Universitat Autònoma de Barcelona

IMPACT OF SERUM PROGESTERONE ON REPRODUCTIVE OUTCOMES IN FROZEN EMBRYO TRANSFER CYCLES

Sofia Gaggiotti-Marre

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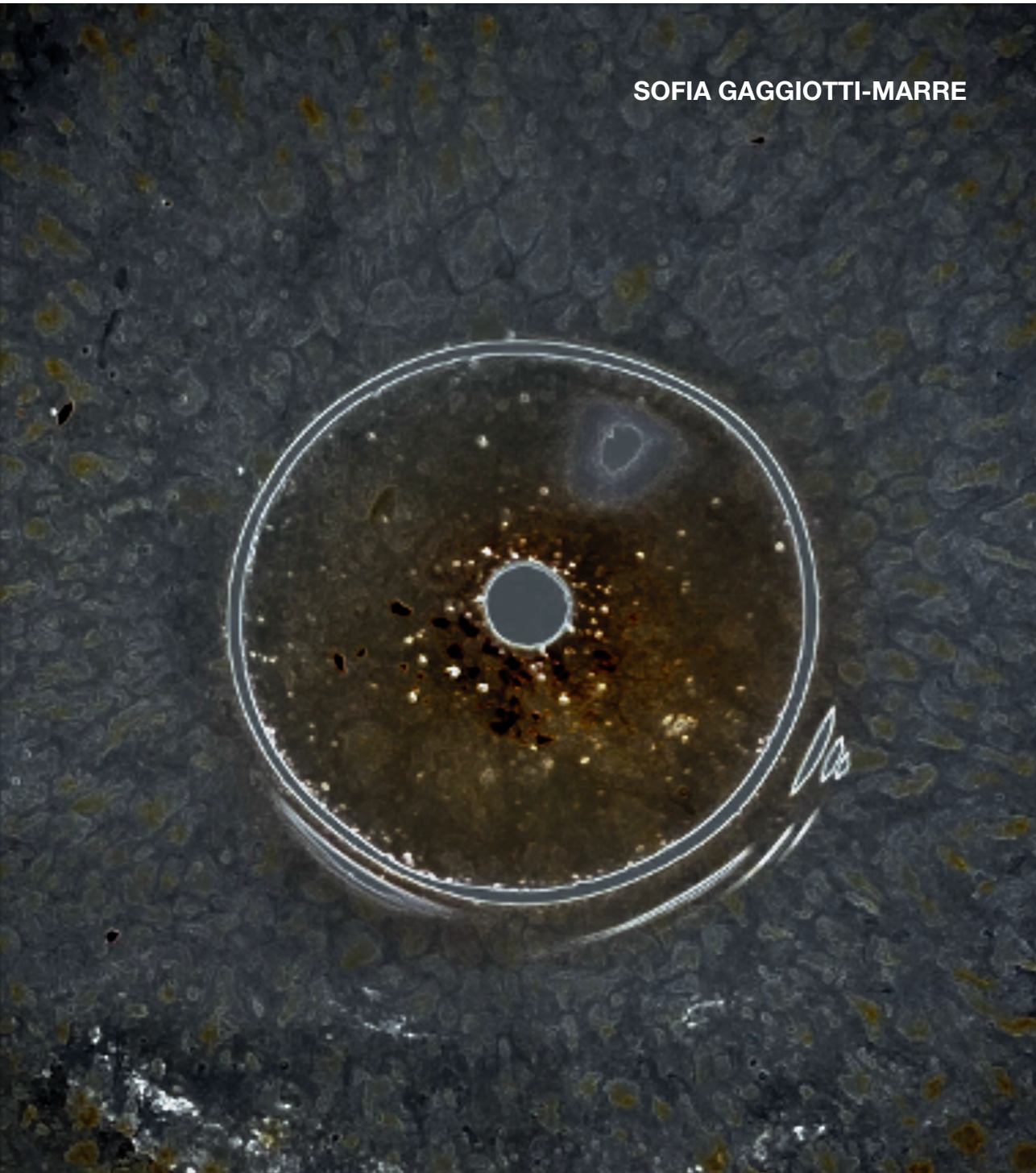

Doctoral Thesis: IMPACT OF SERUM PROGESTERONE ON REPRODUCTIVE OUTCOMES IN FROZEN EMBRYO TRANSFER CYCLES

A thesis submitted by Sofia Gaggiotti-Marre for the PhD degree of Pediatrics, Obstetrics and Gynecology (Faculty of Medicine, Autonomous University of Barcelona)

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We confirm that Sofia Gaggiotti-Marre has conducted the studies presented in the thesis 'Impact of serum progesterone on reproductive outcomes in frozen embryo transfer cycles' under our supervision.

The present thesis has been structured following the normative for PhD theses as a compendium of publications and has been conducted at the Reproductive Medicine Unit of Dexeus University Hospital, Barcelona.

Barcelona, March 2021

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INTRODUCTION

NATURAL FECUNDATION IN THE HUMAN SPECIES

THE MENSTRUAL CYCLE

Like most processes in the human body, the menstrual cycle resembles a delicate clock-like machine that requires an optimal environment to work, not only in the reproductive tract, but throughout the whole body. It usually lasts between 25 and 30 days. By convention, day 1 of the menstrual cycle is the first day of menstruation. The menstrual cycle can be divided into three stages: the follicular phase, in which follicular growth takes place; the ovulatory period, when the final maturation of the oocyte and its release occur; and the luteal phase, in which the corpus luteum secretes hormones in preparation for embryo implantation. Although in a typical cycle the follicular phase lasts approximately 14 days, its length can be variable; whereas the luteal phase is remarkably constant and lasts 12-15 days. If the egg is not fertilized and implantation does not occur, a new cycle begins. If implantation occurs, the luteal phase is prolonged and becomes the progestational phase of the pregnancy (Ferin *et al.*, 1993).

Hormonal markers

There are four main hormonal markers that characterize the menstrual cycle: two of pituitarian origin, the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH), and two of ovarian origin, oestradiol (E2) and progesterone (P). These hormones can be monitored in the blood circulation by assay methods. The regulation and mechanism of the cycle is complex and other hormones and substances are involved. However, for the purpose of this thesis the focus will mainly be on the four abovementioned hormones.

Both LH and FSH are glycoproteins and consist of two subunits (α and β). α subunit is common to LH, FSH, human chorionic gonadotropin (hCG) and thyroid-stimulating hormone (TSH). The β subunits are different and therefore characterize each hormone.

For any given hormone, there is pulsatility and variation in its secretion patterns, and there are circulating variations within individual cycles and even within one same woman (Filicori *et al.*, 1984). Therefore, the menstrual cycle requires a precise coordination of events that take place within distant organs of the body, such as the brain, pituitary gland, ovaries and reproductive tract.

The menstrual cycle can be summarised in Figure I, where the hormonal variations, as well as the ovarian and the endometrial components, are outlined throughout the three phases of the cycle. This diagram, albeit simplified, is essential for the understanding of the menstrual cycle and great part of the human natural conception, which in turn is essential for the understanding of the human conception *in vitro.*

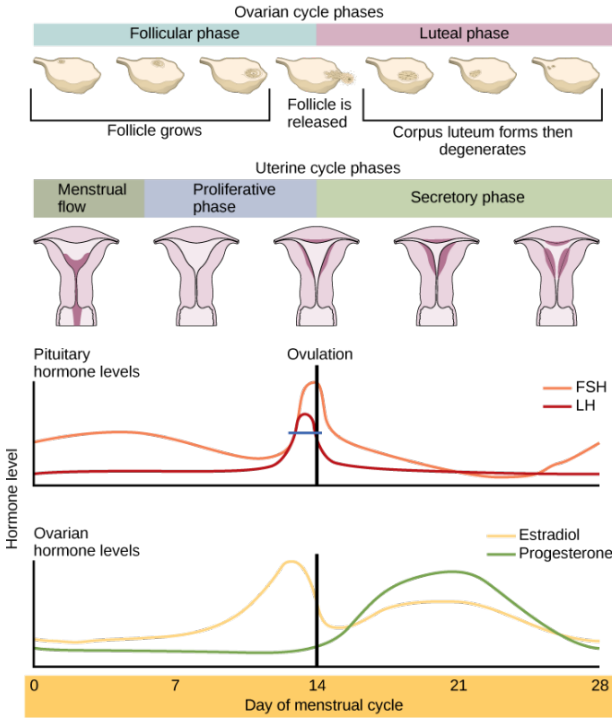

Figure I: The menstrual cycle. *Adaptation from Carr and Wilson, 1987*

The Neuroendocrine component: the hypothalamic-pituitary-ovarian axis.

The hypothalamic gonadotropin-releasing hormone (GnRH) is a small neuropeptide consisting of 10 amino acids (decapeptide). Via the hypothalamic-hypophyseal portal circulation through the portal veins, GnRH is released in pulses, which in turn produces the release of the gonadotropins LH and FSH from the pituitary gland. GnRH interacts with its plasma membrane receptor, allowing for gonadotropin release with the help of the ion calcium. Depending on the stage of the menstrual cycle and the endocrine milieu, the sensitivity of the GnRH receptor and pulse varies. The pulsatility frequency increases during the late follicular phase of the cycle culminating in the LH surge preceding ovulation, whereas a slow frequency stimulates the release of FSH. During the secretory phase, frequency decreases secondary to an increase in circulating oestrogen and progesterone, due to an inhibitory feedback mechanism.

Therefore, an intermittent or pulsatile pattern of GnRH release is crucial for normal gonadotropin function (Johnson, 2007).

There are extensive feedback mechanisms, both negative and positive that exist within the hypothalamic-pituitary-ovarian axis. During the early

follicular phase FSH begins to increase promoting follicular recruitment and growth. LH secretion is inhibited by the increasing levels of oestrogen produced by maturing follicles. However, 36-48 hours before ovulation, the oestrogen feedback mechanism becomes positive initiating an LH surge, which is essential for ovulation. During the luteal phase, the secretion of LH and FSH are inhibited due to high circulating levels of oestrogen, progesterone and inhibin (Johnson, 2007).

Ovary component

The human ovary is an heterogenous tissue in constant change, which cycles are measured by weeks. Histologically, the ovary has 2 main sections: the outer cortex and the inner medulla. A germinal layer coats the entire ovary, made of cuboidal epithelial cells. The oocytes are found locked inside follicles, in the cortex, inside the stroma. The stroma is made of spindle-shaped fibroblasts that respond to hormonal stimulation (LH and hCG) by producing androgens. The medulla is where the ovarian vasculature is found and is primarily loose stromal tissue.

During the 16^{th}-20^{th} weeks of gestation, the number of oocytes reaches 6-7 millions, its maximum. Simultaneously and with a maximum level around the 5^{th} month of pregnancy, there is an oocyte atresia followed by a rapid

follicular atresia. When a female is born, there are around 2 millions of oocytes and during puberty, only 300.000 available for ovulation. Of these, only 400 or 500 will ovulate. The oocytes stay in a 'resting' state in the first meiotic prophase (primordial follicles) to become primary or preantral follicles, after birth and until ovulation (Fritz and Speroff, 2011)

Follicular phase

The early follicular phase in humans is the time when the ovary is the least hormonally active, resulting in low serum oestradiol and progesterone concentrations. The negative feedback effects of oestradiol, progesterone, and inhibin from the luteal phase of the preceding cycle results in a late luteal/early follicular phase increase in GnRH pulse frequency and a subsequent increase in serum FSH concentrations. FSH stimulates the growth of the primordial follicles, with 5-15 of them able to grow at a time. One of them will reach maturity, whilst the other ones will degenerate, a phenomenon called atresia (Johnson, 2007).

FSH and LH bind to their receptors in the follicular granulosa cells, that begin to appear in the late preantral and early antral follicles. FSH is responsible for follicular recruitment and is sufficient for initial follicular growth. The theca synthesizes androgens under the influence of LH, which are converted into

oestrogen by FSH-induced aromatase in the neighbouring granulosa cells of selected growing follicles. Thus, there is a massive increase in oestrogen biosynthesis (Johnson, 2007).

The follicular response to gonadotrophins is regulated by growth factors and autocrine-paracrine peptides. Insuline-like growth factors and inhibins support follicular progression, acting in cooperation with FSH and androgens, whereas others (MIH, TNFα, leptin and IGFBP's) depress follicular development or promote atresia.

Ovulation

The expanding antral follicle requires an LH surge to trigger ovulation. This surge is triggered by increased peripheral oestradiol levels and a small increase in progesterone, and it represents a switch from negative feedback control of LH secretion by ovarian hormones to a sudden positive feedback effect (Johnson, 2007). This results in a rapid release of LH and FSH, which boosts fluid accumulation in the follicle, disruption of the gap-junctions between the oocyte and cumulus cells and resumption of the meiosis in the oocyte, as shown in figure II (Dozortsev and Diamond, 2020).

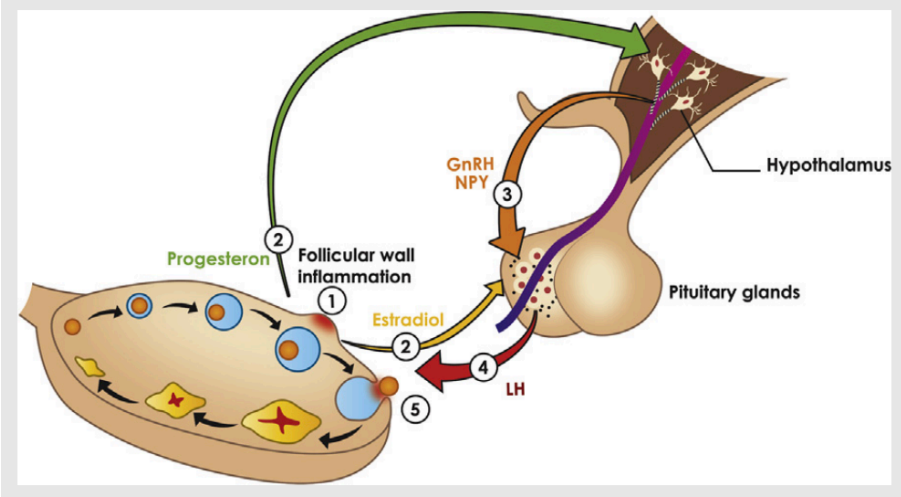

Figure II: Ovulation triggers. *Reproduced from Dozortsev and Diamond, 2020*

The outer cells of the granulosa layer stop converting androgens to oestrogens and instead synthesize progesterone. These cells lose the ability to bind oestrogen and FSH but gain the capacity to bind progestogens. By becoming responsive to LH to produce progesterone and in addition having a positive feedback mechanism, there is an exponential rise in progesterone levels from the follicle just prior to ovulation. The rising progesterone levels have three important consequences: decreased growth of the less mature follicles, ovulation itself, and the promotion of the transition to the progestogenic phase of the ovarian cycle (Johnson, 2007).

The post ovulatory follicle is composed of a fibrin core, surrounded by several collapsed layers of granulosa cells, enclosed within a fibrous outer thecal capsule; the corpus luteum.

Luteal phase

The membrane propria between the granulosa and thecal layers break down and blood vessels invade. The granulosa cells hypertrophy to form large lutein cells, which contain mitochondria, smooth endoplasmic reticulum, lipid droplets, Golgi apparatus and a carotenoid pigment called lutein. This transformation is called luteinisation and is associated with an increase in progesterone production up to 20 times that seen in the follicular phase. The thecal cells form smaller lutein cells which produce progesterone and androgens and appear richer in LH receptors. Progesterone, 17α hydroxyprogesterone and small amounts of 17β oestradiol are produced by the corpus luteum. Inhibin A is also produced which in turn stimulates progesterone production and oxytocin (Johnson, 2007).

In the late luteal phase, in the absence of a fertilized oocyte, a decrease in LH secretion results in a gradual fall in progesterone and oestradiol production by the corpus luteum. If, however, the oocyte becomes fertilized, it may implant in the endometrium several days after ovulation. The early embryo

begins to secrete hCG, which maintains the corpus luteum and progesterone production.

THE ENDOMETRIUM

The endometrium is one of the most astonishing human structures, it is the only tissue in the human body capable of increasing its size up to 12 times, get destroyed repeatedly on a monthly basis and regrow without leaving any scars.

The endometrial phases of the menstrual cycle can be divided into: menstrual flow (early follicular phase), proliferative (mid-late follicular phase) and secretory (luteal phase) as represented in Figure I.

The endometrium consists of epithelial, stromal and vascular elements which undergo complex changes in growth, morphology and function in anticipation of pregnancy. The endometrium thickens in response to oestrogen, and becomes receptive towards embryo implantation in response to the secreted progesterone following ovulation and formation of the corpus luteum (Noyes *et al.*, 1950; Strauss *et al.*, 2019). In the mid-late secretory phase of the menstrual cycle, there is a differentiation and secretory transformation of the glandular epithelial cells followed by

decidualisation of the stromal compartment. Implantation can occur when the endometrium is considered receptive to a functional blastocyst. Studies have suggested that the window of endometrial receptivity extends from post-ovulatory days 6-10 (corresponding to cycle day 20-24). If synchrony between the endometrium and the embryo is impaired, this could lead to failed implantation and pregnancy loss (Jones, 1949).

Implantation

Implantation is a critical step in the establishment of successful pregnancy, requiring synchronization between the developing embryo (blastocyst) and the endometrium (Figure III). Bidirectional communication utilising endocrine, paracrine and autocrine signals exist between the embryo and endometrium.

Following ovulation, provided that sperm reach the oocyte and interact with its zona pellucida (a glycoprotein layer surrounding the oocyte) fertilisation may occur. The fertilised oocyte (zygote) undergoes cell division. After around 5 days, the developing embryo reaches the blastocyst stage. The blastocyst comprises an inner cell mass (ICM) which subsequently forms the foetus, and an outer layer of cells (trophoblast) which gives rise to extra-embryonic structures, such as the placenta (Hobson *et al.*, 2012).

Six days after fertilisation, the blastocyst, containing 100-200 cells, hatches form the zona pellucida exposing its outer aspect of syncytial trophoblasts to the adjacent luminal epithelium of the endometrium. Implantation in humans involves three stages: apposition of a competent blastocyst on a receptive endometrium, adhesion of the embryo to the epithelium, and penetration of the embryo with invasion of uterine vasculature (Ochoa-Bernal and Fazleabas, 2020) .

