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Research Article

Evaluation of a Possible Association between Estradiol and Progesterone Levels and Ectopic Pregnancy in Low Risk Women Undergoing IVF/ICSI

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Abstract

Introduction: Several independent risk factors of Ectopic Pregnancy (EP) have been described to date. Nevertheless, estradiol and progesterone have not been related to ectopic pregnancy, although there is biological rationale to think about them as possible candidates. Our aim was to correlate the incidence of EP with levels of estradiol (E2) and progesterone (P4), measured on two days (hCG day, and seven days later (hCG+7)), including the differences and ratios of these concentrations, between the two time-points.

Material and methods: Retrospective cohort study of 578 patients undergoing fresh embryo transfer after IVF (100 cycles), ICSI (508 cycles) and IVF/ICSI (64 cycles) without risk of EP, between January 2005 and December 2015. We evaluated EP incidence (10 ectopic pregnancies) in fresh embryo transfers according to estradiol and progesterone levels on hCG day (hCGd) and seven days later (hCG+7) and their variation between both. The proportions were compared using the chi-square test or Fisher's exact test and the means were compared using T-test or ANOVA. To determine the accuracy of each studied variable receiver-operating curves were built.

Results: We identified a trend towards an increased risk of EP as progesterone levels rose on hCGd ($p=0.020$) and an association of progesterone values >1.89ng/ml on hCGd with EP (OR 6.8). An increased risk of EP when the difference of estradiol between hCGd and hCG+7 was either <39pg/mL, or >745pg/mL ($p=0.001$) was also found. ROC analysis only resulted significant for a moderate/good predictive ability for progesterone values on hCGd (AUC: 0.694).

Discussion: We conclude that these measurements are sufficient to identify patients at a high risk of EP. These hormone levels on specific days, leading us to define proper strategies to prevent EP risks in IVF. Further studies should design to prove this hypothesis.

Introduction

Ectopic Pregnancies (EP) arise in 1% of spontaneous conceptions [1], increasing to an incidence of 2-5% after In Vitro Fertilization (IVF) [2]. The origin of EP is unknown, although a number of risk factors have been identified. These include previous ectopic pregnancy [3], pelvic surgery or previous tubal surgery, peritonitis, tubal factors (pelvic inflammatory disease, or hydrosalpinx) [4], a previous genital C. trachomatis infection [5], recurrent miscarriage [6], being a heavy smoker, (defined as someone who smokes more than 10 cigarettes per day [7], advanced maternal age [8], Mullerian anomalies [9], adenomyosis [10], or multiple myomas [11]. In an infertile population, the main risk factor is the tubal factor, which markedly increases the rate of ectopic pregnancy (RR: 1.25) [2].

During natural reproduction, the embryo migrates from the Fallopian tube to the endometrium before implanting. However, in IVF, the embryo is directly and artificially placed into the uterine cavity. Subsequently, uterine peristalsis can displace the embryo into the Fallopian tube, or cornua, as demonstrated for 11 of 112 patients for whom the uterine peristaltic wave frequency was measured during the mock embryo transfer [12].

Uterine peristalsis is demonstrably stronger during cycles of controlled ovarian hyper stimulation compared to natural cycles, with the peristaltic wave frequency correlating positively with levels of serum estradiol (E2), and negatively with progesterone (P4) levels. This phenomenon, incurred by a physiologic range of steroid hormone concentrations [13], suggests a potentially relevant conditioning effect at the embryo implantation site. Supra-physiologic levels of E2 and P4 can modify endometrial receptivity, and have a detrimental effect on the luteal phase [14].

Hormonal factors appear to modify smooth muscle activity, and could underlie certain cases of EP not associated with structural tubal or uterine abnormalities [15]. Furthermore, those patients with polycystic ovarian syndrome (PCOS), who would be expected to manifest higher E2 levels, experience higher EP rates after fresh embryo transfer compared to non-PCOS patients [16].

Since IVF first began, it has been postulated that an uncalculated percentage of embryos transferred into the uterine cavity migrate to the Fallopian tubes, that, under normal conditions, are propelled back into the uterine cavity [17]. In support of this are data from an experimental study to evaluate embryo transfer, in which a radio-opaque dye moved to the Fallopian tubes in 38.2% of cases [18].

The controlled Fallopian tube cilia beat plays a role in facilitating the transport of the embryo to its implantation site. Interestingly, in vitro studies with Fallopian tube epithelial samples showed that P4 and E2 modify ciliary activity, offering a plausible explanation for the association between hormone levels and EP. Higher progesterone levels may cause ciliary dysfunction and EP [19]. While the effect of E2 on receptors in the tubal epithelium remains unknown, E2 has been shown to be involved in abnormal embryo implantation in the Fallopian tube [20]. Furthermore, P4 alone slows ciliary activity, and its concomitant use blocked the effect of E2 in ovum transport [21].

E2 and P4 levels are typically elevated during IVF, especially on the day on which hCG is administered. The largest series on this issue speculated that ovarian stimulation is associated with an increased risk of EP [22]. However, we currently lack studies with sufficient statistical power (due primarily to low EP prevalence) with which to associate luteal phase hormone levels with EP.

Our aim was to evaluate the influence of the absolute concentrations of E2 and P4, together with their variation and ratios on hCGd and seven days later (hCG+7), on the incidence of EP. We only included women with no history of factors related with EP.

Capsule: Ectopic pregnancies are more frequent when early luteal estradiol levels vary markedly compared to the day of hCG and when progesterone levels are elevated on that day.

