Human Reproduction, Vol.36, No.3, pp. 683–692, 2021
Advance Access Publication on December 19, 2020 doi:10.1093/humrep/deaa322

human reproduction

ORIGINAL ARTICLE *Infertility*

Impact of low serum progesterone levels on the day of embryo transfer on pregnancy outcome: a prospective cohort study in artificial cycles with vaginal progesterone

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Submitted on March 9, 2020; resubmitted on October 14, 2020; editorial decision on October 29, 2020

STUDY QUESTION: Is there a serum progesterone (P) threshold on the day of embryo transfer (ET) in artificial endometrium preparation cycles below which the chances of ongoing pregnancy are reduced?

SUMMARY ANSWER: Serum P levels <8.8 ng/ml on the day of ET lower ongoing pregnancy rate (OPR) in both own or donated oocyte cycles.

WHAT IS KNOWN ALREADY: We previously found that serum P levels <9.2 ng/ml on the day of ET significantly decrease OPR in a sample of 211 oocyte donation recipients. Here, we assessed whether these results are applicable to all infertile patients under an artificial endometrial preparation cycle, regardless of the oocyte origin.

STUDY DESIGN, SIZE, DURATION: This prospective cohort study was performed between September 2017 and November 2018 and enrolled 1205 patients scheduled for ET after an artificial endometrial preparation cycle with estradiol valerate and micronized vaginal P (MVP, 400 mg twice daily).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Patients ≤50 years old with a triple-layer endometrium ≥6.5 mm underwent transfer of one or two blastocysts. A total of 1150 patients treated with own oocytes without preimplantation genetic testing for aneuploidies (PGT-A) (n = 184), own oocytes with PGT-A (n = 308) or donated oocytes (n = 658) were analyzed. The primary endpoint was the OPR beyond pregnancy week 12 based on serum P levels measured immediately before ET.

MAIN RESULTS AND THE ROLE OF CHANCE: Women with serum P levels <8.8 ng/ml (30th percentile) had a significantly lower OPR (36.6% vs 54.4%) and live birth rate (35.5% vs 52.0%) than the rest of the patients. Multivariate logistic regression showed that serum P < 8.8 ng/ml was an independent factor influencing OPR in the overall population and in the three treatment groups. A significant negative correlation was observed between serum P levels and BMI, weight and time between the last P dose and blood tests and a positive correlation was found with age, height and number of days on HRT. Multivariate logistic regression showed that only body weight was an independent factor for presenting serum P levels <8.8 ng/ml. Obstetrical and perinatal outcomes did not differ in patients with ongoing pregnancy regardless of serum P levels being above/below 8.8 ng/ml.

LIMITATIONS, REASONS FOR CAUTION: Only women with MVP were included. Extrapolation to other P administration forms needs to be validated.

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WIDER IMPLICATIONS OF THE FINDINGS: This study identified the threshold of serum P as 8.8 ng/ml on the day of ET for artificial endometrial preparation cycles necessary to optimize outcomes, in cycles with own or donated oocytes. One-third of patients receiving MVP show inadequate levels of serum P that, in turn, impact the success of the ART cycle. Monitoring P levels in the mid-luteal phase is recommended when using MVP to adjust the doses according to the needs of the patient.

STUDY FUNDING/COMPETING INTEREST(S): None.

TRIAL REGISTRATION NUMBER: NCT03272412.

Key words: artificial cycle / serum progesterone / hormonal replacement therapy / pregnancy outcome / luteal phase support

Introduction

Artificial endometrial preparation with HRT is frequently used for frozen embryo transfer (FET) and egg donation cycles (Groenewoud *et al.*, 2018). However, there is insufficient evidence to recommend any one protocol for endometrial preparation (Ghobara *et al.*, 2017) and there are no clear guidelines for HRT. While estrogen administration is tailored to patient need (Labarta *et al.*, 2017), the same progesterone (P) dose is given to all patients without individualization of luteal phase support (LPS) since an optimal P exposure before embryo transfer (ET) has not been well established (van de Vijver *et al.*, 2016). P doses and administration forms (i.e. vaginal or subcutaneous) are instead chosen based on patient and doctor preferences (Vaisbuch *et al.*, 2014).

There is likely a minimum serum P concentration below which ET success rates are significantly lower when using natural micronized vaginal P (MVP) in artificial endometrial preparation cycles. Most studies on the relation between serum P and pregnancy outcome are retrospective (Yovich *et al.*, 2015; Alsbjerg *et al.*, 2018; Cédrin-Durnerin *et al.*, 2019; Gaggiotti-Marre *et al.*, 2019) except one published by our group (Labarta *et al.*, 2017), which analyzed the relationship between serum P levels on the day of ET and ongoing pregnancy rate (OPR). Patients with serum $P < 9.2$ ng/ml, which corresponded to the 25th percentile, had a 20% lower OPR than those with higher values ($P < 0.05$) in an oocyte donation setting. Despite all patients having received natural MVP at 400 mg twice daily, one in every four patients had inadequate serum P levels.

We sought to determine whether these findings could be extrapolated to the infertile population undergoing artificial cycles for ET, including treatments with own or donated eggs. Moreover, we utilized a larger sample size to define the critical threshold of serum P on the day of ET in HRT cycles that significantly alters the OPR and live birth rates (LBRs). We also identified intrinsic factors that may be predictive of having low serum P levels; finally, we determined whether these low levels influence obstetric or neonatal outcomes.