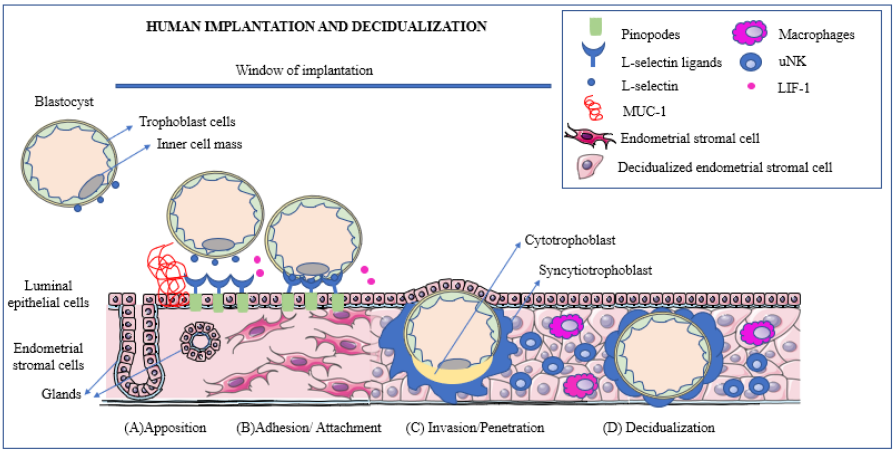

Figure III. Physiologic Events of Embryo Implantation and Decidualization in Human and Non-Human Primates. *Reproduced from Ochoa-Bernal and Fazleabas, 2020*

Regulation of implantation

Regulation of early implantation is a complex process mediated and coordinated by several growth factors, cytokines, adhesion molecules and

steroid hormones within the uterus and the pre-implantation blastocyst. The cyclical features of endometrial proliferation and differentiation are the consequence of sequential exposure to oestradiol and progesterone, produced by the developing ovarian follicle and corpus luteum respectively (Chauchereau *et al.*, 1992). The preovulatory increase in secretion of 17β-estradiol promotes proliferation and differentiation of uterine epithelial cells through their nuclear oestrogen receptors (mainly ER-α and ER-β) (Kastner *et al.*, 1990). A subsequent rise in progesterone secretion which acts primarily through its receptors (mainly PR-A and PR-B), results in the activation or repression of target genes (Kastner *et al.*, 1990) inducing, among other responses, the differentiation of stromal cells (Norwitz *et al.*, 2001). Steroid hormones are therefore required to coordinate the receptivity of the endometrium; both oestrogen and progesterone are necessary for endometrial receptivity, and progesterone expression is essential for implantation and maintenance of early pregnancy.

Many growth factors are expressed in the luminal epithelium during the implantation window and are often increased at the site of embryo apposition, including vascular endothelial growth factors (VEGF), among many others (Dey *et al.*, 2004). VEGF induces endothelial cell proliferation

and increases vascular permeability. It is expressed throughout the menstrual cycle with peak levels in the glandular epithelium during the secretory phase. Oestrogen has been demonstrated to increase VEGF expression (Shifren *et al.*, 1996).

Immune tolerance

Immune tolerance of the invading trophoblast tissue by the maternal immune system is one of the most perplexing functions of implantation. Trophoblasts are presumed to be essential to this hemi-allograft tolerance because they lie at the maternal-foetal interface where there is direct contact with the maternal immune system. Maternal decidual lymphocytes are abundant in the uterus during pregnancy with the majority being CD56+ natural killer (NK) cells, which have low cytotoxic activity, unlike peripheral blood lymphocytes (Bulmer *et al.*, 1991). Progesterone is also responsible for regulating the migration and proliferation of immune and inflammatory cell populations in the endometrium (Choi *et al.*, 2000). Furthermore, the maternal immune system is modulated by progesterone via control of cytokine production. In normal pregnancies there is a shift in the decidua from cellular immune response (Th1 cytokines) to humoral immunity (Th2 cytokines) which may be driven by the hormonal stimuli associated with

pregnancy. Immunological recognition of pregnancy results in up-regulation of progesterone receptors on activated lymphocytes (Szekeres-Bartho *et al.*, 1990). In the presence of progesterone, lymphocytes of pregnant women synthesize progesterone-induced blocking factor (PIBF), which mediates both the immunomodulatory and anti-abortive properties of progesterone (Szekeres-Bartho *et al.*, 1990).

ASSISTED REPRODUCTION

The estimated number of couples affected with infertility was around 48.5 million in 2010. This number increased from 42 million in 1990 (Mascarenhas *et al.*, 2012). In Spain alone, the latest national report indicated a total of 50.263 in vitro fertilisation cycles (IVF) during 2017 (SEF, 2017).

There are many known causes of infertility, such as male factor, endometriosis, tubal factor, ovulation factors and advanced maternal age. Other times, the cause of infertility is unknown. There are different approaches to deal with infertility, depending on the cause. However, the most common and effective method remains IVF.

The most known association to infertility related to advanced maternal age and natural declining fecundity is mainly attributed to the presence of

chromosomal anomalies (aneuploidy) within the aging oocytes. As maternal age increases, so does the frequency of aneuploid embryos (Figure IV), the miscarriages and the inability to produce a healthy offspring (Franasiak and Upham, 2014).

Once natural conception is understood, IVF follows the same steps from follicle recruitment to just before implantation, by transferring a developed embryo into the uterine cavity.

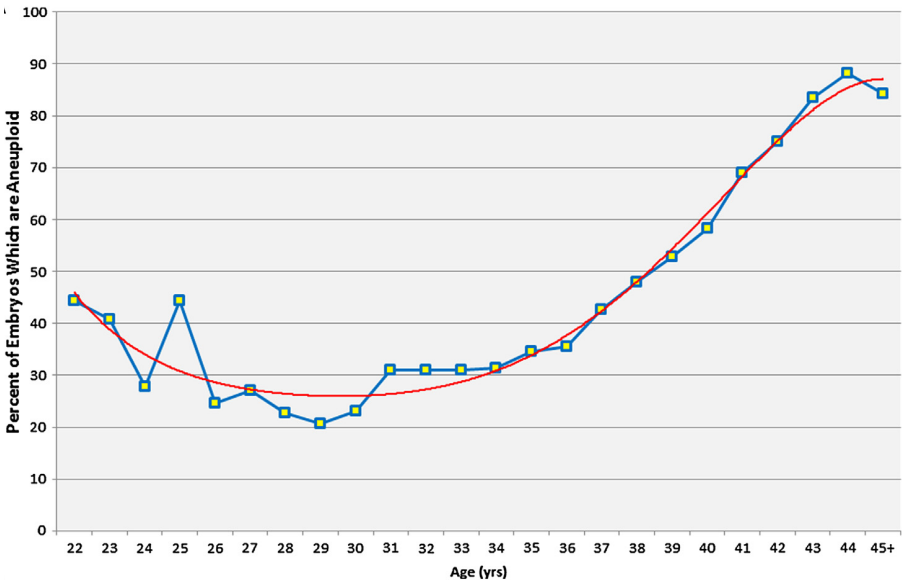

Figure IV: Embryo aneuploidy according to maternal age. *Reproduced from Franasiak and Upham, 2014*

OVARIAN STIMULATION AND OOCYTE RETRIEVAL

As previously stated, a normal ovulation cycle produces only one oocyte, but the number can be boosted significantly (to 10-20 oocytes) by administering a short course of gonadotropins in order to support the development of multiple follicles. Final oocyte maturation is induced by either exogenous hCG or a bolus of GnRH agonist to trigger an endogenous LH surge. The follicular growth is periodically followed via ultrasound and blood test, and ovulation is triggered accordingly, once follicles grow up to 17-19mm. Right before ovulation, oocyte retrieval is performed using an ultrasound-guided long and thin needle, which is inserted into each follicle and follicular fluid is aspirated, along with the microscopic oocytes within.

In parallel, sperm are obtained from the male partner or from a sperm bank and oocytes are inseminated by conventional IVF or intracytoplasmic sperm injection (ICSI), according to sperm sample quality.

EMBRYO SELECTION AND TRANSFER

Fertilised oocytes are usually incubated for 2 to 7 days, from cleavage-stage embryos to blastocysts, according to each centre's standardised operating procedures. Developing embryos can then be transferred into the uterine

cavity (fresh embryo transfer) or vitrified for a subsequent cycle (frozen embryo transfer).

Embryos for transfer are selected according to multiple parameters. One of the classifications for unification of the embryo evaluation is the one proposed by then Spanish Association for the Study of Reproductive Biology (ASEBIR), in which embryos are divided into 4 categories (A, B, C, D) according to morphologic and development criteria, being A the best quality embryos with higher implantation potential (ASEBIR, 2015).

The preimplantation genetic testing (PGT) allows the genetic characterization of the embryos before transfer. The first aim of PGT was to provide the option of embryo selection to couples with inherited genetic diseases or chromosomal rearrangements (Handyside *et al.*, 1990). This strategy was later suggested for the selection of euploid embryos in order to reduce the time to pregnancy and live birth in patients with high risk of having aneuploid embryos (recurrent implantation failure, recurrent miscarriages, severe male factor, previous affected pregnancies or advanced maternal age) (ESHRE Committee, 2020). Currently, the recommended and most implemented methodology for PGT-A consists on a multiple-cell (5-10) trophectoderm

biopsy and its analysis by comprehensive chromosome screening techniques (CCS) followed by a deferred euploid embryo transfer (Sermon *et al.*, 2016).

The improvements in the incubation and vitrification processes have allowed clinicians to increasingly adopt a frozen embryo transfer (FET) strategy. Assisted reproduction techniques (ART) reports from the last decade in both the United States and Spain, clearly evidence a trend towards an increase in FET compared to fresh ones. Precisely, while FET in 2010 represented only 22.9% and 26.3% of all embryo transfers in the United States and Spain respectively, by 2017 this was increased to 69.4% and 53.8% (SEF, 2017; "ART Success Rates | CDC," 2020). FET was initially indicated for patients with increased risk of ovarian hyperstimulation syndrome (OHSS). This syndrome can be severe and is more likely to happen to patients with a high number of recovered oocytes who get pregnant after a fresh embryo transfer. Later on, authors described improved reproductive outcomes of IVF treatment in patients undergoing FET (Shapiro *et al.*, 2008). These finding were related to the supraphysiological hormonal levels achieved during the ovarian stimulation, which may negatively affect the endometrium for implantation (Ubaldi *et al.*, 1997; Horcajadas *et al.*, 2005; Roque *et al.*, 2017). Specifically, the rise in late follicular progesterone level was thought to be negative for

successful implantation (Bosch *et al.*, 2010). Altogether, a recent meta-analysis (Roque *et al.*, 2019) described significant increase in live birth rate (LBR) when elective FET was performed compared with fresh embryo transfer in the overall IVF/ICSI population. Specifically, in the sub-group analysis, such increase was observed in the hyper-responder group and in PGT-A cycles. Regarding safety, the risk of moderate/severe OHSS was significantly lower with FET than with fresh embryo transfer, whereas the risk of pre-eclampsia increased with FET (Roque *et al.*, 2019). Still, the FET approach is the only one available for the use of supernumerary embryos for couples in which the first embryo transfer (either fresh or frozen) failed or desire another child, as well as for patients opting for embryo donation. Furthermore, techniques such as PGT have also highly benefited from this approach, in which the preimplantation embryo is ideally biopsied at the blastocyst stage and subsequently vitrified to allow for chromosomal analysis (Rodriguez-Purata *et al.*, 2016; Sermon *et al.*, 2016).

ENDOMETRIAL PREPARATION

Once an embryo is selected for transfer, either fresh or frozen, uterine receptivity and adequate hormonal environment equivalent to a natural luteal phase needs to be ensured for a successful pregnancy.

For fresh embryo transfer

Luteal phase support is widely recognized as a critical component of fresh IVF cycles, due to ovarian stimulation effectively inducing a luteal phase defect. Whereas in natural ovulatory cycles the progesterone level rises with luteinization and remains stable during the luteal phase, ovarian stimulation is associated with a gradual decline in serum progesterone after the maturation trigger injection and oocyte recovery (Smitz *et al.*, 1988; Hutchinson-Williams *et al.*, 1990). Although this progesterone fall is multifactorial, it is probably due to LH release inhibition by the supraphysiological steroid hormone concentrations due to multifollicular maturation (Fauser and Devroey, 2003). Typically, these patients undergo exogenous administration of progesterone from the day of oocyte retrieval until a few weeks after pregnancy is confirmed.

For frozen embryo transfer

Endometrial preparation for frozen embryo transfer can be achieved through different ways, depending on whether the patient has regular cycles, patient's preferences or internal protocols in each centre. This transformation can be achieved either via a natural cycle or an artificial endometrial preparation.

- Natural endometrial preparation is achieved by monitoring the endogenous ovulatory cycle of the patient via ultrasound and hormonal parameters (LH peak) and programming the embryo transfer accordingly. This approach requires no external medication (Groenewoud *et al.*, 2016), and may be preferable to some patients, although it requires multiple appointments for an adequate cycle monitoring. In some occasions, in order to ensure ovulation an adequately program the embryo transfer, a natural-modified cycle can be used, in which an hCG bolus is administered when a dominant follicle is detected.
- Artificial endometrial preparation requires exogenous oestrogen treatment for around 15 days and progesterone administration for 3-6 days before embryo transfer in order to achieve both adequate endometrial priming and serum hormonal values resembling the natural ovulatory cycle (Groenewoud *et al.*, 2018). Once the endometrium reaches > 6mm, adequate transformation is presumed and embryo transfer can be scheduled.

PROGESTERONE

As mentioned above, the hormone progesterone plays a key role in the endometrial preparation for embryo transfer. In natural cycles, it is secreted from the corpus luteum in order to prepare the endometrium to its secretory stage for implantation. At the same time progesterone acts on the vaginal epithelium and makes the cervical mucus thicker and impenetrable to sperm. During implantation and pregnancy, progesterone decreases the maternal immune response to allow for the acceptance of the pregnancy. Furthermore, progesterone decreases uterine contractility, contributing to prevention of preterm labour (Di Renzo *et al.*, 2016).

Like other steroid hormones, progesterone is synthesized from pregnenolone, derived from cholesterol. Progesterone in turn is the precursor of the mineralocorticoid aldosterone, cortisol and androstenedione. Androstenedione can be converted to testosterone, estrone and oestradiol. Approximately 30 mg of progesterone per day are secreted from the ovaries in women, while the adrenal glands produce about 1 mg of progesterone per day (Fritz and Speroff, 2011).

Progesterone binds extensively to plasma proteins, including albumin (50–54%) and transcortin (43–48%). The metabolism of progesterone occurs mainly in the liver. Progesterone has an elimination half-life of approximately 5 minutes. The metabolism of progesterone is complex, and it may form as many as 35 metabolites when orally ingested. Endogenous progesterone is metabolized approximately 50% into 5α-dihydroprogesterone in the corpus luteum, 35% into 3β-dihydroprogesterone in the liver, and 10% into 20α-dihydroprogesterone (Di Renzo *et al.*, 2016).

NATURAL CYCLES

Progesterone is initially produced by one or multiple corpora lutea in either spontaneous conceptions, IVF cycles with transfer of fresh embryos, or FET under natural or modified-natural ovulatory cycles. Implanting embryo's hCG rescues the corpus luteum which remains the principal source of progesterone until the placenta matures and becomes the predominant source of progesterone. Csapo's classic luteectomy studies in spontaneous conceptions defined a narrow 11-day window for the luteo-placental shift between gestational ages of 7 weeks, when abortion followed excision of the corpus luteum, and 8 weeks 4 days, when luteectomy no longer led to the loss of pregnancy (Csapo *et al.*, 1973). Administration of intramuscular (IM)

progesterone at 200 mg/day was found to sustain early pregnancy despite luteectomy during this window of vulnerability (Csapo *et al.*, 1973).

Luteal phase defect (LPD) is defined as a corpus luteum defective in progesterone production (Jones, 1976) or an abnormal endometrial response to adequate levels of progesterone exposure (Usadi *et al.*, 2008). Midluteal serum P < 10 ng/ml has also been established as a more liberal definition for LPD (Jordan *et al.*, 1994).

ARTIFICIAL CYCLES

The timing of placental maturity in pregnancies established through FET on hormone replacement treatment (HRT) without a corpus luteum has not been defined. In these pregnancies, exogenous hormonal support must first permit implantation and then maintain the early gestation until the placenta matures and secretes sufficient quantity of progesterone to maintain the pregnancy. In the absence of a corpus luteum, progesterone replacement presents a clinical challenge on account of both the large dosage and the long duration of the treatment required to reach physiologic levels. At implantation and in the first trimester of spontaneous pregnancies, progesterone production is about 50-55 mg/day but in mid-trimester and the third trimester it doubles and quadruples, respectively (Little and Billiar,

1972). Mean circulating progesterone level in spontaneous pregnancies is in the range of 25-30 ng/mL until 11 weeks when it climbs steeply to a peak of about 180 ng/mL near term (Tulchinsky and Hobel, 1973).

In the setting of FET on hormone replacement, no single optimal progesterone replacement protocol has been developed let alone universally adopted.

EXOGENOUS PROGESTERONE. PHARMACOKINETICS AND PHARMACODYNAMICS

Exogenous progesterone can be administered in various ways. The most generally accepted forms for exogenous P administration are parenteral (intramuscular or subcutaneous) and vaginal. The intramuscular (IM) and vaginal routes are widely used in the USA, whereas in Europe there is no commercialised IM progesterone. On the contrary, there is a recent subcutaneous (Psc) formulation available, in addition to vaginal.

The advantages and disadvantages of the different routes of progesterone administration continue to be debated. While the vaginal route provides high endometrial tissue content *in vivo* as well as *ex vivo* through the diffusion of progesterone from the vaginal to the uterine circulation, known as the uterine first pass, the circulating progesterone levels remain sub-physiologic

below 15 ng/mL (Miles *et al.*, 1994; Cicinelli *et al.*, 2000). A pharmacokinetic analysis comparing a single dose of 200 mg of micronized P vaginally versus 50 mg IM showed that serum progesterone reached plateau within 4 hours at about 6 and 15 ng/mL, respectively (Miles *et al.*, 1994). After 6 days of 200 mg of micronized P vaginally every 6 hours and 50 mg of IM P every 12 hours, the mean circulating levels were 11.9 and 69.8 ng/mL, respectively (Miles *et al.*, 1994). However, the endometrial concentration of P was 11.5 ng/mL with the vaginal administration, compared to 1.4 ng/mL with the IM. Still, both groups achieved similar endometrial secretory transformation, thus a low threshold is needed. The low serum and high endometrial progesterone values with the vaginal administration can be explained by a limited carrying capacity of the uterine first pass. This also explains why serum P levels do not rise as much despite higher doses of vaginal P administration. In contrast, when vaginal oestradiol is administered, at doses of 2-8 mg/day, high serum levels are observed because the dose does not exceed the carrying capacity of the vaginal venous plexus (Tourgeman *et al.*, 2001). When IM injections are administered, there is a low endometrial content of P despite high circulating levels. This could be related to a reverse gradient and direction of diffusion from the uterine artery with higher levels to the venous plexus.

Studies comparing the clinical results of the different P administrations are mixed. A retrospective cohort study of women undergoing transfer of day 3 cryopreserved embryos found lower clinical pregnancy and live birth rates with Crinone 8% (90 mg) vaginal gel twice per day compared to women receiving IM progesterone 50 mg/day (Kaser *et al.*, 2012). Another retrospective cohort study using the same protocols but with vitrified/warmed blastocysts found no difference in clinical pregnancy, spontaneous abortion and live birth rates (Shapiro *et al.*, 2014). In a randomized controlled trial (RCT) of FETs of vitrified/warmed blastocysts, an interim analysis demonstrated that women conceiving on just vaginal P (Endometrin) 200 mg twice daily had higher pregnancy loss rate and lower ongoing pregnancy rate than women conceiving on either IM or combined vaginal and IM progesterone (Devine *et al.*, 2018). Consequently, the vaginal only arm of the RCT was discontinued.

Table I summarises the characteristics and pharmacodynamics of the main different types of P available.

