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The pathophysiological role of estrogens in the initial stages of pregnancy: molecular mechanisms and clinical implications for pregnancy outcome from the periconceptional period to end of the first trimester

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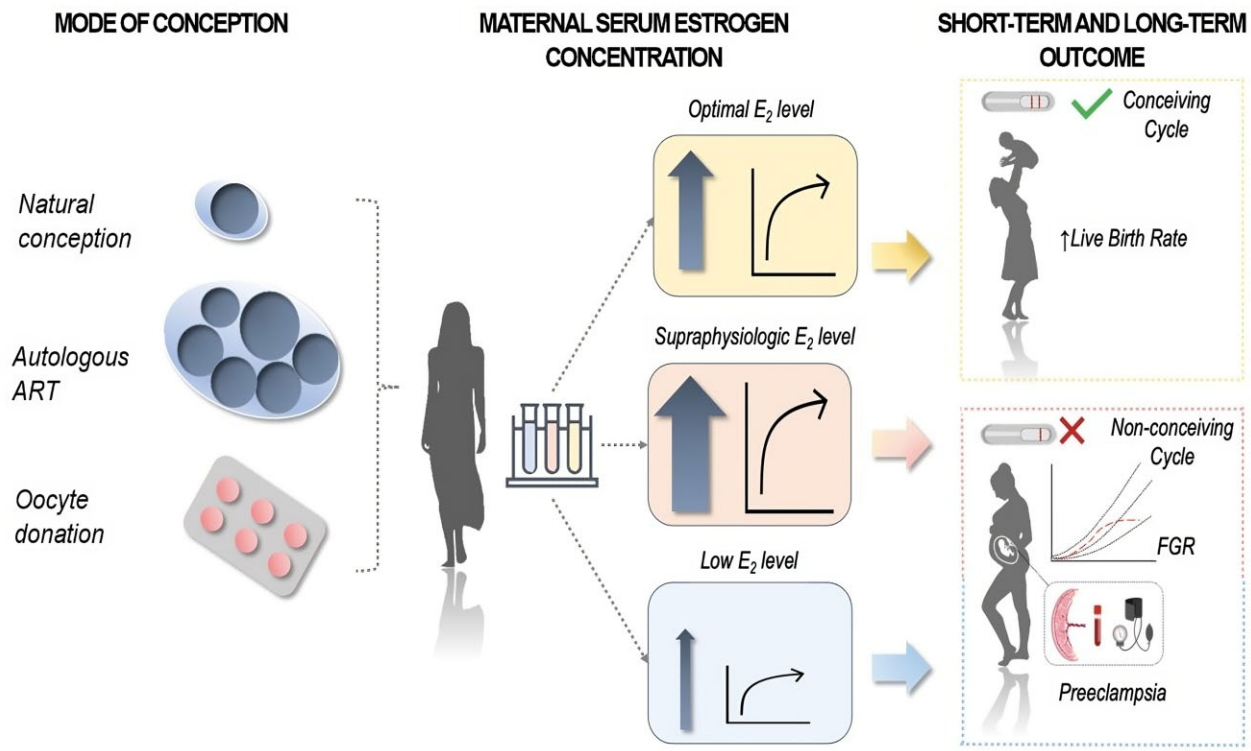
GRAPHICAL ABSTRACT



The impact of method of conception and serum estrogen levels on pregnancy outcome. FGR, fetal growth restriction; E2, estradiol.

ABSTRACT

BACKGROUND: Estrogens regulate disparate female physiological processes, thus ensuring reproduction. Altered estrogen levels and signaling have been associated with increased risks of pregnancy failure and complications, including hypertensive disorders and low birthweight babies. However, the role of estrogens in the periconceptional period and early pregnancy is still understudied.

OBJECTIVE AND RATIONALE: This review aims to summarize the current evidence on the role of maternal estrogens during the periconceptional period and the first trimester of pregnancies conceived naturally and following ART. Detailed molecular mechanisms and related clinical impacts are extensively described.

SEARCH METHODS: Data for this narrative review were independently identified by seven researchers on Pubmed and Embase databases. The following keywords were selected: 'estrogens' OR 'estrogen level(s)' OR 'serum estradiol' OR 'estradiol/estrogen concentration', AND 'early pregnancy' OR 'first trimester of pregnancy' OR 'preconceptional period' OR 'ART' OR 'In Vitro Fertilization (IVF)' OR 'Embryo Transfer' OR 'Frozen Embryo Transfer' OR 'oocyte donation' OR 'egg donation' OR 'miscarriage' OR 'pregnancy outcome' OR 'endometrium'.

OUTCOMES: During the periconceptional period (defined here as the critical time window starting 1 month before conception), estrogens play a crucial role in endometrial receptivity, through the activation of paracrine/autocrine signaling. A derailed estrogenic milieu within this period seems to be detrimental both in natural and ART-conceived pregnancies. Low estrogen levels are associated with non-conception cycles in natural pregnancies. On the other hand, excessive supraphysiologic estrogen concentrations at time of the LH peak correlate with lower live birth rates and higher risks of pregnancy complications. In early pregnancy, estrogen plays a massive role in placentation mainly by modulating angiogenic factor expression—and in the development of an immune-tolerant uterine micro-environment by remodeling the function of uterine natural killer and T-helper cells. Lower estrogen levels are thought to trigger abnormal placentation in naturally conceived pregnancies, whereas an estrogen excess seems to worsen pregnancy development and outcomes.

WIDER IMPLICATIONS: Most current evidence available endorses a relation between periconceptional and first trimester estrogen levels and pregnancy outcomes, further depicting an optimal concentration range to optimize pregnancy success. However, how estrogens co-operate with other factors in order to maintain a fine balance between local tolerance towards the developing fetus and immune responses to pathogens remains elusive. Further studies are highly warranted, also aiming to identify the determinants of estrogen response and biomarkers for personalized estrogen administration regimens in ART.

Keywords: estrogens / steroid hormones / pregnancy / implantation / endometrial receptivity / inflammation / infertility / ART

Introduction

Medical practice sometimes relies on assumptions or anecdotical evidence stemming from limited scientific proof that may ultimately result in inadequate interventions or therapies in clinical settings. We believe that, in view of being one of the most important biological factors throughout a woman's reproductive life and beyond, estrogens deserve more attention in the study of the early events surrounding reproduction, and especially with respect to ART.

Estrogens are a heterogeneous group of steroid compounds that regulate disparate female physiological processes and ensure reproduction. Sex hormone concentrations are strictly dependent on the woman age and hormonal stage: estradiol (E2) predominates throughout life, estriol (E3) mainly during pregnancy, and estrone (E1) in the postmenopausal period (Orzołek *et al.*, 2022). E2, the main product of the ovarian follicles, is the most abundant and potent estrogen, acting as a key mediator of the pathophysiological changes of the female reproductive tract.

While the role of estrogens during pregnancy has been extensively described in late pregnancy and parturition, few human studies have investigated their function during the periconceptional period and the first trimester of pregnancy. Furthermore, maternal sex steroid concentrations during pregnancy have been associated with health risks in both the mother and the offspring later in life, mainly predicting the risk of steroid-sensitive cancers in the mother, and atopic, cancer and neurodevelopmental abnormalities in children (Toriola *et al.*, 2011). This further highlights the urgency to fill the knowledge gap on physiological estrogen concentrations in early pregnancy, as well as the feto-maternal determinants of the hormonal milieu. Such knowledge would be of relevance in light of the increasing rates of autologous and heterologous ART pregnancies, currently accounting for around 3% of livebirths in industrialized countries (Wyns *et al.*, 2022). ART include IVF-embryo transfer (IVF/ET and frozen embryo transfer (FET)). These techniques apply to both autologous and heterologous gametes. IVF/ET is the most common technique and involves three steps: oocyte collection from the ovary, its fertilization with semen *in vitro*, and the subsequent transfer of the embryo into the uterus. To increase the success of the procedure, multiple oocytes are obtained through controlled ovarian stimulation with parenteral gonadotropins administration. Different stimulation protocols are available, based on patients' characteristics and experience of the physician. Once follicles reach at least 18 mm in diameter at the ultrasound evaluation, a trigger is administered to mimic the LH peak and induce ovulation. After 36 h, transvaginal ultrasound-guided needle aspiration is performed. Oocyte fertilization happens *in vitro*: in case of male infertility, ICSI may be used to overcome the problem. The last step of the procedure is the ET, where one or more embryos, suspended in a drop of culture medium, are placed into the uterine cavity. Supernumerary embryos are generally cryopreserved and transferred in subsequent cycles, if pregnancy is not achieved on the first attempt (Goldberg *et al.*, 2007; Sallam and Rizk, 2012). Among heterologous techniques, oocyte or embryo donation from young donors represents a standard and effective treatment for age-related infertility or premature ovarian failure. Oocyte donation (OD) involves egg retrieval from the donor, insemination with semen of the recipient's partner, and *in vitro* culture and transfer of cleaved embryos into the recipient's uterus. The stimulation of the oocyte donor is similar to the standard IVF protocol. ET in the recipient may be performed both in a natural cycle, if the woman has an adequate ovarian function, or to a prepared endometrium. Endometrial preparation is achieved by replacing endogenous steroid with E2 administered both orally (4–6 mg/day) or transdermally (50–100 mg every 72 h). When endometrial thickness reaches at least 7 mm, ET may be performed (Barbieri *et al.*, 2010; Melnick and Rosenwaks, 2018; Kaser *et al.*, 2019).