Material and Methods

In a retrospective cohort study, we reviewed the fresh embryo transfers in our IVF programme using the IVI Vigo and Almeria databases. From January 2005 to December 2014, we included 578 women under their 40 (672 cycles), with no history of factors related to EP who underwent an IVF cycle without PGS. These factors include previous EP, pelvic surgery or previous tubal surgery, peritonitis, tubal factors (pelvic inflammatory disease or hydrosalpinx), a previous genital infection by C. trachomatis, recurrent miscarriage, heavy smoker (defined as someone who smokes more than 10 cigarettes per day), advanced maternal age (≥40 years old), Müllerian anomalies, adenomyosis, or multiple myomas. All these factors, as well as a Body Mass Index (BMI) of higher than 30 kg/m2, were considered exclusion criteria. All patients had undergone a normal hysterosalpingography and/or a laparoscopy to confirm normal tubes. Cycles using donated oocytes were excluded. An Institutional review board approval for this study was granted by our ethical committee. Even though it is not mandatory, due to the study design, we registered it at clinicaltrials.gov (NCT02793089).

As is clinically routine in IVI Vigo and Almeria, we measured E2 and P4 levels on hCGd, and during the early luteal phase in all patients in order to establish their treatment schedule [23,24]. We studied the correlation of EP risk with both E2and P4 levels, as well as with the ratio of E2/P4. These correlates were derived for each hormone, or ratio of hormones, for both time points (hCGd and hCG+7), including an added category of their variation from one time point to the other. E2/P4 ratios were calculated as (E2 x P4)/1000 level. Due to the absence of pre-established clinical thresholds, cycles were categorized into quartiles and deciles depending on E2 and P4 values. Cycles were groupedde pending on the quartile to which they belonged, showing either central (2nd+3thquartile),or eccentric values (1st+4rd quartile). Cycles were additionally classified according to their decile categorization, with the uppermost values included in the upper decile, and the lowest, in the bottom decile.

Ovarian Stimulation

For controlled ovarian hyper-stimulation, Gonadotropin Releasing Hormone (GnRH) antagonist protocols were used, as previously described [25].

Gonadotropin was given once ovarian quiescence had been confirmed. Daily doses of GnRH antagonist (Cetrotide, 0.25 mg; Merck-Serono, Madrid, Spain or Orgalutran, 0.25 mg; Merck Sharp & Dohme Limited, Hertfordshire, UK) were administered until the leading follicle reached 14 mm in mean diameter. Once three or more follicles reached 17 mm in diameter, hCG (Ovitrelle, 250 µg; Merck-Serono, Madrid, Spain) was administered, and, as before, oocyte retrieval was scheduled for 36 hours later. Patients with less than 3 follicles over 17mm, were excluded of the study.

The Luteal phase was supported with the administration of 600 mg (200 mg x 3) of vaginal P4 (Progeffik, 200mg; Effik, France) pessaries [26].

Serum E2 and P4 levels were also measured on the morning of hCG administration, and again 7 days later. Samples were tested with a micro particle enzyme immunoassay Access System (Beckman Coulter S.A., Madrid, Spain). The serum E2 kit had a sensitivity of 23 pg/mL, and comparison with mass spectrometry showed a correlation coefficient of 0.99. The serum P4 kit had a sensitivity of 0.08 ng/mL, and a correlation coefficient of 0.978.

We increased the doses of progesterone to 1200 mg/day when the level was lower than 10 ng/mL. Additionally, in patients with a severe E2 decline (<200pg/mL), 4mg of estradiol valerate at a daily dosewere added [24].

Oocyte pickup, fertilization, and embryo culture

Oocytes were collected by follicle aspiration. Beinga nine-year retrospective study (i.e. a prolonged investigation, prone to changes in materials), two different media were employed by the clinics. From 2005 to 2009, all procedures were performed using Vitro life media (Kungsbacka, Sweden), and from 2010 to 2014, all gametes and embryos were handled and cultured in Global (Life Global, Canada) media. To summarise, the oocytes were aspirated, washed in a buffered media then cultured in media for fertilization at 37ºC, under a 6.0% CO_2 and 20.0% O_2 atmosphere for 3h before oocyte denudation. Granulosa cells were then removed from oocytes by

mechanical pipetting in 1:1 hyaluronidase (80 IU/ml), in buffered media, prior to ICSI.

ICSI was carried out one hour after oocyte denudation, under an Olympus IX71 microscope, in buffered media, at X400 magnification. Then microinjected oocytes were cultured in standard incubators at 37.0ºC, 6.0% CO_2, and 20.0%O2 atmosphere. When classic IVF was performed, oocytes were suspended in 50 microlitre drops and inseminated with 100,000 sperm cells, as previously described [27]. Embryo selection was performed according to the recommendations stated by the Asociación para el Estudio de la Biología de la Reproducción (ASEBIR) [28]. Assisted Hatching was not performed in any embryo.

Embryo transfer

Embryo Transfer (ET) was performed either on day 3, or day 5 of embryo development after oocyte pick up. A maximum of three embryos were transferred, as per Spanish legislation, although single and double embryo transfers were performed on almost all patients.

We made an analysis to establish our own threshold of premature luteinization. Values were considered unacceptably high, when one standard deviation values exceed up the media [29]. To confirm this, we studied 2,144 samples from patients from our clinic using the Access 2 Immunoassays System (Beckman Isaza, Madrid, Spain). On hCGd, we found an average of P4 of 1.236 ng/mL and a standard deviation of 0.754. Therefore, the premature luteinization was considered above 1.99ng/mL.