Table I: Main characteristics and pharmacodynamics of the different types of progesterone available.

	Vaginal Tablet 200mg	Subcutaneous 25mg	Intramuscular 50mg	Vaginal gel 90mg
Form	Tablets	Aqueous	In oil	Gel
Posology	Every 8h	Daily	Daily	Every 12h
Cmax (ng/mL) Tmax (ng/mL) T½ (h) AUC (ng·h/mL)	11.3 ± 4.0 10.2 ± 2.4 13.7 ± 1.05 64.1 ± 27.9	57.84 ± 13.55 0.92 ± 0.42 13.06 ± 7.08 337.65 ± 91.58	20.0 ± 5.3 8.20 ± 2.74 28.05 ± 16.87 320 ± 67	10.51 ± 0.46 7.67 ± 3.67 25.91 ± 6.15 133.26 ± 14.61
Side effects	Discharge, vaginal infection	Bruising, edema, pain	Pain, local soreness, sterile abscess	Perineal pain, bloating, cramps

Data extracted from Levine and Watson, 2000; Sator et al., 2013; Paulson et al., 2014; Cometti, 2015

SERUM PROGESTERONE IN ARTIFICIAL ENDOMETRIAL PREPARATION CYCLES

There is growing interest by many recent publications aiming at finding an optimal serum P level around the time of ET or early pregnancy. Although most authors conclude that low serum P levels in early pregnancy are detrimental in terms of reproductive outcomes, the methodology of the different studies call for more robust evidence in this regard. Also, all previously published data show serum P measurements either the day of ET or after, where little intervention may be possible at this point. Table II summarises previous studies that have suggested serum P threshold levels within different luteal phase support regimens in HRT cycles.

Table II. Comparison of published studies regarding serum progesterone levels in artificially prepared cycles

Reference	n	Route	Dose	Threshold	Measure	Embryos	Outcome (%)	Design
Brady et al., 2014 J Assist Reprod Genet	229	IM	50-100 mg/day	>20 ng/mL	Day of ET	Fresh, donor, D3	LBR (54 vs 51)	Retrospective
Kofinas et al., 2015 J Assist Reprod Genet	213	IM	50-75 mg/day	<20 ng/mL	Day of ET	FET own, euploid, blastocyst	LBR (49 vs 65)	Retrospective
Yovich et al., 2015, RMBO	529	Vaginal	400 mg/8h	22-31 ng/mL	Day of ET	FET, own + donor, blastocyst	LBR (50 vs <41)	Retrospective
Labarta et al., 2017, Hum Reprod	211	Vaginal	400 mg/12h	>11 ng/mL	Day of ET	FET, donor, blastocyst	OPR (53 vs 43)	Prospective
Basnayake et al., 2017, Aust N Z J Obstet Gynecol	1580	Vaginal	Various	>15 ng/mL	Day 16 P	FET, own + donor, D3 or blastocyst	LBR (27 vs 11)	Retrospective
Alsbjerg et al., 2018, RMBO	244	Vaginal	90 mg (gel)/8h	>11 ng/mL	Day of bhCG test	FET, own, blastocyst	OPR (51 vs 38)	Retrospective

IM: intramuscular; P: progesterone; ET: embryo transfer; FET: frozen embryo transfer; LBR: live birth rate; OPR: ongoing pregnancy rate

Still, there are multiple questions regarding the luteal phase in FET cycles:

- Is there a minimum progesterone level that should be reached for improved reproductive outcomes?
- How prevalent are low serum P levels among women undergoing FET cycles?
- Which is the advantage of serum determination the day before FET?
- Which factors can predict low progesterone levels?
- If a low plasma progesterone level is detected, is there a possible strategy to overcome this?

OBJECTIVES

1. To analyse and establish whether there is a cut-off point for serum progesterone levels for improved reproductive outcomes measured the day before frozen embryo transfer in women undergoing:
    a. Artificially prepared FET cycles
    b. Natural FET cycles
2. To evaluate whether there are variables that could predict the low serum P levels in some patients undergoing FET.
3. To evaluate a possible individualised strategy that could improve the outcomes for women with low serum progesterone levels the day before frozen embryo transfer.

RESULTS

LOW SERUM PROGESTERONE THE DAY PRIOR TO FROZEN EMBRYO TRANSFER OF EUPLOID EMBRYOS IS ASSOCIATED WITH SIGNIFICANT REDUCTION IN LIVE BIRTH RATES

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ORIGINAL ARTICLE

Low serum progesterone the day prior to frozen embryo transfer of euploid embryos is associated with significant reduction in live birth rates

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ABSTRACT

A retrospective cohort study was performed to examine whether, in artificial endometrial preparation for frozen embryo transfer (FET) cycles, progesterone (P) levels the day prior to embryo transfer of euploid embryos have an impact on pregnancy outcomes. In a private university clinic, 244 FETs between January 2016 and June 2017 were analyzed. Endometrial preparation was achieved with estradiol valerate and vaginal micronized progesterone. Serum P and estradiol levels the day prior to embryo transfer were measured. A multivariable analysis to assess the relationship between serum P level and pregnancy outcomes was performed, adjusted for confounding variables. Mean P value was 11.3 ± 5.1 ng/ml. Progesterone levels were split in quartiles: Q1: ≤ 8.06 ng/ml; Q2: 8.07–10.64 ng/ml; Q3: 10.65–13.13 ng/ml; Q4: > 13.13 ng/ml. Patients included in the lower P quartile had a significantly higher miscarriage rate and significantly lower live birth rate (LBR) compared to the higher ones. A low serum P level (≤ 10.64 ng/ml) one day before FET is associated with a lower pregnancy and LBR following FET of euploid embryos.

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Progesterone; euploid embryo transfer; miscarriage; live birth rate; FET

Introduction

The outcomes of frozen embryo transfer (FET) have substantially improved over the last decade, due to the improvements in the cryopreservation process [1].

Artificial endometrial preparation is typically accomplished by the administration of estradiol (E2) supplementation and exogenous progesterone (P) in order to transform the endometrium into a secretory one, mimicking a natural cycle [2,3]. Despite the lack of a standard protocol for hormone replacement therapy (HRT) [4,5], the importance of progesterone for an adequate endometrial transformation, embryo implantation and maintenance of pregnancy remains unquestionable. A debate exists regarding the optimal duration [6] and dose [7] of P supplementation in relation to pregnancy rates and early pregnancy loss, in addition to the optimal serum P levels on the day of ET among women undergoing FET cycles [8–10].

One early study showed that low P levels on mid-luteal phase (2–3 days after ET) may result in low pregnancy rates [11], while other studies described a deleterious effect of high *p* values on LBR [9]. However, serum P might not reflect neither the actual absorption nor the level of endometrial support [12].

Recently, a prospective study demonstrated a significant detrimental effect of low P serum level the day of ET on pregnancy evolution in oocyte reception cycles [10]. Still it is unclear the clinical value or serum P measurement on the day of ET, given that at this point no intervention is possible. Many factors could account for these contradictory findings, especially unknown embryo euploidy.

Embryo aneuploidy is one of the main aspects related to IVF failure, causing implantation failure, miscarriages and affected pregnancies [13]. Preimplantation genetic testing for aneuploidies (PGT-A) may allow the de-selection of aneuploid embryos for transfer, and improve final outcomes [14].

The current study aims to determine the association, if any, between serum P levels, measured one day prior to ET, and pregnancy outcome, in women undergoing transfer of frozen euploid blastocysts (FEET).

Materials and methods

Study design

A retrospective analysis of 244 FEET cycles performed at a private University Clinic between January 2016 and June 2017 was undertaken. The trial registration number for the study is NCT03395665. The study was approved by our Institutional Review Board.

Study population

Inclusion criteria were women who underwent FEET in the described period. Patients with known uterine abnormalities, oocyte recipient cycles and FET cycles of mosaic embryos were excluded. FEET that end in an ectopic pregnancy were omitted.

Study protocol

Briefly, all IVF cycles were performed under ovarian stimulation with gonadotropins and pituitary suppression with GnRH analogs (agonists or antagonists) [15]. Mature oocytes were

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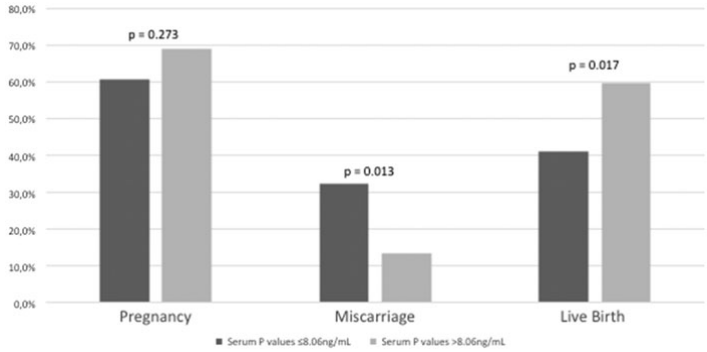

Figure 1. Pregnancy outcomes for serum progesterone ≤8.06 ng/mL *vs* progesterone >8.06 ng/mL. P: serum progesterone value.

microinjected 40 h after hCG administration. Embryos were cultured in a time-lapse incubator using single-step culture media (LifeGlobal®). PGT-A procedure was carried out as previously described [16]. Embryos that reached the blastocyst stage were biopsied and frozen immediately afterwards using the vitrification method [17].

Chromosomal analysis was performed by a-CGH using commercially available kits and software (SurePlex® DNA Amplification System, 24Sure® Microarray Pack, BlueFuseMulti®, Illlumina®) following the manufacturer instructions. Euploid embryos were transferred in a subsequent cycle.

Endometrial preparation in frozen embryo transfer (FET)

Endometrial preparation in PGT-A FET has been described elsewhere [15]. In short, patients received treatment with 2 mg/8h E2 valerate (Progynova®, Schering) for 12–14 days followed by vaginal micronized P (Utrogestan®, Seid) treatment at 200 mg/8h from the night of day 15 until plasma β-hCG determination. A depot GnRH agonist was administered in the midluteal phase of the preceding cycle at clinician's discretion. During the late morning of day 4 of P treatment, the day prior to FEET, a blood sample was obtained between 4–6 h after the last P dose, and immediately analyzed. Hormone determinations of E2 and P were performed with Roche's Cobas reagents in the Cobas e-411 analyzer, an electrochemiluminescence immunoassay.

Transvaginal ultrasound was used to assess endometrial thickness. Only extremely low E2 (< 75 pg/ml) and P values (< 5 ng/ml), or endometrial thickness <5 mm were considered for cycle cancelation. Euploid embryo transfer was performed under ultrasound guidance as previously reported [18,19].

Statistical analysis

Serum P levels were evaluated as a categorical and continuous variable. For categorical analyses, P was divided into quartiles (Q) according to the 25th, 50th, and 75th percentiles. Progesterone was also grouped according to the median and according to groups performed from quartiles. Associations of P with pregnancy outcomes (pregnancy rate, miscarriage and LBR) were evaluated using the Chi-square test. In parallel, a logistic regression was fitted to estimate the OR and the 95% CI for quartile comparison.

Multivariable logistic models were fitted for each outcome after adjusting for confounding variables (maternal age, endometrial thickness, embryo quality, E2, score of transfer, and number of blastocysts transferred).

Mean ± standard deviation was reported for continuous variables, and number and percentage were reported for categorical variables.

The statistical analysis was performed using IBM SPSS Statistics v22.0 software.

Results

A total of 210 women underwent 244 cycles of frozen-thawed euploid blastocysts transfer. Patient's mean age was 38.0 ± 3.3 years and had a BMI of 24.7 ± 4.2 kg/m2. Indications for PGT-A were: advanced maternal age, recurrent miscarriage, implantation failure, and severe male factor. Preferably, one blastocyst was transferred. E2 and P values the day prior to ET were 205.5 ± 92.4.0 ng/ml and 11.3 ± 5.1 ng/ml, respectively, and endometrial thickness was 10.2 ± 1.7 mm.

Patient's BMI, PGT-A indication, mean number of blastocysts transferred, E2 and endometrial thickness values showed no statistically significant differences in terms of pregnancy outcomes.

P values were divided into quartiles (Q). The serum P intervals for each quartile were: Q1 ≤ 8.06 ng/ml; Q2: 8.07–10.64 ng/ml; Q3: 10.65–13.13 ng/ml; Q4 > 13.13 ng/ml. No significant differences in pregnancy rate were found between quartiles. Patients with serum $P \leq 8.06$ ng/ml (Q1) the day prior to ET had a significantly higher miscarriage rate compared to Q3 ($p = .021$) and Q4 ($p = .013$) and a significantly lower LBR compared to Q3 ($p = .048$) and Q4 ($p = .007$). When comparing Q1 with the remaining quartiles (Q2 + Q3 + Q4), patients with p values ≤8.06 ng/ml had a miscarriage rate of 32.4% (12/37) versus 13.5% (17/126) for those with $P > 8.06$ ng/ml ($p = .013$). Similarly, patients with $P \leq 8.06$ ng/ml had a LBR of 41.0% (25/61) versus 59.6% (109/183) for those with $P > 8.06$ ng/ml ($p = .017$) (Figure 1).

When results were grouped by median P values, patients with $P \leq 10.64$ ng/ml had a statically significant higher miscarriage rate ($p = .007$) and lower LBR ($p = .029$) compared to $P > 10.64$ ng/ml (Table 2).

In relation to the median analysis for the P values, the multinomial logistic regression model adjusted for confounding variables showed a significantly higher miscarriage rate with P values ≤10.64 ng/ml with OR: 3.49 95% CI [1.41–8.65] and a significantly lower LBR with P values ≤10.64 ng/ml with OR: 0.57 95% CI [0.34–0.97].

Table 1. Pregnancy outcomes for each progesterone value quartile.

Quartile (P value ng/ml)	Pregnancy	*p*-value	Miscarriage	*p*-value	Live birth	*p*-value
Q1:≤8.06	60.7% (37/61)	.577	32.4% (12/37)	.021	41.0% (25/61)	.046
Q2: 8.07–10.64	68.9% (42/61)		21.4% (9/42)		54.1%(33/61)	
Q3: 10.65–13.13	65.6% (40/61)		10.0% (4/40)		59.0% (36/61)	
Q4:>13.13	72.1% (44/61)		9.1% (4/44)		65.6% (40/61)	

P: serum progesterone level.

Table 2. Pregnancy outcomes for median serum progresterone values.

Median (P value ng/ml)	Pregnancy	*p*-value	Miscarriage	*p*-value	Live birth	*p*-value
≤10.64	64.8% (79/122)	.587	26.6% (21/79)	.007	47.5%(58/122)	.029
>10.64	68.9% (84/122)		9.5% (8/84)		62.3%(76/122)	

P: serum progesterone level.

Discussion

Our study for the first time demonstrates that P levels one day before FET are a strong determinant of treatment success following the transfer of frozen thawed euploid blastocysts. Patients with serum $P < 10.64$ ng/ml the day prior to ET had a significantly higher miscarriage rate and lower LBR, after FEET under artificial endometrial preparation.

Our results are in line with a previous study measuring P levels on the day of ET of non-genetically tested embryos showing similar results [10], further supporting the idea that low progesterone level on the day of ET (or the day before) may increase miscarriage rates in FET cycles.

The current study has two major differences as compared with the study by Labarta *et al* [10]. First of all, we focused only on FETs of genetically-tested embryos, practically eliminating one of the strongest confounders (embryo euploidy status), increasing the external validity of our findings. Secondly, we measured P levels one day before (and not on the same day) of embryo transfer, allowing future studies the adoption of measures to increase serum P levels.

The impact of serum P in FET has been previously studied, suggesting that luteal phase P supplementation improves LBR, whereas data regarding miscarriage rate is mixed [20,21]. Attempts made to find the optimal serum P values range the day of ET have produced contradictory results [8–11].

In the current study, the vaginal route for P supplementation was chosen given its better steady-state serum level compared to intramuscular and oral administration, as well as higher implantation rates in FET cycles [22]. A previous study showed that, despite the lower serum P levels after vaginal administration in comparison to the intramuscular route, P levels at the site of the endometrium were higher [23], with no differences in histologic, ultrasonographic or immunocytochemical receptor analyses after 7 days of treatment [23]. Finally, vaginal P supplementation has been proven to be more convenient for patients [24].

Our results demonstrating a relationship between P values and pregnancy outcomes by quartile categorization, is similar to others [10].

The significance of lower serum P values in relation to higher miscarriage rates are difficult to interpret. Estradiol and progesterone levels are critical modulators of immune reactions during pregnancy and play a key role in inducing peripheral tolerance [25]. It could be speculated that a certain serum P values should be attained to allow for adequate immunological environment to reduce pregnancy loss, although lower serum P levels are sufficient to allow implantation to occur.

In the natural cycle, P is secreted during the luteal phase in order to prepare the uterus for implantation. Through two receptors, PR-A and PR-B, P controls and ensures correct endometrial epithelial proliferation, stromal differentiation, local immune response and angiogenesis, altogether allowing embryo implantation [26]. P decreases active uterine contractions to ensure a correct embryo attachment [27] and has been associated with pinopode development, with a positive correlation between pinopode abundance and implantation success [28]. Once implantation occurs, other unidentified factors may be important for the maintenance of the early stages of pregnancy, which could account for the observed deleterious effect of lower P among patients with higher miscarriages.

A major limitation of the current study is its retrospective design, which precludes to draw conclusions regarding how to improve pregnancy outcomes in patients with very low serum P levels. A previous study concluded that doubling the vaginal progesterone gel supplementation during FET in patients with oligomenorrhea could decrease the early pregnancy loss rate [7], although serum P levels were not subsequently analyzed.

In the current study endometrial pattern was not evaluated, although a previous study failed to show any influence on implantation or pregnancy rate in FEET [29].

The inclusion of patients undergoing FET of euploid embryos is a clear strength of our work as compared to previous studies.

The clinical implications of our findings could suggest that a minimum P values appears to be associated with better pregnancy outcomes under these treatment conditions, even though adequate endometrial development has been observed with vaginal progesterone despite low serum P concentrations [30]. Owing to this finding, different approaches could be examined in the future in order to evaluate their effect on increasing serum P values and finally improving the outcomes, such as increasing P dose supplementation, or adding subcutaneous or intramuscular P when these values are observed. Finally, cycle cancelation and a change in endometrial preparation could also be considered in an attempt to achieve better P values in a subsequent cycle.

In this regard, a prospective randomized controlled trial should be performed aiming at detecting and treating these patients at risk of embryo loss.

In conclusion, our study demonstrates a clear association with serum P and the clinical outcomes following the transfer of euploid embryos in artificially prepared FET. Future research needs to focus on identifying ways to increase P levels in these women and evaluate whether such an increase would be eventually interpreted in better outcomes.

Disclosure of interest

The authors report no conflict of interest

Trial registration number: NCT03395665

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LOW PROGESTERONE LEVELS ON THE DAY BEFORE NATURAL CYCLE FROZEN EMBRYO TRANSFER ARE NEGATIVELY ASSOCIATED WITH LIVE BIRTH RATES

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human reproduction

ORIGINAL ARTICLE ***Infertility***

Low progesterone levels on the day before natural cycle frozen embryo transfer are negatively associated with live birth rates

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STUDY QUESTION: Are progesterone (P) levels on the day before natural cycle frozen embryo transfer (NC-FET) associated with live birth rate (LBR)?

