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ORIGINAL ARTICLE ***Reproductive endocrinology***

Late follicular phase progesterone elevation during ovarian stimulation is not associated with decreased implantation of chromosomally screened embryos in thaw cycles

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STUDY QUESTION: What is the impact of a late follicular phase progesterone elevation (LFPE) during controlled ovarian hyperstimulation (COH) on embryonic competence and reproductive potential in thaw cycles of preimplantation genetic testing for aneuploidy (PGT-A) screened embryos?

SUMMARY ANSWER: Our study findings suggest that LFPE, utilizing a progesterone cutoff value of 2.0 ng/ml, is neither associated with impaired embryonic development, increased rate of embryonic aneuploidy, nor compromised implantation and pregnancy outcomes following a euploid frozen embryo transfer (FET) cycle.

WHAT IS KNOWN ALREADY: Premature progesterone elevation during COH has been associated with lower pregnancy rates due to altered endometrial receptivity in fresh IVF cycles. Also, increased levels of progesterone (P) have been suggested to be a marker for ovarian dysfunction, with some evidence to show an association between LFPE and suboptimal embryonic development. However, the effect of LFPE on embryonic competence is still controversial.

STUDY DESIGN, SIZE, DURATION: Retrospective cohort analysis in a single, academic ART center from September 2016 to March 2020. In total, 5244 COH cycles for IVF/PGT-A were analyzed, of those 5141 were included in the analysis. A total of 23 991 blastocysts underwent trophectoderm biopsy and PGT analysis. Additionally, the clinical IVF outcomes of 5806 single euploid FET cycles were evaluated.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Cohorts were separated in two groups: Group 1: oocytes retrieved from cycles with normal P levels during ovulation trigger ($P \leq 2.0$ ng/ml); Group 2: oocytes retrieved after cycles in which LFPE was noted ($P > 2.0$ ng/ml). Extended culture and PGT-A was performed. Secondly, IVF outcomes after a single euploid FET were evaluated for each cohort.

MAIN RESULTS AND THE ROLE OF CHANCE: Four thousand nine hundred and twenty-five cycles in Group 1 were compared with 216 cycles on Group 2. Oocyte maturity rates, fertilization rates and blastulation rates were comparable among groups. A 65.3% ($n = 22\,654$) rate of utilizable blastocysts was found in patients with normal P levels and were comparable to the 62.4% ($n = 1337$) observed in those with LFPE ($P = 0.19$). The euploidy rates were 52.8% ($n = 11\,964$) and 53.4% ($n = 714$), respectively, albeit this difference was not statistically significant ($P = 0.81$). Our multivariate analysis was fitted with a generalized estimating equation (GEE) and no association was found with LFPE and an increased odds of embryo aneuploidy (adjusted odds ratio 1.04 95% CI 0.86–1.27, $P = 0.62$). A subanalysis of subsequent 5806 euploid FET cycles (normal P: $n = 5617$ cycles and elevated P: $n = 189$ cycles) showed no differences among groups in patient's BMI, Anti-Müllerian hormone (AMH), endometrial thickness at FET and number of prior IVF cycles. However, a

significant difference was found in patient's age and oocyte age. The number of good quality embryos transferred, implantation rate, clinical pregnancy rate, ongoing pregnancy rate, multiple pregnancy rate and clinical pregnancy loss rates were comparable among groups. Of the registered live births (normal P group: $n = 2198$; elevated P group: $n = 52$), there were no significant differences in gestational age weeks (39.0 ± 1.89 versus 39.24 ± 1.53, $P = 0.25$) and birth weight (3317 ± 571.9 versus $3\ 266 \pm 455.8$ g, $P = 0.26$) at delivery, respectively.

LIMITATIONS, REASONS FOR CAUTION: The retrospective nature of the study and probable variability in the study center's laboratory protocol(s), selected progesterone cutoff value and progesterone assay techniques compared to other ART centers may limit the external validity of our findings.

WIDER IMPLICATIONS OF THE FINDINGS: Based on robust sequencing data from a large cohort of embryos, we conclude that premature P elevation during IVF stimulation does not predict embryonic competence. Our study results show that LFPE is neither associated with impaired embryonic development nor increased rates of aneuploidy. Embryos obtained from cycles with LFPE can be selected for transfer, and patients can be reassured that the odds of achieving a healthy pregnancy are similar to the embryos exposed during COH cycles to physiologically normal P levels.

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Key words: premature progesterone elevation / premature luteinization / preimplantation genetic testing / ovarian stimulation / embryo development / early luteal progesterone elevation / frozen embryo transfer

Introduction

Premature progesterone elevation, more aptly referred to as late follicular phase progesterone elevation (LFPE), is a commonly observed phenomenon occurring during controlled ovarian hyperstimulation (COH) for ART (Yong *et al.*, 1992; Bosch *et al.*, 2010). The exact pathophysiology behind the progesterone (P) elevation remains speculative among reproductive specialists. It has been proposed that increased P production during COH cycles may be a result of granulosa cell exposure to supra-physiologic gonadotropin levels or an increased P biosynthesis by the overly active '3 β-hydroxysteroid' dehydrogenase pathway triggered by multiple preovulatory follicle recruitment in response to exogenous gonadotropin administration (Schneyer *et al.*,2000; Thuesen *et al.*, 2014; Oktem *et al.*, 2017). Oddly, P elevation cannot be prevented with the utilization of GnRH analogs in every treatment case, and some researchers have described a prevalence of LFPE ranging from 1% to 46% in stimulated ovarian cycles depending on the cutoff P level utilized (Venetis *et al.*, 2013; Vanni *et al.*, 2017a,b). Aside from ovarian stimulation, LFPE has been associated with various patient profile characteristics such as BMI, patient's ethnicity, ovarian reserve metrics, the degree of ovarian response to exogenous gonadotropins, the number of recruited follicles during COH, dosage and duration of stimulation cycles and/or other factors (Griesinger *et al.*, 2013; Andersen and Ezcurra, 2014; Kaponis *et al.*, 2018).