Therefore, pharmacological manipulation of the female sex hormone regulation system has long been leveraged in ART, in order to mimic menstrual cycle hormone fluctuations in the setting of IVF/ET protocols. However, the lack of high quality randomized controlled trials has hampered the definition of standardized protocols to ensure ART success and no definitive consensus has been reached among the scientific community to date (Mackens *et al.*, 2017; Glujovsky *et al.*, 2020).

Today the treatment with exogenous estrogens in heterologous and autologous reproduction is not supported by sufficient scientific evidence and appears empirical at best. Our hypothesis is that women seeking pregnancy with ART would benefit from a personalized regimen of endometrial stimulation as a crucial step to maximize reproductive success. This review aims to summarize the current understanding of the role of estrogens in the physiopathology of the periconceptional period (here defined as the critical time window starting 1 month before conception) and early pregnancy, detailing the underlying molecular mechanisms affecting the endometrium before and after embryo implantation, as well as during the first trimester.

Methods

Data for this narrative review were identified by seven researchers (A.Z., A.I., F.P., C.S., C.F., M.B., and V.S.) on PubMed and Embase databases from June 2021 to October 2022. The search strategy included the following keywords: 'estrogens' OR 'estrogen level(s)' OR 'serum estradiol' OR 'estradiol concentration', AND 'early pregnancy' OR 'first trimester of pregnancy' OR 'periconceptional period' OR 'Assisted Reproduction technology (ART)' OR 'In Vitro Fertilization (IVF)' OR 'Embryo Transfer' OR 'Frozen Embryo Transfer' OR 'oocyte donation' OR 'egg donation' OR 'miscarriage' OR 'pregnancy outcome' OR 'endometrium'. English language and original data studies, regardless the sample size, were taken into consideration.

Estrogens and their receptors

There are three major types of estrogens in women: E1, E2, and E3. E2 is the most potent estrogen and, along with progesterone, is a master regulator of the changes occurring during the menstrual cycle, whereas E3 becomes the primary estrogen during late pregnancy (Cui *et al.*, 2013). In women, all these estrogen types are synthesized from androgens, namely testosterone and androstenedione, by the enzyme aromatase in the ovaries, or the corpus luteum upon ovulation. Although other organs may produce estrogens, and minor estrogen types that do not require aromatase do exist, they will not be covered in this review.

Unlike other mammals, the primates' placenta becomes the primary source of estrogens during pregnancy (Pepe *et al.*, 2018). However, the primate placenta does not express the cytochrome P450 17A1 (steroid 17-alpha-hydroxylase/17,20 lyase) which participates in corticoid and androgen biosynthesis. Therefore, the placenta cannot convert c21-steroids (pregnenolone and progesterone) into estrogen precursors c19-steroids (dehydroepiandrosterone—DHEA—and androstenedione), leading to the placental dependence on estrogen precursors from maternal and fetal adrenal glands. As a result of extensive 16-hydroxylation of c19-steroids within the fetus, large quantities of E3 are produced by the placenta during human pregnancy. Likewise, the synthesis of a fourth estrogen, estetrol (E4), takes place in the fetus and its concentration is much higher in the fetal than the maternal circulation. While the production and preferential excretion of E3 and E4 was proposed to protect the fetus from the effects of the more potent E2, their function remains to be clarified and this review will focus mainly on E2.

Two receptors account for estrogens' activity in the endometrium, namely estrogen receptor (ER) 1 (ERα) and 2 (ERβ), whose tissue distribution differs across multiple organs (Heldring *et al.*, 2007). ERα is primarily expressed in the uterus, and to a lesser extent in the skin, ovaries, testis, and gut; conversely, ERβ expression has been detected in the ovaries, prostate, colon, kidneys, cardiovascular system, and central nervous system (Brandenberger *et al.*, 1997). Notably, ERβ has been described by some groups to be the sole ER expressed in specific cell types within the endometrium, such as the endothelium and uterine natural killer (uNK) cells, which are the most abundant subset of immune cells in the endometrium (Taylor and Al-Azzawi, 2000; Critchley *et al.*, 2001; Henderson *et al.*, 2003). Notwithstanding, the number of studies investigating the potential roles of ERβ in the endometrium is remarkably modest.

Moreover, although ER expression varies over time during the menstrual cycle, being more prominent in the nuclei of the epithelial layer of the endometrium during the proliferative phase, mRNAs specific for both ER types have been reported in glandular epithelial, stromal, and myometrial cells of the human uterus during all stages of the menstrual cycle (Xu *et al.*, 2021). Three ERα and four ERβ isoforms, generated by alternative splicing of pre-mRNA from *ER1* and *ER2* genes, have been described. Although their role has only marginally been elucidated so far (Jia *et al.*, 2015; Yu *et al.*, 2022), several reports indicate that ER isoforms can differentially modulate estrogen signaling and, as a consequence, impact target gene regulation (Ramsey *et al.*, 2004; Elhasnaoui *et al.*, 2021; Costa *et al.*, 2022). Upon binding estrogen, ERα and ERβ dimerize and move to the cell nucleus, exerting their effect by activating or suppressing the transcription of a plethora of different genes, which are regulators of various physiological processes (Mal *et al.*, 2020) including uterine development and fertility, blastocyst implantation and the early stages of embryo development (Park *et al.*, 2012; Vasquez and DeMayo, 2013). Although responsible for several physiological functions in both females and males, studies performed on ERα and ERβ double knockout mice show that life is feasible even without E2 action, regardless of ubiquitous ER expression in almost all anatomical areas (Lubahn *et al.*, 1993; Dupont *et al.*, 2000; Weihua *et al.*, 2000). Besides, E2 is indispensable for reproductive function in females, but only ERα seems to be crucial in maintaining fertility (Lubahn *et al.*, 1993). Thus, the key task of ERβ is thought to counteract undesired ERα-mediated actions of E2.

The periconceptional stage

Every successful pregnancy starts with embryo implantation in the uterine endometrium. The crucial steps required to prepare a receptive endometrium suitable for the implantation and the subsequent growth of a competent blastocyst are tightly regulated by a number of biochemical factors, including a proper hormonal milieu, and by a strict cross-talk between blastocyst and endometrium (Zhang *et al.*, 2013). The exposure of the endometrium to the fluctuation of ovarian sex hormones during the menstrual cycle periodically creates and maintains a receptive environment for further blastocyst development. However, limited data are available on the role of estrogens in fertile women during the periconceptional period, here defined as the critical time window starting one month before conception, and most of our understanding relies on animal models and *in vitro* cell systems. Previous studies investigated the reference values of serum E2 during natural menstrual cycles (Stricker *et al.*, 2006; Anckaert *et al.*, 2021), but only few focused on its concentration at the LH peak of natural conceiving cycles (Lenton *et al.*, 1982; Stewart *et al.*, 1993). At peak maturation, a single oocyte is estimated to secrete 200–300 pg/ml of E2 (Speroff and Fritz, 2011). Owing to the higher number of retrieved oocytes in IVF/ET cycles (Fig. 1), serum E2 concentration at time of the LH surge in natural conception cycles is significantly lower compared to autologous ART conception. In the following paragraphs, we compiled all available evidence in an attempt to better characterize the potential impact of such difference on pregnancy outcome.