SUMMARY ANSWER: Regular ovulatory women undergoing NC-FET with serum P levels <10 ng/ml on the day before blastocyst transfer have a significantly lower LBR than those with serum P levels >10 ng/ml.

WHAT IS KNOWN ALREADY: The importance of serum P levels around the time of embryo transfer in patients undergoing FET under artificial endometrial preparation has been well established. However, no study has analyzed the importance of serum P levels in patients undergoing FET under a true natural endometrial preparation cycle.

STUDY DESIGN, SIZE, DURATION: This was a retrospective cohort study including 294 frozen blastocyst transfers under natural cycle endometrial preparation at a university-affiliated fertility centre between January 2016 and January 2019.

PARTICIPANTS/MATERIALS, SETTING, METHODS: All patients had regular menstrual cycles and underwent NC-FET with their own oocytes. Only patients who had undergone serum P measurement between 8 am and 11 am on the day before FET were included. Patients did not receive any external medication for endometrial preparation or luteal phase support. Patients were divided into two groups according to serum P levels below or above 10 ng/ml on the day before FET. Univariate analysis was carried out to describe and compare the cycle characteristics with reproductive outcomes. To evaluate the effect of P, a multivariable logistic model was fitted for each outcome after adjusting for confounding variables.

MAIN RESULTS AND THE ROLE OF CHANCE: Mean serum P levels on the day before FET were significantly higher in patients who had a live birth compared to those who did not (14.5 ± 7.0 vs 12.0 ± 6.6 ng/ml, 95% CI [0.83; 4.12]). The overall clinical pregnancy rate (CPR) and LBR were 42.9% and 35.4%, respectively. Patients in the higher P group (>10 ng/ml) had a higher LBR (41.1% vs 25.7%: risk difference (RD) 15.4%, 95% CI [5; 26]) and CPR (48.6% vs 33.0%: RD 15.6%, 95% CI [4; 27]). Patients with higher serum P levels on the day before FET (63% of patients) had an improved LBR (odds ratio: 1.05; 95% CI [1.02; 1.09]). Women with serum P levels <10 ng/ml on the day before FET (37% of patients) had significantly higher weights (62.5 ± 9.9 vs 58.1 ± 7.1 kg, 95% CI [1.92; 6.90]) and BMI (22.9 ± 3.6 vs 21.6 ± 2.7 kg/m^2, 95% CI [0.42; 2.25]) compared to patients with P levels >10 ng/ml.

LIMITATIONS, REASONS FOR CAUTION: The main limitation of our study is its retrospective design. Other potential limitations are the detection of LH surge through urine testing and the inclusion of patients who did and did not undergo preimplantation genetic testing for aneuploidies. The protocol used in our institution for monitoring NC-FET does not look for the onset of progesterone secretion by the corpus luteum, and a slow luteinisation process or delay of corpus luteum function cannot be ruled out.

WIDER IMPLICATIONS OF THE FINDINGS: We provide evidence that a minimum serum P threshold (P >10 ng/ml) might be required for improved reproductive outcomes in NC-FET. This result suggests that there are different mechanisms by which P is produced and/or distributed by each patient. This study also provides an excellent model to evaluate the impact of luteal phase defect through

NC-FET. A prospective evaluation to assess whether P supplementation should be individualised according to patient's needs is necessary to support our findings.

STUDY FUNDING/COMPETING INTEREST(S): No external funding was used, and there are no competing interests.

Key words: serum progesterone / natural cycle / frozen embryo transfer / luteal phase defect / live birth rate

Introduction

Progesterone (P) is essential to develop the endometrium and allow embryo implantation and pregnancy achievement. The repercussions of P deficiency have been well established from early studies. In 1973, Csapo described an immediate P fall in patients in whom a luteectomy was performed during the first 7 weeks of pregnancy and an inability to maintain the pregnancy in these patients (Csapo *et al.*, 1973). Luteal phase defect (LPD) is defined as a corpus luteum defective in P production (Wallach and Jones, 1976) or an abnormal endometrial response to adequate levels of P exposure (Balasch *et al.*, 1992; Usadi *et al.*, 2008). Mid-luteal serum P <10 ng/ml has been established as a more liberal definition for LPD (Jordan *et al.*, 1994).

P also plays a key role in the endometrial transformation prior to frozen embryo transfer (FET). This transformation can be achieved either via a natural cycle or via an artificial endometrial preparation. Natural endometrial preparation is achieved by endogenous progesterone secretion by the corpus luteum in an ovulatory cycle and requires no external medication (Groenewoud *et al.*, 2016), making this strategy preferable to some patients. In artificial endometrial preparation, exogenous oestrogen and progesterone are administered to achieve both adequate endometrial priming and serum hormonal values resembling the natural ovulatory cycle (Groenewoud *et al.*, 2018).

Accumulating evidence supports the importance of serum P levels around the time of embryo transfer (ET) in patients undergoing FET with artificial endometrial preparation, i.e. hormone replacement cycles (HRT). In this aspect, our group has recently described a minimum serum P value of 10.64 ng/ml on the day before frozen blastocyst transfer, below which there seems to be clear detrimental reproductive effects (Gaggiotti-Marre *et al.*, 2019). Our results were in line with other authors also describing a detrimental reproductive effect of low serum P levels around the time of FET or during early pregnancy with HRT cycles (Labarta *et al.*, 2017; Alsbjerg *et al.*, 2018; Cédrin-Durnerin *et al.*, 2019). In views of the growing evidence regarding the importance of serum P around the time of ET, we hypothesised that studying the effect of low serum P before FET in patients under a natural cycle could provide an interesting model to understand LPD.

Taking into account that patients undergoing a natural cycle FET (NC-FET) do have a corpus luteum with a pulsatile physiological secretion of progesterone throughout the luteal phase (Filicori *et al.*, 1984), little emphasis has been given to luteal phase support for these patients. However, NC-FET attempts for an embryo-endometrial dialogue to occur in patients with a physiological P secretion through the corpus luteum.

To our knowledge, no research group has aimed at determining an optimal P threshold for improved reproductive outcomes in patients undergoing NC-FET. Based on all the above, and considering that FET under a natural cycle requires close patient monitoring and no external medication, we set out to evaluate the effects of possible P deficiency in patients with regular ovulatory cycles, undergoing NC-FET.

The aim of the present study is to evaluate the importance of serum P levels on the day before NC-FET with regard to live birth rates (LBR).

Materials and Methods

Study setting

A retrospective observational study at a private university medical centre was performed between January 2016 and January 2019.

Subjects

A total of 294 FET under a natural endometrial cycle were included. Inclusion criteria were patients with regular menstrual cycle who underwent NC-FET with their own oocytes and had serum P levels measured between 8 am and 11 am on the day before ET. Eligible patients underwent an FET at the blastocyst stage, with or without preimplantation genetic testing for aneuploidies (PGT-A).

Patients with uterine abnormalities or mosaic ET, oocyte recipient cycles and patients with serum P extraction taken after 11 am were excluded.

Ethics

The study was approved by our Institutional Review Board (approval number: CIOG012020115/06).

Endpoints

This study analyzed the relationship between serum P values on the day before ET and LBR, defined as the delivery of a living infant after 22 weeks of gestation, as the primary end point. Secondary end points included clinical pregnancy rate (CPR), defined as an ultrasonographical visible gestational sac, and miscarriage rate, defined as a clinical pregnancy loss before 22 completed weeks of gestational age (Zegers-Hochschild *et al.*, 2017).

Study protocol

Embryos were cultured in a single-step culture media (LifeGlobal®, USA) in a time-lapse incubator with 5% oxygen concentration. Embryos that reached the blastocyst stage (D5–D7) were either immediately frozen or biopsied for PGT-A and frozen afterwards using the vitrification method (Solé *et al.*, 2013).

Eligible patients underwent NC-FET without the use of any external medication (Groenewoud *et al.*, 2018). In brief, clinicians performed a daily vaginal ultrasound for each woman starting on cycle days 10–14, depending on the length of their menstrual cycle, to monitor follicular growth. Once the leading follicle had reached 16 mm in diameter, patients underwent a daily ovulation urine tests for LH surge detection

and a daily ultrasound. Blastocysts were warmed and transferred 6 days after the LH surge under ultrasound guidance, as previously described (Coroleu *et al.*, 2002).

Estradiol (E2) and P measurements were performed on the day before ET, between 8 am and 11 am, using an electrochemiluminescence immunoassay (Cobas® e-411 analyser; Roche Diagnostics, Germany). For E2, the lower limit of detection was 5 pg/ml with intra- and inter-assay variations of 2.4–8.5% and 2.5–11.9%, respectively. For P, the lower limit of detection was 0.05 ng/ml, with intra- and inter-assay variations of 1.2–11.8% and 3.6–23.1%, respectively.

Statistics

Serum P levels were evaluated as a continuous and categorical variable. For categorical analyses, P was divided into two groups ($P < 10$ ng/ml vs $P \geq 10$ ng/ml), according to previous definition of LPD (Jordan *et al.*, 1994). Continuous variables were expressed as mean, standard deviation, median and interquartile range, and categorical variables were expressed as frequencies and percentages.

Univariate analysis was carried out to describe and compare the cycle characteristics with reproductive outcomes. *T*-test or Mann–Whitney *U*-test was applied for continuous variables, and Chi-square test or Fisher's test was applied for categorical variables. Normality distribution was analyzed by the Kolmogorov–Smirnov test and Boxplot, and 95% CIs for differences between means or proportions were calculated.

Finally, to evaluate the effect of P, a multivariable logistic model was fitted to analyze the LBR adjusting by age, use of PGT-A and the interaction of both variables. Akaike information criterion (AIC) was used to decide the best model without interaction. *P* values <0.05 were considered statistically significant. Statistical analyses were performed with IBM© SPSS© Statistics v 22 and R software (Team, 2018).

Results

Patient's demographics and cycle parameters for the 294 FET cycles meeting inclusion criteria were comparable according to serum P levels on the day before NC-FET below or above 10 ng/ml, except for weight and BMI (Tables I and II). Patients with serum P levels <10 ng/ml on the day before FET (37% of the patients) had significantly higher weights compared to patients with P levels >10 ng/ml (62.5 ± 9.9 vs 58.1 ± 7.1 kg, 95% CI [1.92; 6.90]). Likewise, women in the lower serum P group had higher BMI measurements compared to those with higher P levels (22.9 ± 3.6 vs 21.6 ± 2.7 kg/m^2, 95% CI [0.42; 2.25]) (Table II). No differences were reported between these two groups according to the number of embryos transferred, embryo quality (data not shown) or the percentage of PGT-A FET.

The overall CPR and LBR were 42.9% and 35.4%, respectively. Patients in the group serum $P > 10$ ng/ml (63% of patients) had a significantly higher CPR (48.6% vs 33.0%: RD 15.6%, 95% CI [4; 27]) and a significantly higher LBR (41.1% vs 25.7%: RD 15.4%, 95% CI [5; 26]) (Fig. 1).

The overall miscarriage rate was 13.5%. There were no significant differences according to P below or above 10 ng/ml and miscarriage (Fig. 1). Patients who suffered a miscarriage had statistically significant higher weight and BMI than those who did not.

Mean serum P levels on the day before FET were significantly higher for patients with a visible gestational sac compared to mean serum P

Table I Patients' demographics and cycle characteristics.

Characteristics	
Age (years)	37.7 ± 4.3
Weight (kg)	59.9 ± 8.6
BMI (kg/m^2)	22.1 ± 3.1
Endometrial thickness (mm)	10.5 ± 2.0
Estradiol (pg/ml)	147.6 ± 70.8
Progesterone (ng/ml)	12.9 ± 6.8
PGT-A	29.2% (86/294)
No PGT-A	70.7% (208/294)
Number of embryos transferred	1.3 ± 0.5

PGT-A, preimplantation genetic testing for aneuploidies.

Table II Cycle characteristics divided into serum progesterone levels <10 and ≥10 ng/ml measured on the day before frozen embryo transfer.

	Progesterone		95% CI
	<10 ng/ml	≥10 ng/ml	
Number	109	185	
Age (years)	37.7 ± 4.3	37.6 ± 4.3	[−0.91; 1.13]
Weight (kg)	62.5 ± 9.9	58.1 ± 7.1	[1.92; 6.90]
BMI (kg/m^2)	22.9 ± 3.6	21.6 ± 2.7	[0.42; 2.25]
Endometrial thickness (mm)	10.7 ± 2.3	10.4 ± 1.8	[−0.24; 0.80]
Estradiol (pg/ml)	142.3 ± 78.0	150.8 ± 66.2	[−26.17; 9.06]
Progesterone (ng/ml)	6.6 ± 2.2	16.6 ± 5.8	[−10.91; −9.05]
PGT-A	25/109 (22.9%)	61/185 (33.0%)	[−0.20; 0.01]
Number of Embryos Transferred	1.4 ± 0.5	1.3 ± 0.5	[−0.05; 0.17]

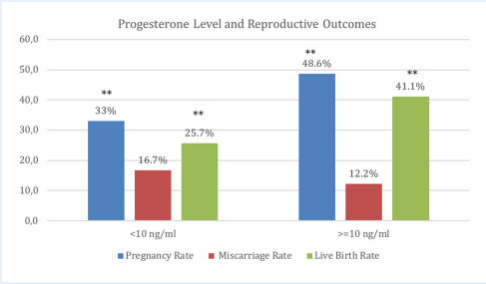

Figure 1 Comparison of reproductive outcomes in patients with serum progesterone levels <10 and >10 ng/ml.

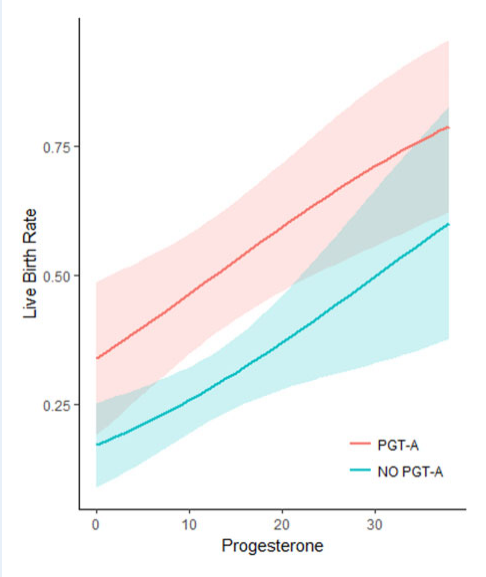

Figure 2 Logistic regression model showing live birth rate according to serum progesterone levels (ng/ml) on the day before blastocyst frozen embryo transfer, comparing patients who did and did not undergo preimplantation genetic testing for aneuploidies (PGT-A).

levels in patients with no visible gestational sac (14.0 ± 6.8 vs 12.0 ± 6.7 ng/ml, 95% CI [0.39; 3.53]). Similar results were observed for patients who had a born infant compared to those who did not (14.5 ± 7.0 vs 12.0 ± 6.6 ng/ml, 95% CI [0.83; 4.12]).

Patients with higher serum P levels on the day before FET had improved LBR (odds ratio: 1.05, 95% CI [1.02; 1.09]), independently of whether they were undergoing FET with or without PGT-A (Table III and Fig. 2).

Table III Logistic regression model showing live birth rate according to serum progesterone levels (ng/ml) on the day before blastocyst frozen embryo transfer, adjusted for patients who did and did not undergo preimplantation genetic testing for aneuploidies and maternal age.

	OR	95% CI
Progesterone	1.05	1.02–1.09
PGT-A	2.49	1.41–4.44
Maternal age	0.95	0.89–1.01

OR, odds ratio.

Discussion

Our study demonstrates for the first time the importance of serum P levels on the day before FET in women undergoing a natural endometrial preparation cycle. According to our results, low serum P levels on the day before ET (<10 ng/ml) are associated with significantly lower CPR and LBR. Although miscarriage rates were higher in women with lower P levels, results did not reach statistical differences, possibly due to sample size.

Our results would support previous findings describing that women with regular ovulatory cycles could be affected by an LPD, due to a corpus luteum being unable to produce enough P or to an endometrium being unable to respond to the circulating P (Jones and Madrigal-Castro, 1970). Other authors have previously described a serum P threshold during the mid-luteal phase of ~10 ng/ml for adequate luteal function in natural cycles (Hull *et al.*, 1982; Jordan *et al.*, 1994). Nonetheless, to our knowledge, no previous study has attempted to investigate whether in an NC-FET, P values below a specific level may be associated with impaired pregnancy outcomes.

Previous studies on FET under HRT with vaginal progesterone administration already revealed that P levels below ≈10 ng/ml prior to ET or during early pregnancy significantly impair pregnancy outcomes (Brady *et al.*, 2014; Labarta *et al.*, 2017; Alsbjerg *et al.*, 2018; Cédrin-Durnerin *et al.*, 2019; Gaggiotti-Marre *et al.*, 2019). Furthermore, Alsbjerg *et al.* (2013) also described improved delivery rates and lower miscarriage rates when vaginal P supplementation was doubled in these patients. In these cases, a deficiency in P absorption would seem to be the plausible cause of the low serum P levels. Distinctively, our results appear to indicate a potentially different mechanism of action, which may be related to an impaired P production by the corpus luteum, given that no exogenous P was administered to these women. Although this topic is still controversial (Practice Committee of the American Society for Reproductive Medicine, 2015), some authors have reported an LPD prevalence of 31% among women with regular ovulatory cycles (Davis *et al.*, 1989), which would be in line with the 37% prevalence of women with serum P < 10 ng/ml observed in our study. This could be related to the fact that many women undergoing IVF treatments may be subfertile and, therefore, may have suboptimal serum P levels in spite of having regular ovulatory cycles. In addition, luteal phase function can be affected by many medical conditions (Wallach and Jones, 1976), which, if undetected, could give the false impression that a woman has a normally functioning ovulatory cycle.

Another explanation for our findings is that low P levels could also be due to a slow luteinisation process or delay in corpus luteum function, as has already been shown in regularly menstruating women (Ecochard *et al.*, 2017). Our research also shows higher miscarriage rates in women with higher weight and BMI. When divided into serum P levels, those patients with serum P <10 ng/ml had a significantly higher weights and BMI measurements than those with serum P >10 ng/ml. In line with this, other authors have published evidence regarding lower serum P levels on the day of ET in overweight and obese women compared to those with normal weight (Brady *et al.*, 2014).

Our findings and the important role of P for adequate endometrial preparation in NC-FET are further supported by two studies (Bjuresten *et al.*, 2011; Veleva *et al.*, 2013), which reported improved LBR for women undergoing NC-FET who received exogenous P as luteal phase support compared with those who did not receive exogenous P. Their results can be explained by the assumption that many women undergoing IVF treatments may have suboptimal corpus luteum function, resulting in a less receptive endometrium during their natural cycles, which could be overcome by luteal phase support. On the contrary, other authors have described that addition of P (Kyrou *et al.*, 2010; Montagut *et al.*, 2016) or hCG (Lee *et al.*, 2017) for luteal support in NC-FET has no effect on pregnancy outcomes.