To date, several publications have described the association between LFPE and IVF outcomes in fresh embryo transfer cases. A number of studies have demonstrated that luteal P rise measured on the day of ovulation trigger leads to lower pregnancy rates (Bosch *et al.*, 2003, 2010; Kolibianakis *et al.*, 2004; Kyrou *et al.*, 2011; Venetis *et al.*, 2013). Although a number of evidence-based studies have shown that LFPE during COH correlates with an adverse impact on endometrial physiology and endometrial receptivity mechanisms (Horcajadas *et al.*, 2007; Labarta *et al.*, 2011; Van Vaerenbergh *et al.*, 2011), fewer studies have evaluated the relationship between P rise with oocyte quality and embryonic reproductive potential.

In an animal model, it has been shown that an increasing P level disrupts normal oocyte maturation and meiosis resumption (Nagai *et al.*, 1993). In human-based models, some studies had demonstrated a deleterious impact of LFPE on embryonic quality (Ubaldi *et al.*, 1995; Huang *et al.*, 2016; Vanni *et al.*, 2017a,b; Racca *et al.*, 2018). However, even with robust published research about oocyte donation cycles (Venetis *et al.*, 2013; Racca *et al.*, 2020), luteal phase start ovarian stimulations (Kuang *et al.*, 2014; Chen *et al.*, 2015; Ubaldi *et al.*, 2016; Pereira *et al.*, 2017; Vaiarelli *et al.*, 2020) and freeze-all strategy during IVF cycles (Wang *et al*, 2017; Baldini *et al.*, 2018); none have yet to show definitive evidence of a direct effect of LFPE on embryonic quality or embryo reproductive potential.

To our knowledge, only one study has analyzed the relationship between LFPE and embryonic quality on cycles utilizing preimplantation genetic analysis. Kofinas *et al.* (2016) reviewed chromosomal composition of embryos derived from cycles notable for follicular phase progesterone elevations. In that analysis, LFPE was not associated with the count of oocytes at retrieval and mean number of euploid embryos per IVF cycle. In addition, there was no difference in single euploid frozen embryo transfer (FET) outcome from embryos derived in cycles with and without LFPE. However, the generalizability of that study's findings is limited to its use of (Microarray-based Comparative Genomic Hybridization)a-CGH as preimplantation genetic testing for aneuploidy (PGT-A) platform, a small sample size of embryos analyzed, and a small number of euploid FET cycles analyzed. Given that there was a disproportionate number of older patients in that study, it needs to be determined whether the study's findings are applicable to the general IVF population. Finally, another limitation of the study by Kofinas is that the authors utilized a 1.5 ng/ml progesterone cutoff value for determining LFPE. It is commonly understood that patients with a serum P level ≥1.5 ng/ml on the day of ovulation trigger administration have an altered endometrial genetic expression pattern and significant endometrial histological changes (Bosch *et al.*, 2010; Van Vaerenbergh *et al.*, 2011; Venetis *et al.*, 2013; Kaponis *et al.*, 2018). Whereas a higher cutoff *P*-value (>2.0 ng/ml) has been demonstrated to not only to affect endometrial receptivity and significantly

impair IVF outcomes in fresh cycles but also to be negatively associated with embryonic development and/or quality (Huang *et al.*, 2016; Vanni *et al.*, 2017a,b).

With the increasing utilization of PGT and/or freeze-all strategies, modern reproductive specialists have become more confident in the presence of an ovarian hyper-response, or may be inclined to prolong the duration of stimulation by delaying the ovulation trigger administration aiming to maximize the number of collectable mature oocytes in poor-responding patients, settings that have been associated with an increased prevalence of LFPE (Kaponis *et al.*, 2018). While these strategies could bypass the effect of LFPE on the endometrium, the influence of LFPE on oocyte and embryo quality is highly debatable; therefore, the objective of our study is to evaluate the impact of LFPE during COH on embryonic quality and euploidy rates, as well to analyze the pregnancy rates and IVF outcomes after the transfer of these embryos in a frozen euploid embryo transfer cycle.

Materials and methods

Study design and patient populations

Main analysis.

The retrospective analysis included infertile patients from a single center who underwent IVF/PGT-A cycle(s) from September 2016 to March 2020. Only cases that underwent COH with a GnRH antagonist protocol were evaluated. For all cases, the level of serum progesterone during COH was measured on the morning of ovulation trigger medication administration. LFPE was defined as serum P $\geq$ 2.0 ng/ml on the day of ovulation trigger. All P serum levels were measured with electro-chemi-luminescence analysis utilizing an in-site 'Cobas e-601'® (Roche Diagnostics, IN, USA) (measuring range = 0.03–60 ng/ml, Intra-assay variation = 1.1% and Inter-assay variation = 0.99). Cohorts were separated into two groups based on serum P levels: (normal P group (P $\leq$ 2.0 ng/ml) and LFPE group (P $\geq$ 2.0 ng/ml)).

All patients underwent COH, ICSI, extended embryo culture, trophectoderm (TE) biopsy and PGT-A. All PGT analyses were performed with next generation sequencing, all embryology laboratory methods were described previously (Hernandez-Nieto *et al.*, 2019). PGT-A screened embryos received a chromosome copy number analysis result and were assigned to the following categories: euploid, aneuploid or inconclusive. Reports of mosaic embryos were considered as aneuploid. Cases involving multiple TE biopsies, patients utilizing donor oocytes, testicular sperm extraction cases and/or patients with known chromosomal rearrangements were excluded from the analysis.

Sub-analysis.

