjusted models), with all analyses repeated in the non-down-regulated subgroup as sensitivity testing; bootstrap 95% CIs for the AUC; and explicit reporting of negative results and inadequate power.

Limitations include the single-centre retrospective design with no natural-cycle control, all cycles being HRT cycles; 16.1% missing P+5 hormone data with reasons unrecorded, which may introduce selection bias; intra-day and inter-individual variability of immunoassays without standardised sampling times [24]; unquantified indications for down-regulation and combined estrogen, leaving residual confounding possible; high collinearity of E_2 across the three timepoints, precluding attribution to any single timepoint; only 23 cycles in the step-up progesterone group; power of only 0.198 for the progesterone regimen comparison, so its null result cannot be interpreted as equivalence; no formal correction for multiple comparisons; absence of endometrial histology and molecular measurements (such as estrogen and progesterone receptor expression), so mechanistic interpretations are inferences based on published literature; and an endpoint of molecular window position without follow-up of pregnancy outcomes after transfer, meaning that the prognostic value of serum markers cannot be assessed within this study.

Conclusions

In HRT cycles undergoing rsERT testing, serum E_2 measured three to five days after progesterone conversion is associated with the transcriptomically defined position of the WOI, and this association remains independent after adjustment for GnRH-a down-regulation, estrogen route and progesterone regimen. The effect is stronger in non-down-regulated cycles (adjusted OR=2.10, AUC 0.652), indicating that the association is not driven by down-regulated cycles. The effect size increases with duration of progesterone exposure (OR rising

from 1.33 at P+0 to 1.96 at P+5), consistent with the sequential estrogen-progesterone mechanism of receptivity establishment, but discriminative ability is moderate (AUC 0.626), so serum E_2 may serve as an adjunctive indicator and cannot replace endometrial biopsy. Serum progesterone concentrations were determined mainly by the dosing regimen and were unrelated to window position. GnRH-a down-regulation was strongly associated with a displaced window (94.1%, adjusted OR=8.58) with no significant interaction with serum E_2, indicating that down-regulation affects window position through a pathway independent of circulating estrogen levels, probably the receptivity abnormalities inherent in its indications. Regimens substantially altered hormone levels (E_2 approximately 50% higher with combined estrogen, P approximately 50% higher with 60 mg progesterone), so serum thresholds not standardised to regimen should not be applied across centres. Prospective studies are needed to validate the value of serum E_2 in selecting women for testing, and molecular mechanisms of window displacement should be investigated together with local endometrial expression of estrogen and progesterone receptors.

Abbreviations

AUC: area under the receiver operating characteristic curve; BMI: body mass index; CI: confidence interval; E_2: estradiol; ERA: Endometrial Receptivity Array; ERalpha: estrogen receptor alpha; FET: frozen embryo transfer; GnRH-a: gonadotrophin-releasing hormone agonist; HRT: hormone replacement therapy; IQR: interquartile range; MDE: minimum detectable effect; OR: odds ratio; P: progesterone; PGT: preimplantation genetic testing; pET: personalized embryo transfer; PGR: progesterone receptor; RIF: recurrent implantation failure; ROC: receiver operating characteristic; rsERT: RNA-sequencing-based endometrial receptivity test; SD: standard deviation; WOI: window of implantation.

Declarations

Ethics approval and consent to participate This study was approved by the Ethics Committee of the Boai Hospital of Zhongshan (Application ID: KY-2022-010-14; date of approval: October 2022). Informed consent was waived because the analysis used retrospectively collected anonymised data. The study was conducted in accordance with the Declaration of Helsinki.

Consent for publication Not applicable.

Availability of data and materials The de-identified dataset analysed during the current study is not publicly available because it contains individual clinical data, but it is available from the corresponding author on reasonable request.

Competing interests The authors declare that they have no competing interests.

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Authors' contributions [To be completed according to the CRediT taxonomy.]

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Tables

Table 1 Baseline characteristics and testing-cycle features by window result

Characteristic	Receptive (n=91)	Displaced (n=220)	P value
Age (years)	35.00 (31.00, 38.00)	34.00 (31.00, 38.00)	0.920
BMI (kg/m2)	22.00 (20.25, 24.00)	22.00 (20.25, 24.75)	0.472
Previous failed transfers of good-quality embryos	2.00 (2.00, 3.00)	2.00 (1.00, 3.00)	0.341
GnRH-a down-regulation, n (%)	2 (2.2)	32 (14.5)	0.003
Single-agent estrogen, n (%)	72 (79.1)	144 (65.5)	0.025
Combined estrogen, n (%)	19 (20.9)	76 (34.5)	
Fixed progesterone 40 mg/day, n (%)	36 (39.6)	72 (32.7)	0.512
Fixed progesterone 60 mg/day, n (%)	49 (53.8)	131 (59.5)	
Step-up progesterone, n (%)	6 (6.6)	17 (7.7)	
PGT cycle, n (%)	20 (23.0)	59 (27.1)	0.454
Predicted hour of implantation (h)	129.00 (123.00, 133.00)	149.50 (144.00, 156.00)	<0.001
Predicted uncertainty interval (h)	3.00 (3.00, 3.00)	3.00 (3.00, 3.00)	0.284

Continuous variables are median (IQR), compared by the Mann-Whitney U test; categorical variables are n (%), compared by the chi-square test with Yates continuity correction for 2x2 tables.