The embryo transfer was not performed if the P4 level onhCGd was ≥ 1.99 ng/mL.

Before ET, we requested that patients drank liquids to keep their bladders full. Trans-abdominal sonography was performed during the procedure to monitor the catheter position in the uterine cavity. A sterile speculum (bivalve speculum) was introduced into the vagina, which was opened to expose the ectocervix. This had previously been cleaned with a sterile cotton swab soaked in flushing media. Embryos were then loaded in an Emtrac Delphin or Emtrac Plus embryo transfer catheter (Gynetics medical products. N.V., Lommel, Belgium), by the embryologist as follows: Firstly, the ET catheter was connected to a 1ml syringe (BD Plastipak, Beckton Dickinson; Madrid)

then flushed with embryo culture medium twice. Secondly, the catheter was filled with embryo culture medium, followed by 5 µl of air, then, 20 µl of culture medium containing the embryos, and finally another 5 µl of air at the tip of the catheter. Once the embryos were loaded into the catheter, the embryologist carried it to the theatre, which is adjacent to the IVF laboratory, and passed it to the gynecologist, who inserted it into the lower uterine segment, passing through the ectocervix. The catheter can be visualized because of its refringence, and was moved to a distance not less than 10mm from the fundus [30]. The syringe plunger was then firmly pressed to release 20 µl of culture medium containing the embryos into the uterine cavity, followed by a 45° rotation, and removal of the catheter. The catheter was then checked in the IVF laboratory to confirm embryo delivery.

ET procedures were classified as easy, moderate, or difficult. Easy transfers were made with the Emtrac Delphin catheter without force, or additional instrumentation. Moderate transfer implied use of the Emtrac Plus Catheter. Finally, a difficult transfer was that in which either the tenaculum was employed, or was a transfer that required more than one ET attempt. In this case, patients were encouraged to either drink more liquids, or empty their bladder, before the next attempt [31].

EP confirmation

The serum β-hCG was either measured 11 days after the blastocyst transfer or 13 days after embryo cleavage transfer. Clinical pregnancy was confirmed two weeks later if the existence of an embryo with a heartbeat was detected with a transvaginal ultrasound. An ongoing pregnancy was defined as a pregnancy that had developed beyond 12 weeks. Early pregnancy loss was defined as that proportion of patients with an initial positive β-hCG, in whom pregnancy failed to develop by 12 weeks of gestation.

EP was suspected when no intrauterine sac was detected with a transvaginal ultrasound at 5.5 weeks of gestational age [32], even if there were no significant abnormalities in the adnexa, and when serum β-hCG was >1000 IU/L [33]. We only consider cases of confirmed EP diagnosis by the presence of an embryo or yolk sac outside of the uterine cavity. [34].

Statistical analyses

For statistical analyses and after confirming the normal distribution of the data herein collected with Shapiro-Wilk Test, the chi-square test, or Fisher's exact test were applied where appropriate to compare proportions and T-test or ANOVA to compare means. The Statistics Package for Social Sciences version 22 was employed for all statistical analyses. Odd ratios and their corresponding 95% Confidence Intervals (CIs) between categories were also computed to provide an estimation of the association strength. Receiver-Operating Curves (ROC) were plotted in order to determine the accuracy of each studied variable in predicting EP.P values of <.05 were considered to be statistically significant.

Results

Demographics, patients and cycles

The mean ages of our female and male patients were 35.7 years 95%CI(35.4-36.0), and 37.8 years 95% CI (37.4-38.2), respectively. The mean female BMI was 23.5 kg/m2 95% CI (23.2-23.8).The prevalence of non-smokers among women included in our study was 86.2%, with light smokers comprising 13.8% of the total cycles. The reasons given for undergoing IV Fincluded:a maternal age of over 37 (58 cases, 8.6%), a low response to conventional ovarian stimulation (83 cases, 12.3%), polycystic ovarian syndrome (100 cases, 14.9%), or mild endometriosis (82 cases, 12.2%).The remaining patients presented no gynecological condition related to female infertility. Fifty-three patients had already had a previous pregnancy (9.1%).

Sperm quality, expressed as mean total motile spermatozoa in fresh ejaculates, was 79.5 million/ejaculate 95% CI (68.6-90.3), falling to 0.67 mill/sample 95% CI (0.44-0.91) after sperm preparation in the laboratory.

The mean (all subsequent values are means) number of stimulation days was9.9 95% CI (9.8-10.1), with a FSH dose of 1826.1 units 95% CI (1754.7-1897.6) and hMG dose of 700.9 units 95% CI

(647.5-754.2).The endometrial thickness was 10.2 mm 95% CI (10.0-10.4) and the number of retrieved oocytes 10.2 95% CI (9.7-10.7),with 8.5 95% CI(8.0-8.9) in metaphase II. In100 cases, fertilization was carried out using the classical IVF method (14.9%), while 508 cases fertilization was though ICSI (75.6%), with both techniques used simultaneously in 64 cases (9.5%).