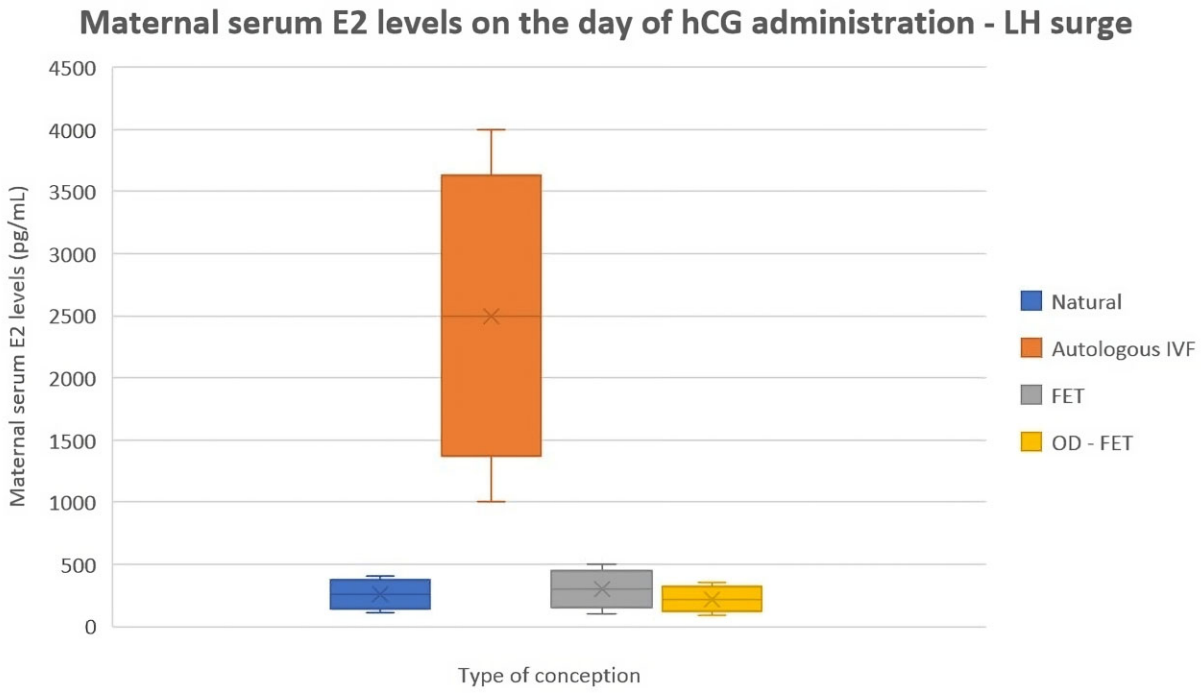


Figure 1. Serum estrogen concentration in women at time of the LH surge in natural conception cycles compared to autologous ART conception and oocyte donation. E2, estradiol; FET, frozen embryo transfer; OD-FET, oocyte-donation FET.

Natural conception

Highly complex and still understudied biological processes occur within the periconceptional period, including follicular development, fertilization, implantation, and early organogenesis, all rigorously orchestrated by hormonal fluctuation, epigenetic changes, and inflammatory signaling (Steegers-Theunissen *et al.*, 2013). Most of the studies are consistent in demonstrating that estrogen levels vary from cycle to cycle in healthy fertile women. In particular, lower serum, urinary, and salivary estrogen concentrations were associated with non-conception cycles compared to conception cycles (Baird *et al.*, 1991; Stewart *et al.*, 1993; Lipson and Ellison, 1996; Baird *et al.*, 1997, 1999; Li *et al.*, 2001; Chen *et al.*, 2003; Venners *et al.*, 2006). Previous literature showed an excellent agreement between serum, urinary and salivary estrogen biomarkers. In particular, urinary metabolites exhibit cyclic patterns similar to serum steroids during the menstrual cycle, but smaller variations between baseline and peak concentrations and about a 1-day delay compared to the serum peak were described (Cekan *et al.*, 1986; Hardiman *et al.*, 1990; Roos *et al.*, 2015). Venners *et al.* (2006) evaluated urinary concentrations of E1 conjugates (E1C) in a population of 347 healthy and fertile women who were attempting to get pregnant naturally, finding higher estrogen concentrations during cycles resulting in clinical pregnancy compared to non-conception cycles (Venners *et al.*, 2006). Lipson and Ellison (1996) previously evaluated salivary estrogen and progesterone levels among conception and non-conception cycles, finding no differences in progesterone concentrations and higher salivary estrogen in conception cycles, especially in the mid-follicular phase (Lipson and Ellison, 1996). In line with these results, Stewart *et al.* (1993) and Baird *et al.* (1997) found higher mid-luteal estrogen concentrations in fertile women with conception compared to non-conception cycles (Stewart *et al.*, 1993; Baird *et al.*, 1997). While the former hypothesizes a possible effect of the preimplantation embryo on ovarian steroid production, the latter suggests that the detected differences in steroid concentrations may reflect a higher quality of cycles resulting in improved chances of conception. Additionally, data on estrogen levels of conception cycles with early pregnancy loss were inconclusive. Baird *et al.* (1991) described similar preimplantation estrogen and progesterone levels among 20 cycles with early pregnancy loss and 20 cycles with successful pregnancy in the same study population, whereas Venners *et al.* (2006) found a trend towards lower urinary E1C levels in 63 early pregnancy loss cycles, especially during the ovulation and the early luteal phase, compared with 266 successful pregnancy cycles (Baird *et al.*, 1991; Venners *et al.*, 2006).

Autologous ART

The available data show controversial results. Five out of the 22 identified studies suggest no correlation between serum E2 levels on the day of luteinizing trigger and pregnancy outcomes. Huang *et al.* (2014) divided 2868 patients stimulated with long GnRH agonist protocol into three groups according to the dynamics of serum E2 levels 12 h before and after hCG administration ($\Delta E2 > 10\%$, $-10\% \leq \Delta E2 \leq 10\%$ and $\Delta E2 < -10\%$) (Huang *et al.*, 2014). In line with Styer *et al.* (2005), no significant difference in live birth rate (LBR) and miscarriage rate (MR) was highlighted and pregnancy outcome seemed not to be influenced by E2 dynamics (Styer *et al.* 2005; Huang *et al.* 2014; Sunkara *et al.* 2015). Similar results were achieved by Zavy *et al.* (2014) who analyzed serum E2 levels on the day of hCG administration in 478 patients undergoing fresh IVF/ET. No difference was detected among the three selected groups (E2 < 2000 pg/ml; E2 2000–4000 pg/ml; E2 > 4000 pg/ml) in LBR and MR, thus suggesting that other factors, including maternal age and embryo quality, seem to be the main determinants of pregnancy outcomes (Zavy *et al.*, 2014).

LBR seemed to be mainly affected by embryo quality also in the retrospective study by Mackens *et al.* (2020), which focused on monitoring serum E2 levels in endometrial preparation protocols of patients undergoing FET. In line with previous results, no significant difference in pregnancy outcome was detected among the three groups defined by serum E2 concentrations at the start of progesterone supplementation (E2 < 144 pg/ml; E2 145–438 pg/ml; E2 > 439 pg/ml) (Mackens *et al.*, 2020). Also, Morales *et al.* (2021), analyzing 181 clinical records, assumed that serum E2 measurements on the day of hCG trigger represent a valid predictor of oocyte retrieval, maturation and subsequent fertilization, but no significant association further existed between the observed E2 concentrations and pregnancy outcome. Among the studies showing a possible association between serum estrogen levels and pregnancy outcome, Kondapalli *et al.* (2012) retrospectively focused on E2 dynamics (ΔE2) before and after hCG administration in 1712 patients undergoing IVF. Participants were divided into three groups ($\Delta E2 > 10\%$, $-10\% \leq \Delta E2 \leq 10\%$, and $\Delta E2 < -10\%$): while LBR was significantly higher in women with $\Delta E2 > 10\%$, no difference resulted in MR (Kondapalli *et al.*, 2012). Despite the retrospective design and heterogeneous stimulation protocols, the adjustment for multiple confounding factors and the large sample size make the results of interest. Similar results were achieved by Wang *et al.* (2017), who retrospectively divided 3393 patients undergoing long GnRH-agonist protocol stimulation into two groups according to serum E2 levels on the day of hCG administration. Significantly increased LBRs were detected in the 'high E2' group (E2 > 3757 pg/ml) versus the 'low E2' group (E2 < 3757 pg/ml), with no differences in delivery mode, gestational age (GA) at birth, birthweight, risk of preterm birth, and fetal malformations between the two groups (Wang *et al.*, 2017). Nevertheless, the poor statistical analysis with no adjustment for any confounding factor makes interpretation of the results difficult. Furthermore, Li *et al.* (2019) observed that only the interval of medium-high E2 concentrations (1000–5000 pg/ml) on hCG administration day correlates with better IVF and pregnancy outcomes. In fact, as previously noted by Steward *et al.* (2015), supraphysiological estradiol levels were associated with lower LBRs and higher MRs. E2 > 5000 pg/ml was additionally identified as an independent risk factor for low birthweight (LBW) newborns, with a reported odds ratio (OR) of 16.8 (Li *et al.*, 2019). These observations may explain the controversial results previously reported, as an inverted U-shaped association between E2 concentrations and IVF/pregnancy outcome probably involves an optimal serum E2 concentration range, outside of which both extremes are determinants of adverse reproductive outcome. Similar results are reported by Joo *et al.* (2010), who retrospectively analyzed 494 fresh IVF/ET cycles. The five groups defined according to serum E2 levels on the day of hCG administration (E2 < 1000 pg/ml; E2 1000–2000 pg/ml; E2 2000–3000 pg/ml; E2 3000–4000 pg/ml; E2 > 4000 pg/ml) highlighted a significant difference in implantation, pregnancy, and LBRs, further suggesting optimal age-dependent ranges of serum E2 (3000–4000 pg/ml for women <38 years and 2000–3000 pg/ml for women ≥38 years) (Joo *et al.*, 2010). Wu *et al.* (2012) added that patients with elevated progesterone and E2 concentrations (E2 > 19124 pmol/ml, i.e. E2 > 5209 pg/ml) on the day of hCG administration have significantly lower LBRs and higher ectopic pregnancy rates, assuming that, when combined with premature elevation of progesterone,

high E2 concentrations may have a potential detrimental effect on pregnancy outcome (Wu et al., 2012).