Table 2 Serum hormones by window result and correlation with predicted hour of implantation

Marker	Receptive	Displaced	*P* value	rho (all)	*P* value	rho (receptive)	*P* value	rho (displaced)	*P* value
P+0 E_2 (pg/mL)	140.88 (112.58, 185.79)	149.66 (120.37, 208.40)	0.086	0.104	0.066	-0.058	0.588	0.064	0.343
P+0 P (ng/mL)	0.35 (0.20, 0.52)	0.33 (0.20, 0.50)	0.415	-0.021	0.719	0.220	0.036	-0.004	0.949
P+0 E2/P	422.63 (269.79, 710.54)	463.77 (307.38, 962.71)	0.106	0.071	0.209	-0.262	0.012	0.042	0.537

Marker	Receptive	Displaced	*P* value	rho (all)	*P* value	rho (receptive)	*P* value	rho (displaced)	*P* value
P+3 E_2 (pg/mL)	134.10 (106.66, 177.53)	160.62 (116.67, 222.37)	0.003	0.214	<0.001	-0.015	0.889	0.166	0.014
P+3 P (ng/mL)	11.08 (8.46, 14.96)	10.95 (8.27, 14.54)	0.604	0.018	0.747	0.027	0.797	0.076	0.263
P+3 E_2/P	12.50 (8.24, 19.71)	15.12 (9.88, 23.30)	0.020	0.129	0.023	-0.058	0.585	0.060	0.377
P+5 E_2 (pg/mL)	133.42 (106.99, 172.61)	163.54 (117.49, 220.61)	0.002	0.228	<0.001	-0.053	0.661	0.157	0.031
P+5 P (ng/mL)	11.35 (9.07, 14.32)	12.36 (9.64, 16.28)	0.131	0.206	0.001	0.075	0.535	0.248	<0.001
P+5 E_2/P	11.26 (8.01, 17.83)	13.94 (8.47, 22.14)	0.114	0.041	0.507	-0.084	0.486	-0.055	0.452

Continuous variables are median (IQR), compared by the Mann-Whitney U test; rho denotes the Spearman correlation coefficient with the predicted hour of implantation. Numbers analysed: P+0 markers, 91 receptive and 220 displaced; P+3 markers, 91 and 218 (309 overall); P+5 markers, 71 and 190 (261 overall, 50 missing).

Table 3 Logistic regression for a displaced window of implantation

Variable	Model	OR	95% CI	P value	n (events)
P+5 E_2 (per SD)	Univariable	1.956	1.183-3.236	0.009	261 (190)
P+3 E_2 (per SD)	Univariable	1.742	1.182-2.566	0.005	309 (218)
P+3 E_2/P (per SD)	Univariable	1.684	1.142-2.482	0.009	309 (218)
P+0 E_2/P (per SD)	Univariable	1.395	0.972-2.003	0.071	311 (220)
P+0 E_2 (per SD)	Univariable	1.330	0.980-1.806	0.068	311 (220)
P+5 E_2/P (per SD)	Univariable	1.291	0.927-1.797	0.131	261 (190)
P+5 P (per SD)	Univariable	1.160	0.868-1.550	0.315	261 (190)
P+3 P (per SD)	Univariable	0.915	0.721-1.162	0.465	309 (218)

Variable	Model	OR	95% CI	P value	n (events)
P+0 P (per SD)	Univariable	0.921	0.725-1.171	0.504	311 (220)
P+0 E_2 (per SD)	All three E_2 timepoints	0.932	0.568-1.529	0.781	259 (189)
P+3 E_2 (per SD)	All three E_2 timepoints	1.591	0.773-3.274	0.207	259 (189)
P+5 E_2 (per SD)	All three E_2 timepoints	1.452	0.741-2.845	0.277	259 (189)
P+5 E_2 (per SD)	Multivariable	2.060	1.178-3.605	0.011	261 (190)
GnRH-a down-regulation (yes vs no)	Multivariable	8.575	1.948-37.753	0.005	261 (190)
Combined estrogen (vs single)	Multivariable	1.156	0.574-2.327	0.685	261 (190)
Progesterone 60 mg (vs 40 mg)	Multivariable	0.743	0.395-1.401	0.359	261 (190)
Step-up progesterone (vs 40 mg)	Multivariable	0.842	0.258-2.751	0.776	261 (190)
P+5 E_2 (per SD)	Reduced multivariable	2.070	1.179-3.633	0.011	261 (190)
GnRH-a down-regulation (yes vs no)	Reduced multivariable	7.907	1.818-34.376	0.006	261 (190)
Combined estrogen (vs single)	Reduced multivariable	1.210	0.609-2.403	0.586	261 (190)

The multivariable model included standardised P+5 E_2, GnRH-a down-regulation, estrogen route and progesterone regimen (60 mg and step-up versus fixed 40 mg as reference); the reduced model excluded progesterone regimen. All models converged. SDs of the markers were: P+5 E_2 151.66 pg/mL, P+3 E_2 141.10 pg/mL, P+0 E2 109.05 pg/mL, P+5 E_2/P 15.56, P+3 E_2/P 17.94, P+0 E_2/P 829.74, P+5 P 6.07 ng/mL, P+3 P 5.73 ng/mL, P+0 P 0.25 ng/mL.