Comparisons between HRT and NC-FET have not shown differences in reproductive outcomes (Yarali *et al.*, 2016; Ghobara *et al.*, 2017), even though serum P levels are not generally evaluated and patients with LPD are not usually identified. This is not the case in the present study as our standard protocol for FET includes measuring serum P level on the day before FET. We believe that this gives us the possibility for individualisation and consideration of P supplementation if deemed appropriate, requiring cycle cancellation for a new attempt through a change in endometrial preparation.

The main strength of our study is its standardised methodology to minimise confounding factors. Serum P was evaluated at a specific and adjusted time range (8 am to 11 am) on the day before FET. The rationale for measuring at that time is that serum P levels are not steady during the day. Secretion of P is highly pulsatile in the mid-luteal phase when P levels are >10 ng/ml, with values varying between 7 and 35 ng/ml (Filicori *et al.*, 1984; Bungum *et al.*, 2013; González-Foruria *et al.*, 2019). Therefore, it is of outstanding importance to standardise the time of determination for all patients. Moreover, all blood samples were analyzed in our laboratory using the same equipment. Another strength of this study is that we included only true natural cycles with no external medication.

The main limitation of the present study is its retrospective design, which can lead to an unidentified or unknown population bias. The inclusion of patients who did and did not undergo PGT-A could provide a possible confounding bias, which may be the reason why maternal age was not found to be associated with impaired LBR in our multivariate regression model. For this reason, the interpretation of this result should be made with great caution, as maternal age is not associated with poorer outcomes when genetically tested embryos are included (Harton *et al.*, 2013). Still, the logistic regression analysis shows that the influence of P levels before FET was similar both with and without PGT-A. Therefore, we could conclude that the low P levels observed before FET could be related to compromised live births in both euploid and untested embryos.

Another possible limitation of our study is the detection of LH surge through urine testing, which may have in fact not detected ovulation (Direito *et al.*, 2013). Also, a 21-hour delay between LH serum peak and urine test has been described, which could also compromise embryo-endometrium synchronisation (Miller and Soules, 1996). Other authors have suggested that P onset detection and measurement could provide a better approach for patient monitoring under an NC-FET (Dong *et al.*, 2014). However, no subsequent prospective studies have been published under this protocol and, to date, there is still no standard strategy for ovulation detection in natural endometrial preparation cycles (Groenewoud *et al.*, 2013). Unfortunately, given that the current management of NC-FET in the protocol used by our institution aims to simplify patient monitoring, the onset of progesterone secretion by the corpus luteum could not be analyzed and a slow luteinisation process or delay of corpus luteum function cannot be ruled out.

The present study demonstrates that serum P levels vary among women with regular ovulatory cycles, suggesting that there are different mechanisms by which P is produced and/or distributed by each patient. Future larger studies are needed to understand and uncover the mechanisms for these findings and to better understand the discrepancies in serum P among these women.

To our knowledge, this is the first study showing a correlation between serum P levels on the day before ET and LBR in women undergoing FET under natural cycle endometrial preparation. This indicates that a minimum serum P threshold might be required to maximise the probability of achieving an ongoing pregnancy in these patients. Measurement of P levels before ET in NC-FET would be advisable to have a chance to correct them if needed. A prospective evaluation is required to assess the usefulness of P supplementation and whether it should be individualised according to each patient's needs, rather than aiming for a one-size-fits-all treatment.

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Authors' roles

B.C. and S.G.-M. conceived and designed the study. All the authors analyzed and interpreted the data. S.G. contributed to the data collection and performed the statistical analysis. M.A., I.G.-F., F.M., M.P., P.N.B., N.P.P. and B.C. revised the article for important intellectual content. SGM, MA and BC wrote the article. All the authors approved the final version of the manuscript.

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Conflict of interest

None declared.

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FACTORS ASSOCIATED WITH SERUM PROGESTERONE CONCENTRATIONS THE DAY BEFORE CRYOPRESERVED EMBRYO TRANSFER IN ARTIFICIAL CYCLES

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Factors associated with serum progesterone concentrations the day before cryopreserved embryo transfer in artificial cycles

BIOGRAPHY

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KEY MESSAGE

Serum progesterone concentrations the day before cryopreserved embryo transfer are independently associated with live birth rates. Body weight, age, time of blood sampling and previous history of low progesterone are determinants of progesterone concentrations when using hormone replacement therapy.

ABSTRACT

Research question: What factors determine serum progesterone concentrations the day before cryopreserved embryo transfer in artificially prepared cycles?

Design: Retrospective cohort study at a university-affiliated fertility centre including infertile women under 45 years old using own oocytes who underwent a total of 685 single cryopreserved blastocyst transfers under hormonal therapy. Determinants that affected live birth rate (LBR) were analysed using a multivariate logistic regression. Univariate analysis and multivariate linear regression were used to evaluate independent factors that affect serum progesterone concentrations.

Results: Age (odds ratio [OR] 0.93; 95% confidence interval [CI] 0.89–0.96), duration of oestradiol (OR 0.96; 95% CI 0.92–0.99), serum progesterone concentrations (OR 1.04; 95% CI 1.01–1.08) and patients who underwent preimplantation genetic testing for aneuploidies (PGT-A) (OR 2.17; 95% CI 1.55–3.03) were independently associated with LBR. After univariate analysis, determinants of progesterone concentrations were: age, weight, history of a previous cryopreserved embryo transfer with serum progesterone concentrations <10 ng/ml, and time of blood extraction. The multivariate linear regression showed that increasing age presented a positive correlation with progesterone concentrations (β = 0.11; 95% CI 0.01–0.20). On the contrary, significant negative correlations with progesterone concentrations were shown for a previous history of serum progesterone value <10 ng/ml (β = –3.13; 95% CI –4.45 to –1.81]), higher weight (β = –0.05; 95% CI –0.08 to –0.01) and the time of blood sampling during the day (β = –0.13; 95% CI –0.25 to –0.01).

Conclusions: This study adds more evidence regarding the importance of serum progesterone concentrations before frozen embryo transfer (FET). It also showed that body weight, age, time of blood sampling and a history of low progesterone are determinants associated with progesterone concentrations before blastocyst FET.

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KEYWORDS

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Live birth rate
Progesterone

INTRODUCTION

The production and secretion of progesterone by the corpus luteum is essential for an adequate endometrial transformation in order to achieve and maintain a pregnancy. Improvements in the cryopreservation process have allowed good reproductive results to be achieved with fewer complications (*Devroey et al., 2011*; *Evans et al., 2014*) such as ovarian hyperstimulation syndrome and multiple pregnancies. In these cases, the secretory transformation of the endometrium can be achieved in a natural, natural-modified or artificial cycle by the exogenous administration of progesterone and oestradiol. Previous studies have demonstrated a detrimental effect of low serum progesterone concentrations around the day of embryo transfer in patients undergoing frozen embryo transfer (FET) with artificial endometrial preparation (*Brady et al., 2014*; *Gaggiotti-Marre et al., 2019*; *Labarta et al., 2017*). Other authors have compared different dosages or routes of progesterone supplementation for these patients (*Asoglu et al., 2019*; *Shapiro et al., 2014*), without finding significant differences in terms of reproductive results. However, while there is a significant correlation between low serum progesterone concentrations and lower live birth rates (LBR) and higher miscarriage rates (*Gaggiotti-Marre et al., 2019*), researchers have not found an explanation for this finding. Whether this correlation is due to intrinsic patient characteristics, cycle aspects or other factors, is yet to be elucidated. There is a lack of evidence to explain the great disparity in inter-patient serum progesterone values shown in these studies, given the exact same treatment is administered to all the women studied.

Given the growing belief that 'one treatment does not fit all', this study sought to find patient and/or cycle parameters that could help predict which women are at risk of having a low serum progesterone value around the time of FET.

MATERIALS AND METHODS

Study design

A retrospective cohort study of 685 single blastocyst transfers was performed at a private university clinic between March 2016 and February 2018.

Study population

The study included 578 infertile women under 45 years old, who underwent an IVF cycle using their own oocytes during the described period. Only FET cycles were included. Patients underwent single-embryo transfer (SET) in the blastocyst stage under artificial endometrial preparation. Some patients underwent more than one FET and each attempt was included in the analysis as an independent event.

Study protocol

Ovarian stimulation and embryology procedures

All IVF cycles were performed under ovarian stimulation with gonadotrophins and pituitary suppression with gonadotrophin-releasing hormone (GnRH) analogues (agonists or antagonists) according to established standard protocols (*Martínez et al., 2016*). After oocyte retrieval, conventional IVF was performed. In cases of male factor and those cycles undergoing preimplantation genetic testing for aneuploidies (PGT-A), mature oocytes were microinjected 40 h after ovulation triggering with human chorionic gonadotrophin or with triptorelin acetate in those cases at risk of ovarian hyperstimulation syndrome. Embryos were cultured in a time-lapse incubator using single-step culture media (LifeGlobal®, USA). Embryos that reached the blastocyst stage (D5–D6) were either immediately cryopreserved, or biopsied for PGT-A and cryopreserved afterwards using the standard vitrification method (*Solé et al., 2013*).

Endometrial preparation

Starting on the second or third day after menstrual bleeding, patients received either 2 mg/8 h oral oestradiol valerate (Progynova®, Schering, Spain) or 150 µg every 3 days transdermal patches (Evopad®, Janssen-Cilag, Spain) for an average of 2 weeks, followed by 200 mg/8 h (at 08:00, 16:00 and 00:00 h) of vaginal micronized progesterone (Utrogestan®, Seid, Spain) until plasma β-human chorionic gonadotrophin (β-HCG) determination. When indicated, a depot GnRH agonist (Decapeptyl®, 3.75 mg, Ipsen Pharma, Spain) injection for pituitary suppression was administered in the mid-luteal phase of the preceding cycle. In case of biochemical pregnancy, all exogenous hormonal treatment was continued until the 10th week of pregnancy.

Serum analysis and ultrasound assessment

On the day prior to embryo transfer, and after 4 days of vaginal progesterone administration, a blood sample was obtained from 08:00 to 19:00 h and immediately analysed. Hormone determinations of oestradiol and progesterone were performed in a single laboratory with an electrochemiluminescence immunoassay (Cobas® e-411 analyser, Roche Diagnostics, Germany). For oestradiol, the lower limit of detection was 5 pg/ml with intra- and inter-assay variation of 2.4–8.5% and 2.5–11.9%, respectively. For progesterone, the lower limit of detection was 0.05 ng/ml, with intra- and inter-assay variation of 1.2–11.8% and 3.6–23.1%, respectively.

Transvaginal ultrasound was performed to assess endometrial thickness and pattern. Only cycles with extremely thin endometrium (<5 mm) were considered for cycle cancellation.

Single blastocyst transfer was performed under ultrasound guidance as previously reported (*Coroleu et al., 2002*; *Kava-Braverman et al., 2017*).

Statistical analysis

Mean and SD were used for continuous variables and frequencies and percentage for categorical variables. All the results expressed were per single blastocyst transfer. A multivariate logistic regression was used to evaluate the effect of the following variables on LBR: age, weight, serum progesterone and oestradiol concentrations the day before FET, type of oestrogen administrated (oral or transdermal), days of oestradiol exposure before FET and the use of previous agonist administration. These effects were submitted with odds ratios (OR) and 95% confidence intervals (CI), respectively. The same factors plus the time of blood sample collection and history of previous artificially prepared FET cycle with progesterone concentrations <10 ng/ml (*Jones, 1991*) were analysed to find any correlations with progesterone concentrations using the Student's *t*-test or Pearson correlation. According to univariate analysis, a multivariate linear regression was carried out to estimate factors associated with progesterone concentrations. A *P*-value <0.05 was considered statistically significant. Statistical analyses were performed with SPSS Statistics for Windows, Version 22 (IBM Corp., Armonk, NY, USA).

TABLE 1 PATIENT DEMOGRAPHICS AND CYCLE CHARACTERISTICS

Age (years)	36.99 ± 4.06
Weight (kg)	61.86 ± 11.02
Progesterone day before transfer (ng/ml)	11.15 ± 4.57
Oestradiol (day before transfer) (pg/ml)	203.08 ± 94.88
Oestrogen (days)[a]	18.20 ± 1.43
Oestrogen type (%, *n*/*N*)	
Transdermal	9.3 (64/685)
Oral	90.7 (621/685)
Previous agonist (%, *n*/*N*)	
No	60.0 (411/685)
Yes	40.0 (274/685)

All data are presented as mean ±SD, unless otherwise stated.

[a] Days of exogenous oestrogen administration until the day of cryopreserved embryo transfer.

Ethical approval

The study was approved by the Institutional Review Board on 16 January 2019 (reference number: 012019011604).

RESULTS

Patient demographics and cycle parameters for the 685 FET cycles meeting inclusion criteria are shown in TABLE 1. Mean ± SD serum progesterone and oestradiol concentrations the day prior to blastocyst transfer were 11.15 ± 4.57 ng/ml and 203.08 ± 94.88 pg/ml, respectively. Mean endometrial thickness was 10.44 ± 1.9 mm (5–20 mm).

Factors associated with LBR in FET cycles

The overall LBR per single cryopreserved blastocyst transfer was 44.8%. The following clinical parameters were significantly associated with LBR: age (OR 0.93; 95% CI 0.89–0.96), duration of oestradiol treatment before FET (OR 0.96; 95% CI 0.92–0.99), serum progesterone concentrations the day before FET (OR 1.04; 95% CI 1.01–1.08) and patients who underwent PGT-A (OR 2.17; 95% CI 1.55–3.03).

Influence of progesterone concentrations the day before FET on LBR

A logistic regression model was performed to show LBR according to serum progesterone concentrations the day prior to blastocyst transfer in patients who did and did not undergo PGT-A, adjusted for age and duration of oestradiol treatment (FIGURE 1). LBR showed a linear increase in both types of cycles (with/without PGT-A) as serum progesterone concentrations rise.

Factors that affect serum progesterone concentrations on the day before FET

Among the factors analysed with univariate analysis: age (R = 0.092; P = 0.017), weight (R = –0.114; P = 0.007), history of a previous FET with serum progesterone concentrations <10 ng/ml (P < 0.001) and time of blood extraction (R = –0.090; P = 0.018) showed a significant correlation to serum progesterone concentrations on the previous day of FET (TABLE 2). A total of 72 cycles presented a history of low serum progesterone values (<10 ng/ml) in a previous FET attempt under the same treatment. In these cycles, the mean (± SD) progesterone values the day before FET was 7.99 ± 2.95 ng/ml, compared with 11.52 ± 4.59 ng/ml in those cycles without such previous history. The further apart the time of blood collection from the latest dose of vaginal progesterone administered, the lower the serum progesterone value (R = –0.090; P = 0.018) (FIGURE 2).

When a multivariate linear regression was performed to correct for potential confounders, increasing age presented a positive correlation to serum progesterone concentrations (β = 0.11; 95% CI 0.01–0.20). On the contrary, significant negative correlations to progesterone concentrations were shown with a previous history of FET with serum progesterone value <10 ng/ml (β = –3.13; 95% CI –4.45 to –1.81), higher weight (β = –0.05; 95% CI –0.08 to –0.01), and delaying the moment of blood sampling during the day (β = –0.13; 95% CI –0.25 to –0.01) (TABLE 3).

DISCUSSION

This is thought to be the first study to analyse clinical factors related to serum progesterone values before FET. The findings demonstrate that weight, age, time of blood sampling and a prior history of low progesterone concentrations (<10 ng/ml) are independent factors associated with serum progesterone concentrations the day before blastocyst FET.

The effect of progesterone concentrations on the LBR following FET has been thoroughly investigated over the last 2 years. This study is in line with current literature (*Brady et al., 2014*; *Cédrin-Durnerin et al., 2019*; *Gaggiotti-Marre et al., 2019*; *Labarta et al., 2017*) showing that progesterone concentrations before FET are an independent factor associated with LBR. Similarly to previous published data on IVF and FET outcomes, other factors associated with LBR in cycles of FET were found such as age (*Devesa et al., 2018*; *Moragianni and Penzias, 2010*; *Younis, 2012*), duration of oestradiol before transfer (*Bourdon et al., 2018*) and decision to pursue PGT-A (*Murphy et al., 2019*; *Neal et al., 2018*).

According to the results of this study, there are specific factors that affect progesterone concentrations on the day prior to embryo transfer. It is extremely important to highlight that some of these factors are associated with altered pharmacokinetics (age, weight and prior history of low progesterone concentrations in a previous FET cycle), while others do not depend on changes in drug absorption or metabolism (timing of blood sampling).

The effect of age on vaginal absorption of progesterone tablets has been previously analysed. In a prospective study by *Levy et al. (2000)*, women >40 years old demonstrated an enhanced rate of absorption of progesterone using vaginal tablets compared with younger patients. In agreement with these previous data, results of this study show that age is positively associated with serum concentrations of progesterone in an independent manner. The thinner and more atrophic vaginal mucosa of older women may lead to increased absorption of vaginal progesterone, explaining these findings.

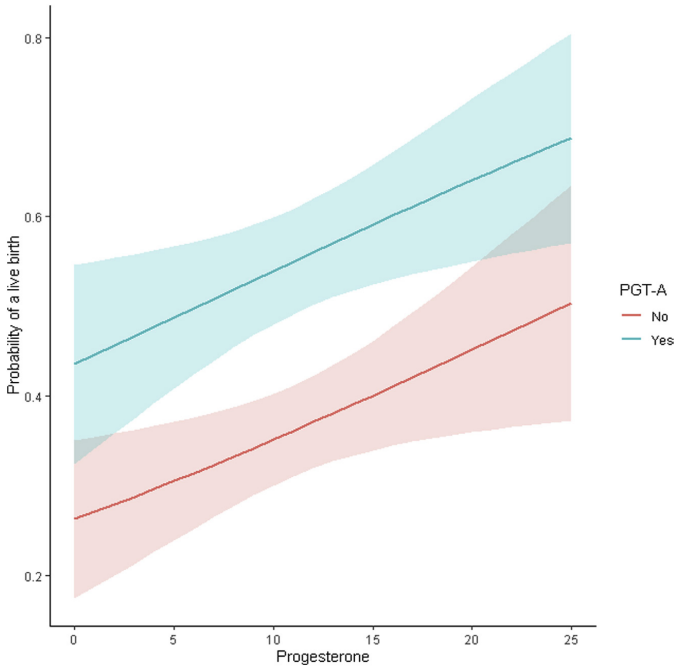

FIGURE 1 Logistic regression model, adjusted for age and duration of oestradiol treatment, showing probability of a live birth according to serum progesterone concentrations (ng/ml) the day before cryopreserved blastocyst transfer. The shading on the figure shows the 95% confidence interval.

TABLE 2 FACTORS ASSOCIATED WITH SERUM PROGESTERONE VALUES THE DAY BEFORE CRYOPRESERVED BLASTOCYST TRANSFER USING UNIVARIATE ANALYSIS

		Correlation coefficient	*P*-value
Oestrogen type			0.103
Oral	11.06 ± 4.62[a]		
Transdermal	12.04 ± 4.07[a]		
Previous agonist			0.146
Yes	10.86 ± 3.91[a]		
No	11.35 ± 4.96[a]		
Oestrogen (days)[b]	18.17 ± 3.7	–0.041	0.286
Progesterone in previous FET cycle			<0.001
<10 ng/ml (*n* = 72)	7.99 ± 2.95[a]		
≥10 ng/ml (*n* = 613)	11.52 ± 4.59[a]		
Oestradiol (pg/ml)	203.08 ± 94.88	–0.054	0.162
Age (years)	36.99 ± 4.06	0.092	0.017
Time extraction (time)	NA	–0.090	0.018
Weight (kg)	61.86 ± 11.02	–0.114	0.007

All data are presented as mean ± SD.