In autologous FET cycles, where exogenous estrogen administration is an essential step for endometrial preparation, Fritz et al. (2017) observed a significant inverse association between LBRs and average and peak serum E2 levels, measured from initiation of the artificial FET cycle to the last level prior to progesterone supplementation (LBRs from 54% for peak serum E2 levels below 234 pg/ml to 9% for peak serum E2 levels above 692 pg/ml) (Fritz et al., 2017). Again, these results highlight that, even in FET cycles and in line with fresh IVF, serum E2 levels should be monitored and held in a relatively narrow range for optimizing endometrial receptivity without detrimental effects of supraphysiological concentrations. Furthermore, Li et al. (2022), in the setting of a retrospective study on 776 FET cycles and in multi-adjusted models including confounding factors, suggested that elevated E2 levels on progesterone-initiation-day may negatively affect pregnancy outcomes only after cleavage-stage embryo transfers and not when transferring blastocysts (Li et al., 2022). Pregnancy outcomes following frozen-thawed blastocyst transfer appear not to be influenced by estrogen levels, as also shown in the study by Choi et al. (2021). Again, these results suggest a shortened receptivity window, caused by supraphysiological E2 levels, that may be missed by a cleavage-stage ET, with no impact on a late-growing blastocyst. This is also confirmed in the retrospective study by Romanski et al. (2021) on patients undergoing natural FET. In this study, frozen blastocysts were transferred in natural cycles without exogenous hormonal stimulation. In this setting, the length of endogenous estrogenic stimulation may have a positive impact on pregnancy outcome: in fact, the authors reported a significantly lower LBR (46.6%) in patients with E2 > 100 pg/ml for <4 days in the follicular phase compared to patients with E2 > 100 pg/ml for more than 4 days (52.0%) (Romanski et al., 2021). This result supports the idea that sustained physiological E2 concentrations can result in adequate endometrial receptivity, unlike the detrimental endometrial effects of prolonged supraphysiological E2 concentrations. Regarding the association between follicular estrogenic concentrations, placentation and pregnancy complications, the results in humans seem concordant when pregnancies are achieved in the setting of high E2 levels after controlled ovarian stimulation for IVF. An exception is the Dunne et al. (2017) study, which did not find associations between estrogen concentrations, early markers of placental development (pregnancy-associated plasma protein-A (PAPP-a)) and composite adverse maternal and neonatal birth outcomes associated with placental dysfunction (i.e. fetal growth restriction, pregnancy-induced hypertension). Farhi et al. (2010) retrospectively reported higher rates (10.1%) of placenta-related pregnancy complications (fetal growth restriction, pregnancy-induced hypertension and abnormal implantation of the placenta) in patients with E2 concentrations above the 75th percentile (=9000 pmol/l) compared with patients with E2 lower than the 75th percentile (1.4%), identifying high E2 concentration on hCG day as the only independent variable associated with increased risks for pregnancy complications in multi-adjusted regression models (adjusted OR 14.1) (Farhi et al., 2010). In line with these results, Pereira et al. (2015) reported a significantly raised risk of LBW newborns in patients undergoing fresh IVF/ET cycles, with peak E2 levels > 3069 pg/ml (Pereira et al., 2015). The same group observed that the risk of LBW increases up to 6.1–7.9 times when serum E2 levels on hCG administration day are above 2500 pg/ml, revealing E2 as an independent predictor for LBW rate with adjusted OR of 10.8 (Pereira et al., 2015). LBW rates in IVF-conceived pregnancies are also reported to be associated with the number of retrieved oocytes during controlled ovarian stimulation. Sunkara et al. (2015) observed that high responder patients (cut-off: >20 follicles retrieved) are significantly more prone to develop fetal growth restriction, as a possible indirect effect of extreme supraphysiological hyper-estrogenism (Sunkara et al., 2015). Tarlatzi et al. (2021), as previously noted by Liu et al. (2017) and Kalra et al. (2011), confirmed the association between supraphysiologic E2 levels and LBW, highlighting a significant difference in birthweight by comparing fresh IVF cycles and FET. Although fetal growth restriction burdens both ART procedures, fresh cycles have shown significantly lower birthweight compared to FET, probably as a consequence of higher periconceptional estrogenic concentrations, which are reported to be related to lower β-hCG levels during the first trimester and abnormal placentation (Kalra et al., 2011; Liu et al., 2017; Tarlatzi et al., 2021).

Finally, Imudia et al. (2012) showed that elevated E2 levels (E2 > 3450 pg/ml) on the day of hCG administration during ovarian stimulation is associated with greater odds of developing pre-eclampsia and small-for-GA infants in singleton pregnancies resulting from IVF cycles. On the other hand, Chen et al. (2020) revealed an opposite association between serum E2 concentration and the risk of pre-eclampsia in fresh IVF/ET-conceived pregnancies, reporting lower estrogenic levels, together with excessive maternal weight gain, as the main risk factor for pre-eclampsia in a multivariate analysis (Chen et al., 2020). Despite the apparently contradictory results, different E2 concentrations are reported as a risk factor: in fact, Chen et al. defined E2 lower than 1200 pg/ml as a risk factor for pre-eclampsia, while Imudia et al. reported high supraphysiological levels (>3450 pg/ml) as a detrimental risk factor.

While all the presented studies are surely limited by the retrospective design and comparisons may be affected by differences in study populations, protocols used for ovarian stimulation or endometrial preparation, and day on which steroid hormone levels were measured, a strict serum E2 concentration window in artificial settings with hormonal manipulation appears to be optimal in order to improve reproductive outcomes in terms of both LBR and placental efficiency. Figure 2 shows the E2 levels on the day of hCG administration described in the literature as adequate for pregnancy progression or at risk for pregnancy complications.

Oocyte donation

Advances in ART have allowed women who cannot use their own oocytes to achieve a pregnancy through OD. OD is associated with the highest success rates among ART procedures and nowadays has become a common treatment option, especially to overcome infertility linked to advanced maternal age. This success has led to an increasing interest in the impact of OD on several aspects of obstetrical care, especially pregnancy outcomes (Savasi et al., 2016). However, despite increasing interest in this ART procedure, many issues remain unclear, such as the hormonal environment in both the donor and recipient, and its possible manipulation. In OD, the donor's gametogenesis and ovarian steroidogenesis are dissociated from the recipient's endometrial development and receptivity, allowing the effects of elevated E2 concentrations on the embryo to be distinguished from those on the endometrium.

Considering endometrial preparation, the most recent meta-analysis concluded that it is not yet possible to recommend one protocol over another in FET cycles (Groenewoud et al., 2013). However, for fresh or FET two methods can be used to attain a

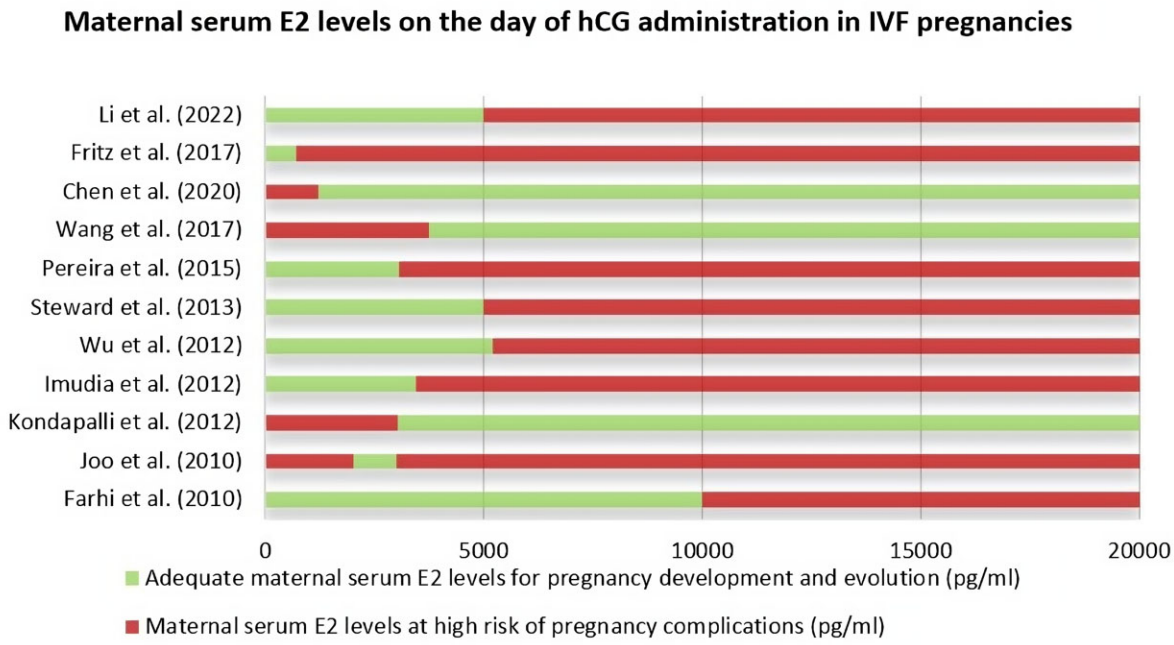


Figure 2. Studies reporting serum estradiol levels in women on the day of hCG administration in IVF pregnancies. The pregnancies were described as adequate for pregnancy development and progression (green line) or at high risk for pregnancy complications (red line). E2, estradiol.