Table 4 Area under the ROC curve for discriminating a displaced window

Marker	n analysed	AUC	95% CI
P+5 E_2	261	0.626	0.552-0.702
P+3 E_2	309	0.606	0.544-0.677
P+3 E_2/P	309	0.584	0.519-0.653

Marker	n analysed	AUC	95% CI
P+5 E_2/P	261	0.564	0.491-0.637
P+0 E_2	311	0.562	0.492-0.639
P+5 P	261	0.561	0.480-0.640
P+0 E_2/P	311	0.558	0.488-0.622
P+3 P	309	0.481	0.410-0.549

491

492 AUC was calculated non-parametrically with 95% CI from 1000 bootstrap resamples; the
493 outcome was a displaced window (yes = 1, no = 0).

494 **Table 5** Effect of endometrial preparation regimens on serum hormones in testing cycles

495

Marker	No down-regulation (n=277)	Down-regulation (n=34)	P value	Single estrogen (n=216)	Combined estrogen (n=95)	P value	40 mg (n=108)	60 mg (n=180)	Step-up (n=23)	P value
P+0 E_2	150.61 (120.77, 209.75)	119.71 (97.01, 139.97)	<0.001	143.75 (115.15, 183.59)	164.89 (125.05, 245.78)	0.004	150.41 (127.09, 212.02)	143.74 (114.32, 198.16)	147.92 (111.95, 176.15)	0.575
P+3 E_2	152.31 (115.81, 211.70)	122.54 (100.10, 169.77)	0.014	139.01 (109.11, 177.39)	198.71 (133.31, 327.23)	<0.001	163.38 (118.27, 222.64)	142.42 (106.28, 196.32)	151.06 (128.98, 234.24)	0.101
P+5 E_2	157.38 (116.98, 219.68)	127.17 (104.25, 167.84)	0.013	134.87 (109.12, 179.02)	206.10 (152.80, 325.41)	<0.001	169.17 (117.94, 223.43)	148.43 (111.51, 200.38)	153.50 (123.87, 276.02)	0.208
P+0 P	0.34 (0.20, 0.51)	0.28 (0.17, 0.49)	0.117	0.35 (0.22, 0.53)	0.29 (0.19, 0.46)	0.014	0.34 (0.20, 0.53)	0.34 (0.21, 0.51)	0.25 (0.17, 0.39)	0.152
P+3 P	10.71 (8.17, 14.70)	12.05 (9.96, 15.76)	0.151	11.16 (8.45, 15.07)	10.52 (7.87, 13.88)	0.226	9.50 (7.53, 11.82)	12.70 (10.10, 16.36)	6.90 (6.05, 8.10)	<0.001

Marker	No down-regulation (n=277)	Down-regulation (n=34)	P value	Single estrogen (n=216)	Combined estrogen (n=95)	P value	40 mg (n=108)	60 mg (n=180)	Step-up (n=23)	P value
P+5 P	11.55 (9.17, 15.51)	14.21 (11.78, 17.48)	0.012	12.93 (10.03, 16.59)	11.09 (8.22, 14.42)	0.007	9.44 (7.20, 12.11)	14.11 (11.27, 17.43)	11.35 (9.66, 12.26)	<0.001
P+0 E_2/P	458.03 (297.33, 892.67)	400.85 (270.61, 786.71)	0.471	416.94 (268.44, 740.22)	558.87 (340.93, 1307.56)	<0.001	472.05 (309.72, 975.13)	446.97 (279.28, 828.92)	464.68 (350.98, 790.84)	0.524
P+3 E_2/P	14.39 (9.77, 22.56)	10.33 (9.12, 17.22)	0.012	12.40 (8.75, 19.31)	19.63 (12.12, 30.89)	<0.001	18.56 (11.23, 24.71)	11.21 (8.08, 16.95)	21.32 (17.24, 46.79)	<0.001
P+5 E_2/P	13.79 (8.60, 21.11)	9.06 (7.38, 14.19)	0.004	10.57 (7.51, 16.44)	19.43 (12.84, 33.23)	<0.001	18.41 (11.74, 28.11)	10.44 (7.46, 16.49)	14.85 (11.44, 23.74)	<0.001

Values are median (IQR); two groups compared by the Mann-Whitney U test and three groups by the Kruskal-Wallis test. The single-agent group comprised 210 oral and 6 transdermal-only cycles. Numbers analysed for P+3 markers were 276 without down-regulation, 33 with down-regulation and 214 single-agent; for P+5 markers, 229, 32, 174 and 87 respectively. The step-up regimen gave 40 mg/day from P+0 to P+2 and 60 mg/day from P+3, so its P+3 value reflects exposure at the 40 mg stage.