The sub-analysis included all patients that completed an IVF/PGT-A cycle followed by synthetic endometrial preparation for a euploid FET cycle from September 2016 to March 2020. Cohorts were established based on the progesterone levels on the day of oocyte ovulation trigger administration during the IVF cycle in which the oocytes were retrieved. These cohorts of patients that underwent a euploid FET cycle were defined as follows: Group A: normal P (P $\leq$ 2.0 ng/ml) and Group B: LFPE (P $\geq$ 2.0 ng/ml). Cases with patients diagnosed with recurrent pregnancy loss, recurrent implantation failure, uterine factor infertility, patients with hydrosalpinx, balanced chromosomal translocations, severe male factor with testicular sperm extraction and/or recipients of donor oocytes were excluded from the analysis.

Additionally, another sub-analysis evaluated euploid FET cycle outcomes based on different P concentrations. We separated subjects into cohorts based on P levels on the day of the ovulation trigger administration during the COH cycle as follows: Group 1: ($\leq$1.0 ng/ml); Group 2: (1.1–2.0 ng/ml); Group 3: (2.1–3.0 ng/ml); Group 4: (3.1–4.0 ng/ml) and Group 5: ($\geq$4.1 ng/ml).

Stimulation protocols.

A flexible GnRH antagonist protocol was used in treatment by all patients. The dose of gonadotropin was individualized for each patient according to age, BMI and antral follicle count (AFC). Recombinant follicle-stimulating hormone and hMG were administered starting on Day 3 of the cycle. Follicle monitoring was carried out routinely. Patients received the GnRH antagonist Cetrorelix acetate 0.25 mg (Cetrotide; Merck-Serono, MA, USA) or Ganirelix acetate 250 µg (Merck Sharp & Dohme, NJ, USA) starting on Day 5 after the onset of stimulation with injectable gonadotropins and/or when at least two follicles measured $\geq$14 mm; and/or serum estradiol was $\geq$800 pg/ml. The GnRH antagonist was administered daily until the day of ovulation trigger administration. When two or more follicles reached 18 mm in diameter, final oocyte maturation was induced with 10 000 IU hCG (Novarel, Ferring pharmaceuticals, Parsippany, NJ, USA), 250–500 µg of recombinant human chorionic gonadotropin (Ovidrel, EMD Serono, Rockland, MA, USA); or in high responders at risk of ovarian hyperstimulation syndrome, a dual trigger with 2 mg of Leuprolide acetate (Lupron, Abbvie Laboratories, Chicago, IL, USA) and 1000 u of hCG. Thereafter patients underwent vaginal oocyte retrieval (VOR) under transvaginal ultrasound guidance 36 h after oocyte maturation was triggered.

Laboratory procedures

Our center-specific embryo culture protocol, TE biopsy technique, embryo grading system and cryopreservation/thawing techniques have been described previously (Hernandez-Nieto *et al.*, 2019; Sekhon *et al.*, 2019). Every euploid FET was performed in a synthetic preparation cycle. For each patient, the uterine cavity was prepared with micronized oral estradiol (Estrace, Teva Pharmaceuticals, NJ, USA) 2 mg twice daily for 4 days, then 2 mg three times daily. After a minimum of 12 days of estradiol administration, transvaginal ultrasonography was performed to assess endometrial lining. When a minimum thickness of at least 7 mm was achieved, 50 mg of intramuscular progesterone in oil (Progesterone injection, Watson Pharma Inc., Parsippany, NJ, USA) was administered daily. For all clinical cases, thawing and transfer of the embryos were carried out on the sixth day of progesterone supplementation regardless of the day of embryo development at time of cryopreservation (Day 5–7). Euploid embryos with the highest morphological grade were selected for transfer. The embryo selection process for transfer has been described previously (Hernandez-Nieto *et al.*, 2019).

Outcome measures

Primary outcomes analyzed included blastulation rate (total number of viable blastocysts over the total number of fertilized oocytes), utilizable

blastocyst rate (number of blastocyst available for TE biopsy and vitrification) and ploidy rates (number of euploid/aneuploid blastocysts over the number of biopsied blastocysts). Embryonic quality was assessed utilizing a site specific modified Gardner blastocyst grading system described previously (Hernandez-Nieto *et al.*, 2019). Embryos were classified in three cohorts based on the morphologic grading of three blastocyst components (Expansion, ICM and TE) at the moment of vitrification as it follows: good quality (expansion = 4 or greater, AA, AB, BA or BB); moderate quality (expansion = 4 or greater + AC, CA, BC or CB) or fair quality: (any expansion grade + CC).

Sub-analysis outcomes included implantation rate (IR) (proportion of intrauterine gestational sacs per embryo transferred), clinical pregnancy rate (CPR) (proportion of cases with ultrasonographically detectable fetal cardiac activity), ongoing pregnancy rate (OPR) (proportion of cases when pregnancy had completed ≥20 weeks of gestation), clinical pregnancy loss rate (CPL) (pregnancy loss detected after the presence of a confirmed gestational sac) and multiple pregnancy (two or more fetal poles with observable cardiac activity after presumed monozygotic splitting) (Zegers-Hochschild *et al.*, 2017).

Statistical methods

Descriptive data were compared by Student's *T*, Mann–Whitney *U*, Chi-squared and ANOVA tests when appropriate. Results were expressed as percentages, means and SDs. Adjusted odds ratios (aORs) with 95% CI's were calculated using a multivariate logistic regression analysis, the models were fitted with a generalized estimating equation (GEE) to account for patients who underwent multiple COH or FET cycles. All variables that showed significance and/or were thought to be clinically relevant were encompassed and adjusted-for as covariates in the models. All *P*-values are two sided with a clinical significance level determined at $P \leq 0.05$.

Power analysis

For the main analysis, a sample size of 519 blastocysts per group was required to detect a 10% difference in euploidy rates with 90% power and alpha of 0.05. Also, a sample size of 477 blastocysts per group was required to have a 90% power to detect a 10% difference in blastulation rates with alpha at 0.05. For the sub-analysis, to detect a difference in IRs from a euploid FET, a sample size of 121 FET's per group was calculated to detect a difference of 15% in IR with 80% power (alpha = 0.05).