receptive endometrium for embryo implantation: through a 'natural cycle' (synchronization of the embryo transfer with recipient's ovulation) or through the administration of exogenous steroids: in the latter case, estrogens are supplied either orally or transdermally from Day 1 of the menstrual cycle in which the embryo transfer will be performed, and estrogen can be administered either in an increasing dose or a constant dose from the beginning of the menstrual cycle. Madero *et al.* (2016) compared a constant versus an increasing E2 dose protocol in 8236 heterologous ETs. No significant difference resulted in terms of LBR, as well as in MR, between the two protocols. No difference has been detected among various routes of estrogen administration. These data, in contrast with previous findings in autologous ART procedures, suggest that the estrogen dose for endometrial preparation does not impact reproductive outcomes as much as other variables, such as alterations of the BMI, embryo quality and oocyte donor's age (Madero *et al.*, 2016).

On the donor side, Bianco *et al.* (2009) compared 58 oocyte donors undergoing controlled ovarian stimulation, distributed into two groups according to E2 levels on the day of hCG administration (E2 < 2000 pg/ml and E2 > 2000 pg/ml). Significantly higher oocyte retrieval was achieved in the 'high E2' group, in line with evidence reported by Peña *et al.* (2002). However, no difference in terms of LBR and other obstetric outcomes occurred following embryo transfer in the recruited recipients (Bianco *et al.*, 2009). Surprisingly, Palmerola *et al.* (2018) observed in 366 OD cycles that low and very low E2 levels on the day of hCG trigger (low E2: 50–100 pg/ml per retrieved oocyte; very low E2 < 50 pg/ml per retrieved oocyte) are common (20.2%) and not associated with poorer pregnancy rates and outcomes. Unlike autologous IVF cycles, serum E2 levels in the donor seem to have no effect on recipient pregnancy outcomes, indicating that the effects of E2 on reproductive outcomes are mainly related to its effect on endometrial preparation and receptivity. Furthermore, these differences may have other explanations, including reduced ovarian reserve, oocyte quality, and the effects of aging in women undergoing autologous IVF/ET cycles (Palmerola *et al.*, 2018).

Unfortunately, very limited evidence is available on this topic. Further investigation is necessary to better understand the mechanisms and clinical implications of estrogenic concentrations on both sides of the donor–recipient couple. On the donor side, as seen in autologous ovarian hyperstimulation, reference E2 ranges should be established in order to maximize oocyte retrieval and embryo quality. On the recipient side, instead, suitable endometrial preparation protocols should be created, with the goal of optimizing implantation and placentation. Considering that OD pregnancies are per se associated with a higher rate of placental disorders of pregnancy compared to other IVF pregnancies, avoiding further risk factors for abnormal trophoblastic invasion is highly warranted (van der Hoorn *et al.*, 2010; Savasi *et al.*, 2016) (Table 1).

Molecular mechanisms

Uterine receptivity falls within a discrete temporal window of the menstrual cycle, although the underlying molecular mechanisms are not fully understood (Critchley *et al.*, 2020). Estrogens participate in both phases of the menstrual cycle, although they are prominent in the follicular phase by promoting epithelial cell proliferation and tissue regeneration upon menses. ERα expression in the endometrium increases during the proliferative phase, to decrease after ovulation during the subsequent secretory phase that is dominated by progesterone. An abnormal increase of local ERα expression during the secretory phase has been associated with infertility, PCOS, and endometriosis (Lessey *et al.*, 2006; Young and Lessey, 2010). For instance, in infertile women local ERα overexpression in the mid-secretory stage was accompanied by reduced levels of glycodelin-A (Dorostghoal *et al.*, 2018), one of the major progesterone-regulated proteins secreted into the uterine luminal cavity by secretory/decidualized endometrial glands, and with immunosuppressive properties required for successful implantation (Seppälä *et al.*, 2007). On the other hand, studies in mice models showed a specific role for estrogen signaling in altering protease activity in the oviduct, a physiological regulation required for successful fertilization and preimplantation embryo

Table 1. Studies evaluating the association between estradiol levels during the periconceptional period and pregnancy outcomes in ART-conceived pregnancies.

Study (year)	Sample size	Age of patients	Stimulation protocol	Type of ART procedure	Type of association between E2 levels during the periconceptional period and pregnancy outcomes
Styer *et al.* (2005)	3653 IVF cycles	31 ± 5 years	GnRH agonist long protocol	Fresh IVF/ET	No association
Farhi *et al.* (2010)	1020 patients 2135 IVF cycles	<38 years	GnRH agonist long protocol	Fresh IVF/ET	Higher placenta-related pregnancy complications for E2 >10.000 pg/ml
Joo *et al.* (2010)	455 IVF cycles	34 ± 4.5 years	GnRH agonist long or short protocol	Fresh IVF/ET	Better pregnancy outcomes for E2 2000–3000 pg/ml Lower IR, PR and LBR
Kondapalli *et al.* (2012)	1712 IVF cycles	21–45 years	GnRH agonist protocol, GnRH antagonist protocol, microdose flare protocol	Fresh IVF/ET	Higher LBR in ΔE2 > 10% group No difference in MR
Imudia *et al.* (2012)	1301 fresh IVF/ET cycles	34 ± 4 years	GnRH agonist protocol or GnRH antagonist protocol	Fresh IVF/ET	Higher risk of pre-eclamspia and SGA for E2 >3450 pg/ml
Wu *et al.* (2012)	2921 patients	31 ± 4 years	GnRH agonist long or short protocol	Fresh IVF/ET	Lower LBR and higher ectopic pregnancy rates for E2 >5209 pg/ml
Steward *et al.* (2014)	71 cycles	33 ± 4 years	LPS protocol	Fresh IVF/ET	Lower LBR and higher MR for E2 >5000 pg/ml Higher risk of LBW for E2 >5000 pg/ml
Huang *et al.* (2014)	2868 patients	31 ± 4 years	GnRH agonist long or short protocol	Fresh IVF/ET	No association
Zavy *et al.* (2014)	478 patients	<40 years	GnRH agonist long or short protocol GnRH antagonist protocol	Fresh IVF/ET	No association
Pereira *et al.* (2015)	5618 patients	35 ± 4 years	GnRH agonist long protocol	Fresh IVF/ET	Higher risk of LBW and SGA for E2 >3069 pg/ml
Sunkara *et al.* (2015)	402 185 IVF cycles	Not mentioned	Not mentioned	Fresh IVF/ET	Higher risk of FGR in high responder patients
Dunne *et al.* (2017)	216 patients	Not mentioned	Not mentioned	Fresh IVF/ET	No association
Wang *et al.* (2017)	3393 patients	<40 years	GnRH agonist long protocol	Fresh IVF/ET	Higher LBR in 'High E2 group'
Chen *et al.* (2020)	622 patients	35 ± 5 years	GnRH agonist long protocol or GnRH antagonist protocol	Fresh IVF/ET	Higher risk of Preeclampsia for low E2 levels
Morales *et al.* (2021)	181 patients	18–56 years	GnRH antagonist protocol	Fresh IVF/ET	No association
Fritz *et al.* (2017)	95 patients 110 FET cycles	30–36 years	GnRH agonist suppression + transdermal or intramuscular estradiol valerate	FET	Lower LBR for E2 >692 pg/ml
Mackens *et al.* (2020)	854 patients 1222 FET cycles	31–34 ± 5 years	Oral estradiol valerate	FET	No association
Romanski *et al.* (2021)	Group 1: 1891 patients Group 2: 2358 patients	Group 1: 35.6 ± 4.3 years Group 2: 36.1 ± 4.1 years	GnRH antagonists or short GnRH agonist protocols	FET	Lower LBR in patients with an elevated estradiol to surge of <4 days compared to patients with an elevated estradiol to surge of >4 days
Li *et al.* (2022)	669 patients 776 FET cycles	31 ± 4 years	Not mentioned	FET	Worse pregnancy outcomes in cleavage-stage embryos in elevated E2 levels
Choi *et al.* (2021)	170 patients 173 FET cycles	18–43 years	Vaginal estradiol	FET	No association between E2 levels in blastocyst embryo-transfer
Kalra *et al.* (2011)	38 626 fresh IVF/ET cycles and 18 166 FET cycles	34 ± 4 years	Not mentioned	Fresh IVF/ET vs FET	Higher risk of LBW in fresh IVF/ET cycles
Liu *et al.* (2017)	3191 FET cycles and 3770 fresh IVF/ET cycles	31 ± 4 years	GnRH agonist protocol Oral estradiol valerate	Fresh IVF/ET vs FET	Higher risk of LBW in fresh IVF/ET cycles
Tarlatzi *et al.* (2021)	2885 fresh IVF/ET cycles and 746 FET cycles	Mean age 32 years	GnRH agonist protocol Oral estradiol valerate	Fresh IVF/ET vs FET	Higher risk of LBW in fresh IVF/ET cycles
Bianco *et al.* (2009)	58 patients (oocyte donors)	21–34 years	Oral micronized estradiol	OD	No association
Madero *et al.* (2016)	8254 patients 8236 heterologous embryo-transfers (oocyte recipients)	Not mentioned	CD vs ID Oral and transdermal estradiol	OD	No association