Table 6 Regimens in relation to window results

Group	n	Displaced window, n (%)	P value	Predicted hour of implantation (h)	P value
GnRH-a down-regulation			0.003		<0.001
No down-regulation	277	188 (67.9)		144.00 (133.00, 151.00)	

Group	n	Displaced window, n (%)	P value	Predicted hour of implantation (h)	P value
Down-regulation	34	32 (94.1)		157.00 (150.25, 161.00)	
Estrogen route			0.025		0.042
Single agent	216	144 (66.7)		144.00 (134.00, 151.25)	
Combined	95	76 (80.0)		145.00 (140.00, 155.00)	
Progesterone regimen			0.512		0.043
Fixed 40 mg/day	108	72 (66.7)		—	
Fixed 60 mg/day	180	131 (72.8)		—	
Step-up	23	17 (73.9)		—	

Displacement rates were compared by the chi-square test (Yates continuity correction for 2x2 tables) and predicted hours by the Mann-Whitney U test (two groups) or Kruskal-Wallis test (three groups). Median predicted hours for the three progesterone regimens were 144.0, 144.0 and 150.0 h respectively.

Table 7 Statistical power and minimum detectable effect

Comparison	Group 1 (%)	Group 2 (%)	n1/n2	Power	MDE (percentage points)
GnRH-a down-regulation x displaced window	67.9	94.1	277/34	0.980	—
Estrogen route x displaced window	66.7	80.0	216/95	0.674	—
Progesterone 60 mg vs 40 mg x displaced window	66.7	72.8	180/108	0.198	—

Two-sided alpha=0.05; power calculated with the unpooled standard error. Power for the progesterone regimen comparison was only 0.198, so its null result cannot be interpreted as equivalence.

Table 8 Sensitivity analysis in the non-down-regulated subgroup (277 cycles) versus the overall cohort (311 cycles)

Analysis	Non-down-regulated subgroup	Overall cohort
P+5 E_2 (pg/mL), receptive vs displaced	133.42 vs 170.06 (P=0.0003)	133.42 vs 163.54 (P=0.002)
P+3 E_2 (pg/mL), receptive vs displaced	134.10 vs 165.46 (P=0.0002)	134.10 vs 160.62 (P=0.003)
P+0 E_2 (pg/mL), receptive vs displaced	142.00 vs 158.00 (P=0.012)	140.88 vs 149.66 (P=0.086)
P+5 P (ng/mL), receptive vs displaced	11.30 vs 11.94 (P=0.209)	11.35 vs 12.36 (P=0.131)
P+5 E_2 OR (univariable, per SD)	2.188 (1.281-3.740), P=0.004	1.956 (1.183-3.236), P=0.009
P+5 E_2 OR (adjusted for regimens)	2.101 (1.193-3.697), P=0.010	2.060* (1.178-3.605), P=0.011
P+3 E_2 OR (adjusted for regimens)	1.852 (1.194-2.873), P=0.006	1.742 (1.182-2.566), P=0.005
P+0 E_2 OR (adjusted for regimens)	1.403 (1.011-1.948), P=0.043	1.330 (0.980-1.806), P=0.068
P+3 E_2/P OR (adjusted for regimens)	1.905 (1.227-2.958), P=0.006	1.684 (1.142-2.482), P=0.009
Displacement across P+5 E_2 quartiles (%)	55.2, 64.9, 77.6, 82.1 (chi-square P=0.007; trend z=3.45, P=0.0006)	63.6, 66.2, 80.0, 81.5 (chi-square P=0.038; trend z=2.74, P=0.006)
AUC for P+5 E_2	0.652 (0.569-0.726)	0.626 (0.552-0.702)
AUC for P+3 E_2	0.637 (0.568-0.705)	0.606 (0.544-0.677)
AUC for P+0 E_2	0.594 (0.525-0.665)	0.562 (0.492-0.639)
P+5 E_2 correlation with predicted hour (rho)	0.301 (P<0.001)	0.228 (P<0.001)
P+3 E_2 correlation with predicted hour (rho)	0.306 (P<0.001)	0.214 (P<0.001)
E_2 x GnRH-a down-regulation interaction	OR=0.953 (P=0.983)	As stated

Within the subgroup, ORs were adjusted for estrogen route and progesterone regimen; the overall-cohort P+5 E_2 value marked with an asterisk is from the multivariable model additionally adjusting for GnRH-a down-regulation, whereas values at other timepoints are

univariable. Numbers analysed within the subgroup were: P+0 markers, 89 receptive and 188 displaced (277 total); P+3 markers, 89 and 187 (276 total); P+5 markers, 69 and 160 (229 total). The interaction term was tested in the overall model. Effect sizes, gradients, discriminative ability and correlation strengths at every timepoint within the subgroup were no weaker than in the overall cohort, indicating that the main findings are not driven by down-regulated cycles.