A mean value (again, all subsequent values are means) of 5.9 oocytes 95% CI (5.7-6.2) were successfully fertilized, and 1.8 embryos transferred 95% CI (1.8-1.9), 1.1 95% CI (1.0-1.2) oocytes frozen. Easy embryo transfers occurred in 589 cases (87.6%), with moderate difficulty encountered in 22 cases (3.3%) and severe difficulties in 4 (0.6%). In 57 cases (8.5%), the ET procedures were not recorded, which were excluded of the study. The majority (79.3%) of embryo transfers were performed at day three of embryo cleavage, and 8 ectopic pregnancies where found in this group, while only two EP where found in those patients who were transferred day 5 embryos. The clinical pregnancy rate per transfer was 46% 95% CI (42.3-49.8), with an implantation rate of 25.6% 95% CI (22.9-28.3). The early pregnancy loss rate per transfer was 7.2% 95% CI (5.3-9.1), and the ectopic pregnancy rate per transfer was 1.5% 95% CI (0.6-2.4).

Comparisons of E2 and P4 levels between EP and no EP (inclusive of negative results and normal pregnancies)

E2 levels on hCGd and hCG+7 and their differences, were comparable in cycles resulting in EP compared to cycles that did not. The mean E2 concentrations on hCGd (for EP positive *vs.* negative cycles) were 1454.7 pg/mL 95% CI (779.0-2130.4) *vs.* 1562.7pg/mL95% CI (1484.56-1640.79). Seven days later (hCG+7), these values were 1024.1 pg/mL95% CI (625.6-1422.6) *vs.* 1176.3 pg/mL95% CI (1109.5-1242.6), with mean differences (between the two time points) of 430.6 pg/mL95% CI (0-899.1) *vs.* 390pg/mL 95% CI (325.4-456.1).

The mean P4 concentrations on hCGd were 1.5 ng/mL95% CI (1.1-2.0) *vs.* 1.1 ng/mL95% CI (1.1-1.1) for cycles with and without EP; these differences were found to be statistically significant (p=0.036). However, values for P4 athCG+7, and the variation between values recorded on hCGd *vs.* hCG+7, were statistically comparable. Specifically, on hCG+7, P4 was 86.2 ng/mL 95% CI (26.6-145.7) *vs.* 81.8 ng/mL 95%C I (76.6-86.9) for EP versus no EP; the mean fluctuation in concentration of P4between both days was 84.6ng/mL 95%CI (25.4-143.9) *vs.* 80.7 ng/mL95% CI (75.6-85.8) for EP positive *vs.* negative and no EP cases, respectively.

Regarding the E2/P4 ratio, all parameters were statistically comparable when comparing cases of EP, and no EP. On hCGd, the relevant mean values were (again for EP positive versus negative cycles) 1020.6 95%CI (605.1-1436.2) *vs.* 1728.1 95%CI (1612.4-1843.9), changing to 1003.8 95% CI (589.4-1418.2) *vs.* 1708.9 95% CI (1593.5-1824.3) for hCG+7. The differences between the time points were 16.8 95%CI (8.1-25.6) *vs.* 19.2 95%CI (17.8-20.6).

E2 and P4 relationship between EP and normal pregnancies (NP)

When we compared the main outcome measures, in cases of EP versus a normal implanted pregnancy, E2 on hCGd was 1454.7pg/mL 95% CI (779.0-2130.4) *vs.* 1583.5pg/mL 95% CI (1468.7-1698.3), decreasing to 1024.1 pg/mL 95% CI (625.6-1422.6) on hCG+7 *vs.* 1304.3pg/mL 95% CI (1200.6-1408.1). The respective differences in concentration were 430.6 pg/mL 95% CI (0-899.1) *vs.* 285.5pg/mL 95% CI (191.7-379.3).

The data for P4 (i.e. EP versus a normal implanted pregnancy) on hCGd were 1.5ng/mL 95% CI (1.1-2.0) *vs.* 1.1ng/mL (1.0-1.2), and 86.2ng/mL 95% CI (26.6-145.7) on hCG+ 7 *vs.* 90.2ng/mL 95% CI (81.6-99.2). The respective variations in concentration over these time points were 84.6ng/mL 95% CI (25.4-143.9) *vs.* 89.3ng/mL 95% CI (80.5-98.1) for EP and NP respectively. The P4 value on hCGd was statistically significant (p=0.036).

Regarding the E2/P4 ratio, a similar relationship was confirmed between groups, with all parameters statistically comparable. The respective values for EP positive cycles versus normal implanted pregnancies were, on hCGd, 1020.6 95% CI (605.1-1436.2) *vs.* 1689.9 95% CI (1561.0-1818.8), on hCG+ 71003. 8 95% CI (589.4-1418.2) *vs.* 1669.7 95% CI (1541.3-1798.0) being the differences 16.8 95% CI (8.1-25.6) *vs.* 20.2 95% CI (17.9-22.5) respectively.

Correlation between E2 and EP

As shown in Table 1, the absolute levels of E2 on hCGd, seemed not to influence the risk of EP, since no trend towards either increased or decreased rates of EP were noted in the quartiles, nor obvious differences in the central/eccentric categories, nor highest or lowest values. In terms of E2 levels found on hCG+7, a similar result was noted, although no EP was registered at values below 338pg/mL, or above 2,304pg/mL.

When taking into account the E2 fluctuation between days, an effect was detected. We noted an increased risk of EP if patients showed variations of below 39pg/mL, or above 745pg/mL, meaning that outlier changes in hormonal concentration exhibited a significantly higher chance of EP. Moreover, we did not identify a single case of EP below a decreased value of -436.0 (between time points).

Correlation between P4 concentration and EP

Interestingly, there is a noticeable trend that was statistically significant, in which elevated P4 levels on hCGd presented an increased risk of EP (Table 2: test for trend p=0.020), although specific inter-group differences were not identified. Those P4 values on hCGd, lying outside of the central values, presented statistically comparable rates of EP. P4 values were particularly related to EP when belonging to the last decile (i.e. extremely high values for P4, in excess of 1.89 ng/mL), with these data providing an OR indicating an approximately 7 fold effect. No link with EP was observed for data falling within the first decile (i.e. below 0.51 ng/mL).

