The impact of estradiol on pregnancy outcomes in letrozole-stimulated frozen embryo transfer cycles

Wendy Y. Zhang, M.D.,[a,b] Rebecca M. Gardner, M.S.,[c] Kristopher I. Kapphahn, M.S.,[c] Maya K. Ramachandran, M.D.,[a] Gayathree Murugappan, M.D.,[b] Lusine Aghajanova, M.D., Ph.D.,[b] and Ruth B. Lathi, M.D.[b]

[a] Department of Medicine, [b] Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, and [c] Quantitative Sciences Unit, Department of Medicine, Stanford University School of Medicine, Stanford, California

Objective: To assess the impact of low estradiol (E2) levels in letrozole-stimulated frozen embryo transfer (FET) cycles on pregnancy and neonatal outcomes.
Design: Retrospective cohort.
Setting: University-affiliated fertility center.
Patient(s): All patients who underwent letrozole-stimulated FET cycles from January 2017 to April 2020 (n = 217). The "Low E2" group was defined as those with E2 serum levels on the day of trigger <10th percentile level (E2 <91.16 pg/mL, n = 22) and the "Normal E2" group was defined as those with E2 serum levels ≥10th percentile level (E2 ≥91.16 pg/mL, n = 195).
Intervention(s): None.
Main Outcome Measure(s): Pregnancy outcomes including rates of clinical pregnancy, clinical miscarriage, and live birth. Neonatal outcomes including gestational age at delivery, birth weight, and Apgar score.
Result(s): The mean ± SD estradiol level was 66.8 ± 14.8 pg/mL for the "Low E2" group compared with 366.3 ± 322.1 pg/mL for the "Normal E2" group. There were otherwise no substantial differences in cycle characteristics such as endometrial thickness on the day of ovulation trigger and progesterone levels in early pregnancy. The "Low E2" group had a significantly higher clinical miscarriage rate (36.4% vs. 8.8%, adjusted odds ratio 8.06) and lower live birth rate (31.8% vs. 57.9%, adjusted odds ratio 0.28). Neonatal outcomes such as gestational age at delivery, mean birth weight, Apgar scores, and incidence of newborn complications were not clinically different between the groups.
Conclusion: Low E2 levels were associated with a significantly higher miscarriage rate and lower live birth rate, suggesting that E2 levels in the follicular phase may have an effect on cycle outcomes. Given the rise in use of FET, further studies are needed to confirm our findings and understand the mechanisms. (Fertil Steril Rep® 2021;2:320–6. ©2021 by American Society for Reproductive Medicine.)
Key Words: Letrozole, endometrial preparation, frozen embryo transfer, estradiol, miscarriage, pregnancy outcomes

Letrozole is a third-generation aromatase inhibitor that reduces androgen conversion into estrogen, generating a negative central feedback on gonadotropin secretion and facilitating follicular development. Its first reported use in assisted reproduction was a 2001 study, which found that letrozole was effective for ovulation induction in anovulatory infertility and for increased follicle recruitment in ovulatory infertility (1). Since then, increasing evidence has proven its safety and efficacy in both ovulation induction and controlled ovarian hyperstimulation (2–5). This mounting evidence has extended to multiple subfertile and infertile populations, including patients with poor ovarian response (6–11), estrogen-sensitive cancers undergoing fertility preservation (12–15), polycystic ovary syndrome (16–21), endometriosis (22–24), unexplained infertility (25, 26), and recurrent implantation failure (27). These studies cite a variety of benefits,

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Reprint requests: Wendy Y. Zhang, M.D., Stanford University School of Medicine, Department of Obstetrics and Gynecology, Division of Reproductive Endocrinology and Infertility, 1195 W Fremont Avenue, Sunnyvale, California 94087 (E-mail: wendyz@stanford.edu).

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including increased implantation, pregnancy, and live birth rates as well as decreased multiple gestation rates, cycle cancellation, side effects, and overall treatment cost.