Regulatory approval

This retrospective analysis was approved by an academic Institutional Review Board (WIRB PRO NUM: 20161791; Study number 1167398). Patient information was de-identified before data analysis.

Results

Of the 5244 IVF/PGT cases initiated during this study, 103 (1.9%) were cancelled before VOR due to poor ovarian response during COH. Of the remaining 5141 cases, 4925 had normal P (≤2.0 ng/ml) and 216 had LFPE (>2.0 ng/ml). Ninety-nine (2.0%) of the normal P group and four (1.9%) of the LFPE cases did not undergo VOR and were excluded from the study's analysis. Of the 5141 COH cycles that were included in the analysis, 4925 consisted of patients with a normal P and 216 with LFPE. Fifty-three patients with normal P (1.1%) and 1 patient with LFPE (0.5%) did not achieve fertilization of oocytes after ICSI. A total of 122 (2.5%) patients with normal P and 6 (2.8%) with LFPE had no embryos reaching the blastocyst stage of development. Lastly, 301 (6.1%) patients with normal P and 8 (3.7%) with LFPE did not have an embryo that met criteria for TE biopsy.

On an unadjusted analysis, significant differences were found in mean patient's age, BMI, serum P the day of ovulation trigger, days of gonadotropins used, day of ovulation trigger, P at trigger, estradiol at trigger, AMH, baseline FSH, AFC and number of retrieved oocytes among cohorts. No differences were found in number of prior IVF cycles and cumulative gonadotropin dosage utilized between groups (Table I). Oocyte maturity rates (76.4% ($n = 58\,703$) versus 76.5% ($n = 3731$), $P = 0.96$); fertilization rates (80.1% ($n = 46\,999$) versus 80.8% ($n = 3016$), $P = 0.70$) and blastulation rates were comparable among groups (73.8% ($n = 34\,676$) versus 71.1% ($n = 2144$), $P = 0.20$), respectively. A 65.3% ($n = 22\,654$) of utilizable blastocysts was found on normal P group and it was comparable to the 62.4% ($n = 1337$) on the LFPE group ($P = 0.19$). The euploidy rates were 52.8% ($n = 11\,964$) and 53.4% ($n = 714$), respectively, albeit this difference was not statistically significant ($P = 0.81$). Also, no difference was found on the aneuploidy rate among groups (41.7% ($n = 9445$) versus 37.9% ($n = 507$), $P = 0.07$), and a significant difference was found in the rate of inconclusive results after PGT among groups (5.5% ($n = 1245$) versus 8.7% ($n = 116$), $P \leq 0.0001$) (Table I). After adjusting for age, BMI, AMH, baseline FSH, days of stimulation and oocytes retrieved per case; no association was found with the presence of LFPE and increasing the odds of embryo aneuploidy (aOR 1.04, 95% CI 0.86–1.27, $P = 0.62$), or with the odds of embryos being reported as inconclusive (aOR 1.12, 95% CI 0.69–1.84, $P = 0.62$).

The percentage of good quality embryos per group was assessed, no differences were found in the percentage of good quality embryos in patients with normal P (61.9%) compared with LFPE patients (65.0%), $P = 0.16$, also no differences were found in the rate of moderate quality (33.0% versus 30.7%, $P = 0.14$) or fair quality embryos (6.3% versus 5.9%, $P = 0.57$), respectively (Fig. 1).

Finally, after evaluating euploidy rates based on the Society for Assisted Reproductive Technology (SART) age group categories, no significant differences were found between embryo euploidy rates among normal and LFPE groups (Fig. 2).

Sub-analysis

Five thousand eight hundred and six euploid FETs were included in the study's sub-analysis, of those cases only 6.8% ($n = 395$) of all FETs were from patients that had only one euploid embryo available for transfer, the remaining patients ($n = 5411$) pursued elective single embryo transfers. In the analysis, 5617 cycles had patients with a normal P (≤2.0 ng/ml) while the remaining 189 cycles included patients with LFPE (>2.0 ng/ml) on day of trigger administration during the COH cycle. On an unadjusted analysis, significant differences were found on patient's age at ET, oocyte age, serum P levels on the day of the FET and P on the day of ovulation trigger administration in the fresh COH cycle where embryos were sourced. No differences were found in patient's BMI, AMH, endometrial thickness at FET and number of

Table I Demographic characteristics, COH parameters and embryologic data comparisons between groups based by serum progesterone on the day of ovulation trigger during COH cycles.

	Normal progesterone (≤2.0 ng/ml)		Late follicular progesterone elevation (>2.0 ng/ml)		*P*-value	Significance
	n = 4925 cycles		*n* = 216 cycles			
	Mean	SD±	Mean	SD±		
Age (years)	36.80	4.31	35.63	4.43	0.0001	*
BMI (kg/m^2)	24.11	4.54	23.13	4.09	0.0008	*
Gravidity	0.96	1.27	0.91	1.33	0.52	
Parity	0.37	0.70	0.30	0.62	0.13	
Prior IVF stimulation cycles	0.46	1.06	0.43	0.79	0.65	
Days of gonadotropins used	8.87	1.42	9.55	1.66	<0.0001	*
Day of ovulation trigger	11.87	1.42	12.55	1.66	<0.0001	*
Gonadotropin cumulative dose (IU)	3672	1282.9	3806	1350.7	0.24	
Progesterone at trigger (ng/ml)	0.89	0.38	2.68	0.95	<0.0001	*
Estradiol at trigger (pg/ml)	2291	1197.8	3229	1530.5	<0.0001	*
Baseline FSH (IU/ml)	6.84	3.18	5.77	2.86	0.0001	*
Anti Müllerian hormone (ng/ml)	3.34	3.64	4.15	3.36	0.0001	*
Antral follicle count	13.62	7.46	16.74	9.87	<0.0001	*
Oocytes retrieved	15.60	9.37	22.58	12.31	<0.0001	*
	N	%	N	%		
Oocyte maturity rate	58 703/76 810	76.4	3731/4877	76.5	0.96	
Fertilization rate	46 999/58 703	80.1	3016/3731	80.8	0.70	
Blastulation rate	34 676/46 999	73.8	2144/3016	71.1	0.20	
Utilizable blastocyst rate	22 654/34 676	65.3	1337/2144	62.4	0.19	
PGT-A results						
Euploidy rate	11 964/22 654	52.8	714/1337	53.4	0.81	
Aneuploidy rate	9445/22 654	41.7	507/1337	37.9	0.07	
Inconclusive report rate	1245/22 654	5.5	116/1337	8.7	<0.0001	*