When analyzing the data for hCG+7, as well as the differences in P4 concentration between both days, there was neither an observable trend, nor any change regardless of the category analyzed, indicating a lack of effect on EP rate.

E2/P4 ratio on hCGd, hCG+7 and variation in EP incidence

As seen in Table 3, the ratio of E2/P4 appeared not to be related to the risk of EP, neither on hCGd, nor hCG+7.When analyzing the differences between both days, we found a trend emerging in that the risk of EP decreased, as the difference in values increased, although this effect was not found to be statistically significant. No EPs occurred when E2/P4 values on hCGd were above 3,131 or below 5.34 on hCG+7.

Table 1: Risk of EP according to E2 concentration on hCGd, hCG+7, and its variation. Data are expressed as proportions or odds ratios (OR) with 95%CI. Regarding all ranges, the value included corresponded always to the higher within the range (example, lower to 857.7, has 857.7 included, and this figure is not included within the subsequent category).

E2 hCGd	Range	Cycles	Ectopic pregnancy	OR	p value
1st Quartile	Lower to 857.7	167	4 (2.3%)	Ref.	
2nd Quartile	857.7-1372.5	169	1 (0.6%)	0.25 (0.03-2.23)	Ns
3rd Quartile	1372.5-2012	168	3 (1.8%)	0.75 (0.16-3.38)	Ns
4th Quartile	2012 to higher	168	2 (1.2%)	0.50 (0.08-2.74)	Ns
Central values (2nd+3rd QT)		337	4 (1.2%)	Ref.	
Eccentric values (1st+4th QT)		335	6 (1.8%)	1.51 (0.42-5.40)	Ns
Lower 90th percentile	Lower to 2859.0	605	9 (1.5%)	Ref.	
Upper 10thpercentile (extreme val.)	2859.0 to higher	67	1 (1.5%)	1.00 (0.13-8.05)	Ns
Upper 90th percentile	552.0 to higher	604	9 (1.5%)	Ref.	
Lower 10th percentile (extreme val.)	Lower to 552.0	68	1 (1.4%)	0.99 (0.13-8.04)	Ns
E2 hCG +7 day	**Range**	**Cycles**	**Ectopic pregnancy**	**OR**	**p value**
1st Quartile	Lower to 561	168	3 (1.8%)	Ref.	
2nd Quartile	561-983	167	3 (1.8%)	1.01 (0.20-5.05)	Ns
3rd Quartile	983-1491	171	1 (0.6%)	0.33 (0.03-3.18)	Ns
4th Quartile	1491 to higher	166	3 (1.8%)	1.01 (0.20-5.07)	Ns
Central values (2nd+3rd QT)		338	4 (1.2%)	Ref.	
Eccentric values (1st+4th QT)		334	6 (1.8%)	1.52 (0.43-5.43)	Ns
Lower 90th percentile	Lower to 2304.0	604	10 (1.6%)	Ref.	
Upper 10th percentile (extreme val.)	2304.0 to higher	68	0 (0%)	NA	Ns
Upper 90th percentile	Higher to 338.0	603	10 (1.6%)	Ref.	
Lower 10th percentile (extreme val.)	338.0 to lower	69	0 (0%)	NA	Ns
E2 variation hCGd – hCGd+7	**Range**	**Cycles**	**Ectopic pregnancy**	**OR**	**p value**
1st Quartile	Lower to 39	165	6 (3.5%)	Ref.	
2nd Quartile	39-328	170	0 (0.0%)	NA	Ns
3rd Quartile	328-745	171	0 (0.0%)	NA	Ns
4th Quartile	745 to higher	166	4 (2.4%)	0.66 (0.18-2.39)	Ns
Central values (2nd+3rd QT)		341	0 (0.0%)	Ref.	
Eccentric values (1st+4th QT)		331	10 (2.9%)	NA	0.001
Lower 90th percentile	Lower to 1321.0	605	9 (1.5%)	Ref.	
Upper 10th percentile (extreme val.)	1321.0 to higher	67	1 (1.5%)	1.00 (0.13-8.04)	Ns
Upper 90th percentile	Higher to -436.0	603	10 (1.6%)	Ref.	
Lower 10th percentile (extreme val.)	-436.0 to lower	69	0 (0%)	NA	Ns

Table 2: Risk of EP depending on the P4 level on hCGd, hCG+7, and its variation. Data are expressed as proportions or odds ratios (OR) with 95%CI.