More recently, there has been growing evidence for the use of letrozole during endometrial preparation (28–33). Supraphysiologic serum levels of estradiol (E2) have been associated with decreased endometrial receptivity (34). Early letrozole controlled ovarian hyperstimulation studies demonstrated that letrozole attained more physiologic E2 serum levels and more favorable endometrial morphology (35). Contemporary studies that have directly examined the use of letrozole for endometrial preparation have similarly demonstrated that, among other effects of letrozole on the endometrium, letrozole-induced reduction in follicular phase E2 serum levels may improve endometrial receptivity and embryo implantation (23, 33, 36). However, the degree of E2 suppression by letrozole is variable among patients, and the impact of this reduced E2 level during endometrial preparation has yet to be studied. Thus, the goal of this pilot study was to assess the impact of low E2 levels in letrozole-stimulated frozen embryo transfer (LTZ-FET) cycles on pregnancy and neonatal outcomes.

MATERIALS AND METHODS

Patients

Our retrospective cohort study included all completed LTZ-FET cycles with autologous oocytes performed at Stanford Fertility and Reproductive Health Center from January 2017 to April 2020. We excluded cycles that included E2 supplementation to avoid confounding the outcomes with non-physiologic E2 levels (n = 53). Thus, our analysis included 177 women who underwent a total 217 LTZ-FET cycles. We defined the "Low E2" group as <10th percentile E2 serum level on the day of ovulation trigger within our cohort (E2 <91.16 pg/mL); the "Low E2" group contained 22 cycles and the "Normal E2" group contained 195 cycles. All demographic, fertility, pregnancy, and neonatal information were collected from the medical records. The demographics and clinical characteristics included the maternal age at FET, body mass index (BMI), gravidity and parity, number of prior miscarriages, number of prior embryo transfer cycles (fresh or frozen), number of embryos transferred for the current cycle, embryo grade, preimplantation genetic testing (PGT) use, smoking status, race/ethnicity, and infertility diagnosis. The Stanford University Institutional Review Board approved the study protocol.

LTZ-FET Treatment

The standard protocol for LTZ-FET started on cycle day 3 with the daily administration of letrozole (5 mg) for 5 days (30, 33). Patients underwent regular ultrasound monitoring until the dominant follicle was ≥18 mm or a positive luteinizing hormone (LH) surge was noted (defined as LH ≥20 mIU/mL), at which point E2, progesterone, and LH serum levels were collected. Ovulation was then triggered or boosted with recombinant human chorionic gonadotropin (hCG, 250 mcg Ovidrel; EMD Serono, Rockland, MA, USA). The FET proceeded only if the endometrial thickness was ≥7 mm, but if the current cycle was a personal best among a history of endometrial thicknesses below the threshold, exceptions were made to proceed; our study included only two such cases (5.6 mm and 6.5 mm), both of which were in the "Normal E2" cohort. Two days after ovulation, micronized progesterone (100 mg, Endometrin; Ferring Pharmaceuticals, Parsippany, NJ, USA) was given vaginally twice daily, and FET was performed 7 days after trigger or 6 days after the LH surge (37). The βhCG serum level was obtained approximately 9 days after FET, and clinical pregnancy was confirmed by the presence of fetal cardiac activity within the gestational sac on transvaginal ultrasound 6–8 weeks after FET. The E2, LH, progesterone, and βhCG serum levels were assayed with the Roche Cobas E411 analyzer (Roche Diagnostics, Santa Clara, CA, USA). Cancelled LTZ-FET cycles were not included.

All embryos transferred were blastocysts derived from autologous oocytes. Blastocysts were graded from AA to DD on the basis of the inner cell mass and trophectoderm morphology. If patients elected PGT of their embryos, biopsy was performed by pipette removal of 5–8 trophectoderm cells from day 5 or day 6 fully expanded blastocysts. Our clinic policy allows for biopsy of embryos with grade CC or higher for PGT.

Study Outcomes

The primary outcomes studied were clinical pregnancy (presence of fetal cardiac activity), clinical miscarriage (pregnancy loss before 20 weeks of gestation), and the live birth rate (live infant born after 24 weeks of gestation). Additional pregnancy outcomes examined were the rates of biochemical miscarriage, ectopic pregnancy, intrauterine fetal demise, cesarean delivery, and preterm delivery (<37 weeks). The secondary aim of our study was to examine neonatal outcomes, which included gestational age at delivery, birth weight, Apgar scores at 1 and 5 minutes, and incidence of newborn complication.

In addition to the E2 serum level on the day of trigger, other cycle characteristics collected were progesterone serum level at trigger, LH serum level at trigger, endometrial thickness at trigger, number of follicles >14 mm at trigger, and progesterone serum level at the first serum βhCG check.