Figure legends

Figure 1 Distribution of the rsERT-predicted hour of optimal implantation. A: by window result; receptive 129.0 (123.0-133.0) h versus displaced 149.5 (144.0-156.0) h, Mann-Whitney U test P<0.001; the dashed line marks 120 h (P+5). B: by progesterone regimen, Kruskal-Wallis test P=0.043.

Figure 2 Serum E_2 (pg/mL) and P (ng/mL) at P+0, P+3 and P+5 in receptive and displaced cycles. Box plots with individual values overlaid; Mann-Whitney U test P values are annotated in each panel.

Figure 3 Scatter plots of predicted hour of implantation against E_2 at P+0, P+3 and P+5. Colours denote window status; Spearman correlation coefficients and P values are given for the overall cohort and for displaced cycles, with the dashed line marking 120 h.

Figure 4 Receiver operating characteristic curves for discriminating a displaced window. AUC was 0.626 for P+5 E_2, 0.606 for P+3 E_2, 0.584 for the P+3 E_2/P ratio, 0.562 for P+0 E_2 and 0.561 for P+5 P; 95% CIs from 1000 bootstrap resamples, details in Table 4.

Figure 5 P+5 E_2 quartile gradient and effect estimates for a displaced window. A: displacement rate by P+5 E_2 quartile (63.6% to 81.5%, chi-square P=0.038, trend z=2.74, P=0.006). B: forest plot of univariable (circles, per SD increase) and multivariable (diamond) ORs for a displaced window; horizontal axis on a logarithmic scale.

Figure 6 Influence of endometrial preparation regimens on hormone levels. A: P+5 E2 by estrogen route (single-agent 134.87 versus combined 206.10 pg/mL, P<0.001). B: P+3 and P+5 P by progesterone regimen; the step-up group was lowest at P+3 while still on 40 mg and rose to between the two fixed-dose groups at P+5. C: median hormone levels at each timepoint by GnRH-a down-regulation status; E_2 was lower and P+5 P higher with down-regulation.

Figure 7 Sensitivity analysis in the non-down-regulated subgroup. A: displacement rate by P+5 E_2 quartile, with a steeper gradient in the subgroup (n=229) than in the overall cohort (n=261) (55.2% to 82.1%, trend z=3.45, P=0.0006). B: adjusted OR per SD increase, subgroup (red) alongside overall cohort (blue); associations for E_2 at each timepoint were retained and stronger within the subgroup. C: displacement in the GnRH-a down-regulated group approaches a ceiling (94.1%); the E_2-by-down-regulation interaction OR was 0.95 (P=0.98), indicating a pathway independent of serum E_2.