[a] Mean progesterone values (ng/ml) ± SD.

[b] Days of exogenous oestrogen administration until the day of FET.

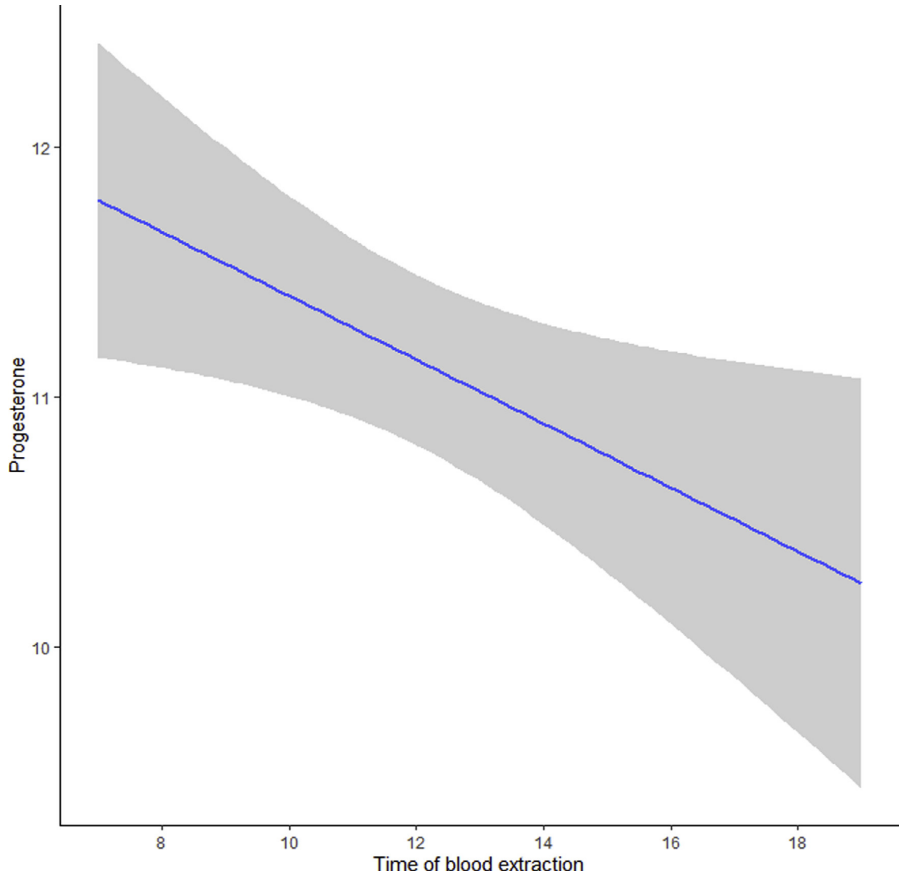

FIGURE 2 Regression model analysis showing the relationship between serum progesterone values (ng/ml) and time of blood extraction on the day prior to embryo transfer (from 08:00 to 19:00 h). $R = -0.090$; $P = 0.018$. The shading on the figure shows the 95% confidence interval.

On the contrary, the effect of body weight on serum progesterone concentrations of artificially prepared cycles is not yet clear. Previous research on 50 post-menopausal women who received 50 or 100 mg/day of vaginal micronized progesterone showed no significant difference in pharmacokinetic behaviour of serum progesterone in relation to weight (*Levy et al., 1999*). However, a more recent study on 229 oocyte recipient cycles using intramuscular progesterone found that serum progesterone concentrations on the day of embryo transfer were lower in overweight and obese women compared with those of normal weight (*Brady et al., 2014*). The findings here are in line with the latter study, showing that body weight is an independent factor that affects serum progesterone concentrations after 4 days of vaginal progesterone administration. These results are biologically plausible as body weight is a determinant factor that influences drug absorption, distribution, metabolism and elimination (*Edelman et al., 2010*).

TABLE 3 MULTIVARIATE LINEAR REGRESSION FOR FACTORS ASSOCIATED WITH SERUM PROGESTERONE CONCENTRATIONS ON THE DAY BEFORE CRYOPRESERVED BLASTOCYST TRANSFER

	β	95% CI
Age	0.11	0.01 to 0.20
Weight	−0.05	−0.08 to −0.01
Time of blood sampling	−0.13	−0.25 to −0.01
Low progesterone (< 10 ng/ml) in previous cycle	−3.13	−4.45 to −1.81

Interestingly, the current analysis showed that previous agonist down-regulation, oestradiol concentrations, type and duration of oestrogen did not affect serum progesterone values.

An abundance of evidence supports that the concentrations of progesterone

are not steady during the day. Intra-day variability of serum progesterone concentrations has been reported not only in the spontaneous cycle of normal women (*Bungum et al., 2013*; *Filicori et al., 1984*; *Fujimoto et al., 1991*; *Kerkhof et al., 2015*), but also in the late follicular and mid-luteal phases of gonadotrophin-stimulated cycles for IVF (*González-Foruria et al., 2019*; *Thomsen et al., 2018*). In artificially prepared cycles, insights from endocrinological studies in progesterone pharmacokinetics show a rapid absorption when using vaginal tablets, reaching mean peak plasma concentrations after 3–6 h, and also a fast mean elimination half-life of 13 h from administration (*Archer et al., 1995*; *Corleta et al., 2004*; *Levy et al., 1999*). The results of this study demonstrate once more that progesterone concentrations vary during the day, even when exogenous hormone replacement is given. These findings perfectly reflect the previous data on vaginal progesterone tablet pharmacokinetics, showing that mean progesterone values are lower, the further from the last administration of vaginal progesterone the blood sample was obtained.

A history of late follicular phase progesterone elevation on the day of ovulation triggering in ovarian stimulation for IVF is the most important factor to predict the same outcome in a subsequent cycle (*Venetis et al., 2016*). In a similar manner, and according to our results using hormone replacement therapy for FET, the history of low progesterone concentrations (<10 ng/ml) under the same treatment is the strongest predictor of progesterone concentrations in a subsequent cycle. Although the origin of serum progesterone concentrations in artificial endometrial preparation cycles for FET is completely different from those of ovarian stimulation cycles, the previous history remains the most determining factor in predicting both clinical outcomes. Thus, the current results suggest that patient intrinsic characteristics, regarding the vaginal absorption of progesterone and the distribution and metabolism of this sex steroid, are of utmost importance in determining serum progesterone concentrations.

The main strength of this study is the novelty of the topic, as it is the first study to analyse clinical factors associated with progesterone concentrations before FET, apart from confirming previous data on the relevance of serum progesterone concentrations in FET cycles. Interestingly, all FET cycles included in the analysis were performed in a single centre, under the same clinical setting and laboratory conditions. All patients underwent the same protocol of vaginal progesterone administration, regarding the doses (200 mg/8 h) and posology (08:00, 16:00 and 00:00 h). In addition, only cycles performed with a patient's own eggs and with single-embryo transfer were included to avoid potential confounders when analysing LBR. Despite a robust and strict design, this work has some shortcomings that need comment. The main limitation is its retrospective design, leading to higher risk of patient selection bias. Using this approach it is not possible to explain what the reasons were for receiving oral or transdermal oestrogens, to undergo more or less days of oestrogens and to undergo GnRH agonists in the preceding cycle. Although patients were advised to administer vaginal tablets of 200 mg of micronized progesterone at three set times of the day (08:00, 16:00 and 00:00 h), there was no way to verify that the treatment was really performed in such a manner and under those precise instructions. Also, even though patients were advised not to have sexual intercourse after starting vaginal progesterone tablets, it was not possible to confirm sexual abstinence during this period. In relation to this, an interesting randomized controlled trial demonstrated that vaginal absorption of progesterone was dramatically reduced in cases of immediate intercourse after vaginal progesterone administration (*Merriam et al., 2015*).

The results of this study have important implications in clinical practice that should be highlighted. First of all, it provides more evidence demonstrating the important effect of serum progesterone concentrations before FET on LBR. As FET is nowadays becoming more widely used in clinical practice (*De Geyter et al., 2018*; *Devesa et al., 2018*; *Moragianni and Penzias, 2010*; *Younis, 2012*), we believe that more efforts should be directed towards improving the chances of success under this approach. Furthermore, the study has shown that certain clinical characteristics of the patient are associated with progesterone concentrations. These findings may help clinicians to personalize the luteal phase support in artificially prepared FET cycles, depending on patient characteristics. In this regard, future research should be directed towards the validation of these results and, more importantly, to individualize endometrial artificial preparation for FET, because so far no cycle regimen has been shown to be superior (*Ghobara et al., 2017*). An interventional study assessing LBR after the addition of more exogenous progesterone when serum concentrations are low before FET would be of remarkable value.

In conclusion, this study confirms previous data showing that serum progesterone concentrations before FET are associated with LBR, and demonstrates that such concentrations depend on certain clinical characteristics of the patient. These findings highlight the importance of future research on the individualization of luteal phase support in artificially prepared FET cycles.

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INDIVIDUALISED LUTEAL PHASE SUPPORT IN ARTIFICIALLY PREPARED FET CYCLES BASED ON SERUM PROGESTERONE LEVELS. A PROSPECTIVE COHORT STUDY

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ORIGINAL ARTICLE ***Infertility***

Individualised luteal phase support in artificially prepared frozen embryo transfer cycles based on serum progesterone levels: a prospective cohort study

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STUDY QUESTION: Does an individualised luteal phase support (iLPS), according to serum progesterone (P4) level the day prior to euploid frozen embryo transfer (FET), improve pregnancy outcomes when started on the day previous to embryo transfer?

SUMMARY ANSWER: Patients with low serum P4 the day prior to euploid FET can benefit from the addition of daily subcutaneous P4 injections (Psc), when started the day prior to FET, and achieve similar reproductive outcomes compared to those with initial adequate P4 levels.

WHAT IS KNOWN ALREADY: The ratio between FET/IVF has spectacularly increased in the last years mainly thanks to the pursuit of an ovarian hyperstimulation syndrome free clinic and the development of preimplantation genetic testing (PGT). There is currently a big concern regarding the endometrial preparation for FET, especially in relation to serum P4 levels around the time of embryo transfer. Several studies have described impaired pregnancy outcomes in those patients with low P4 levels around the time of FET, considering 10 ng/ml as one of the most accepted reference values. To date, no prospective study has been designed to compare the reproductive outcomes between patients with adequate P4 the day previous to euploid FET and those with low, but restored P4 levels on the transfer day after iLPS through daily Psc started on the day previous to FET.

STUDY DESIGN, SIZE, DURATION: A prospective observational study was conducted at a university-affiliated fertility centre between November 2018 and January 2020 in patients undergoing PGT for aneuploidies (PGT-A) IVF cycles and a subsequent FET under hormone replacement treatment (HRT). A total of 574 cycles (453 patients) were analysed: 348 cycles (leading to 342 euploid FET) with adequate P4 on the day previous to FET, and 226 cycles (leading to 220 euploid FET) under iLPS after low P4 on the previous day to FET, but restored P4 levels on the transfer day.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Overall we included 574 HRT FET cycles (453 patients). Standard HRT was used for endometrial preparation. P4 levels were measured the day previous to euploid FET. P4 > 10.6 ng/ml was considered as adequate and euploid FET was performed on the following day (FET Group 1). P4 < 10.6 ng/ml was considered as low, iLPS was added in the form of daily Psc injections, and a new P4 analysis was performed on the following day. FET was only performed on the same day when a restored P4 > 10.6 ng/ml was achieved (98.2% of cases) (FET Group 2).

MAIN RESULTS AND THE ROLE OF CHANCE: Patient's demographics and cycle parameters were comparable between both euploid FET groups (FET Group 1 and FET Group 2) in terms of age, weight, oestradiol and P4 levels and number of embryos transferred. No statistically significant differences were found in terms of clinical pregnancy rate (56.4% vs 59.1%: rate difference (RD) −2.7%, 95% CI [−11.4; 6.0]), ongoing pregnancy rate (49.4% vs 53.6%: RD −4.2%, 95% CI [−13.1; 4.7]) or live birth rate (49.1% vs

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52.3%: RD −3.2%, 95% CI [−12; 5.7]). No significant differences were also found according to miscarriage rate (12.4% vs 9.2%: RD 3.2%, 95% CI [−4.3; 10.7]).

LIMITATIONS, REASONS FOR CAUTION: Only iLPS through daily Psc was evaluated. The time for Psc injection was not stated and no serum P4 determinations were performed once the pregnancy was achieved.

WIDER IMPLICATIONS OF THE FINDINGS: Our study provides information regarding an 'opportunity window' for improved ongoing pregnancy rates and miscarriage rates through a daily Psc injection in cases of inadequate P4 levels the day previous to FET (P4 < 10.6 ng/ml) and restored values the day of FET (P4 > 10.6 ng/ml). Only euploid FET under HRT were considered, avoiding one of the main reasons of miscarriage and implantation failure and overcoming confounding factors such as female age, embryo quality or ovarian stimulation protocols.

STUDY FUNDING/COMPETING INTEREST(S): No external funding was received. B.C. reports personal fees from MSD, Merck Serono, Ferring Pharmaceuticals, IBSA and Gedeon Richter outside the submitted work. N.P. reports grants and personal fees from MSD, Merck Serono, Ferring Pharmaceuticals, Theramex and Besins International and personal fees from IBSA and Gedeon Richter outside the submitted work. The remaining authors have no conflicts of interest to declare.

TRIAL REGISTRATION NUMBER: NCT03740568.

Key words: frozen embryo transfer / progesterone / euploid embryo / hormone replacement treatment / preimplantation genetic testing

Introduction

Frozen embryo transfer (FET) is increasingly adopted in modern IVF. The ratio between FET and fresh embryo transfer in ART cycles has increased both in Europe and USA: from 28% to 40.3% (2010–2015) and from 22.9% to 69.4% (2010–2017), respectively (De Geyter *et al.*, 2018; ART Success Rates | CDC, 2020). Among the many factors that have contributed to such change, the pursuit of an ovarian hyperstimulation syndrome free clinic has been determinant. Improvements in the vitrification and warming processes and the excellent cryosurvival rates have turned FET in our main tool for preventing this complication (Devroey *et al.*, 2011). Moreover, a freeze all strategy has proven to provide excellent or even better pregnancy rates (PRs), not only in high (Chen *et al.*, 2016) but also in normal responders (Shi *et al.*, 2018; Vuong *et al.*, 2018; Wei *et al.*, 2019; Stormlund *et al.*, 2020). Furthermore, techniques such as preimplantation genetic testing (PGT) have also highly benefited from FET, in which the preimplantation embryo is ideally biopsied at the blastocyst stage and subsequently vitrified to allow for chromosomal analysis (Rodriguez-Purata *et al.*, 2016; Sermon *et al.*, 2016).

While ART have rapidly evolved in the areas of embryo culture, vitrification and understanding of the embryo development, little progress has been achieved regarding endometrial preparation for FET. Undoubtedly, correct implantation requires a good quality embryo and a suitable decidualised endometrium. In order to achieve an adequate environment for implantation, endometrial transformation for FET can be achieved through a natural cycle (NC-FET) or an artificial preparation (AC-FET). Artificial cycles require hormone replacement treatment (HRT) with oestradiol and progesterone (P4). However, there is not a single standardised treatment described for optimal endometrial preparation and no protocol has proven superiority in terms of reproductive outcomes (Ghobara *et al.*, 2017; Groenewoud *et al.*, 2018).

Although artificial preparation is the most convenient method to schedule FET cycles, recent reports have highlighted a potentially detrimental effect of low P4 levels prior to FET on miscarriage and live birth rates (LBRs). These results have been observed both in homologous and oocyte recipient FET cycles (Labarta *et al.*, 2017; Cédrin-Durnerin *et al.*, 2019; Volovsky *et al.*, 2020), but also in FET cycles of embryos that had undergone PGT for aneuploidies (PGT-A) (Gaggiotti-Marre *et al.*, 2019).

Nonetheless, despite the accumulating reports on the value of pre-transfer P4 levels on pregnancy outcomes, to our knowledge, no prospective study has been published up to date aiming at overcoming this risk factor. Additional P4 supplementation may be a way to improve reproductive outcomes in these patients. The current prospective study aims to investigate whether patients with low serum P4 levels the day before euploid FET under standard HRT can benefit in terms of ongoing pregnancy and miscarriage rates (MRs) from an individualised luteal phase support (iLPS) consisting in the addition of a daily subcutaneous P4 injection (Psc).

Materials and methods

Study setting

A prospective observational study was performed at a university-affiliated fertility centre between November 2018 and January 2020 in patients undergoing PGT-A IVF cycles and a subsequent FET under HRT.

The www.clinicaltrials.gov registration number is NCT03740568.

Sample size calculation

Sample size calculation was based on previous studies (Alsbjerg *et al.*, 2018; Cédrin-Durnerin *et al.*, 2019; Gaggiotti-Marre *et al.*, 2019), according to which the estimated percentage of patients with low progesterone levels that needed Psc supplementation was 46%. The study hypothesis was that the ongoing pregnancy rate (OPR) in the group with normal P4 levels would be 54%, equivalent to the group with low P4 levels receiving Psc. Based on this assumption we calculated that, by using a two-sided 95% confidence interval in an equivalence study design, at least 592 patients (46% in the supplementation group and 54% in the standard group) are needed in order to exclude a difference between the standard and supplemental groups, with an

equivalence limit set at the level of 10%, which we considered clinically relevant.

Endpoints

The primary endpoint in this study is to compare OPR, defined as the ultrasound confirmation of a foetus with heart activity beyond 12 weeks of pregnancy per transfer, between patients with adequate P4 before FET under standard HRT to those with initial low P4 before FET and restored value after additional P4 supplementation through a daily Psc injection (iLPS).

Pregnancy rate (PR) (defined as a rise in serum beta hCG concentration >25 UI/L per transfer), clinical pregnancy rate (CPR) (defined as the presence of at least one gestational sac in ultrasound per transfer) and MR (defined as the spontaneous loss of an intra-uterine pregnancy prior to 12 completed weeks of gestational age) between both groups were considered as secondary endpoints. Biochemical pregnancy rate (BP), defined as a pregnancy diagnosed only by the detection of beta hCG in serum per transfer, LBR, defined as the number of deliveries that resulted in a live born neonate per transfer, were also included in the analysis. We also considered as secondary endpoints the % of rescued cycles (defined as cycles were a normal P4 level was achieved after iLPS) and percentage of cancelled FET due to lack of response to iLPS (defined as cycles were a normal P4 level was not achieved after iLPS).