Materials and methods

Design and setting

This prospective cohort study is registered in clinicaltrials.gov (NCT03272412) and was approved by the Institutional Review Board of IVI RMA Valencia, Spain. The study was conducted at IVI RMA Valencia between September 2017 and November 2018.

Study population

The study enrolled 1205 infertile patients scheduled for ET under an artificial endometrial preparation treatment with HRT, of which 1150 were ultimately included. Among these, 658 cases (57.2%) were oocyte donation cycles, whereas 492 (42.8%) were own oocyte cycles with 308 preimplantation genetic testing for aneuploidies (PGT-A) treatments and 184 regular FET. Participating women were ≤50 years old with adequate endometrial pattern (triple layer) and thickness (≥6.5 mm) after estrogen treatment in the proliferative phase and LPS with only MVP (400 mg twice daily for 5 days) before ET. One or two blastocysts were transferred. We wanted to reflect the broad range of patients encountered in clinical practice, so all patients meeting eligibility criteria for ET were included in the study. Patients with uterine or adnexal anomalies did not undergo ET according to our protocol, so these patients were not recruited for the study.

Endpoints

The primary endpoint was the OPR in patients who underwent ET after endometrial preparation artificial cycles based on serum P levels measured on the day of ET. Secondary endpoints were (i) the critical threshold of serum P on the day of ET below which pregnancy rates were significantly lower, (ii) pregnancy outcomes according to the threshold, (iii) factors that determine serum P levels and (iv) the relationship between serum P levels and obstetric outcomes.

Pregnancy outcome was determined by a positive β-hCG test (serum levels of β-hCG > 10 IU/ml 11 days after ET); clinical pregnancy was defined as the presence of at least one gestational sac on ultrasound; implantation was defined as the presence of a gestational sac per embryo transferred; miscarriage rate was defined as any pregnancy loss before Week 12, including biochemical miscarriage with a positive β-hCG test without evidence of a gestational sac and clinical miscarriage after confirmation of an intrauterine gestational sac; ectopic pregnancy was defined as a gestational sac located outside the uterine cavity; OPR was defined as the presence of at least one viable fetus beyond Week 12; and LBR was defined as the number of deliveries that resulted in at least one live born neonate.

Sample size

Based on our previously published results, it was estimated that 25% of the population could show inadequate serum P levels on the day of ET (Labarta *et al.*, 2017). The study population was calculated to detect a 10% difference in OPR between two groups according to serum P levels (expected to be 40% in the suboptimal serum P group and 50% in the optimal serum P group). By accepting an alpha risk of 0.05 (95% CI) and a beta risk of 0.2 (80% statistical power) in a two-sided test, and a ratio of 3:1 between groups (serum P levels >p25 vs ≤p25), 1050 patients were needed to determine a statistically

significant difference. A drop-out rate of 15% was anticipated, so 1205 patients were needed. The ARCSINUS approximation was used for this calculation (Casagrande *et al.*, 1978).

Study protocol

Endometrial preparation

Only patients who underwent egg donation cycles using fresh embryos were given a GnRH agonist (Decapeptyl® 3.75 mg IM, single dose, Ipsen Pharma, Barcelona, Spain) administered in the mid-luteal phase of the previous menstrual cycle, or a GnRH antagonist (0.25 mg/day) for 5 days from the first day of menstruation (Orgalutran® 0.25 mg/0.5 ml SC, single dose, Merck Sharp & Dohme, Madrid, Spain) (Vidal *et al.*, 2018). After transvaginal ultrasound to confirm ovarian quiescence, estrogen treatment commenced on Days 2–3 of menstruation. Estradiol was administered orally at either 6 mg/day of estradiol valerate (Progynova®, Bayer Hispania, Barcelona, Spain; Meriestra®, Novartis, Barcelona, Spain) or transdermally with two patches of 75 µg estradiol hemihydrate (Evopad®, Janssen Cilag, Madrid, Spain) every 48 h. After 10–14 days on estrogens, a vaginal two-dimensional (2D) ultrasound was performed to measure endometrial thickness (EMT) and to confirm a triple-layer pattern, and a blood sample was drawn for estradiol (E2) and P determinations to ensure that no spontaneous ovulation had occurred. If EMT was ≥6.5 mm, the endometrial pattern was trilaminar, and serum P < 1.0 ng/ml, ET was scheduled. LPS began 5 days before ET with MVP at a dose of 400 mg twice daily (Utrogestan®, SEID, Barcelona, Spain or Progeffik®, Effik, Madrid, Spain). The last dose before ET (10th dose) was administered on the morning of ET. If pregnancy occurred, hormone treatment was maintained until pregnancy Week 12 in accordance with routine practice.

Selecting patients

All eligible patients at our clinic were offered participation in the study and those interested provided written informed consent. A blood test was performed 1–2 h before ET to determine serum E2 and P levels.

Hormone measurement

Hormone measurements taken the day of ET (E2 and P) were blinded to the doctor, embryologist, and patient and the results were not available until the end of the study. Blood samples were analyzed by an electrochemiluminescence immunoassay (Cobas® e411 analyzer, Roche diagnostics GmbH, Germany). Intra- and inter-assay coefficients of variation for the P determinations were 1.2–11.8% and 3.6–23.1%, respectively, for *P*-values between 0.22 and 51.6 ng/ml. Sensitivity was 0.03 ng/ml. The intra- and inter-assay coefficients of variation for E2 determinations were 2.4–9.5% and 2.5–11.9%, with a measurement range of 25.4–2932 pg/ml. Sensitivity was 5 pg/ml.