Statistical Analysis

The study data were captured and managed in Stanford's REDCap electronic data tool (38), and the raw data were analyzed by biostatisticians who were not a part of the data collection.

Because of the lack of prior research on the impact of E2 on LTZ-FET outcomes, we defined our "Low E2" threshold of <10th percentile on the basis of the handful of prior studies that examined the effect of E2 on FET outcomes (39–41). Given the paucity of prior studies to inform the E2 thresholds, we performed a supplemental analysis of higher E2 cutoff points at the 25th and 50th percentiles to see if the trends found in our study persisted.

Patient and cycle characteristics for the "Low E2" and "Normal E2" groups were compared using absolute standardized differences (ASDs), which measure the difference in means or proportions between two groups in units of standard deviations (42). Absolute standardized differences values of 0.2, 0.5, and 0.8 correspond to small, moderate, and large differences, respectively. Multivariable logistic regression models were used to determine the differences in the pregnancy outcomes between the two groups while adjusting for maternal age at FET, BMI, number of previous miscarriages, embryo grade (categorized into AA, AB/BA, BB, and any C), endometrial thickness on the day of trigger, race/ethnicity, male factor infertility, and use of PGT. We used generalized estimating equations (GEEs) to account for the correlation between cycles per patient. We calculated adjusted odds ratios (aORs) and 95% confidence intervals (CIs) to evaluate the relative odds for live births, clinical pregnancies, and clinical miscarriages for the "Normal E2" group vs. the "Low E2" group. Additional pregnancy outcomes and neonatal outcomes were compared between the two groups using ASDs only.

Analyses were performed using the R statistical software version 3.6.2, and GEE analyses were performed using library geepack (43–46). All statistical tests were two-sided and performed at the .05 significance level.

RESULTS

Participant and Cycle Characteristics

Our pilot study included 177 women who underwent a total of 217 LTZ-FET cycles between January 2017 and April 2020. The 21 women in the "Low E2" group had 22 cycles, whereas the 156 women in the "Normal E2" group had 195 cycles. The "Low E2" group's mean age ± SD at FET was 37.2 ± 2.9 years, which was similar to the "Normal E2" group's mean age of 36.2 ± 4.0 years. The cohort's overall mean BMI was 24.1 kg/m^2. There were moderate differences between the two cohorts in the grade of embryos transferred, the participant's race/ethnicity, and the incidence of male factor infertility diagnoses. Otherwise, there were no substantial differences between the two groups with respect to BMI, gravidity and parity, history of prior miscarriages, number of prior embryo transfer cycles, number of embryos transferred, use of PGT, smoking status, and non-male factor infertility diagnoses (Table 1).

The mean E2 level at trigger was 66.8 ± 14.8 pg/mL in the "Low E2" group compared with 366.3 ± 322.1 pg/mL in the "Normal E2" group (Table 2). Other cycle characteristics on the day of ovulation trigger—which included progesterone and LH serum levels, endometrial thickness, and number of follicles >14 mm—were similar between the two groups. In addition, there were no substantial differences in the progesterone levels at the time that βhCG was collected 9 days after FET.

Pregnancy Outcomes

The clinical pregnancy rate was 50% for the "Low E2" group compared with 64.1% for the "Normal E2" group (ASD = 0.377). The "Low E2" group had a substantially higher rate of clinical miscarriage (36.4% vs. 8.8% for "Normal E2", ASD = 0.70) and a lower live birth rate (31.8% vs. 57.9% for "Normal E2", ASD = 0.54). The two groups had similar rates of biochemical miscarriage, ectopic pregnancy, intrauterine fetal demise, cesarean delivery, and preterm delivery (Table 2).

After implementing GEE in a multivariable logistic regression adjusting for maternal age at FET, BMI, number of prior miscarriages, embryo grade, endometrial thickness, race/ethnicity, male factor infertility, and use of PGT, the differences in pregnancy outcomes persisted (Table 3). The "Low E2" group had a significantly higher odds of clinical miscarriage (aOR 8.06, 95% CI 1.36, 47.61; $P = .021$) and significantly lower odds of live birth (aOR 0.28, 95% CI 0.10, 0.81; $P = .019$) compared with the "Normal E2" group (Table 3). In addition, regression models demonstrated that cycles with PGT use were less likely to result in clinical miscarriage, cycles that used embryos with at least one "C" grade were less likely to result in a clinical pregnancy compared with cycles with AA grade embryos, and thicker endometrial lining was associated with lower odds of clinical miscarriage.