Study protocol

Both ovarian stimulation protocols and PGT-A technique have been previously described elsewhere. Briefly, ovarian stimulation was performed under gonadotrophins and pituitary suppression with gonadotrophin-releasing hormone analogues (agonists or antagonists) according to established standard protocols (Alvarez *et al.*, 2019). Mature oocytes were microinjected 40 h after hCG or GnRH agonist trigger, upon indication. Embryos were cultured in a time-lapse incubator (Geri®, Merck, Germany) using single-step culture media (G-TL™, Vitrolife, Sweden). All developing embryos on Day 3 had their zona pellucida opened. Hatching blastocysts were biopsied using laser thermolysis (Veiga *et al.*, 1997) and vitrified immediately afterwards using Kitazato methodology (Kitazato Medical Group, Japan). Preimplantation genetic testing aneuploidies analysis was performed by next generation sequencing using the VeriSeq™PGS—MiSeq® platform from Illumina® (USA) following the manufacturer's protocols and guidelines. Embryo quality and grading is determined by morphologic and development criteria (ASEBIR, 2015). Euploid embryos were transferred in a subsequent cycle (Parriego *et al.*, 2007).

Endometrial preparation

Hormonal replacement under standard protocol (Martínez *et al.*, 2011) was used for endometrial preparation and FET. In brief, patients underwent treatment with either 2 mg/8 h oral oestradiol (E2) valerate (Progynova®, Schering, Spain) or 150 µg every 3 days transdermal patches (Evopad®, Janssen-Cilag, Spain) for 12–14 days. Vaginal micronized P4 treatment at 200 mg/8 h was started from the night of Day 15 (D0) until the day of plasma β-hCG determination (D14). The day prior to FET (D4) a vaginal ultrasound to assess endometrial thickness and a blood analysis for E2 and P4 were performed.

Serum analysis

Blood samples were obtained and processed in our laboratory for E2 and P4 measurements, using an electrochemiluminescence immunoassay (Cobas® e-411 analyser, Roche diagnostics, Germany). For E2, the lower limit of detection was 5 pg/ml with intra and interassay variation of 2.4–4.6% and 4.3–9.9%, respectively. For P4, the lower limit of detection was 0.03 ng/ml, with intra and interassay variation of 1.5–2.7% and 3.7–5.5%, respectively.

Patient selection

Only patients undergoing FET of an euploid blastocyst between the established time period were included. Patients who underwent mosaic FET and those who did not follow our standard supplementation protocol were excluded.

All patients undergoing FET of an euploid embryo were prospectively followed up and categorized into two groups according to their serum P4 values one day before FET: low P4 (<10.6 ng/ml) and adequate P4 (>10.6 ng/ml) (Fig. 1). The cut-off value to define low and adequate progesterone was stated at 10.6 ng/ml, in relation to a previous retrospective study (Gaggiotti-Marre *et al.*, 2019) in which 244 euploid FET were included under HRT, and patients with serum P4 < 10.6 ng/ml the day before FET had significantly higher MR (26.6% vs 9.5%, $P = 0.007$) and lower LBR (47.5% vs 62.3%, $P = 0.029$) than those with serum P4 > 10.6 ng/ml.

Treatment plan

Patients were treated as shown in Fig. 1. Patients with adequate serum P4 level (P4 > 10.6 ng/ml) on D4 (Group 1) continued standard P4 supplementation treatment (vaginal micronized P4 200 mg every 8 h) until serum β-hCG determination. Embryo warming and transfer were performed on the following day (D5) (FET Group 1) under ultrasound guidance as previously described (Coroleu *et al.*, 2002).

Group 2 was defined in patients with low serum P4 level (P4 < 10.6 ng/ml) on D4. In this group a daily subcutaneous P4 injection of 25 mg (Prolutex® 25 mg, IBSA, Spain) was added to HRT on the same day. Patients underwent a second serum P4 analysis on D5. Embryo warming and FET were performed only in case P4 level on D5 was >10.6 ng/ml (FET Group 2). Embryo transfer was cancelled in those patients in which P4 level on D5 was <10.6 ng/ml.

The treatment was continued in the same regimen until around gestational week 10 if pregnancy was confirmed.

Ethical approval

Patients signed an informed consent form. The study was approved by our Institutional Review Board: number 172018101003.

Statistical analysis

Continuous outcomes were presented as mean and standard deviation whereas categorical outcomes were presented as frequencies and percentages.

Univariate analysis was carried out to describe and compare the cycle characteristics and reproductive outcomes between the two groups of progesterone. *T*-test or Mann Whitney *U* test were applied for continuous variables and Chi-square test or Fisher's test for categorical variables. Normality distribution was analysed by the

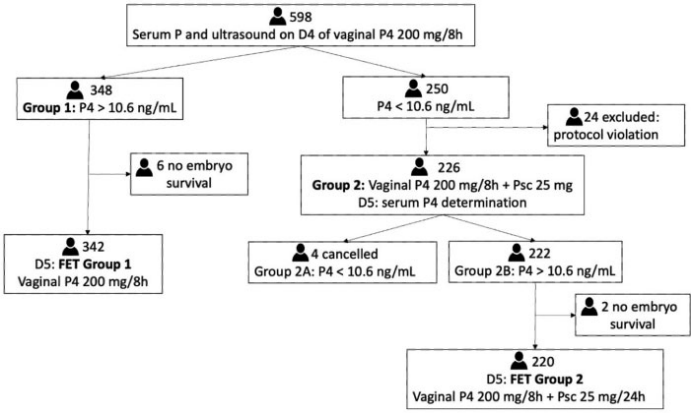

Figure 1. Flowchart showing patient distribution into groups according to serum progesterone levels on the day previous to frozen embryo transfer. P4: progesterone, FET: frozen embryo transfer, D4: day previous to frozen embryo transfer, D5: day of embryo transfer.

Kolmogorov–Smirnov test and Boxplot. The 95% confidence intervals for differences between proportions were calculated for main outcomes (PR and OPR). All tests were two tailed, and $P < 0.005$ was considered statistically significant. Statistical analyses were performed with IBM© SPSS© Statistics v 22 software.

Results

Patients' demographics and cycle characteristics

A total of 598 FET cycles were included in the study. Although most of women with low serum P4 levels received Psc as per protocol, 24 patients undergoing FET cycles did not proceed with Psc and were excluded from the analysis. These patients did not receive treatment either because they were remotely located with no access to medication before the embryo transfer or because they were not willing to initiate Psc (despite being advised so) either for convenience or cost reasons. A total of 574 FET cycles (453 patients) were finally considered for analysis.

Patient's demographics and cycle parameters for the 574 FET cycles meeting inclusion criteria and for the two groups are described in Table I. In summary, the mean age of all intended mothers was 39.7 ± 3.8 years and mean weight was 63.4 ± 11.4 kg. The mean serum P4 level the day before FET was 12.9 ± 6.9 ng/ml. Patients and cycle characteristics were comparable between the group with initial adequate P4 level (Group 1) and the group with low initial P4 who received additional P4 supplementation (Group 2). Group 1 included 58.2% (348) patients. Group 2 included 37.7% (226) women, who received an additional Psc injection. On the following day, 98.2% (222/226) had reached serum P4 levels >10.6 ng/ml and FET was performed. Overall, only four FET cycles were cancelled (1.8%) due to inadequate serum P4 levels despite additional P4 treatment.

Two FET cycles from 222 in Group 2 and six from 348 in Group 1 were not performed as embryos did not survive the warming process.

Reproductive outcomes

Reproductive outcomes were similar between FET Group 1, with initial adequate P4 level, and FET Group 2, with a restored adequate P4 level after additional treatment with Psc (Fig. 2).

The PR and CPR in FET Group 1 was 62.3% (213/342) and 56.4% (193/342) compared to 64.5% (142/220) and 59.1% (130/220) in FET Group 2 (rate difference (RD) −2.2%, 95% CI [−10.8; 6.3]; RD −2.7%, 95% CI [−11.4; 6.0]). Similarly, the OPR was comparable between FET Group 1 (49.4% [169/342]) and FET Group 2 (53.6% [118/220]) respectively (RD −4.2%, 95% CI [−13.1; 4.7]).

Miscarriage rate was 12.4% (24/193) in FET Group 1, compared to 10.8% (14/130) in FET Group 2 (RD 1.6%, 95% CI [−6.1; 9.4]), with no statistically significant differences. There were also no significant differences according to biochemical pregnancy rate that were 5.85% (20/342) and 5.45% (12/220) in FET Group 1 and FET Group 2 respectively.

Finally, we also did not find significant differences according to LBR between FET Group 1 (49.1% [168/342]) and FET Group 2 (52.3% [115/220]) (RD −3.2%, 95% CI [−12; 5.7]).

The 24 FET with P4 < 10.6 ng/ml excluded from the study for protocol violation as no Psc was added, albeit small in sample, had poor reproductive success, with an OPR of 20.8% (5/24) and MR of 37.5% (3/8).

All four cancelled cycles due to unrestored P4 despite additional Psc underwent FET in a subsequent cycle under HRT with both vaginal and Psc treatment. All women achieved serum P4 level >10.6 ng/ml the day before FET and FET was performed.

Table I. Patients' demographics and cycle characteristics.

	Overall (n = 574)	Group 1 (n = 348)	Group 2 (n = 226)	*P* value
Age (years)	39.7 ± 3.8	40.0 ± 3.9	39.2 ± 3.6	0.021
Weight (kg)	63.4 ± 11.4	63.0 ± 11.4	64.0 ± 11.3	0.387
Endometrial thickness (mm)	10.5 ± 1.9	10.5 ± 1.9	10.5 ± 2.0	0.980
Oestradiol (pg/ml)	221.4 ± 99.0	220.9 ± 101.2	222.1 ± 95.6	0.894
Number of embryos transferred	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.2	0.125
Good quality embryos (A + B)*	0.6 ± 0.5	0.6 ± 0.5	0.5 ± 0.5	0.073

Group 1: Patients with adequate serum P4 level (P4 > 10.6 ng/ml) on the day before frozen embryo transfer (D4).
Group 2: Patients with low serum P4 level (P4 < 10.6 ng/ml) on the day before frozen embryo transfer (D4) who received additional daily subcutaneous P4 injection.
*According to ASEBIR's morphological scoring system (ASEBIR, 2015).

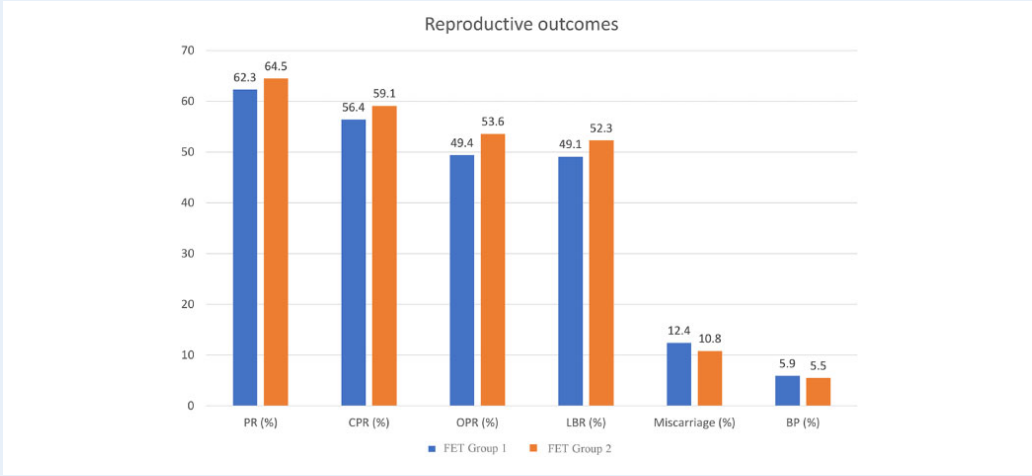

Figure 2. Reproductive outcomes. CPR: clinical pregnancy rate, PR: pregnancy rate, OPR: ongoing pregnancy rate, LBR: live birth rate, BP: biochemical pregnancy rate. Blue: FET Group 1; Orange: FET Group 2.

Discussion

To our knowledge this is the first study providing evidence that an individualised LPS can result in a very high OPR and LBR in patients undergoing euploid FET cycle under HRT in cases of low serum P4 levels prior to embryo transfer. In this context, addition of daily Psc injection to our standard HRT in patients with low P4 levels (<10.6 ng/ml) the day prior to euploid FET (D4) results in excellent OPR and LBR, similar to those in women with adequate initial P4 levels (>10.6 ng/ml).

Serum P4 levels and FET has become a main topic in ART. Recent retrospective studies have described P4 levels as an independent prognostic factor associated not only with OPR (Boynukalin *et al.*, 2019), but also with LBR (Cédrin-Durnerin *et al.*, 2019; González-Foruria *et al.*, 2020) in patients undergoing FET. In fact, previous studies have demonstrated a detrimental effect of low P4 levels around the time of embryo transfer on reproductive outcomes in women undergoing FET under HRT. Altogether, the mixed data and the retrospective basis of these studies called for a prospective design comparing the reproductive outcomes between FET under standard HRT and FET under iLPS when low P4 serum level is registered prior to FET.

Even though there is no clear consensus concerning the optimal P4 threshold in FET, one of the most accepted reference values is around 10 ng/ml (Labarta *et al.*, 2017; Cédrin-Durnerin *et al.*, 2019; Gaggiotti-Marre *et al.*, 2019), which correlates to an adequate P4 production by the corpus luteum in a natural cycle (Hull *et al.*, 1982; Jordan *et al.*, 1994). In most of the recent publications on this topic, serum P4 is measured on the day of embryo transfer (Brady *et al.*, 2014; Labarta

et al., 2017; Cédrin-Durnerin et al., 2019) or the day of pregnancy test (Alsbjerg et al., 2018), both timepoints at which little or no intervention is possible before transferring the embryo. However, in a previous study by our group we determined the optimal cut-off value for serum progesterone not on the day of embryo transfer or the day of pregnancy test, but on the day prior to blastocyst transfer (Gaggiotti-Marre et al., 2019), a timepoint at which an individualised LPS can be initiated. Based on our results, this cut-off has been set at 10.6 ng/ml and patients with levels beyond this value were supplemented with a daily 25 mg. Psc injection. The percentage of patients with low serum P4 values appears to be relatively constant among studies published up to date. In one study, 37% of the patients under HRT for FET had a serum P4 value the day of FET below 10 ng/ml (Cédrin-Durnerin et al., 2019) whereas in another 25% had levels below 9.2 ng/ml on the day of the embryo transfer (Labarta et al., 2017), following vaginal administration of 200 mg micronized progesterone every 8 h and 400 mg every 12 h, respectively. While both studies have shown that low P4 levels are associated with compromised PRs, Cédrin-Durnerin et al. (2019) also found that doubling the vaginal P4 dosage from the day of FET did not improve the reproductive outcome. Similarly, other reports (Archer et al., 1995; Paulson et al., 2014) also described a limited beneficial effect of increasing the vaginal dosage of P4, probably due to a rate-limited absorption by the vaginal epithelium. Likewise, Brady et al. (2014) described detrimental effects of P4 < 20 ng/ml the day of embryo transfer in oocyte recipients under HRT with intramuscular (IM) P4 replacement. They also did not report improved outcomes when additional IM dosages were prescribed to these patients with low P4 levels. Similarly, a recent retrospective study (Alur-Gupta et al., 2020) conclude that increasing doses of IM P when P4 levels are lower than 15 ng/ml give similar outcomes to patients with P4 levels >15 ng/ml. On the other hand, Alsbjerg et al. (2013) did report improved reproductive outcomes when vaginal P4 was doubled in patients undergoing FET under HRT, or when additional rectally administered P4 was provided (Alsbjerg et al., 2020).

In the present study, the percentage of cycles with low serum P4 progesterone levels (<10.6 ng/ml) was 37.8% (226 cycles). Among them in 140 cycles (61.95%) P4 levels were between 8 and 10.6 ng/ml and 86 cycles (38.05%) with P4 levels <8 ng/ml. All these cycles fulfilled the criteria for iLPS through the addition of daily 25 mg Psc injection. Most of them (98.2%) reached adequate serum P4 levels with the administration of only one dosage of Psc. This can be explained by the pharmacokinetics of the two different routes for P4 administration, given that while the vaginal route has been shown to provide a rapid endometrial absorption and local effect via the uterine first-pass effect (Miles et al., 1994), it also yields lower circulating levels due to its shorter half-life (Miles et al., 1994; Levy et al., 1999; Cicinelli et al., 2000). Thus, addition of P4 through a parental route could be an option to rapidly and effectively increase the serum P4 levels in case of low values after only vaginal progesterone exposure.

Up to date, literature regarding the best route for P4 replacement is mixed. In terms of reproductive outcomes, while some authors describe better results in women receiving IM P4 supplementation compared to only vaginal (Haddad et al., 2007; Kaser et al., 2012; Devine et al., 2018), others do not confirm these results (Williams et al., 2000; Shapiro et al., 2014; Wang et al., 2015). Still, a combined treatment with different routes seems a plausible option to ensure adequate P4 exposure for patients that fail to achieve sufficient serum P4 levels under one selected treatment. In fact, there is published evidence on improved reproductive outcomes when the combined route is used compared to only vaginal (Feinberg et al., 2013; Devine et al., 2018). In this regard, Psc has proven its efficacy for both endometrial preparation and luteal phase support in ART and FET (Baker et al., 2014; Lockwood et al., 2014; Turkgeldi et al., 2020), providing higher serum P4 levels than the vaginal route (Sator et al., 2013; Paulson et al., 2014) and a good acceptance, comfort and ease of use among patients (Venturella et al., 2018). We could also hypothesize about a possible lower subendometrial wave activity under Psc that has been described when P4 was switched to the IM route during the three days before FET compared to those who continued on the vaginal route (Casper, 2014), although a recent randomized clinical trial did not confirm this data (Klement et al., 2018).

Another possible explanation behind the biological rationale of our study could be related to what we could define as an 'opportunity window' in which additional parenteral P4 administration may offer an advantage when is provided before FET but no later than hCG test. In this regard, Delcour et al. (2019) describe no improved outcomes when IM P4 is administered after hCG test. On the contrary, we have to note the low ongoing pregnancy (20.8%) rate and high MR (37.5%) observed in the 24 patients that did not strictly follow the iLPS protocol. Altogether, the present study provides the advantages of both administration routes (vaginal and subcutaneous) with reduced discomfort compared to the IM administration, which requires training and can cause pain in the site of injection, skin inflammation or even sterile abscesses (Penzias, 2002; Phy et al., 2003).

One of the main strengths of present study is its prospective design in a single centre, under the same standardised clinical setting, treatment and laboratory conditions. Also, the inclusion of only chromosomally normal embryos avoids one of the main reasons of miscarriage and implantation failure (Marconi et al., 2003) and overcomes confounding factors such as female age (Harton et al., 2013; Rubio et al., 2017), embryo quality or ovarian stimulation protocols. The determination of P4 the day before FET allowed an iLPS through an alternative route for P4 supplementation according to our own data in a previous study (Gaggiotti-Marre et al., 2019). In this regard, other authors have recognized that serum P4 analysis on the transfer day may be too late, as doubling vaginal dosage did not influence in ongoing or LBRs, and advise on the possibility of cancelling FET with such low levels (Cédrin-Durnerin et al., 2019). In this sense, our study does not only provide an alternative route for additional P4 supplementation, but also introduces for the first time the possibility of rescuing cases of P4 deficiency along the 'opportunity window' (before the FET). This approach could provide an individualised strategy based on each patient's need.