IVF laboratory

Intracytoplasmic sperm injection was used in all cases. Either fresh or vitrified oocytes were used in oocyte donation cycles since there are no differences in pregnancy rates between them (Cobo *et al.*, 2008; Rienzi *et al.*, 2010). Likewise, ET was performed with fresh or thawed blastocysts.

Embryo quality was classified according to the Spanish ASEBIR (Asociación para el estudio de la biología de la reproducción) classification (Pons, 2015) and only embryos graded A to C were transferred. All ETs were performed by senior gynecologists under transabdominal ultrasound guidance.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics v25 software (SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as mean and SD, whereas categorical variables were expressed as percentages. Patients were classified into 10 groups according to the deciles of serum P. OPR was calculated in each group to detect a critical threshold related to the probability of OPR. Categorical variables were compared with a χ^2 test, and a Student's *t*-test was used to compare the continuous variables between the two groups defined by the threshold.

To analyze the influence of patient characteristics on serum P on the day of ET, multivariate logistic regression analysis was performed. Variables that were correlated in a univariable analysis with serum P levels were included (age, height, weight, BMI, time frame between the last dose of MVP and blood tests and days on HRT before ET). Similarly, to analyze the net impact of low serum P on OPR, a multivariate logistic regression analysis was conducted and adjusted for the following variables: age, weight, height, serum E2 in the proliferative phase, EMT, number of embryos transferred in previous cycles, number of embryos transferred in the current cycle, quality of the transferred embryo, day of blastocyst development (Day 5 or 6) and donor age in oocyte donation cycles. All variables were considered potential confounding when the *P*-value was <0.2 in univariate analysis. Multivariate logistic regression analysis was performed both in the general population and in each of the three groups according to the type of treatment (own oocytes without PGT-A, own oocytes with PGT-A and donated oocytes).

To evaluate the predictive capability of serum P on OPR, the receiving operating characteristic (ROC) curve was described and the AUC was calculated. The optimal threshold was defined according to sensitivity and specificity to predict OPR.

Results

Descriptive analysis

Of the 1205 eligible patients, 1150 were analyzed. The reasons for exclusion were withdrawal (n = 20), change in the exogenous P dose (n = 25) and failure to undergo blood testing (n = 10). This meant that 55 patients (4.6%) could not be included in the analysis.

The mean overall population age was 39.6 ± 4.6 years, mean BMI was 23.7 ± 4.2 kg/m^2 and mean EMT was 8.8 ± 1.5 mm when receiving estrogen therapy. A mean of 1.1 ± 0.3 blastocysts were transferred (88% were single ETs). Ongoing pregnancy outcome was reported in 1148 patients while live birth outcome was available for 1125 patients.

The overall OPR was 49.0% (95% CI: 46.2–51.9) and the LBR was 47.0% (95% CI: 44.1–49.9).

Clinical outcome according to serum P on the day of embryo transfer

The mean serum P level on the day of ET was 12.1 ± 7.0 ng/ml. Patients were divided in 10 groups by deciles of serum P and OPR

was calculated in each group (Fig. 1). Additional results of positive beta-hCG, LBRs and miscarriage rates according to the deciles of serum P are shown in Supplementary Fig. S1.

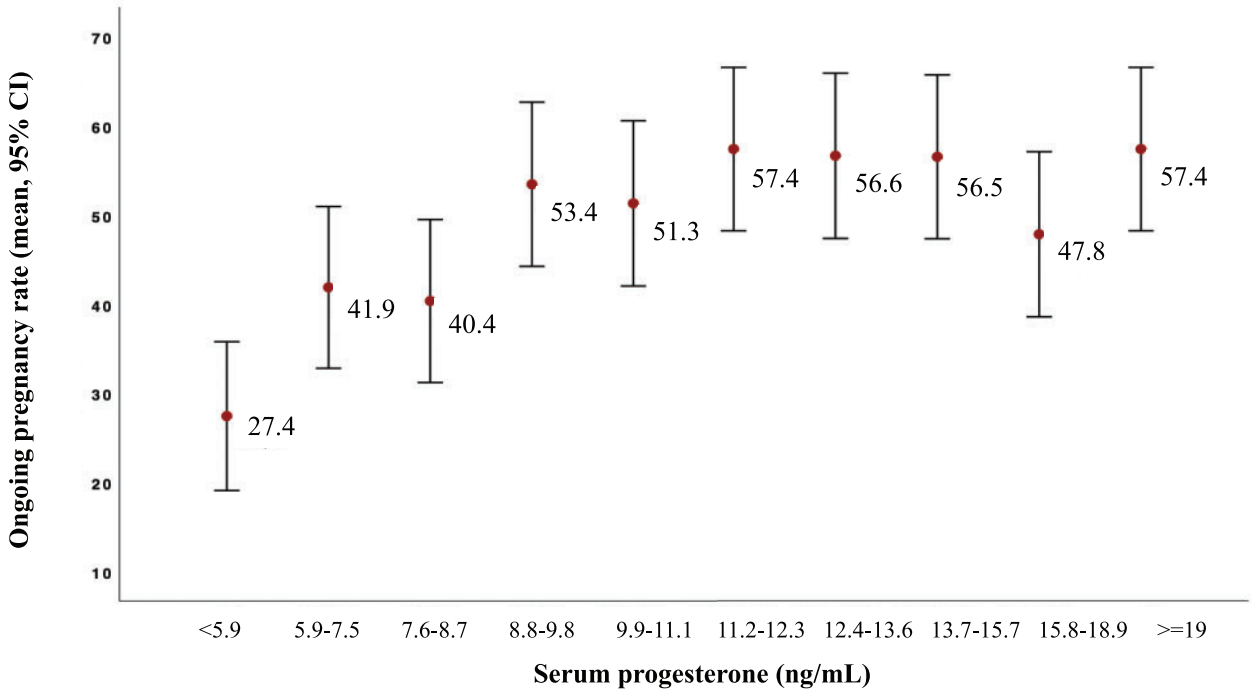
Considering the lower limit of the 95% CI of the overall OPR (46.2%) as a fair rate, a critical cutoff of 8.8 ng/ml was observed. This cutoff corresponded to the 30th percentile. Table I shows the baseline clinical data and results in patients whose serum P levels fell below or above 8.8 ng/ml, according to the type of treatment. Overall, patients with serum P levels <8.8 ng/ml yielded a significantly lower OPR of 36.6% vs 54.4%; crude odds ratio (OR), 95% CI: 0.49 (0.35–0.63); $P < 0.001$; lower LBR of 35.5% vs 52.0%; OR (95% CI): 0.51 (0.39–0.66); $P < 0.001$ and higher clinical miscarriage rate at 13.5% vs 23%; OR (95% CI): 1.9 (1.2–2.9); $P = 0.006$) (Fig. 2). Crude OR for comparison of OPRs reached statistical significance in the group with own oocytes with PGT-A and in the group with donated oocytes, as shown in Fig. 3.