P4 hCGd	Range	Cycles	Ectopic pregnancy	OR	p value
1st Quartile	Lower to 0.72	171	1 (0.6%)	Ref.	
2nd Quartile	0.72-1.0	162	1 (0.6%)	1.05 (0.06-16.94)	Ns
3rd Quartile	1.0-1.44	166	2 (1.2%)	2.06 (0.27-2.72)	Ns
4th Quartile	1.44 to higher	156	6 (3.7%)	6.57 (0.78-55.6)	Ns
Central values (2nd+3rd QT)		328	3 (0.9%)	Ref.	
Eccentric values (1st+4th QT)		327	7 (2.1%)	2.34 (0.60-9.13)	Ns
Lower 90th percentile	Lower to 1.89	611	6 (1.0%)	Ref.	
Upper 10th percentile (extreme val.)	1.89 to higher	61	4 (6.3%)	6.8 (1.87-24.8)	0.01
Upper 90th percentile	Higher to 0.51	603	10 (1.6%)	Ref.	
Lower 10th percentile (extreme val.)	0.51 to Lower	69	0 (0%)	NA	Ns
P4 D+7 day	**Range**	**Cycles**	**Ectopic pregnancy**	**OR**	**p value**
1st Quartile	Lower to 40.4	236	6 (2.5%)	Ref.	
2nd Quartile	40.4-60.0	100	0 (0%)	NA	Ns
3rd Quartile	60.0-105.2	159	2 (1.2%)	0.49 (0.10-2.50)	Ns
4th Quartile	105.2 to higher	165	2 (1.2%)	0.48 (0.10-2.39)	Ns
Central values (2nd+3rd QT)		259	2 (0.8%)	Ref.	
Eccentric values (1st+4th QT)		401	8 (2.0%)	2.58 (0.54-12.26)	Ns
Lower 90th percentile	Lower to 153.4	607	8 (1.3%)	Ref.	
Upper 10th percentile (extreme val.)	153.4 to higher	65	2 (3.0%)	2.34 (0.49-11.23)	Ns
Upper 90th percentile	Higher to 33.7	606	9 (1.5%)	Ref.	
Lower 10th percentile (extreme val.)	33.7 to Lower	66	1 (1.5%)	1.02 (0.13-8.18)	Ns
P4 variation hCGd+7 – hCGd	**Range**	**Cycles**	**Ectopic pregnancy**	**OR**	**p value**
1st Quartile	Lower to 39.2	158	5 (3.0%)	Ref.	
2nd Quartile	39.2-58.8	162	1 (0.6%)	0.20 (0.02-1.70)	Ns
3rd Quartile	58.8-105.3	161	2 (1.2%)	0.40 (0.07-2.07)	Ns
4th Quartile	105.3 to higher	161	2 (1.2%)	0.40 (0.07-2.07)	Ns
Central values (2nd+3rd QT)		323	3 (0.9%)	Ref.	
Eccentric values (1st+4th QT)		320	7 (2.1%)	0.43 (0.11-0.66)	Ns
Lower 90th percentile	Lower to 153.28	609	8 (1.3%)	Ref.	
Upper 10th percentile (extreme val.)	153.28 to higher	63	2 (3.1%)	2.42 (0.50-11.63)	Ns
Upper 90th percentile	Higher to 33.62	608	9 (1.5%)	Ref.	
Lower 10th percentile (extreme val.)	33.62 to lower	64	1 (1.5%)	1.06 (0.13-8.47)	Ns

Table 3: Risk of EP depending on the E2/P4 ratios on hCGd, hCG+7, and their variation. Data are expressed as proportions or odds ratios (OR) with 95% CI.

E2/P4 hCGd	Range	Cycles	Ectopic pregnancy	OR	p value
1st Quartile	Lower to 820.7	161	5 (3.0%)	Ref.	
2nd Quartile	820.7-1319.15	164	2 (1.2%)	0.39 (0.08-2.07)	Ns
3rd Quartile	1319.15-2134.5	162	3 (1.8%)	0.60 (0.14-2.55)	Ns
4th Quartile	2134.5 to higher	167	0 (0 %)	NA	Ns
Central values (2nd+3rd QT)		326	5 (1.5%)	Ref.	
Eccentric values (1st+4th QT)		329	5 (1.5%)	0.99 (0.28-3.46)	Ns
Lower 90th percentile	Lower to 3131.57	606	10 (1.6%)	Ref.	
Upper 10th percentile (extreme val.)	3131.57 to higher	66	0 (0%)	NA	Ns
Upper 90th percentile	Higher to 536.3	609	8 (1.3%)	Ref.	
Lower 10th percentile (extreme val)	536.3 to lower	63	2 (3.1%)	2.42 (0.50-11.63)	Ns
E2/P4 hCG+7 day	**Range**	**Cycles**	**Ectopic pregnancy**	**OR**	**p value**
1st Quartile	Lower to 8.32	166	2 (1.2%)	Ref.	
2nd Quartile	8.32- 14.14	163	4 (2.4%)	2.03 (0.37-11.23)	Ns
3rd Quartile	14.14-23.44	166	2 (1.2%)	1.00 (0.14-7.19)	Ns
4th Quartile	23.44 to higher	165	2 (1.2%)	1.01 (0.14-7.24)	Ns
Central values (2nd+3rd QT)		329	6 (1.8%)	Ref.	
Eccentric values (1st+4th QT)		331	4 (1.2%)	0.66 (0.19-2.37)	Ns
Lower 90th percentile	Lower to 38.97	606	9 (1.5%)	Ref.	
Upper 10th percentile (extreme val.)	38.97 to higher	66	1 (1.5%)	1.02 (0.13-8.18)	
Upper 90th percentile	Higher to 5.34	605	10 (1.6%)	Ref.	
Lower 10th percentile (extreme val)	5.34 to lower	67	0 (0%)	NA	Ns
E2/P4 variation hCGd – hCGd+7	**Range**	**Cycles**	**Ectopic pregnancy**	**OR**	**p value**
1st Quartile	Lower to 799.59	157	5 (3.1%)	Ref.	
2nd Quartile	799.59-1302.67	162	2 (1.2%)	0.39 (0.07-2.04)	Ns
3rd Quartile	1302.67-2094.10	161	2 (1.2%)	0.39 (0.07-2.04)	Ns
4th Quartile	2094.10 to higher	162	1 (0.6%)	0.20 (0.02-1.68)	Ns
Central values (2nd+3rd QT)		323	4 (1.2%)	Ref.	
Eccentric values (1st+4th QT)		320	6 (1.8%)	1.51 (0.42-5.42)	
Lower 90th percentile	Lower to 3088.15	607	10 (1.6%)	Ref.	
Upper 10th percentile (extreme val.)	3088.15 to higher	65	0 (0%)	NA	Ns
Upper 90th percentile	Higher to 520.49	609	8 (1.3%)	Ref.	
Lower 10th percentile (extreme val)	520.49 to lower	63	2 (3.1%)	2.41 (0.52-11.63)	Ns