*Statistical significance, $P < 0.05$.
COH, controlled ovarian hyperstimulation.

previous IVF cycles among groups (Table II). The number of good quality embryos transferred, IR, CPR, OPR, multiple pregnancy rate, clinical loss rate and biochemical pregnancy rates were comparable among groups (Table II).

Of the registered live births 72.2% ($n = 2250$) (normal P group: $n = 2198$; LFPE group: $n = 52$) from all the ongoing pregnancies to the date of termination of this study ($n = 3117$), there were no significant differences in the gestational age (weeks) (39.0 ± 1.89 versus 39.2 ± 1.53, $P = 0.25$) or birth weight at delivery (3317 ± 571.9 g versus 3266 ± 455.8 g, $P = 0.26$), respectively (Table II).

After adjusting for age, BMI, AMH, endometrial thickness at FET, day of blastocyst biopsy and embryo quality; no association was found between embryos exposed to elevated P during COH and lower odds of implantation (aOR 0.74, 95% CI 0.31–1.76, $P = 0.50$). Likewise, no association with lower odds of clinical pregnancy (aOR 0.71, 95% CI 0.45–1.13, $P = 0.15$) and ongoing pregnancy (aOR 0.94, 95% CI 0.50–1.75, $P = 0.85$) were observed. Last, no association was found with increased odds of biochemical pregnancy (aOR 1.47, 95% CI 0.95–2.28, $P = 0.07$) or clinical pregnancy loss (aOR 1.05, 95% CI 0.57–1.96, $P = 0.85$) among study groups.

When reviewing IVF outcomes based on different P threshold groups, a significant difference was found in IRs among cohorts: Group 1 (≤1.0 ng/ml) = 74.1%; Group 2 (1.1–2.0 ng/ml) = 75.4%; Group 3 (2.1–3.0 ng/ml) = 77.1%; Group 4 (3.1–4.0 ng/ml) = 87.5%; and Group 5 (≥4.1 ng/ml) = 52.0%, $P = 0.04$. No significant differences were found in, CPR, OPR and CPL rates between cohorts (Table III).

Discussion

In an era of freeze all, PGT-A, and FET cycles, LFPE, utilizing a progesterone cutoff value of 2.0 ng/ml, does not seems to represent an obstacle to embryo implantation potential. To our knowledge, this is the largest analysis to date to analyze blastulation rates, euploidy rates and FET outcomes of euploid embryos obtained from cycles exposed to elevated levels of P during COH. Our study findings suggest that LFPE is neither associated with impaired embryonic development, increased rate of embryonic aneuploidy, nor compromised implantation and pregnancy outcomes following an FET cycle.