Figure 1 Distribution of the rsERT-predicted transfer hour

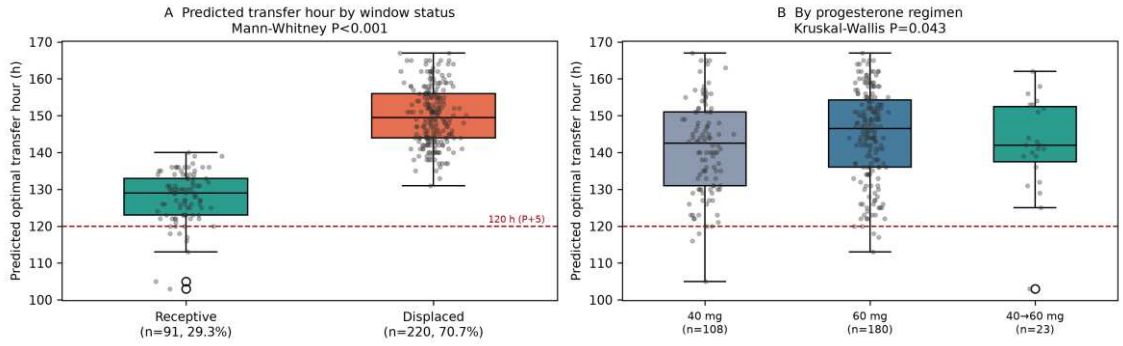


Figure 1 Distribution of the predicted hour of implantation

Figure 2 Serum estradiol and progesterone at three timepoints by window status

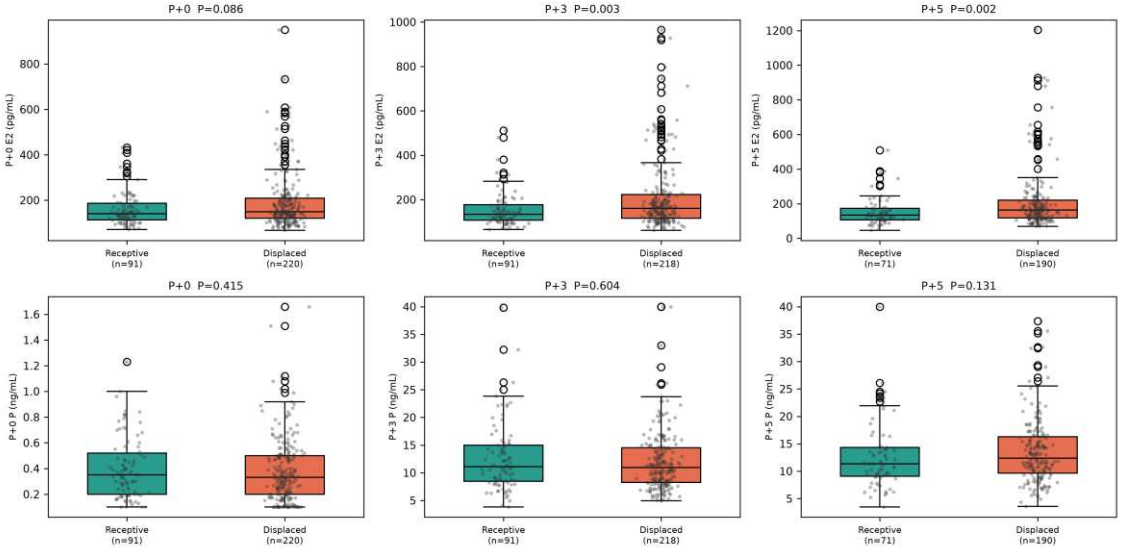


Figure 2 Hormones at three timepoints by window status

Figure 3 Relationship between serum estradiol and the predicted transfer hour

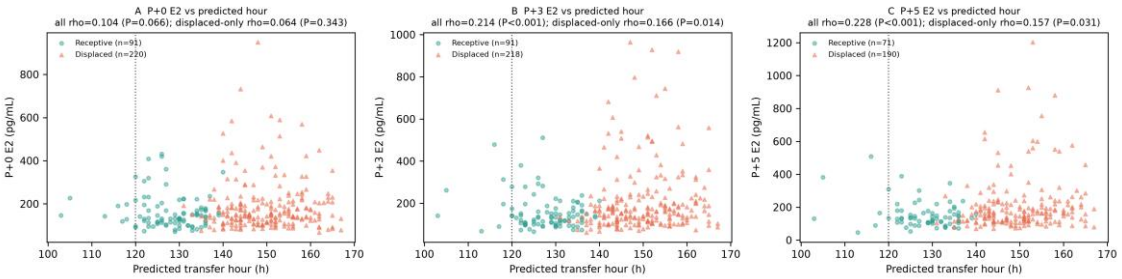
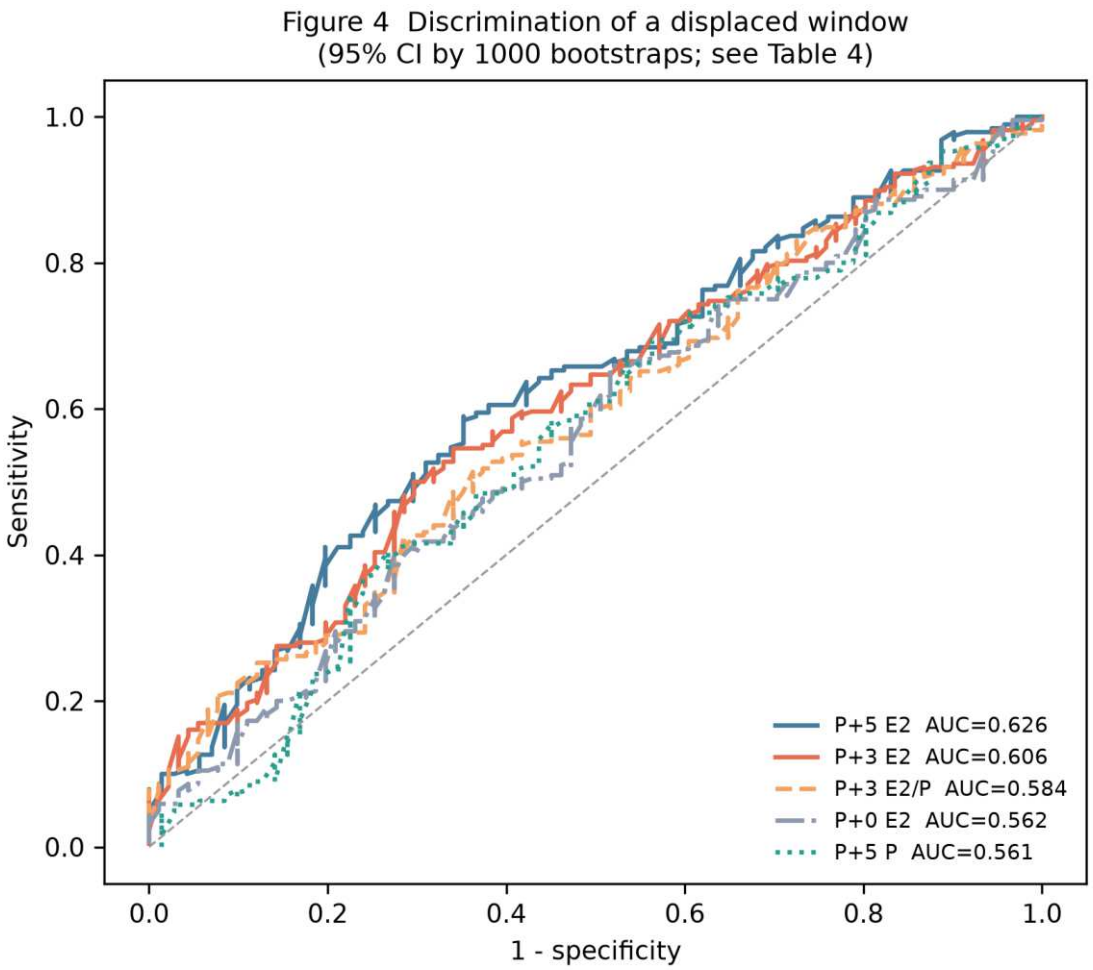