Multivariate logistic regression showed that serum P below 8.8 ng/ml remained an independent factor for decreasing OPRs in the three different populations after adjusting for all confounding variables (Table II). The resulting adjusted OR (95% CI) for ongoing pregnancy was 0.49 (0.37–0.64; $P < 0.001$) and for live birth 0.52 (0.40–0.69; $P < 0.001$) for patients with serum P levels <8.8 ng/ml on the day of ET.

Exploratory analysis of factors related to serum P levels on the day of embryo transfer

Correlation analysis identified which variables could impact serum P levels. Pearson's coefficient (r) showed a significant association ($P < 0.05$) of serum P levels with BMI ($r = -0.16$), weight ($r = -0.13$), time between last dose of MVP and blood tests ($r = -0.07$), age ($r = 0.07$), height ($r = 0.07$) and days on HRT until ET ($r = 0.08$). Among them, multivariate logistic regression showed that only body weight remained statistically significant for presenting serum P levels below the critical threshold ($P = 0.02$). Patients with a serum P level of <8.8 ng/ml had a significantly higher body weight than the rest (65.9 vs 63.4 kg, $P = 0.001$). The mean time between the last dose of P and blood sample was 341 ± 140 min. Mean serum P was 12.9 ± 8.6 ng/ml vs 11.5 ± 5.4 ng/ml when the last dose of P was within below or above 6 h, respectively ($P = 0.007$). This difference did not affect OPR; 50.3% vs 44.3%, $P = 0.08$.

The ROC curve showed a significant predictive value of serum P levels on the day of ET for both OPR and LBR, with an AUC (95% CI) of 0.58 (0.55–0.62) for both outcomes ($P < 0.001$). The serum P threshold of 8.8 ng/ml offered a sensitivity of 77.6% for OPR,



Percentiles	p10	p20	p30	p40	p50	p60	p70	p80	p90
Progesterone	5.9	7.6	8.8	9.9	11.2	12.4	13.7	15.8	19

Figure 1. Ongoing pregnancy rate according to the deciles of serum progesterone (P) on the day of embryo transfer. Data are expressed as mean, 95% CI.

Table I Comparison of baseline clinical characteristics based on serum P level on the day of embryo transfer below or above 8.8 ng/ml, denoted as the critical threshold.