E2 and P4 levels on hCGd, hCG +7 and their predictive value

ROC curve analyses revealed (Supplemental Figure 1) that neither E2, P4 (inclusive of differences for both hormones over time), or their ratios (E2/P4), on either test day, provided any predictive power, except for a moderate to good predictive ability for P4 value, on the day of administration of hCG. For this metric (P4), the accuracy of its predictive power (the area under the curve (AUC) was almost 0.7.

Discussion

We found that EP occurred more frequently in fresh IVF cycles when an excessive fluctuation in hormone levels occurred at the early luteal phase compared to hCGd in patients without known EP risk factors. High P4 levels on hCGd were also associated to an increased risk of EP, with significant risks caused by extremely high values of P4.

The considerable diversity of individuals undergoing assisted reproductive treatments limits our ability to pinpoint the factors that predispose EP. This study was conducted with a selected population without any known EP risk factors, rather than a random sample, and it could explain why an IVF cycle without any apparent risk factors ends in EP.

We measured hormonal levels on day +7 from hCGd, as it corresponds to the peak level of P4 during luteal phase. Although it is controversial, several studies support taking the measurement of E2 and P4 during the luteal phase as they have a potential predictive value for P4 in IVF/ICSI [35]. A value of 9.4 ng/mL as single serum progesterone measurement in the luteal phase is a criterion of a potentially fertile cycle ("ovulation") [36]. Furthermore, a serum P4 levels between 3 and 10ng/ml have been considered as low concentrations [37]. Some authors have found a beneficial effect to add estradiol to progesterone to support the luteal phase in IVF agonists cycles [38,39].

Conversely, there is no threshold for estradiol during luteal phase. A recent study shows that supplementation with P4 plus E2 for luteal phase is associated with a higher clinical pregnancy rate than progesterone alone in women undergoing IVF. [40].

When E2 ratio (day 0/day 8) was>5 (corresponding to 80% decline), implantation and pregnancy rates decreased significantly [24].

Agreed hormone thresholds that constitute high/low E2 and P4 levels at the mid luteal phase are unavailable. Therefore, to demonstrate the hormone variability for our patient cohort, they were categorized into four groups, based on percentiles. Patients in either of the outlying quartiles (the first or fourth) were considered to demonstrate aberrant levels of hormone variation between hCGd, and hCG+7. While we are aware that we lost information by categorizing in this fashion, we also gained accuracy with this strategy.

According to our data, a wide variation of E2values between hCGd and hCG+7 is associated with EP. In either group, when E2 variation markedly increased or declined steeply (eccentric values, Table 1), the risk of EP was high. Specifically, altered levels of E2 of less than 39 pg/mL, or greater than 745 pg/mL (i.e. sharply divergent from the mean), were associated to significantly higher risks of EP.

In terms of high concentrations of E2, a supra-physiologic hormonal milieu could alter uterine contractile patterns as E2 levels rise at hCG+7 [41]. However, as yet, we do not know why a decrease of E2 would lead to a higher risk of EP. We would hypo the size that a sharp serum fall in E2 could modify endometrial receptivity, providing a plausible explanation for the link between low E2 levels and EP.

The effect of E2 and P4 concentrations around the time of implantation as a cause of EP is not a novel proposition. In the early eighties, an unexplained spike in the incidence of EP was described in relation to ovarian stimulation, with the suggestion that an altered tubal peristalsis was to blame, due to elevated hormone concentrations [42,43]. This hypothesis would be plausible if the embryo moves outside of the uterine cavity after its transfer, causing its retention in the Fallopian tube. Supra-physiological E2 levels may cause disorganized uterine contractility, which could expel the embryos from the uterine cavity [44].

Uterine peristalsis exerts control over fluid migration after ET, with fluid displaced into the Fallopian tubes or the cornua of the uterus in 9.8% of the cases. Also, the frequency of peristaltic waves significantly correlates with the distance the fluid moves [12]. Embryos placed in the intrauterine cavity using a combination of soft catheter and ultra sound do not always remain in situ [45]. Once the embryo reaches the tube, it should transit to the uterine cavity under the effects of regulatory mechanisms including endocrine regulation, mainly by ovarian steroids, as well as neuronal and paracrine regulation [46]. E2 treatment accelerates ovum transport, whereas P4 slows the process, and induces a decrease in uterine peristalsis, especially for the cervix-fundus waves, and during the luteal phase [47]. We did not find any relationship between the insemination technique and EP risk.