In the secondary analyses with higher E2 cutoffs at the 25th and 50th percentiles, the "Low E2" group was still significantly associated with lower live birth rates, whereas the clinical miscarriage odds progressively decreased in association with the higher E2 thresholds and was not statistically significant at either cutoff (Supplemental Table 1, available online). Additionally, the pattern of lower clinical pregnancy rates with "Low E2" reached statistical significance at the 50th percentile E2 threshold (aOR 0.49, 95% CI 0.25, 0.93; $P = .029$) (Supplemental Table 1), suggesting that the data may be underpowered to detect differences in clinical pregnancy rates.

Neonatal Outcomes

The mean gestational age was 259.9 ± 5.4 days for the "Low E2" neonates compared with 253.1 ± 10.8 days for the "Normal E2" neonates (ASD = 0.80). This large ASD was mainly driven by the differences in standard deviations between the two distributions and unlikely to be clinically significant given the similar rates of prematurity between the two cohorts. There were no notable differences in the other neonatal outcomes: birthweight, Apgar score at 1 and 5 minutes, and newborn complications. The "Low E2" group had no newborn complications—likely because of the small sample size (n = 7 live births)—whereas the "Normal E2" group had five cases of complications (out of 103 live births): jaundice and urosepsis, ankyloglossia, neonatal intensive care unit admission for prematurity (born at 30 weeks), and two neonatal intensive care unit admissions for respiratory distress. It is unclear whether these complications were related to the FET process.

DISCUSSION

Our pilot study is the first to examine the impact of low E2 levels during LTZ-FET on pregnancy and neonatal outcomes. We found that "Low E2" pregnancies were eight times more likely to result in a miscarriage and 72% less likely to result

TABLE 1

Patient demographics and baseline clinical characteristics.

	Normal E2 ≥10th percentile	Low E2 < 10th percentile
Clinical characteristics for all cycles	**N = 195**	**N = 22**
Age at FET (years)	36.2 ± 4.0	37.2 ± 2.9
Maternal BMI (kg/m^2)	24.1 ± 5.1	23.5 ± 5.0
Gravidity	1.3 ± 1.3	0.9 ± 1.0
Parity	0.4 ± 0.7	0.4 ± 0.6
Nulliparous	134 (68.7)	15 (68.2)
Number of prior abortions	0.8 ± 1.1	0.6 ± 0.8
Number of prior embryo transfers cycles	0.7 ± 1.1	0.5 ± 0.9
Number of embryos transferred	1.1 ± 0.2	1.0 ± 0.0
Embryo grade		
AA	83 (42.6)	9 (40.9)
AB/BA	42 (21.5)	8 (36.4)
BB	39 (20.0)	3 (13.6)
Any C	17 (8.7)	2 (9.1)
Unknown	14 (7.2)	0 (0.0)
PGT used	143 (73.3)	18 (81.8)
Patient characteristics; not duplicated for patients with multiple cycles	**N = 156**	**N = 21**
Smoker		
Never	144 (92.3)	20 (95.2)
Former	11 (7.1)	1 (4.8)
Current	1 (0.6)	0 (0.0)
Race/Ethnicity		
Asian American	70 (44.9)	14 (66.7)
White	61 (39.1)	6 (28.6)
African American	2 (1.3)	0 (0.0)
Hispanic/Latino	12 (7.7)	0 (0.0)
Other	2 (1.3)	1 (4.8)
Unknown	9 (5.8)	0 (0.0)
Infertility Diagnosis		
Male factor	38 (24.4)	10 (47.6)
DOR	34 (21.8)	8 (38.1)
PCOS	24 (15.4)	1 (4.8)
Other ovulatory dysfunction	14 (9.0)	2 (9.5)
RPL	9 (5.8)	2 (9.5)
Endometriosis	7 (4.5)	2 (9.5)
Uterine/Tubal	16 (10.3)	2 (9.5)
Single gene disorder	6 (3.8)	0 (0.0)
Lesbian or single female	7 (4.5)	0 (0.0)
Unexplained	34 (21.8)	4 (19.0)
Other	5 (3.2)	1 (4.8)

Note: Data are presented as mean ± SD or number (percentage). BMI = body mass index; DOR = diminished ovarian reserve; E2 = estradiol; FET = frozen embryo transfer; PCOS = polycystic ovary syndrome; PGT = preimplantation genetic testing; RPL = recurrent pregnancy loss.