	Own oocytes no PGT-A (n = 184)			Own oocytes with PGT-A (n = 308)			Oocyte donation (n = 658)		
	P < 8.8 ng/ml	P ≥ 8.8 ng/ml	*P*	P < 8.8 ng/ml	P ≥ 8.8 ng/ml	*P*	P < 8.8 ng/ml	P ≥ 8.8 ng/ml	*P*
Number (%) of patients	57 (31%)	127 (69%)		99 (32.1%)	209 (67.9%)		188 (28.6%)	470 (71.4%)	
Age	35.6 ± 3.3	35.1 ± 3.3	0.290	37.4 ± 3.6	37.9 ± 3.4	0.268	41.5 ± 4.5	41.7 ± 3.9	0.603
BMI	24.2 ± 3.4	23.0 ± 3.5	**0.034**	24.5 ± 5.0	23.1 ± 3.7	**0.014**	24.5 ± 4.8	23.6 ± 4.2	**0.019**
Weight (kg)	65.5 ± 9.9	62.8 ± 11.1	0.114	66.7 ± 13.7	62.8 ± 11.4	**0.018**	65.7 ± 13.1	63.8 ± 11.4	0.096
Height (m)	1.64 ± 0.1	1.64 ± 0.1	0.543	1.64 ± 0.1	1.64 ± 0.1	0.844	1.63 ± 0.1	1.64 ± 0.1	0.133
Proliferative phase									
Serum E2 (pg/ml)	236 ± 156	230 ± 168	0.809	220 ± 149	273 ± 315	0.115	271 ± 185	291 ± 283	0.371
Serum P (ng/ml)	0.12 ± 0.17	0.13 ± 0.17	0.828	0.09 ± 0.11	0.15 ± 0.17	**<0.001**	0.21 ± 0.23	0.19 ± 0.20	0.317
Endometrial thickness (mm)	9.0 ± 1.8	9.0 ± 1.5	0.996	8.9 ± 1.5	8.8 ± 1.6	0.324	8.6 ± 1.5	8.8 ± 1.6	0.117
Luteal phase									
Serum E2 (pg/ml)	257 ± 134	232 ± 145	0.279	252 ± 215	237 ± 173	0.521	224 ± 118	250 ± 181	**0.034**
Serum P (ng/ml)	6.5 ± 1.5	13.8 ± 5.4	**<0.001**	6.5 ± 1.9	14.3 ± 10.0	**<0.001**	6.4 ± 1.9	14.8 ± 5.7	**<0.001**
Days on HRT until ET	16.7 ± 3.4	16.6 ± 3.1	0.908	16.6 ± 3.5	16.9 ± 3.2	0.531	18.4 ± 4.2	18.9 ± 4.4	0.124
Time between last P dose and blood test (min)	334 ± 160	346 ± 120	0.624	346 ± 115	336 ± 158	0.732	349 ± 158	336 ± 132	0.354
No. embryos transferred	1.3 ± 0.4	1.2 ± 0.4	0.315	1.1 ± 0.3	1.1 ± 0.3	0.761	1.1 ± 0.3	1.1 ± 0.3	0.665
Proportion of single embryo transfer (% SET)	75.4%	81.9%	0.325	91.8%	92.8%	0.817	86.6%	87.9%	0.695
No. previous embryos transferred	1.6 ± 2.1	1.3 ± 1.7	0.299	0.9 ± 1.5	0.6 ± 1.3	0.064	2.2 ± 3.4	1.4 ± 2.2	**0.004**
Embryo quality of the best embryo transferred									
A (%)	9.8	10.7	0.709	3.9	3.7	0.943	13.8	23.1	**0.008**
B (%)	56.1	62.1		68.8	71.0		63.1	62.3	
C (%)	34.1	27.2		27.3	25.3		22.5	14.6	
Gestational outcome (%)									
Positive beta-hCG test (>10 IU/l)	52.6	70.1	**0.030**	52.5	68.4	**0.008**	56.4	68.2	**0.005**
Implantation	44.7	59.4	0.076	42.9	60.0	**0.005**	43.0	53.7	**0.001**
Clinical pregnancy	43.9	61.4	**0.036**	42.4	61.2	**0.002**	50.0	62.0	**0.007**
Ongoing pregnancy	38.6	53.5	0.079	34.3	54.1	**0.001**	37.2	54.7	**<0.001**
Live birth	36.8	51.2	0.080	34.3	53.1	**0.002**	35.7	51.8	**<0.001**
Biochemical miscarriage	16.7	12.4	0.546	19.2	10.5	0.144	11.3	8.5	0.437
Clinical miscarriage	16.0	15.4	1.000	19.0	11.7	0.297	26.6	13.8	**0.007**

Data are expressed as mean ± SD or n (%). Bold entries mean that the *P*-value was < 0.05, which is statistically significant.
PGT-A, preimplantation genetic testing for aneuploidies; E2, estradiol; P, progesterone; ET, embryo transfer.

whereas specificity was 37.1%. The optimal serum P threshold at which sensitivity and specificity for OPR were both >50% was 10.4 ng/ml (63.6% sensitivity, 50.1% specificity).

Obstetrical and perinatal outcomes according to serum P below or above 8.8 ng/ml on the day of embryo transfer

A total of 555 pregnant women remained pregnant beyond Week 20. Of them, 529 had a confirmed live birth; there were two stillbirths and one perinatal death; and no birth was confirmed for the remaining 23. Serum P levels below or above 8.8 ng/ml on the day of ET did not influence obstetrical or perinatal outcomes in the overall population; e.g. percentages of term deliveries (86.1% vs 85.4%), normal birth weight (83.6% vs 91.3%), pregnancy-associated hypertension (12.9% vs 9%), gestational diabetes (8.1% vs 7.4%), risk of preterm labor (4.8% vs 4.9%) and bleeding during the first (32.3% vs 24.6%) or second/third trimester (2.4% vs 2.8%). Notably, serum P levels <8.8 ng/ml trended toward higher risk of pregnancy-associated hypertension in oocyte donation cycles but did not reach statistical significance (15.7% vs 8.2%, $P = 0.07$). This was not seen in treatments with own oocytes (8.9% vs 9.9%, $P = 1.00$).