The high ratio between E2/P4 can increase the muscular tone of the isthmus, facilitating the retention of the embryo in the tube, whereas a low ratio may reduce tubal peristalsis delaying embryo transportation [15]. However, in our study we could find no correlation between the E2/P4 ratio and EP. We did however find a correlation between P4 levels on hCGd and EP risk. This finding is in agreement with previous studies, which demonstrated that patients with elevated E2 and P4 on hCGd have significantly higher EP rates compared to the unexposed group [48]. Although controversial, some studies show adverse outcomes in Assisted Reproductive Techniques (ART) when the P4 level is high in the late follicular phase of ovarian stimulation [25]. We do not yet know the mechanisms whereby increased P4 during the late follicular phase increases the risk of EP, but we would propose two theories. First, the premature endometrial maturation induced by P4 could lead to a non-receptive status provoked by an altered gene expression [49,50]. This may cause implantation at other locations. Secondly, uterine peristalsis may guide the embryo away from the uterus, with the embryo unable to return from the Fallopian tube. Exposure to a high P4 concentration during the late follicular phase could alter tubal peristalsis, as has been shown in experimental studies, leading to the retention of the embryo in the Fallopian tube [19,21]. However, this hypothesis is yet to be proven given that we neither measured uterine nor tubal peristalsis in the different groups. Additionally, the finding of a very low EP rate in treatments under constant hormonal conditions reinforces our theory.

Multiple studies have shown that EP is less common after frozen ET compared to fresh [51,52]. Incidence rates of 1.8% in fresh IVF cycles, and 1.4% in fresh ICSI cycles have been reported, versus 0.8% for frozen cycles [52]. To explain these findings, authors have raised the possibility that controlled ovarian hyper-stimulation (COH) adversely effects the endometrium. Meanwhile, the incidence of EP is lower in oocyte recipients, whose treatment is conducted without exposure to a supra-physiologic hormone milieu [53]. Interestingly, the number of oocytes retrieved [54], and embryos transferred, have been associated with a high risk of EP in a recent retrospective analysis [55]. Similarly, patients with Polycystic Ovarian Syndrome (PCOS), who likely generate a higher E2 level, manifest a higher EP rate after fresh embryo transfer compared to non-PCOS patients [16]. High levels of E2 at the early luteal phase may be the result of an elevated level of corpus luteum-generated hormones in high responders. In support of this hypothesis, we found a higher number of oocytes generated in those outlier groups categorized as highly divergent in terms of hormone fluctuation, while a lower number of oocytes were generated by the central categories that display less dramatic hormonal variations. Consequently, we would suggest that the number of oocytes constitutes one possible component of an elevated EP rate.

Several studies [53,54,56] report the incidence of EP associated with ART based on large national databases. These types of studies are often less reliable, as they are hampered by in accuracies linked to non-owner databases. Therefore, the lack of information available for clinical protocols and processes for embryo transfer might impact the outcome. In our study, all patients were followed-up by the same gynaecological team in just two clinics.

Our study does, however, have some limitations. First, it was conducted retrospectively, making several factors difficult to control. Although transfers were performed by the same gynaecology and embryology team, we could not control interpersonal variation. The accuracy of the study would probably be greater if the transfers had been carried out by only one gynaecologist and embryologist. We did not analyse the incidence of ectopic pregnancy on the degree of difficulty on embryo transfer because only 3.9% of cycles were considered not easy. Therefore, the sample size is not big enough to reach a conclusion. The quantities of culture medium and air used to transfer the embryos were consistent.

Due to the long term included in this study, two different culture media were used by the clinics. A recent retrospective study pointed out that EP might, in part, be influenced by the culture media used [57]. However, we found no differences in the EP rate between the two culture media employed (data not shown).

Many authors have pointed out a different ectopic pregnancy incidence according to embryo stage transference .Some groups have associated blasyocyst transference with lower incidence of ectopic pregnancy [52,58-59]. Others, have stated out that there is no relationship between the embryo stage at the moment of the transference and ectopic pregnancy [2,60-61]. Finally others had found lower risk of Ectopic Pregnancy when transferring cleavage stage embryos [62-63].

Our study has not been designed for analyzing the influence between embryo cleavage stage and Ectopic Pregnancy. It is an issue which can't be excluded, indeed, but if we observe the numbers of ectopic pregnancy in our study, we can´t find any statistically significant difference (in cleavage stage we found 8/482 off ectopic pregnancy, it is a 1,5% , and for blastocyst stage, we found 2 EP out of 133 transference, what means a 1,6% of ectopic pregnancy).

We analyzed a specifically low risk group, to avoid bias induced by other possible causes of EP. As might be expected, our conclusions cannot therefore be generalized to the IVF population as a whole. Future efforts should therefore focus on studying, simultaneously, hormone levels and uterine peristalsis, as tubal peristalsis is still unable to be measured in vivo. Randomized prospective blind or experimental studies are also now required to confirm our findings.

EPs are potentially life-threatening scenarios that occur more frequently after assisted reproduction than in spontaneous pregnancies. Given their potential severity, any efforts to prevent them are desirable. This research provides new mechanistic data for EPs in the setting of IVF. The concentration of E2, and its associated effects in the early luteal phase, could provide new insights into the management of ET. We suggest that patients with high risk EP should have their E2 and P4 levels evaluated during the luteal phase. In the case these values change markedly, it would be appropriate to defer frozen embryo transfer.

To our knowledge, this is the first study to specifically examine the association between hormone level variation and the risk of EP. Possible implications for uterine peristalsis and tubal function in influencing EP risk are still unproven.

In conclusion, we find that aberrant E2 variation between the late follicular phase, and early luteal phase (hCGd and hCG+7), is associated with an elevated risk of EP. A high P4 level on hCGd is also linked to a risk of EP in fresh embryo transfers.

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