FET, frozen embryo-transfer; IVF/ET, IVF/embryo-transfer; E2, estradiol; IR, implantation rate; PR, pregnancy rate; LBR, live birth rate; MR, miscarriage rate; LBW, low birthweight; CD, constant dose; ID, increasing dose; OD, oocyte donation.

development, which is impaired in transgenic mice lacking ERα (Winuthayanon *et al.*, 2015). Therefore, a strict spatio-temporal regulation of estrogen signaling is needed during the early stages of blastocyst implantation and survival. Likewise, local ERβ expression seems to be upregulated by estrogens and downregulated by progesterone in mice (Wada-Hiraike *et al.*, 2006). Although widely used, the mouse model exhibits some limitations and discrepancies compared to humans, and observations should be carefully considered (Ajayi and Akhigbe, 2020). In fact, in humans such changes are less prominent compared to ERα changes across the menstrual cycle, and the role of ERβ in the pathophysiological events surrounding reproduction remains poorly investigated (Lecce *et al.*, 2001; Hapangama *et al.*, 2015).

Based on this evidence, a possible explanation proposed during the past two decades relies on the 'implantation window' theory (Simón *et al.*, 1996). While local ovarian (i.e. follicular fluid) estrogen concentrations are better defined, local uterine estrogen concentrations are understudied, thus making speculation on local steroid effects incomplete. Nevertheless, studies investigating serum and endometrial specimens were performed in patients with endometriosis and controls, eventually revealing tissue concentrations similar to the serum levels during the menstrual cycle (Huhtinen *et al.*, 2014). At the cellular level, estrogens might prime the functional activation of the endometrium facilitating a receptive/permissive phase, then maintained by paracrine/autocrine signals, rather than a direct regulatory action (Ghosh *et al.*, 1994; Simón *et al.*, 1996; Borini *et al.*, 2001; Niu *et al.*, 2008). Following this theory, estrogens would need to reach the threshold in order to trigger the morphological and biological changes resulting in endometrial receptivity, in both the epithelium and stromal compartments, favoring both embryo attachment and implantation maintenance, respectively (Simón *et al.*, 1996; Borini *et al.*, 2001; Niu *et al.*, 2008). This might explain the apparent discrepancies observed *in vivo* between estrogen levels and pregnancy/implantation rate, and thickness of endometrium (Sauer *et al.*, 1990; De Ziegler *et al.*, 1991).

Following an estrogenic trigger, a number of effector molecules are secreted either by direct action of estrogens or via paracrine cell signaling, including growth factors, cytokines, and tissue remodeling factors, such as matrix metalloproteases and their inhibitors, along with cell surface and adhesion molecules, which may facilitate embryo attachment and decidua invasion (Simón *et al.*, 1996). Among these, here we mention selected molecules that have long been implicated in the early events of reproduction, also based on their relevance to clinical observations. The glycoprotein leukemia-inhibiting factor (LIF) is produced by the endometrium during the secretory phase of the menstrual cycle under the control of both E2 and progesterone (Aghajanova, 2004). LIF is a pleiotropic cytokine of the IL-6 family, with effects on multiple cells of different lineages. LIF was shown to inhibit embryonic stem cell differentiation *in vitro* and is essential for embryo implantation in mice. Although, endometrial fluids and tissue from infertile women display reduced levels of LIF, and LIF receptors are present in the human blastocyst, its crucial role remains to be demonstrated in human pregnancy (Lass *et al.*, 2001).

Changes in the composition of the uterine sugar molecules expressed on the surface of epithelial cells, i.e. glycocalyx, have also been observed during the peri-implantation period. Expression of the large transmembrane glycoprotein mucin 1 (MUC-1) achieves peak levels during the secretory phase and is lost at the site of embryo attachment, perhaps triggered directly or indirectly by the blastocyst upon interacting with the endometrium (Brayman *et al.*, 2004). Although a clear role of estrogens in the regulation of MUC-1 has yet to be demonstrated, this process may affect endometrial receptivity by modulating the embryo's access to adhesion molecules, e.g. cadherins and integrins, which would be otherwise masked by larger molecules such as MUC-1. In line with this hypothesis, women who suffer recurrent spontaneous miscarriages display reduced levels of MUC-1 in endometrial fluids, suggesting a role for defects in the MUC-1 release system in infertility (Hey *et al.*, 1995). On the other hand, extremely low levels of MUC-1, as well as of glycodelin A, in endometrial biopsies have also been associated with recurrent implantation failure (Bastu *et al.*, 2015), thus highlighting the importance of location, as well as timing, in the study of early reproduction.

Immune cells react to the hormonal fluctuations of the menstrual cycle, both at the peripheral and locally at the uterine level, although the underlying molecular mechanisms are not fully elucidated (Oertelt-Prigione, 2012). The main immune cell types involved in such fluctuation and, later on, in pregnancy are uNK cells, macrophages and T lymphocytes, whose contribution consist of approximatively 60%, 25%, and 10% of the leukocytes found locally in the decidua, respectively (Trundley and Moffett, 2004). However, the percentages reported herein may greatly vary throughout the menses. Typically, uNK proliferate during the follicular phase, to reach a peak during the late follicular phase and part of the luteal phase (Lee *et al.*, 2010). This is compatible with the observation proposed by Borzychowski *et al.* (2003) that uNK cells require an estrogen priming to proliferate and reach their functional stage, although they respond to both estrogens and progesterone once activated (Kalkunte *et al.*, 2008). During the follicular phase, uNK cells contribute to the endometrium tissue and vascular remodeling by secreting vascular endothelial growth factor (VEGF) and chemokines in an estrogen-dependent manner, ultimately resulting in endometrial receptivity (Gibson *et al.*, 2015; Wang *et al.*, 2021). Decidual macrophages are endometrium-resident specialized cells that are involved in the menstrual cycle and during pregnancy (Tian *et al.*, 2020). Unlike uNK cells, which robustly proliferate during the follicular phase and are reduced in numbers during the late luteal phase and throughout the gestation (Aghaeepour *et al.*, 2017), decidual macrophages are thought to maintain an overall fairly constant number (Tian *et al.*, 2020). Unexpectedly, during the estrogen-driven follicular phase, the implantation window and at the very beginning of the pregnancy, decidual macrophages build a pro-inflammatory environment by secreting IL-1, following the estrogen peak (Mathur *et al.*, 1979). Such a transitory pro-inflammatory environment was confirmed by observations reported in the mouse model. Indeed, within hours of the insemination, uterine cells synthesize the granulocyte macrophage colony-stimulating factor (GM-CSF) (Robertson *et al.*, 1992), a cytokine that promotes proliferation, maturation and activation of macrophages, which were found mainly skewed towards the pro-inflammatory classical phenotype M1 at this early stage (Jaiswal *et al.*, 2012). Moreover, in an *in vitro* experiment performed on cultured human endometrial cells, the expression of IL-8, which serves to both promote leukocyte recruitment and activate neutrophils, was increased in an estrogen dose-dependent manner (DeLoia *et al.*, 2002), as confirmed *in vivo* (Arici *et al.*, 1998). It is controversial how this transitory pro-inflammatory environment could be beneficial. It was proposed that this may support the blastocyst during the 'break through' of the endometrial tissue, in order to invade (Mor *et al.*, 2011). On the other hand, it could promote menses in the absence of implantation (DeLoia *et al.*,

2002). Finally, T lymphocytes are subjected to estrogens fluctuations. The T regulatory cell (Treg) subpopulation expands during the follicular phase, reaching a pre-ovulatory mid-cycle peak, to then significantly decrease during the luteal phase (Oertelt-Prigione, 2012). Low estrogen levels were correlated with a lower Treg activity, rather than a lower frequency (Arruvito *et al.*, 2007), suggesting that Treg proliferation is not entirely dependent on estrogens. The extent and the magnitude of a direct effect of estrogens on immune cells has yet to be defined.