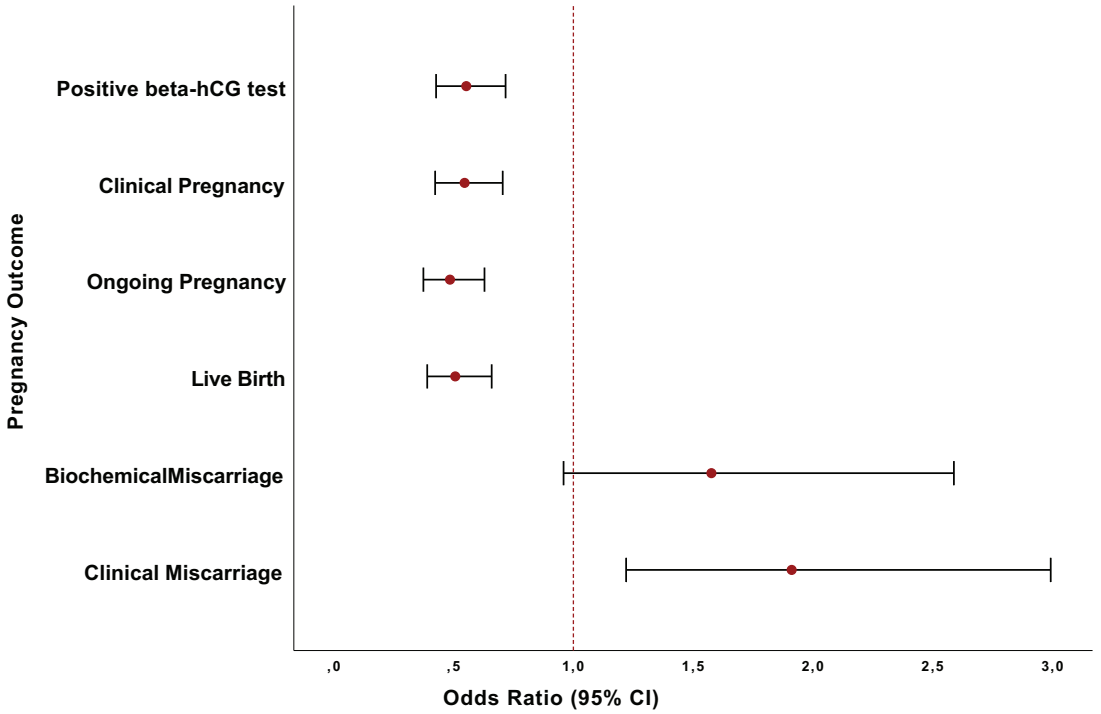


Figure 2. Crude odds ratios for the clinical outcomes in patients with serum P levels <8.8 ng/ml on the day of embryo transfer compared to patients with higher levels. All differences were statistically significant except for biochemical miscarriage.

Discussion

This prospective study aimed to analyze the relationship between serum P levels on the day of ET in artificial cycles and pregnancy outcomes. Our results confirm that low serum P levels on the day of ET lead to worse pregnancy outcomes. In fact, regardless of the type of treatment (own oocytes with or without PGT-A and oocyte donation), the impact of serum P on the day of ET is present after adjusting for all possible confounding factors. Two important messages can be obtained from this study. First, all patients who receive MVP in HRT cycles need to reach a minimum of 8.8 ng/ml circulating P to maintain pregnancy, regardless of the origin of the oocytes. Second, 30% of patients receiving MVP at a dose of 400 mg twice daily are below the optimal P level.

This study allowed us to better define the critical threshold of serum P values needed in artificial endometrial preparation cycles to maintain OPRs above 45%. The threshold hereby defined (8.8 ng/ml) is slightly different to the one described in our previous study (9.2 ng/ml) (Labarta *et al.*, 2017). The goal of the present study was to better define this threshold; for that purpose, a much larger population (1150 in the current study vs 211 in the previous one) of every-day patients was included.

Our research focused on women using natural MVP for LPS. Thus, applicability to other forms of P administration needs to be validated. In fact, information about the percentage of patients with inadequate serum P levels using other administration routes (e.g. rectal, oral, subcutaneous or intramuscular) is lacking. Serum P can be measured only when using natural-like progestogens, such as MVP. Indeed, synthetic progestogens like dydrogesterone make P measurements futile because the molecule completely differs and will not be detectable by this test (Griesinger *et al.*, 2019).

Serum P levels vastly differ when using MVP compared to subcutaneous or intramuscular P, due to different pharmacokinetics (PK) and pharmacodynamics (PD) of the distinct compounds (Miles *et al.*, 1994). Compared to injected P, MVP leads to lower serum P levels and higher intrauterine P levels due to the first uterine pass effect (Bulletti *et al.*, 1997). Moreover, steady levels are more stable when using vaginal P (Duijkers *et al.*, 2018), which facilitates its measurement and interpretation. Although no direct correlation between uterine and serum P levels have been found in PK and PD studies (Paulson *et al.*, 2014), current evidence showing an association between serum P levels and OPR or LBR demonstrates the relevance of serum P levels in determining pregnancy success (Yovich *et al.*, 2015, Labarta *et al.*, 2017, Alsbjerg *et al.*, 2018; Cédrin-Durnerin *et al.*, 2019; Gaggiotti-Marre *et al.*, 2019). We hypothesize that one of the reasons for this relationship is the immunomodulatory role of P in early pregnancy stages (Shah *et al.*, 2018), which favors embryo tolerance and prevent miscarriage. This is not a direct effect on the uterus, but rather a systemic effect that requires adequate P levels in the bloodstream to have positive effects on maintaining pregnancy.

Since the first published study that demonstrated a relation between serum P levels and pregnancy outcome in artificial cycles