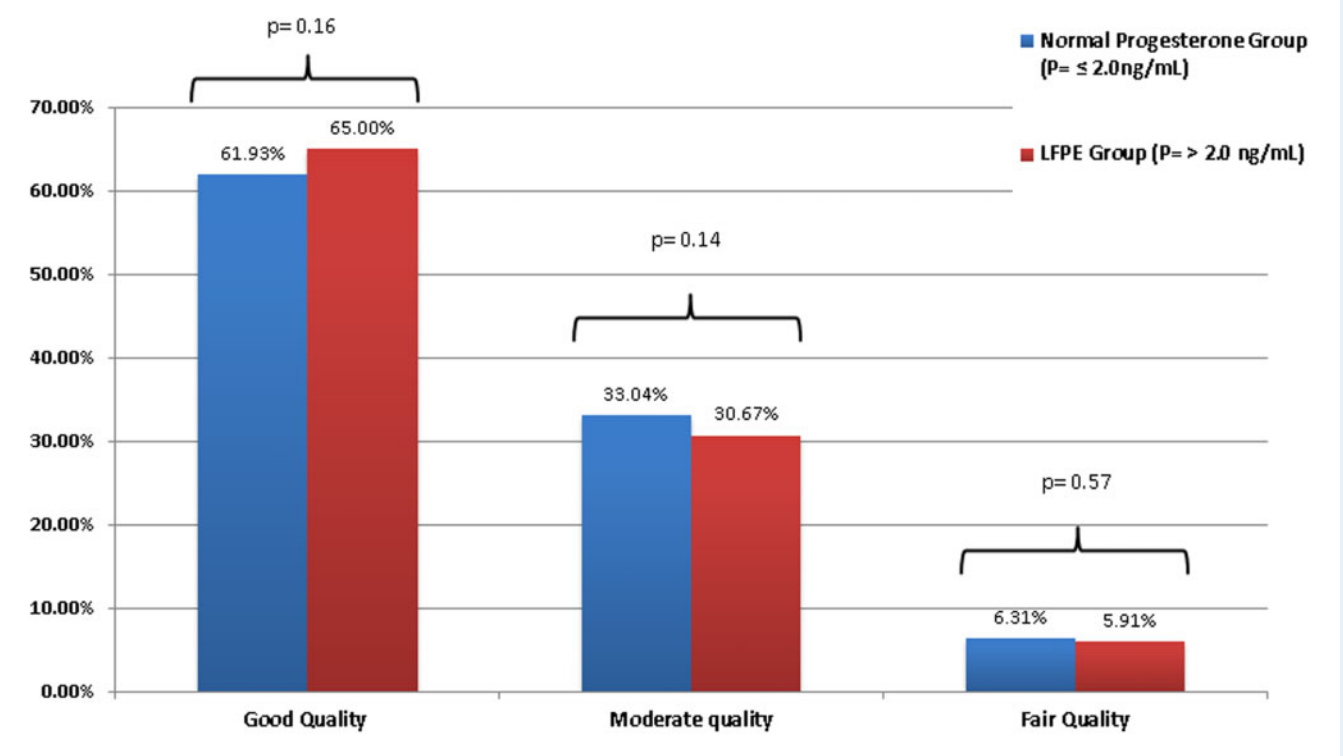

Figure 1. Embryo quality grading proportions based on progesterone levels at oocyte maturation trigger during COH. Data presented as percentage of good, moderate and fair embryo quality grades. Color coding based on progesterone at COH cycles: blue: normal progesterone (<2.0 ng/ml); red: LFPE (≥2.0 ng/ml). *Statistical significance (χ^2 test) $P \leq 0.05$. LFPE, late follicular phase progesterone elevation.

Our findings are consistent with the study published by Kofinas *et al.* (2016). That study showed patients who were exposed to LFPE had no effect on the number of oocytes retrieved, count of utilizable embryos or a decrease in rate of euploid embryos. Also, similar to our analysis, that study observed no negative association with CPR and OPR among patients exposed to LFPE.

Additionally, our study's analysis investigated other important clinical outcomes such as IR, CPL, biochemical pregnancy and multiple pregnancy rates. Furthermore, our study is the first demonstrating that there is no association with LFPE and abnormal birthweight and/or gestational weeks at delivery after a euploid FET.

Our study findings oppose a number of clinical impressions about LFPE and IVF pregnancy outcomes. We reason that our study's findings differ from previously published studies due to its inclusion of euploid FET cycles. Prior researchers mainly evaluated only fresh IVF cycles, which could explain for the adverse influence of LFPE on endometrial quality, subsequent asynchrony between embryo and endometrium, and altered gene expression during the window of implantation; rather than an inferred impairment on oocytes or embryonic development (Bosch *et al.*, 2010; Elgindy, 2011; Van Vaerenbergh *et al.*, 2011; Lahoud *et al.*, 2012; Venetis *et al.*, 2015; Kofinas *et al.*, 2016; Lawrenz and Fatemi, 2017).

A possible detrimental effect on oocyte meiotic maturation and pronuclear development has been correlated with increased P levels within an animal study (Nagai *et al.*, 1993). However, our study found no negative association in oocyte recovery rates, oocyte maturity, fertilization, blastocyst conversion and utilizable blastocyst rates among oocytes exposed to altered hormonal milieu *in vivo* during COH. Furthermore, the euploidy rates were generally comparable among cohorts ($P = 0.19$), even when we sub-divided them based on different SART age categories (Fig. 2), which suggests LFPE is not associated with sub-optimal oocyte development even in later stages of a woman's reproductive life.

Notably, a few studies overlooked the impact of supra-physiologic P levels on embryonic quality by means of morphological grading (Huang *et al.*, 2016; Vanni *et al.*, 2017a,b). Those studies suggested that good quality embryos were negatively correlated with P levels during COH. Although the generalizability of their findings is problematic, as these studies examined cleavage stage embryo quality and/or lacked significant sample sizes within their analysis. Our study included only oocytes that were fertilized and cultured to blastocyst stage. We observed that blastulation rates (73. 8% versus 71.1%, $P = 0.20$) and the number of utilizable blastocysts were not different among groups (65.3% versus 62.4%, $P = 0.19$). Furthermore, when analyzing blastocyst quality, the rates of good quality (61.9% versus 65.0%, $P = 0.16$); moderate quality (33.0% versus 30.7%, $P = 0.14$) and fair quality (6.3% versus 5.9%, $P = 0.57$) blastocysts were similar, regardless of embryo exposure to LFPE at time of ovulation trigger administration.

When analyzing IVF outcomes after single euploid FETs our study shows that there is no association with lower odds of implantation, OPRs and no association with increased odds of biochemical and CPL in patients that presented LFPE during their COH cycles. After