The early stages of pregnancy

Although the associations between maternal sex steroid levels and short-term (pregnancy loss, pre-eclampsia) and long-term health outcomes (risk of maternal and offspring steroid-sensitive cancers, autistic spectrum and atopic disorders in children) are becoming progressively evident, the role of estrogens in early naturally conceived pregnancies is still poorly understood (Holl *et al.*, 2009). From promoting endometrial cell proliferation and embryo implantation, to modulating maternal cardiovascular adaptations to pregnancy, the effect of estrogens on trophoblast development and function remains controversial (Chang and Lubo, 2008; Burton *et al.*, 2009; King and Critchley, 2010; Baud and Berkane, 2019).

Natural conception

Unexplained large variations in E2 levels have been detected among pregnant women. Estrogen concentrations in early natural pregnancy are strongly correlated with GA ($r = 0.71$) (Toriola *et al.*, 2011). Our group built GA-specific reference intervals for maternal E2 concentrations during early singleton natural pregnancies, further providing a mathematic model for E2 assessment through an equation that takes into account progesterone concentrations and GA ($\log E2 = 1.96 + 0.01 \times GA + 0.004 \times$ progesteron) (Grossi *et al.*, 2019). In this study, women aged at least 18 years, without any known disease or drug consumption, as well as with no previous obstetric adverse outcomes (i.e. pre-eclampsia, intrauterine growth restriction) and carrying a singleton ongoing pregnancy (5^{+0}–13^{+6} gestational weeks) with no obstetric complications, were enrolled and considered as a 'physiological' reference optimizing the external validity of the results. Data examining the associations between feto-maternal characteristics and estrogen concentrations during the first trimester of natural pregnancies showed higher E2 levels in younger nulliparous women carrying a female fetus (Toriola *et al.*, 2011). In particular, E2 concentrations were 6% lower, 16% lower, and 9% higher in case of age higher than 30 years, multiparity, and a female fetus, respectively, whereas no associations were detected with maternal smoking (Toriola *et al.*, 2011). More recently, a multi-centre American study on 548 singleton pregnancies showed a 1–2% decrease in estrogen levels for every unit increase in maternal BMI, whereas no associations were detected between early estrogen levels and gestational weight gain or dietary fat intake in a prospective Swedish study, thus indicating a likely independence of estrogen levels from maternal nutritional habits (Lof *et al.*, 2009; Barrett *et al.*, 2019). Despite being surprising, the authors hypothesize that the inverse association between BMI and estrogens could be mainly related to the fetoplacental origin of these steroids, that tend to be more diluted in the circulation of heavier mothers compared to normal weight controls. Finally, estrogen concentrations were found to be significantly lowered by alcohol use during pregnancy (Troisi *et al.*, 2008).

Estrogens stimulate endometrial cell proliferation, myometrium thickness, uterine vascularization, and contraction force. As discussed above, E2 may represent a key factor in establishing and maintaining pregnancy (Verma *et al.*, 2019; Zhang *et al.*, 2019; Deng *et al.*, 2022). In fact, E2 levels during early natural pregnancy may reflect the quality of the dominant follicle and the proper development and function of the corpus luteum (Salazar and Calzada, 2007; Gao *et al.*, 2008). In line with this hypothesis, serum E2 concentrations were significantly lower in pregnant women undergoing spontaneous abortion than in those with a normal pregnancy (Gao *et al.*, 2008). More recently, E2 concentrations showed a marked deviation in pregnancies evolving to miscarriage compared to normal pregnancies, reflecting a deficiency in the ovarian response (Whittaker *et al.*, 2018). It has been hypothesized that E2 could prevent allogeneic fetal rejection by acting as a potent local immunomodulator, possibly explaining increased rates of miscarriage in case of E2 deficiency.

During pregnancy, the placenta represents the main site of estrogen release through the conversion of both maternal and fetal adrenal androgen precursors. Estrogens may play a role in vascular adaptations and development in both uterus and placenta. Firstly, on the uterine side, adaptations to pregnancy involve the growth of an arterial and venous axis in order to lower uterine vascular resistance and increase capacitance. Additionally, changes in the uterine vascular walls at cellular level (via endovascular cytotrophoblast invasion) and in matrix composition (impacting on vascular distensibility through changes of the extracellular matrix composition caused by metalloproteinases) are essential for modifying local vascular resistance and increasing blood flow to the intervillous space. The regulation of uterine vascular remodeling by estrogens has been suggested by the correlation between steroid plasma levels and uteroplacental blood flow modifications during pregnancy and the follicular phase of the ovarian cycle (Bernstein, 2002). To corroborate this hypothesis, exogenous estrogen administration to non-pregnant ovariectomized ewes induces uterine vasodilation and increases uterine blood flow, which was a reversible effect after the administration of ER inhibitors (Magness *et al.*, 2005; Mandalà, 2020). ERs were detected both in endothelial and vascular smooth muscle of the uterine artery and are thought to modulate vasculature contractility and myogenic tone both directly (via transcriptional factor activity) and indirectly (i.e. via a nitric oxide (NO)-dependent pathway), eventually driving the described local adaptations to pregnancy (Chang and Lubo, 2008). Again, ovariectomized Guinea pigs injected with E2 showed uterine vascular and cellular changes similar to those seen in pregnancy (Makinoda and Moll, 1986). Secondly, estrogens, more specifically E2, may drive angiogenesis and vascular development on the placental side in multiple ways. Estrogens are thought to induce placental angiogenesis and vasodilation mainly by increasing vasoactive mediator (NO and prostacyclin) and angiogenic factor release (VEGF, placental growth factor: PlGF) (Rosenfeld and Rivera, 1978; Cullinan-Bove and Koos, 1993; Shifren *et al.*, 1996; Caulin-Glaser *et al.*, 1997; Hisamoto *et al.*, 2001; Simoncini *et al.*, 2002; Hervé *et al.*, 2006; Johnson *et al.*, 2006). *In vitro* human and animal studies demonstrated that E2 led to enhanced endothelial cell proliferation and migration, thus resulting in new vessel proliferation (Powazniak *et al.*, 2009; Zhang *et al.*, 2016; Liu *et al.*, 2018). The same models additionally demonstrated that the suppression of estrogen in the first stages of pregnancy through specific aromatase inhibitors (letrozole) led to a marked suppression of maternal estrogen concentrations, increased androgen levels, reduced placental vessel network and pro-angiogenic gene expression, eventually

resulting in increased pregnancy loss and reduced birthweight. This suggests an estrogen-dependent placental vasculogenesis and development as early as the first half of pregnancy (Albrecht *et al.*, 2004; Albrecht and Pepe, 2010; Haneda *et al.*, 2021). Moreover, estrogens may guide placental development by involving structural uterine reorganization of the extracellular matrix, as indicated by the correlation between local expression of metalloproteinases and maternal estrogen concentrations, as well as by the induced upregulation of metalloproteinase expression by estrogen in animal models (Schäfer-Somi *et al.*, 2005; Dang *et al.*, 2013).

Since proper vascular remodeling and maternal adaptation to pregnancy represent crucial determinants of appropriate placentation and pregnancy progression, a potential association between estrogen levels and the development of human placental insufficiency diseases has been widely investigated (Fig. 3). Whereas previous studies reported conflicting results on the association between low maternal blood estrogen concentrations and the development of pre-eclampsia, recent research has consistently shown lower serum estrogen levels in women diagnosed with pre-eclampsia compared to controls (Rahman *et al.*, 1975; Notation and Tagatz, 1977; Rosing and Carlström, 1984; Zamudio *et al.*, 1994; Hertig *et al.*, 2010; Jobe *et al.*, 2013). Although lower, estrogen serum concentration did not correlate with disease severity (Salas *et al.*, 2006; Hertig *et al.*, 2010; Bussen and Bussen, 2011; Jobe *et al.*, 2013). Nevertheless, whether the E2 deficiency takes part in the pathogenic process or rather is a consequence of the pre-eclamptic insult is still a matter of debate. The lower estrogen concentrations of pre-eclamptic women can be linked to abnormal activity of the aromatase as a consequence of chronic hypoxia or abnormal hormonal signaling (i.e. increased leptin in obese pre-eclamptic women decreases placental estrogen production) (Coya *et al.*, 2006; Perez-Sepulveda *et al.*, 2015). Promising results have been shown using a therapeutic approach with short-term estrogens in ameliorating placental oxidative stress and clinical features of pre-eclampsia (Djordjević *et al.*, 2010; Babic *et al.*, 2018).

Autologous ART

Limited data are available on this topic. The hormonal profile in the mid-luteal phase and initial stages of ART-conceived pregnancy has mainly been studied to find an early predictor of IVF/ET outcome as an alternative to β-hCG. Kumbak *et al.* (2006) and Sonntag *et al.* (2013) observed that serum E2 levels were significantly higher in conception cycles than in non-conception cycles from the very beginning of the luteal phase (4–8 days after ET). (Kumbak *et al.*, 2006; Sonntag *et al.*, 2013). Similar results emerged from the Vanderlelie *et al.* (2003) study, which showed that E2 concentrations on Day 6 after ET were significantly lower in those patients with no conception (Vanderlelie *et al.*, 2003). Further analysis of the concentration of E2 on Day 6 found that patients who conceived had only a 7.9% chance of pregnancy success when E2 levels were below 600 pg/ml (Vanderlelie *et al.*, 2003). In line with these findings, Melnick *et al.* (2016) highlighted a correlation between serum E2 levels on the 28th cycle day and pregnancy outcomes: LBRs were higher in those groups with medium (E2 51–100 pg/ml) and medium-high (E2 > 100 pg/ml) E2 concentrations. Instead, biochemical pregnancy rates were higher in the low estrogen group (E2 < 50 pg/ml), suggesting that estrogen support is fundamental in early gestational phases (Melnick *et al.*, 2016).