Figure 3 Hormones in relation to predicted hour



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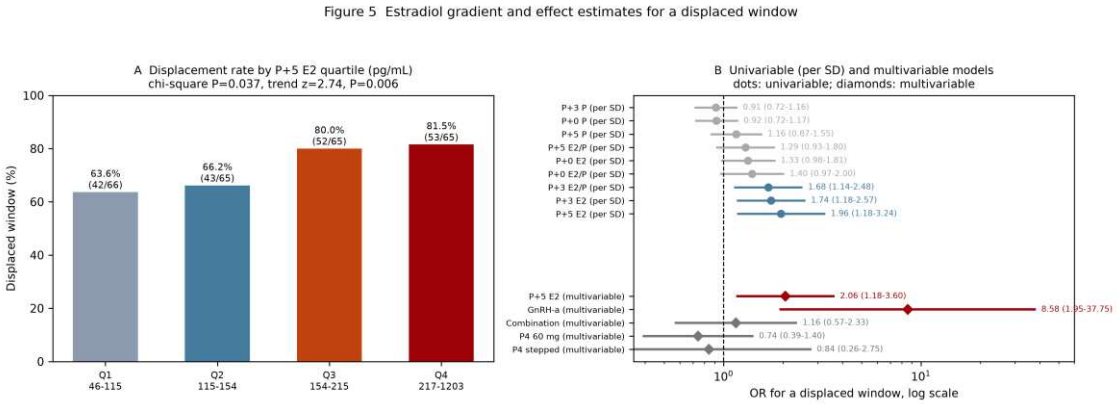
566 Figure 4 ROC curves for discriminating a displaced window



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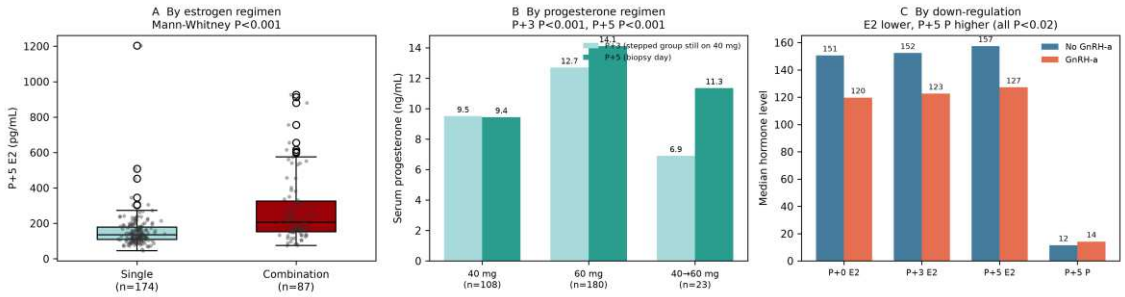
568 Figure 5 E2 quartile gradient and effect estimates

Figure 6 Medication regimens shape serum hormone levels in the testing cycle



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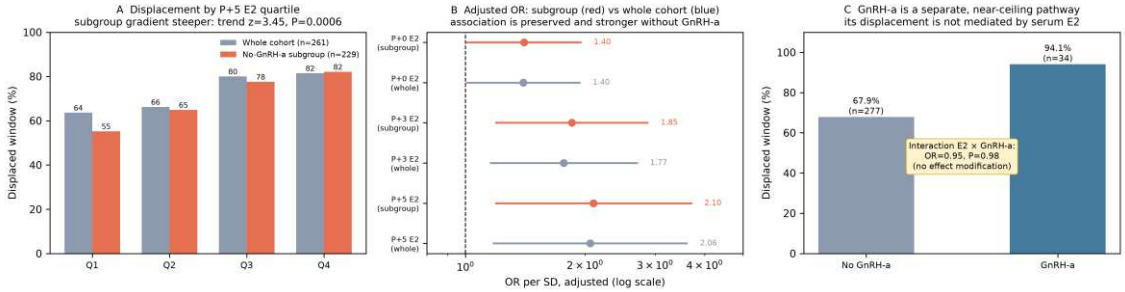
570 Figure 6 Influence of regimens on hormone levels

Figure 7 Sensitivity analysis in the non-down-regulated subgroup confirms the estradiol–window association



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572 Figure 7 Sensitivity analysis in the non-down-regulated subgroup