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in a live birth compared with "Normal E2" cycles. Once live birth was achieved, however, the "Low E2" neonates had no substantial differences in adverse outcomes, with similar rates of prematurity, mean birthweights, Apgar scores, and incidence of complications. The findings from our study could prove potentially valuable in guiding both patient-counseling and clinical decision-making. Our study suggests that clinicians need to consider E2 levels during LTZ endometrial preparation when deciding whether to proceed with FET.

Despite the increased use of LTZ in assisted reproduction, most studies have examined its use in ovulation induction and controlled ovarian hyperstimulation. There are only a few studies that have investigated the use of LTZ for endometrial preparation before FET, and none have examined the effect of E2 suppression during such preparation. Furthermore, a recent 2020 meta-analysis by Chen et al. (47) demonstrated that all LTZ-FET studies were conducted outside the United States, which arguably limits the applicability of such data to patients seeking treatment within the United States. In the largest LTZ-FET study, Tatsumi et al. (32) in 2017 compared letrozole with natural and hormone replacement treatment FET cycles; they found that letrozole use improved the clinical pregnancy and live birth rates as well as reduced the miscarriage rates compared with the natural and hormone replacement treatment groups. However, their study did not account for multiple characteristics that could have significantly impacted their outcomes, including the cause of infertility, prior pregnancy history, embryo quality, duration of letrozole intake, and cycle characteristics such as E2 and progesterone serum levels. Additionally, a significant portion

TABLE 2

Comparison of cycle characteristics, pregnancy outcomes, and neonatal outcomes between the "Normal E" and "Low E" groups.

	Normal E2 N = 195	Low E2 N = 22	ASD[a]
Cycle characteristics			
Peak E2 level at trigger (pg/mL)	366.3 ± 322.1	66.8 ± 14.8	1.31
Progesterone level at trigger (ng/mL)	0.53 ± 0.38	0.55 ± 0.44	0.05
Progesterone level at first βhCG check (ng/mL)	39.6 ± 15.2	35.7 ± 17.9	0.24
LH level at trigger (mIU/mL)	20.8 ± 19.7	15.2 ± 14.4	0.32
Endometrial thickness (mm)	8.9 ± 1.4	8.5 ± 1.2	0.29
Number of follicles >14 mm	1.5 ± 0.9	1.3 ± 0.7	0.22
Pregnancy outcomes			
Clinical pregnancy	125 (64.1)	11 (50.0)	0.29
Biochemical miscarriage	20 (13.4)	2 (15.4)	0.06
Ectopic pregnancy	4 (2.7)	0 (0.0)	0.24
Clinical miscarriage	11 (8.8)	4 (36.4)	0.70
Intrauterine fetal demise	1 (0.9)	0 (0.0)	0.13
Live birth	113 (57.9)	7 (31.8)	0.54
Cesarean delivery	61 (48.8)	7 (636)	0.12
Preterm delivery	7 (6.7)	0 (0.0)	0.38
Neonatal outcomes			
Gestational age at delivery (d)	253.1 ± 10.8	259.9 ± 5.4	0.80
Birth weight (grams)	3257.7 ± 520.4	3272.7 ± 301.3	0.04
Apgar score at 1 min	8.1 ± 1.0	7.8 ± 1.0	0.21
Apgar score at 5 mins	8.9 ± 0.4	9.00 ± 0.0	0.33
Child's Sex			0.17
Ambiguous	1 (1.0)	0 (0.0)	
Female	39 (37.9)	3 (42.9)	
Male	63 (61.2)	4 (57.1)	
Newborn complications	5 (5.0)	0 (0.0)	0.32

Note: Data are presented as mean ± SD or number (percentage). ASD = absolute standardized difference; βhCG = β-human chorionic gonadotropin; E2 = estradiol; LH = luteinizing hormone.
[a] Absolute Standardized Difference: 0.2 = small difference; 0.5 = medium difference; 0.8+ = large difference.

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TABLE 3

Adjusted odds ratios (aORs) for "Low E2" pregnancy outcomes.