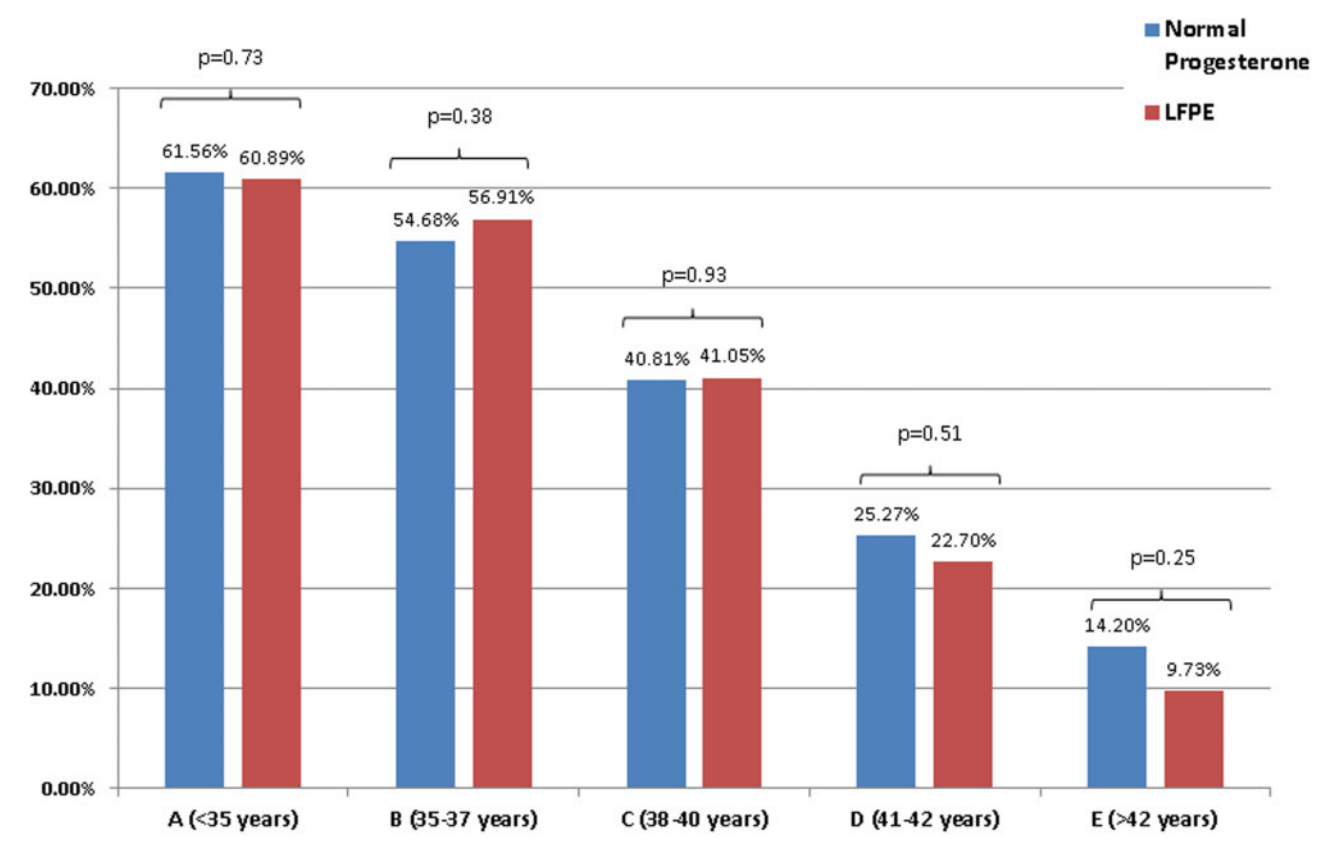

Figure 2. Proportions of euploid embryos classified by SART age group based on progesterone at COH cycles groups. Data presented as years (maternal SART age groups) and percentage of euploid blastocysts. Color coding based on progesterone at COH cycles: blue: normal progesterone (<2.0 ng/ml); red: LFPE (≥2.0 ng/ml). *Statistical significance (ANOVA) $P \leq 0.05$.

analyzing FET implantation, clinical, OPRs and CPLs by observing multiple P concentrations cohorts we found a significant difference in IR in embryos exposed to very high P concentrations (Group 5 ≥ 4.1 ng/ml). That cohort was found to have reduced IR (52.0%) when compared to all other P concentration categories (Group 1 (0–1.0 ng/ml) = 74.1%; Group 2 (1.1–2.0 ng/ml) = 75.4%; Group 3 (2.1–3.0 ng/ml) = 77.1% and Group 4 (3.1–4.0 ng/ml) = 87.5%, $P = 0.04$). We acknowledge the possibility of compromising the cohort sample sizes and the decreased power to detect significant effects when performing a sub-analysis that sub-categorizes P concentrations, as it may inadequately compare these groups differences (Table III) so this comparative analysis has to be interpreted with caution. Still, on the multivariate analysis after adjusting for potential confounders such as age, BMI, AMH, endometrial thickness, day of embryo biopsy and blastocyst quality at ET, we found no association with LFPE and lower odds of implantation (aOR 0.74, 95% CI 0.31–1.76, $P = 0.50$) or with any other IVF outcome analyzed.

Our study is not without limitations. The retrospective nature of the analysis increases the chances of selection bias; although the calculated sample size and power analysis showed that our included analysis population is sufficient to detect significant differences in the main outcomes of the study. Also, we utilized an adjusted analysis for potential confounders and important clinical factors such as age, BMI, days of stimulation, gonadotropin dose utilized, oocytes retrieved and endometrial thickness at ET.

An important fact to consider about the reproducibility of any study involving serum P testing is the difficulty of interpreting and comparing findings among different studies due to multiple factors directly associated to the P essay. These caveats include: the utilization of different P essay techniques; multiple discrepancies regarding the timing of P measurements and the different P concentrations as a result of intra-day variability through the human circadian cycle (Kaponis *et al.*, 2018; Gonzalez-Foruria *et al.*, 2019; Shanker *et al.*, 2019); different utilization of commercial essays and equipment across research centers or clinical practices (Lawrenz *et al.*, 2018), external factors such as immunoassay cross-reactivity (Elecsysanalytics, 2007); and/or patients utilizing medications or oral supplements that have been described could modify or interfere with the serum P concentration measurements (Tietz, 1995; Fleming, 2008; Weissman *et al.*, 2011; Franasiak *et al.*, 2017). For our analysis, we aimed to eliminate this confounding bias by evaluating all P samples in the same laboratory, utilizing a reliable and modern assay that was consistently utilized under identical conditions and within the relative same time interval during COH monitoring.

Another limitation is the progesterone cutoff value utilized in our study for determining LFPE; this cutoff value was based on prior publications that have shown a significant detrimental effect on IVF

Table II Demographic characteristics of patients that underwent a single euploid FET analyzed by group of progesterone levels at ovulation trigger during COH cycles.