The main limitation of our study is that a single serum P4 determination was performed without a specific time interval since the last vaginal dose administration or the first subcutaneous injection. Our group has recently published that lower P4 levels on the day prior to FET are in relationship with the further apart the time of blood collection from the latest dose of vaginal progesterone administration ($R = -0.090$; $P = 0.018$) (González-Foruria et al., 2020). However, the exact time of injection was not stated in the present study. Another limitation is the lack of serum P4 determinations on the day of β-hCG testing or once the pregnancy is achieved. Patients continued on either only vaginal or both regimens from the day of FET until

β-hCG testing, but no additional determinations were performed in order to ensure adequate P4 exposure during the first weeks of pregnancy. Treatment discontinuation was individualised but not strictly defined, usually at around 10th week of pregnancy.

Another important limitation of the current prospective study is that, per protocol, Psc supplementation was adopted for patients with serum progesterone levels <10.6 ng/mL, given that several previous reports in our setting demonstrated that such values are likely to be associated with lower PRs (Gaggiotti-Marre *et al.*, 2019). However, caution is needed because we haven't proven that low progesterone levels were also associated with inferior PRs in the current study. Consequently, our finding that iLPs through Psc supplementation results in excellent PRs in the patients with P4 levels below 10.6 ng/mL, may only indirectly support that iLPS improves pregnancy outcomes, given the absence of evidence that the patients without the adjustment would have had lower pregnancy or higher MRs.

In summary, this is the first prospective study to provide an individualised strategy for P4 replacement treatment in patients undergoing euploid FET with low P4 serum level the day prior to transfer. Our results suggest a minimum P4 threshold to improve reproductive outcomes in FET under HRT with vaginal progesterone, which, if detected, can be overcome in most cases by the addition of a daily subcutaneous shot. Such a benefit could be provided not only by the different routes of P4 administration to ensure adequate P4 exposure for patients, but also taking into account the 'opportunity window' related to adding P4 before the embryo transfer. Furthermore we cannot neglect the high patient's satisfaction in regard to Psc, especially as compared with the side effects associated with IM administration. Based on our findings we demonstrate that the approach described in the present study could provide clinicians a standardised and individualised protocol for luteal phase replacement in women undergoing FET HRT, securing excellent PRs even in cases of low serum P4 levels. Undoubtedly more studies are needed to confirm whether iLPS through the addition of daily Psc is the optimal treatment for cases of low P4 levels around the time of FET.

In conclusion, according to our results, iLPS through Psc co-administration with vaginal P4 in cases of low serum P4 values before FET under HRT can result in excellent OPRs and LBRs. Although our study design is not a randomized trial and thus cannot prove superiority of co-treatment with Psc with vaginal progesterone vs only vaginal progesterone in women with low levels, it is unclear whether such a study should be considered ethically appropriate today, especially taking into account the consistent and accumulating evidence demonstrating very low PRs in women with low serum P4 levels who continue treatment only with vaginal P4.

Data availability

Derived data supporting the findings of this study are available from the corresponding autor (M.A) on reasonable request. Data cannot be shared for ethical/privacy reasons. This research was performed under the auspices of the Càtedra d'Investigació en Obstericia i Ginecologia of the Department of Obstetrics, Gynaecology and Reproductive Medicine, Dexeus University Hospital, Universitat Autònoma de Barcelona.

Authors' roles

BC, MA and SGM conceived and designed the study. All the authors analysed and interpreted the data. SG and IR contributed to data collection and performed the statistical analysis. IGF, FM, MP, LC, NPP and BC revised the article for important intellectual content. SGM, MA and NPP wrote the article. All the authors approved the final version of the manuscript.

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Conflict of interest

B.C. reports personal fees from MSD, Merck Serono, Ferring Pharmaceuticals, IBSA and Gedeon Richter outside the submitted work. N.P. reports grants and personal fees from MSD, Merck Serono, Ferring Pharmaceuticals, Theramex and Besins International and personal fees from IBSA and Gedeon Richter outside the submitted work. The remaining authors have no conflicts of interest to declare.

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DISCUSSION

The main results from this thesis could be summarized into: 1. A low serum P value (< 10.6 ng/mL) the day before FET is an independent factor for lower LBR and higher miscarriage rates; 2. This detrimental effect can be observed both in women undergoing FET under artificial and natural cycles; 3. There are identifiable factors associated to an increased risk of lower serum P levels before FET: weight, age, time of blood sampling and a prior cycle with low progesterone level. 4. It is possible to provide an individualized strategy through the addition of P via a different route in these women and improve their reproductive outcomes.

Whereas several studies have analysed the type of endometrial preparation for FET and pregnancy outcomes (Gelbaya *et al.*, 2006; Hill *et al.*, 2010; Groenewoud *et al.*, 2013, 2016; Hatoum *et al.*, 2017), no author has identified any superior way over another in terms of pregnancy outcomes, and up until recently, little focus has been given to serum P levels around FET. In this regard, this thesis provides an alternative view for FET: to focus on the serum P level before FET, where an intervention is still possible, rather than on finding a universal protocol for all patients.

The fact that most IVF programs are privately funded, being both financially and emotionally demanding as well as time-consuming, further supports the idea that patients deserve the best and most individualized strategy that fits into their schedule, budget and expectations.

SERUM PROGESTERONE CUT-OFF

Is there a minimum progesterone level that should be reached for improved reproductive outcomes?

Our publications have determined that a serum P below ≈10ng/mL on the day before FET in women undergoing both HRT and natural cycle FET is associated to detrimental reproductive outcomes (Gaggiotti-Marre *et al.*, 2019, 2020).

In terms of women undergoing FET under HRT with vaginal progesterone administration, other authors have similarly revealed that P levels below ≈10ng/mL around the time of ET or during early pregnancy significantly impair pregnancy outcomes (Brady *et al.*, 2014; Labarta *et al.*, 2017, 2020; Alsbjerg *et al.*, 2018; Cédrin-Durnerin *et al.*, 2019; Gaggiotti-Marre *et al.*, 2019). Our study (Gaggiotti-Marre *et al.*, 2019) divided patients into quartiles according to their serum P levels the day before FET (Q1 < 8.06 ng/mL, Q2:

8.07 – 10.64 ng/mL, Q3: 10.65 – 13.13 ng/mL, Q4 > 13.13 ng/mL). Patients in the Q1 group had the most detrimental reproductive results. When the results were grouped by median P values, patients in the P < 10.64 ng/mL had significantly higher miscarriage rates and lower LBR compared to patients with P > 10.64 ng/mL (26.6% vs 9.5% and 47.5% vs 63.3%). Unlike previously published studies, our data on HRT-FET cycles, provide information only on cycles that underwent PGT-A. In this regard, the inclusion of only chromosomally normal embryos overcomes one of the main reasons of miscarriage and implantation failure (Marconi *et al.*, 2003), controlling for other confounding factors such as female age (Harton *et al.*, 2013; Rubio *et al.*, 2017), embryo quality or ovarian stimulation protocols.

One limitation of our study was its retrospective design, in which there was no intervention in order to evaluate a possible approach for these patients. Therefore, and owing to these findings, we conducted a prospective study in which patients with low serum P the day prior to FET received additional P administration through daily subcutaneous injections (Psc) (Alvarez *et al.*, 2021), which is discussed in more detail below.

On the other hand, in terms of natural cycles, other authors had previously described a serum P threshold during the mid-luteal phase around 10 ng/mL

for adequate luteal function (Hull *et al.*, 1982; Jordan *et al.*, 1994), but no study had yet demonstrated a correlation between serum P levels on the day before ET and LBR in women undergoing FET under a natural cycle endometrial preparation (NC-FET). Although the most common way to prepare the endometrium for a FET in our setting is under HRT, given its convenience in terms of programming the ET both for patients and for clinicians, the natural cycle preparation is also an excellent strategy for patients undergoing FET, provided they have regular cycles. Furthermore, many authors have recently described possible obstetrical adverse effects of the artificial endometrial preparation, in part due to the absence of other substances produced by the corpus luteum, which is lacking under this regime (Conrad *et al.*, 2019).

In this sense, we wanted to study whether a serum P cut-off the day before FET in patients undergoing a NC-FET would also be associated to detrimental reproductive outcomes, so we conducted a retrospective cohort study including 294 FET cycles under a NC-FET cycle during January 2016 and January 2019 (Gaggiotti-Marre *et al.*, 2020). Our study also showed a clear correlation between lower serum P levels the day before FET and detrimental reproductive outcomes. In this sense, patients with serum P levels < 10 ng/mL the day prior to FET had lower LBR compared to those with serum P > 10

ng/mL (25.7% vs 41.1%: RD 15.4%, 95% CI [5; 26]). This correlation was maintained independently of whether patients underwent PGT-A. Altogether, this study further supported the idea that a minimum serum P threshold may be necessary in order to adequately sustain a pregnancy or, in other words, a low serum P level may be a risk factor for detrimental reproductive outcomes in women undergoing FET. Although this study was also limited by its retrospective design, the fact that the natural cycle is not the predominant protocol for endometrial preparation in FET, made a prospective approach complicated to execute. However, a future prospective multicentric study could overcome this issue.

The importance of determining a cut-off value is crucial in order to first detect the issue and subsequently attempt at providing an adequate solution for these patients.

Overall, both presented papers concluded that serum P determination before FET, both in patients under HRT and a natural cycle, can become an excellent tool to detect patients at risk of having diminished chances of a live birth, and that a plausible cut-off would be of around 10 ng/mL.

INCIDENCE OF LOW SERUM PROGESTERONE LEVELS

How prevalent are low serum P levels among women undergoing FET cycles?

The percentage of patients with low serum P values appears to be relatively constant among studies published up to date. In a study by Cédrin-Durnerin, 37% of the patients under HRT for FET had a serum P value the day of FET below 10 ng/mL, following vaginal micronized P administration of 200 mg every 8 hours (Cédrin-Durnerin *et al.*, 2019). Labarta *et al* described that 25% of their population had levels below 9.2 ng/mL (Labarta *et al.*, 2017) and around 30% had values below 8.8 ng/mL in a subsequent study (Labarta *et al.*, 2020), both of them with P detection the day of the embryo transfer, following vaginal administration of 400 mg every 12 hours. Similarly, other authors have reported a luteal phase defect prevalence of 31% among women with regular ovulatory cycles (Davis *et al.*, 1989).

Since the first study presented in this thesis in relation to FET under HRT, provided a quartile and median analysis, the incidence of patients with low serum P was 50% (Gaggiotti-Marre *et al.*, 2019). In the second presented study, in which patients underwent NC-FET, the prevalence was 37% (Gaggiotti-Marre *et al.*, 2020). Similarly, the latest presented study provided

a 37.8% prevalence of women with low serum P the day before FET (Alvarez *et al.*, 2021).

Altogether these data suggest a stable and yet elevated prevalence of women that predictably will have lower chances at having new-born.

SERUM PROGESTERONE DETERMINATION BEFORE FROZEN EMBRYO TRANSFER

Which is the advantage of serum determination the day before FET?

One of the novelties in our studies is that we have determined the serum P levels the day before FET (Figure V).

In most of the recent publications on this topic, serum P is measured on the day of embryo transfer (Brady *et al.*, 2014; Labarta *et al.*, 2017, 2020; Cédrin-Durnerin *et al.*, 2019) or the day of pregnancy test (Alsbjerg *et al.*, 2018), both timepoints at which little or no intervention is possible before transferring the embryo.

In this regard, other authors have recognized that serum P analysis on the transfer day may be too late. Cédrin-Durnerin found that doubling vaginal dosage after the day of FET did not influence in ongoing or live birth rates, and advised on the possibility of cancelling FET with such low levels (Cédrin-

Durnerin *et al.*, 2019). Brady *et al* (2014) did not report improved outcomes when additional IM dosages were prescribed to patients with low P levels the day of ET, under IM P treatment. Likewise, Delcour *et al*. also described no improved outcomes when IM P was administered after hCG test in patients under only vaginal P treatment (Delcour *et al.*, 2019). On the other hand, improved reproductive outcomes in patients undergoing FET have been observed provided that vaginal P dosages were doubled from the very beginning of HRT (Albsjerg *et al*, 2013). However, pharmacokinetic studies have described limited beneficial effect of increasing the vaginal dosage of P on serum P levels (Archer *et al.*, 1995; Paulson *et al.*, 2014).

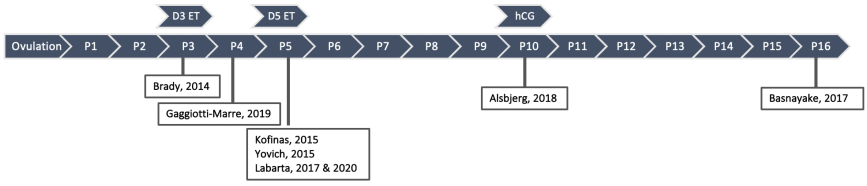


Figure V. Day of serum progesterone determination among different publications

P: progesterone supplementation day; D: day; ET: embryo transfer; hCG: pregnancy test

In this sense, we introduce for the first time the possibility of rescuing cases of P deficiency during an "opportunity window" (before the FET), considering P supplementation if deemed appropriate or even cycle cancelation for a new attempt through a change in endometrial preparation.

INDIVIDUALISED STRATEGY BASED ON SERUM PROGESTERONE LEVELS

Which factors can predict low progesterone levels?

The detection of a determinant that causes a detrimental effect in the reproductive outcomes forces us as clinicians to adopt any possible measure to correct or adapt the treatment in order to provide the best possible chances for our patients. In this regard, there are several other known determinants that can jeopardize a FET cycle, such as age (Moragianni and Penzias, 2010; Younis, 2012; Devesa *et al.*, 2018) and duration of oestradiol before transfer (Bourdon *et al.*, 2018).

In terms of serum P levels, we found specific factors that affect progesterone concentrations on the day prior to embryo transfer, by a multivariate linear regression analysis (González-Foruria *et al.*, 2020): age is positively associated with serum levels of progesterone (β = 0.11; 95% CI 0.01-0.20), patients with higher body weight present lower serum P concentrations (β = -0.05; 95% CI

-0.08 to -0.01), mean progesterone values are lower the further from the last administration of vaginal progesterone (β = -0.13; 95% CI -0.25 to -0.01) and, finally, a history of low progesterone levels (<10 ng/mL) under the same treatment is the strongest predictor of a low progesterone level in a subsequent cycle (β = -3.13; 95% CI -4.45 to -1.81). Among these factors, all but the delay in blood sampling are related to treatment pharmacokinetics. Our results have been further supported by a later prospective cohort study (Labarta *et al.*, 2020). Altogether this research provides a promising opportunity for individualized luteal phase support (iLPS) in this specific population.

If a low plasma progesterone level is detected, is there a possible strategy to overcome this?

In terms of dosage and route for P administration, we have provided a practical and plausible plan for rescuing those patients with low serum P levels, through the administration of additional daily subcutaneous P injection from the day before of FET. We demonstrated that most patients (98.2%) with initial low serum P levels (< 10.6 ng/mL) reached adequate serum P levels on the day of FET with the additional administration of only

one dosage of Psc, achieving similar reproductive outcomes to those patients with initial adequate serum P levels (Alvarez *et al.,* 2021).

In conclusion, it is possible to provide an individualized strategy for luteal phase support in women undergoing FET cycles. A single serum analysis of P on the day before FET permits us to detect those patients at risk of lower pregnancy rates and correct these cases via additional treatment. Furthermore, other predictable factors for lower P levels include age, weight, time of blood sampling and a prior cycle with low progesterone level, which can also become crucial as an attempt to provide an individualized treatment strategy.

FUTURE PERSPECTIVES

I believe that this thesis provides enough evidence to support the implementation of an individualized strategy according to serum P levels the day before FET.

One limitation of this protocol is that not all IVF clinics or settings have access to rapid tests or have the opportunity to obtain the blood test results within a few hours or even before FET. Also, patients may be travelling from abroad or from a different city or village to undergo fertility treatments, which makes

this approach inconvenient or not accessible to everyone. One way to attempt at overcoming these situations could be to try to detect the possible predictable factors for lower P levels, such as elevated weight or previous history of low serum P, if known, and anticipate to the possibility of a lower P level through the administration of additional P via another route or a higher dosage.

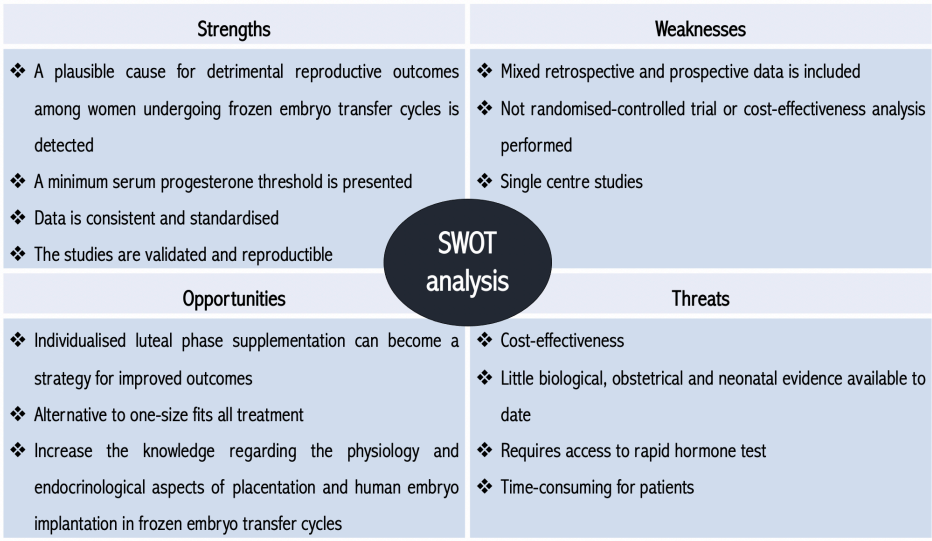
Another important aspect to take into account is the fact that the current available efficient routes for P administration for luteal phase support or luteal phase replacement are only parenteral or vaginal. This is a long treatment, which requires multiple daily dosages for over 2 months, and women have many complaints regarding both routes. On one hand, the parental administration comes with an injection, sometimes painful or uncomfortable, and the vaginal route is often associated to discharge or even vaginal infections in occasions. The investigation and design of a more comfortable route that is as efficient as the available ones seems a priority, given the large (and increasing) number of women that require or will require this treatment.

Finally, larger future studies are needed to adequately individualise and detect which patients may require additional treatment. Ideally, it would be

fantastic to be able to anticipate this need and avoid multiple blood tests and time-consuming appointments.

SWOT ANALYSIS

Lastly, a SWOT analysis is presented (Figure VI) in order to summarize the Strengths, Weaknesses, Opportunities, and Threats of this thesis.



CONCLUSIONS

1. Serum progesterone determination the day before FET can be a useful and valid tool to predict reproductive outcomes in patients undergoing FET cycles.
2. A serum P level below 10.6 ng/mL the day before FET is associated to higher miscarriage and lower live birth rates in women undergoing artificially prepared FET cycles.
3. A serum P level below 10 ng/mL the day before FET is associated to detrimental reproductive outcomes in women undergoing FET cycles under a natural endometrial preparation.
4. There are predictable factors associated to serum P levels before FET: weight, age, time of blood sampling and a prior cycle with low progesterone level.
5. An individualized strategy through the addition of P via a different route in women with low serum P the day before FET can improve their reproductive outcomes.

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