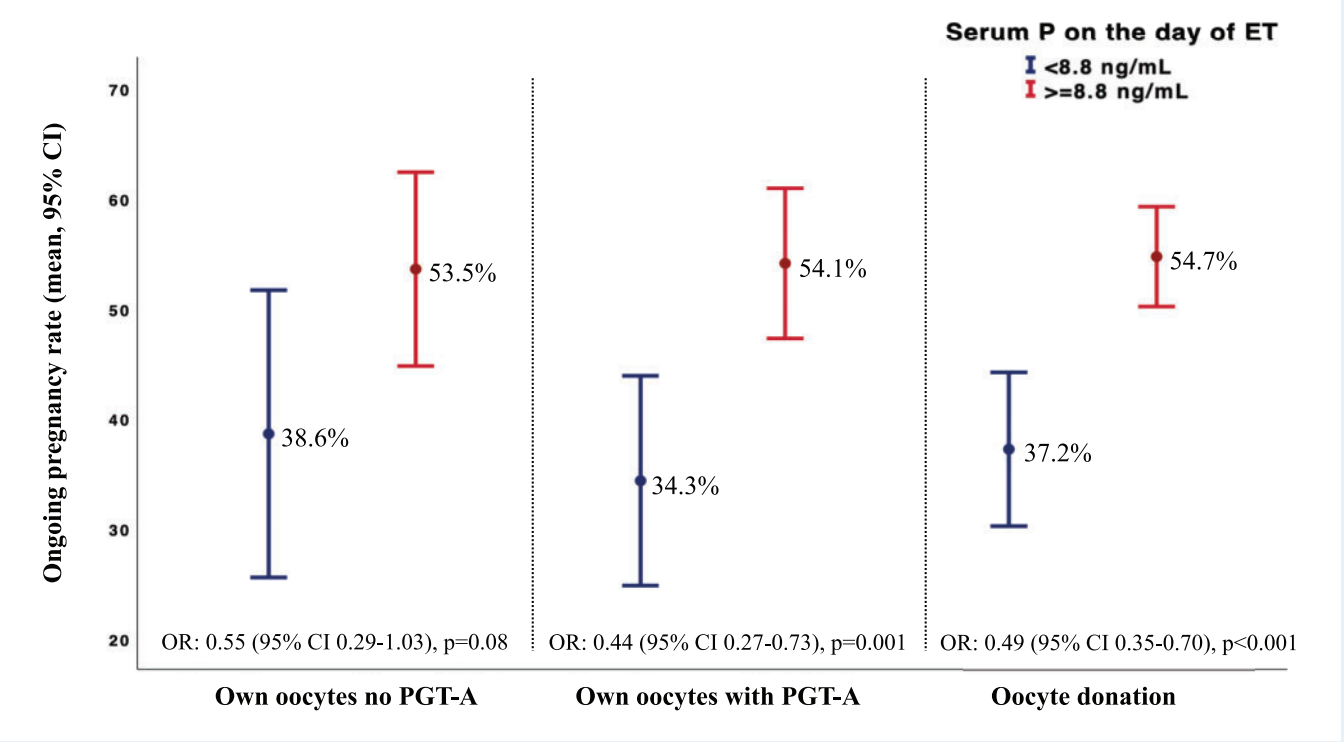


Figure 3. Mean ongoing pregnancy rate (95% CI) and crude odds ratios in the three treatment groups based on serum P level on the day of ET below or above 8.8 ng/ml. ET, embryo transfer.

Table II Multivariate logistic regression evaluating the effect of variables on ongoing pregnancy rate.

	Own oocytes no PGT-A (n = 184)		Own oocytes with PGT-A (n = 308)		Oocyte donation (n = 658)		Total (1150)	
	Adjusted OR (95% CI)	*P*	Adjusted OR (95% CI)	*P*	Adjusted OR (95% CI)	*P*	Adjusted OR (95% CI)	*P*
Age	0.95 (0.85–1.05)	0.325	0.98 (0.91–1.10)	0.593	0.97 (0.93–1.02)	0.217	0.98 (0.95–1.01)	0.099
Weight	1.03 (0.99–1.06)	0.114	1.02 (1.00–1.04)	0.061	1.00 (1.00–1.01)	0.680	1.01 (1.00–1.02)	0.124
Height	0.21 (0.00–51.7)	0.578	1.08 (0.03–46.4)	0.970	2.95 (0.19–45.2)	0.437	1.77 (0.24–13.06)	0.577
E2 proliferative phase	1.00 (1.00–1.00)	0.695	1.00 (1.00–1.00)	0.403	1.00 (1.00–1.00)	0.045	1.0 (1.00–1.00)	0.039
Endometrial thickness	1.13 (0.91–1.39)	0.276	0.99 (0.86–1.15)	0.920	1.12 (1.00–1.26)	0.045	1.07 (0.99–1.16)	0.098
No. embryos previously transferred	0.98 (0.82–1.17)	0.832	0.96 (0.81–1.15)	0.686	0.83 (0.76–0.91)	<0.001	0.88 (0.83–0.94)	<0.001
No. embryos transferred	1.05 (0.45–2.44)	0.917	2.57 (0.94–6.99)	0.065	2.09 (1.23–3.52)	0.006	1.80 (1.22–2.65)	0.003
Quality of transferred embryo	2.32 (1.31–4.12)	0.004	0.73 (0.45–1.19)	0.210	1.83 (1.37–2.44)	<0.001	1.59 (1.28–1.97)	<0.001
Day of blastocyst development (d5 vs d6)	0.56 (0.27–1.17)	0.122	0.80 (0.45–1.41)	0.434	0.99 (0.63–1.56)	0.977	0.79 (0.58–1.08)	0.140
Donor's age					0.95 (0.92–0.99)	0.009		
P < 8.8 ng/ml the day of ET	**0.49 (0.24–0.99)**	**0.048**	**0.38 (0.23–0.65)**	**<0.001**	**0.54 (0.37–0.78)**	**0.001**	**0.49 (0.37–0.64)**	**<0.001**

Results were calculated in each type of treatment and in the overall population. Data are presented as odds ratio (OR) and 95% confidence interval. Bold entries mean that the *P*-value was < 0.05, which is statistically significant. Impact of low serum progesterone levels on the day of embryo transfer on pregnancy outcome. A prospective cohort study in artificial cycles with vaginal progesterone.