	Adjusted odds ratio (aOR)[a]	95% CI	*P*-value
Pregnancy outcomes: "Low E2" defined as < 10th percentile (n = 22)			
Clinical pregnancy	0.52	(0.18, 1.51)	.23
Clinical miscarriage	8.06	(1.36, 47.61)	.021
Live birth	0.28	(0.10, 0.81)	.019

Note: aOR = adjusted odds ratio; CI = confidence interval; E2 = estradiol.
[a] Primary pregnancy outcomes were adjusted for the following confounders: maternal age at frozen embryo transfer, maternal body mass index, number of previous miscarriages, embryo grade (categorized into AA, AB/BA, BB, and any C), endometrial thickness on the day of trigger, race/ethnicity, male factor infertility, and use of preimplantation genetic testing.

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of the embryos transferred were in the cleavage stage. Similarly, in the most recent LTZ-FET study by Zhang et al. (30) in 2019, most of their FET cycles used cleavage-stage embryos, which limits the applicability to the current clinical settings in the United States, where most transfers are of blastocyst-stage embryos.

Nevertheless, studies generally favor the use of LTZ during endometrial preparation, indicating that it achieves an endometrial hormonal profile that is similar to that of normal spontaneous ovulation (28–33). This is hypothesized to be accomplished through LTZ's reduction of estrogen serum levels during the early follicular phase, which subsequently upregulates endometrial estrogen receptors, increases endometrial sensitivity to estrogen increase, and prevents premature progesterone action; this results in enhanced endometrial proliferation (23, 33, 34, 36). In addition, letrozole has been associated with increased integrin expression in the mid-secretory endometrium, which may improve endometrial receptivity (23, 48).

However, given that our understanding of endometrial preparation with LTZ is in its early stages, it stands to reason that letrozole's inhibition of estrogen biosynthesis could have negative consequences on cycle outcomes. In the follicular phase of spontaneous cycles, physiologic E2 serum levels usually reach 250–400 pg/mL (49, 50), but it is our clinical experience that patients have quite variable responses to

LTZ and that their levels often fall below these values. In one of the earliest LTZ-FET studies, Hu et al. (33) noted that the lowest E2 level required for successful pregnancy in their group of patients was 431 pmol/L (117 pg/mL). Under the same letrozole protocol as our study, their cohort (n = 40) had a mean E2 level at trigger of 1,806.3 pmol/L (492 pg/mL). However, 26 out of the 40 letrozole cycles had additional human menopausal gonadotropin stimulation, resulting in a higher mean number of mature follicles than that in our study. Subsequent LTZ-FET studies did not report follicular E2 levels.

Thus, our cohort study is unique in several aspects. To our knowledge, it is the first study in the United States to examine the clinical outcomes of LTZ-FET, and the first study worldwide to examine the impact of preovulatory E2 during such a protocol. We studied all consecutive, completed LTZ-FET cycles since the protocol was first initiated at our academic center in 2017 to minimize selection bias. Although most LTZ-FET studies have limited their study populations to polycystic ovary syndrome or other ovulatory dysfunctions, we included all infertility diagnoses to improve generalizability. Additionally, all the embryos in our study were blastocysts to reflect the current practice models in the United States. Last, we accounted for a key factor that could further improve generalizability and significantly influence pregnancy and neonatal outcomes: the use of PGT. Preimplantation genetic testing use has been rapidly on the rise and is now used in >40% of all cycles in the United States (51, 52), which highlights the importance of accounting for PGT use when investigating pregnancy and neonatal outcomes (53–56).

The main limitation of our study was the sample size, given that our participant pool was from a single academic center. As evident in the supplemental analysis, the study was potentially underpowered to detect significant differences in outcomes such as clinical pregnancy rates until the "Low E2" sample size increased with a higher E2 cutoff. The lack of a unified medical record system in the United States poses a significant challenge to accessing detailed pregnancy and neonatal medical records for a larger cohort of women who have undergone LTZ-FETs (57). Most of the patients in our cohort were Asian and Caucasian, potentially limiting the generalizability to other ethnic groups. Thus, further studies are needed to confirm our findings and to understand the mechanisms behind these differences in outcomes.

CONCLUSION

In conclusion, low E2 levels during LTZ-FET were associated with a statistically significant increase in the odds of clinical miscarriage and a decrease in the odds of live birth. However, after live birth was achieved, low E2 levels were not associated with adverse neonatal outcomes. This study not only provides emerging data that may guide clinical decision-making, but also highlights the need to further investigate the impact that the follicular E2 changes due to letrozole have on pregnancy outcomes.

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