573 # References

574 **1. Norwitz ER, Schust DJ, Fisher SJ. Implantation and the survival of early pregnancy. N Engl J**

575 **Med. 2001;345(19):1400-8.**

576 **2. Lessey BA, Young SL. What exactly is endometrial receptivity? Fertil Steril. 2019;111(4):611-7.**

577 **3. Prapas Y, Prapas N, Jones EE, Duleba AJ, Olive DL, Chatziparasidou A, et al. The window for**

578 **embryo transfer in oocyte donation cycles depends on the duration of progesterone therapy. Hum**

579 **Reprod. 1998;13(3):720-3.**

580 **4. Sebastian-Leon P, Garrido N, Remohi J, Pellicer A, Diaz-Gimeno P. Asynchronous and**

581 **pathological windows of implantation: two causes of recurrent implantation failure. Hum Reprod.**

582 **2018;33(4):626-35.**

583 **5. Cakiroglu Y, Tiras B. Determining diagnostic criteria and cause of recurrent implantation failure.**

584 **Curr Opin Obstet Gynecol. 2020;32(3):198-204.**

6. Cimadomo D, Craciunas L, Vermeulen N, Vomstein K, Toth B. Definition, diagnostic and therapeutic options in recurrent implantation failure: an international survey of clinicians and embryologists. Hum Reprod. 2021;36(2):305-17.

7. 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(5):1264-72.

8. Diaz-Gimeno P, Horcajadas JA, Martinez-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(1):50-60.e15.

9. Ruiz-Alonso M, Blesa D, Diaz-Gimeno P, Gomez E, Fernandez-Sanchez 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.

10. Mahajan N. Endometrial receptivity array: clinical application. J Hum Reprod Sci. 2015;8(3):121-9.

11. He A, Zou Y, Wan C, Zhao J, Zhang Q, Yao Z, 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.

12. Chen J, He A, Zhang Q, Zhao J, Fu J, Li H, et al. 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. Front Endocrinol (Lausanne). 2022;13:1009161.

13. Xu Y, Du J, Zou Y, Lin X, Chen Y, Ma L, et al. Precise hourly personalized embryo transfer significantly improves clinical outcomes in patients with repeated implantation failure. Front Endocrinol (Lausanne). 2024;15:1408398.

14. Enciso M, Aizpurua J, Rodriguez-Estrada B, Jurado I, Ferrandez-Rives M, Rodriguez E, et al. The precise determination of the window of implantation significantly improves ART outcomes. Sci Rep. 2021;11(1):13420.

15. Ruiz-Alonso M, Gomez C, Stankewicz T, Castellon JA, Diez-Juan A, Gomez E, et al. Clinical

outcomes following endometrial receptivity assessment-guided personalized euploid embryo transfer in patients with previous implantation failures. Sci Rep. 2025;15(1):16967.

16. Bellver J, Santos-Ribeiro S, Cervello I, Pellicer A, Remohi J. Obesity affects endometrial receptivity by displacing the window of implantation. Reprod Sci. 2021;28(11):3171-80.

17. Xu B, Li Z, Zhang H, Jin L, Li Y, Ai J, et al. Serum progesterone level effects on the outcome of in vitro fertilization in patients with different ovarian response: an analysis of more than 10,000 cycles. Fertil Steril. 2012;97(6):1321-7.e4.

18. Venetis CA, Kolibianakis EM, Bosdou JK, Tarlatzis BC. Progesterone elevation and probability of pregnancy after IVF: a systematic review and meta-analysis of over 60 000 cycles. Hum Reprod Update. 2013;19(5):433-57.

19. Gaggiotti-Marre S, Alvarez M, Gonzalez-Foruria I, Parriego M, Garcia S, Martinez F, et al. Low progesterone levels on the day before natural cycle frozen embryo transfer are negatively associated with live birth rates. Hum Reprod. 2020;35(7):1623-9.

20. Alvarez M, Gaggiotti-Marre S, Martinez F, Coll L, Garcia S, Gonzalez-Foruria I, et al. Individualised luteal phase support in artificially prepared frozen embryo transfer cycles based on serum progesterone levels: a prospective cohort study. Hum Reprod. 2021;36(6):1552-60.

21. Melo P, Wood S, Petsas G, Chung Y, Easter C, Price MJ, et al. The effect of frozen embryo transfer regimen on the association between serum progesterone and live birth: a multicentre prospective cohort study (ProFET). Hum Reprod Open. 2022;2022(4):hoac054.

22. Casper RF, Yanushpolsky EH. Optimal endometrial preparation for frozen embryo transfer cycles: window of implantation and progesterone support. Fertil Steril. 2016;105(4):867-72.

23. 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(11):2861-70.

24. Gonzalez-Foruria I, Rodriguez I, Martinez F, Rodriguez-Purata J, Montoya P, Rodriguez D, et al. Clinically significant intra-day variability of serum progesterone levels during the final day of oocyte maturation: a prospective study with repeated measurements. Hum Reprod. 2019;34(8):1551-8.