	Normal progesterone (≤2.0 ng/ml)		Late follicular phase progesterone elevation (>2.0 ng/ml)		*P*-value
	n = 5617 FET cycles		*n* = 189 FET cycles		
	Mean	SD	Mean	SD	
Serum progesterone at ovulation trigger on COH cycle (ng/ml)	0.88	0.37	2.95	1.66	<0.0001*
Maternal age at FET (years)	36.34	3.99	35.68	3.77	0.02*
Maternal age at oocyte retrieval (years)	35.78	3.96	35.20	3.68	0.04*
BMI (kg/m^2)	24.02	4.47	23.66	4.63	0.28
Anti Müllerian hormone (ng/ml)	3.49	3.83	3.80	3.14	0.32
Endometrial thickness at transfer (mm)	9.62	2.16	9.48	2.11	0.37
Progesterone at embryo transfer (ng/ml)	28.31	12.82	33.14	17.53	0.0004*
Delivery birthweight (g)	3317.37	571.89	3266.01	455.79	0.26
Gestational age at delivery (weeks)	39.00	1.89	39.24	1.53	0.25
	n/N	%	*n/N*	%	
Top morphologic embryo quality at ET	4028/5617	71.71	145/189	76.72	0.42
Implantation rate	4015/5617	71.48	134/189	70.90	0.92
Clinical pregnancy rate	3449/4190	82.32	109/143	76.22	0.11
Ongoing pregnancy rate	3020/4190	72.08	97/143	67.83	0.65
Multiple pregnancy rate	84/4190	2.00	2/143	1.40	0.67
Clinical loss rate	429/4190	10.24	12/143	8.39	0.65
Biochemical loss rate	732/4190	17.47	33/143	23.08	0.1

*Statistical significance, $P < 0.05$.
COH, controlled ovarian hyperstimulation; FET, frozen embryo transfer.

Table III Clinical IVF outcomes after a single euploid FET cycle among cohorts based on progesterone levels on the day of ovulation trigger administration during controlled ovarian hyperstimulation cycles.

	Progesterone group (ng/ml)										
	1 (≤1.0)		2 (1.1–2.0)		3 (2.1–3.0)		4 (3.1–4.0)		5 (>4)		
Progesterone group	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*P* value
Implantation rate	2835/3826	74.1	1377/1827	75.4	94/122	77.1	14/16	88	13/25	52.0	0.04*
Clinical pregnancy rate	2323/2835	81.9	1145/1377	83.2	71/94	76	11/14	79	8./13	61.5	0.12
Ongoing pregnancy rate	2036/2835	71.8	999/1377	72.6	66/94	70	9/14	64	7./13	53.9	0.88
Clinical pregnancy loss rate	287/2835	10.1	146/1377	10.6	5/122	4.1	2/14	14	1./13	7.7	0.67

*Statistical significance, $P < 0.05$.
FET, frozen embryo transfer.

outcomes (Bosch *et al.*, 2010; Van Vaerenbergh *et al.*, 2011; Venetis *et al.*, 2013; Kaponis *et al.*, 2018), while multiple other studies have looked at different cutoff values (Bosch *et al.*, 2010; Elgindy *et al.*, 2011; Lahoud *et al.*, 2012; Kofinas *et al.*, 2016; Lawrenz and Fatemi, 2017). To date, there is still no consensus on the exact cutoff of serum P to determine when to recommend a specific clinical action or treatment modification during COH, like previously reported 'rescue' strategies such as 'freeze-all embryos' instead of proceeding with a fresh ET, utilization of different stimulation protocols, corticosteroid supplementation and step-down stimulation approaches, among many other strategies (Lawrenz and Labarta, 2018; Hussein *et al.*, 2019). However, these clinical unknowns are beyond the objectives of our analysis.

Finally, during the FET analysis we included multiple cycles for patients and not only the first FET for each patient. During the time of the study, some patients underwent multiple transfers wherein some

had implantation failure or successful pregnancies and thereafter returned for a subsequent embryo transfer. By including this group of patients we acknowledge the risk of introducing bias among the population analyzed. Although, in order to account for this potential bias, we performed a multivariate analysis model fitted with a GEE with an exchangeable correlation structure which statistically corrects for this repeated measures. By employing this model we were allowed to assess the known and unknown possible correlations between the variables included in the model over the whole populations analyzed, accounting for the same patient appearing multiple times on different cycles on the same database.

Future human *in vitro fertilization* studies assessing the genetic expression pathways and molecular dynamics of dividing blastomeres by exposing gametes and/or embryos to nonphysiological hormonal milieu during COH should be performed in order to assess the real effect of premature progesterone elevations over oocyte fertilization, blastomere division, embryonic development and reproductive potential. Based on the current knowledgebase about LFPE and its relationship with IVF outcomes, we recommend reproductive specialists to adopt a personalized medicine approach for each patient. In that matter, reproductive specialists could take into consideration prior patient's reproductive history and other clinical factors to determine an optimal ovarian stimulation protocol and embryo transfer plan, aiming to improve patient's chances to achieve building a healthy family during ART treatments.

In patients presenting LFPE during COH, a freeze-all strategy and delayed embryo transfer with or without PGT is recommended as a therapeutic approach to overcome the detrimental effects of LFPE within the endometrium that occurs during a fresh IVF cycle. Patients can expect to optimize implantation potential by employing an FET cycle strategy.

Based on robust sequencing data from a large cohort of embryos, we conclude that progesterone elevation during IVF stimulation does not predict embryonic competence. Our study results show that LFPE is neither associated with impaired embryonic development nor increased rates of aneuploidy. Embryos obtained from cycles with LFPE can be selected for transfer, and patients can be reassured that the odds of achieving a healthy pregnancy are similar to the embryos exposed during COH cycles to physiological normal P levels.

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Authors' roles

All the authors of this manuscript have made substantial contributions to the conception or design of the study or the acquisition, analysis or interpretation of data for the study and have contributed to drafting the work or revising it critically for important intellectual content. All authors have approved the final version to be.

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Conflict of interest

Dr A.B.C. is advisor or board member of Sema4 (Stakeholder in data), Progyny and Celmatix. The other authors have no conflicts of interest to declare.

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