(Yovich et al., 2015), several studies have found similar results. However, they were all retrospective analyses that reached the same conclusion: a minimum threshold of serum P levels needs to be reached to optimize outcomes in artificial cycles when using MVP (Alsbjerg et al., 2018; Cédrin-Durnerin et al., 2019; Gaggiotti-Marre et al., 2019). Only one large retrospective study does not agree with this statement, which reported that patients with serum P levels <10 ng/ml obtained similar results to those with good levels (Volovsky et al., 2020). Nevertheless, this study presented a major flaw because patients with lower P levels (<8 ng/ml) were supplemented with more exogenous P (no details of route and added doses were provided) from the day of ET onward. Therefore, the net effect of low P levels could not be fully analyzed because it would have biased their conclusions, as later suggested (Alsbjerg et al., 2020).

Our prospective design has the advantage of performing blinded P determinations and avoiding the risk of modifying LPS according to hormonal measurements. This is often the case with other retrospective studies, which makes it difficult to interpret the results (Volovsky et al., 2020). In our study, patients with later modified LPS (according to the doctor's criteria, and mainly due to bleeding) were excluded from analysis.

Although serum P levels are associated with pregnancy outcome, they do not show a high predictive value for ongoing pregnancy as seen on the ROC curve analysis. This implies that P is not a single predictor of treatment success because other factors, such as embryo quality, determine the cycle's fate. After adjusting for all the confirmed confounding factors, including embryo quality, serum P below the optimal threshold was still associated with outcome in the three groups according to the type of treatment. According to our recent experience, in almost 80% of patients (unpublished data), levels of P are similar in subsequent cycles if doses of exogenous P are not modified. This could be a plausible explanation of the findings in oocyte donation recipients with low serum P. In fact, they showed a higher number of embryos previously transferred, which could have failed due to this reason; whereas patients with a good level of serum P had less previous failed attempts and had the possibility of doing an elective transfer of a grade A embryo. We do not know if this is the cause or the consequence, but it makes sense that these patients may have more implantation failure due to insufficient progesterone exposure and are conducting a second or third ET.

In this study, we identified variables related to serum P levels. Although some variables were significantly correlated, the strength of the relation was very weak as all absolute Pearson's *r* values were below 0.2. The only variable to remain significant in the multivariate regression analysis was body weight, which showed a negative correlation. The difference in body weight between patients with low or adequate levels of P was 2.5 kg, which is not clinically relevant, considering that this did not impact pregnancy outcome. Indeed, weight had no impact on PK when using MVP (Levy et al., 1999). A recent retrospective analysis in 685 FET cycles confirmed our results, observing a negative correlation between weight and serum P levels (González-Foruria et al., 2020). These interpersonal variations could be due to a variable capacity of absorption, clearance and different distribution in fat tissue.

The decrease in mean serum P levels with increased time interval is small and did not affect clinical outcomes. The difference in the threshold level (9.2 ng/ml) between our first study (Labarta et al., 2017) and the current one (8.8 ng/ml) is not due to the timing of measurement (mean time 5.7 h in the current study). Regarding obstetrical outcomes, we found no differences between the two groups of P levels. Interestingly, the prevalence of hypertensive disorders during pregnancy was not statistically different between patients with low and good P levels. This suggests that the higher prevalence of hypertensive disorders in artificial cycles vs natural cycles might be related to something else, perhaps relaxin levels, rather than to serum P levels as suggested (Conrad et al., 2019; von Versen-Höynck et al., 2019).

One limitation of our study is that no cleavage-stage ETs were included, so we cannot ensure that the threshold is the same for this approach. Yet, PK studies demonstrate that steady levels of P are reached after 6 h of progesterone exposure and can be maintained by continued dosing. Thus, we hypothesize that the same threshold can be used for Days 2–3 ETs. Indeed, measurement of serum P on Day 4, the day before blastocyst transfer, is related to pregnancy outcome and shows a similar serum P cutoff (Gaggiotti-Marre et al., 2019).

Another limitation could be the heterogeneity in the population included (own oocytes + PGT-A, own oocytes without PGT-A, oocyte donation), and for this reason we have shown the results separately in these three groups.

In summary, we confirm the relevance of serum P levels when using natural MVP in HRT cycles, not only in oocyte donation cycles as previously published (Labarta et al., 2017) but also in cycles with own oocytes. Research into individualized luteal phase supplementation (iLPS) (Labarta, 2020) should be prioritized as not all patients may benefit from a one-size-fits all protocol. Questions about the best dose and route of administration of exogenous P according to individual characteristics and type of cycle are still remaining. Future studies are needed to demonstrate if iLPS is clearly effective in this particular set of patients with inadequate levels of serum P.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Authors' roles

All authors made substantial contributions to data acquisition, revised the article and approved the final version. The first and last authors significantly contributed to the study conception and design, performed statistical analyses and data interpretation and drafted the article.

Funding

No external funding was either sought of obtained for this study.

Conflict of interest

E.L. received a grant from Ferring in 2020, has provided consultancy services for MSD and Ferring Pharmaceuticals and is part of the Ferring Pharmaceuticals LIFE program and Merck Global program for Fertility Innovation Leaders. During the past 12 months, she has received honoraria from Angelini/IBSA, Merck, MSD and Ferring Pharmaceuticals for lecturing. E.B. received a grant from Finox in 2016, has given lectures or provided consultancy services for MSD, Merck, IBSA and Ferring Pharmaceuticals. F.C. has received honoraria from MSD and Ferring Pharmaceuticals for lecturing. Rest of authors have nothing to declare. C.R.-V. received a grant from the Spanish Ministry of Education, Culture and Sport in 2019 for the National Programme for Training University Lecturers (FPU).

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