Indeed, during the late first trimester, steroid sex hormones concentrations are significantly higher in in IVF, after pregnancies conceived after controlled ovarian stimulation + ET, compared to natural pregnancies. As already observed in the late 1980s and 1990s, and corroborated by Sun *et al.* (2019), higher steroid sex hormones concentrations influence the placenta metabolomic profile, biophysical aspects (i.e. the uterine artery Doppler velocimetry) and epigenetic reprogramming. However, so far, no conclusive literature supports a clear-cut scenario. Low levels of E2 are required for normal trophoblastic invasion in human pregnancies to avoid adverse pregnancy outcomes (Yovich *et al.*, 1985; Johnson *et al.*, 1993; Inversetti *et al.*, 2018; Sun *et al.*, 2019).

Comparing fresh IVF/ET cycles and FET, E2 levels at 4 and 8 gestational weeks are significantly higher in the former group,

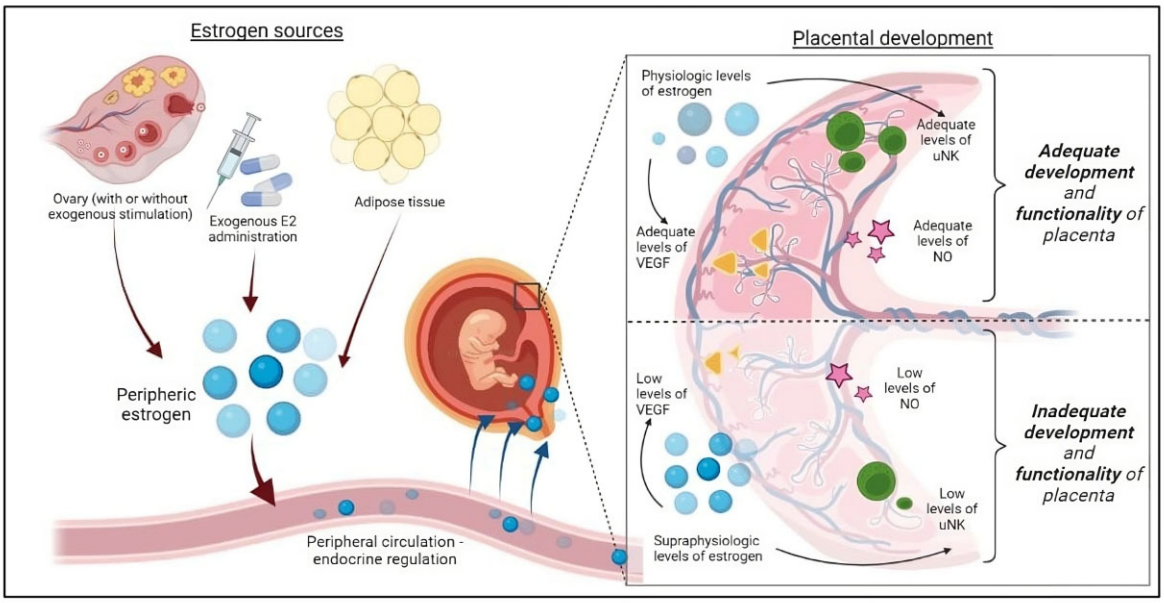


Figure 3. Influence of estrogen levels on appropriate placentation and pregnancy progression. The schematic represents crucial determinants of the development of human placental insufficiency diseases and compares the effects of adequate and supraphysiologic E2 levels on vascular remodeling and angiogenesis at the materno-fetal interface. E2, estradiol; VEGF, vascular endothelial growth factor; NO, nitric oxide; uNK, uterine natural killer.

suggesting that the effect of controlled ovarian stimulation is stronger than any other endometrial preparation protocol. This may be related to the important role of the corpus luteum in hormone production during the first weeks of gestation: multiple corpora lutea can be found in patients undergoing oocyte retrieval, while the exogenous hormone administration for endometrial preparation used during IVF protocols produces pregnancy with no corpus luteum (Conrad et al., 2019). However, as previously mentioned, elevated estrogenic concentrations are associated with increased LBW rates in autologous ART pregnancies: better pregnancy outcomes were detected in FET cycles than in fresh ones (Hu et al., 2014). Moreover, as observed by Johnson et al. (1993) and Póvoa et al. (2018), E2 concentration during late first trimester (>8 gestational weeks) is greater in multiple pregnancies than in singleton ones (Johnson et al., 1993; Póvoa et al., 2018). Maternal serum E2 level at 8 weeks of gestation increased with the number of fetuses, showing a direct correlation with the number of placentas, the main responsible for hormone production from the 8th to 10th gestational week.

In conclusion, as mentioned above, high estrogenic concentrations seem negatively associated with offspring birthweight. Evidence from animal and human models indicated that certain transient environmental influences could produce persistent changes in epigenetic marks, such as on imprinted genes, which are implicated in the regulation of fetal growth and development. As well as endocrine disruptors that can alter DNA methylation, resulting in transcriptional changes, high maternal E2 levels might induce epigenetic modifications, leading to LBW in a dose-dependent manner (Hu et al., 2022). However, evidence on this topic is still very limited and heterogeneous. Further studies could highlight optimal estrogenic levels to minimize adverse outcomes of pregnancy and a possible use of E2 concentration as an early marker of ART and pregnancy success.

Oocyte donation

A review of the literature has highlighted only a few studies, mainly from our study group. Mandia et al. (2020) compared serum E2 levels in four different groups (55 OD pregnancies, 48 autologous fresh IVF/ET pregnancies, 10 autologous FET pregnancies and 122 naturally conceived pregnancies) during the late first trimester of pregnancy (from 11 + 0 to 13 + 6 gestational weeks). Unexpectedly, significantly lower E2 concentrations were detected in OD pregnancies compared to natural conception or autologous IVF pregnancies. However, no difference in pregnancy outcomes and complications emerged (Mandia et al., 2020).

Owing to the lack of evidence, this result could be variously explained: first, we could hypothesize that the smaller placental volumes detected in OD placentas could imply a lower estrogen release compared to natural and autologous IVF pregnancies (Rizzo et al., 2016; Mandia et al., 2020); and second, we could speculate that oral estrogen administration might inhibit placental production, in a sort of feedback mechanism that is common in many endocrine systems. Undoubtedly, placental function in OD is reduced compared to naturally conceived and autologous IVF pregnancies: as observed by Savasi et al. (2015), first trimester placental markers, such as free β-hCG and PAPP-A, are significantly altered, suggesting a reduced crosstalk at the fetal–maternal interface (Savasi et al., 2015). Moreover, exogenous steroid supplementation may not replace the total absence of a corpus luteum during the very early stages of an OD pregnancy, as well as playing a part in a negative feedback regulation on placental function. Eventually, altered serum estrogen levels during the late first trimester may induce improper modelling of uterine arteries, resulting in pregnancy complications, such as pre-eclampsia and fetal growth restriction (Jauniaux et al., 1992; Mandia et al., 2020). As for periconceptional stages, further investigation is needed to find an optimal range for estrogenic concentrations during the first trimester. This may improve pregnancy outcomes in OD programs, reducing the risk of feto-maternal complications (Table 2).

Molecular mechanisms

Upon interacting with the endometrium, cytotrophoblast and syncytiotrophoblast cells are generated that facilitate penetration of the blastocyst into the decidua and allow materno-fetal exchanges, as well as sex hormone production (Albrecht and Pepe, 2010). These cells will eventually engage uterine stromal, endothelial, and immune cells to promote tissue remodeling. Among the most important and studied morphological changes occurring during early pregnancy is the development of a uterine vascular network aimed at facilitating blood flow to the feto-placental unit. The role of estrogens in enhancing uteroplacental flow is well established and may not only reflect changes in vascular reactivity, E2 being a potent vasodilator via the induction of endothelial NO synthase (eNOS) and subsequent NO release, but also enhanced angiogenesis (Berkane et al., 2017). In fact, although hypoxia is a potent stimulus of angiogenesis, multiple studies point at a crosstalk between estrogens and angiogenic factors as a determinant of placental vascularization. ERβ is thought to have a role in the control of the angiogenic and vascular changes that happen during embryo implantation and early placentation as well as for the maintenance of pregnancy (Su et al., 2012). The VEGF gene contains estrogen responsive elements and is upregulated by estrogens that in turn upregulate VEGF receptor 1 (VEGFR-1) and additional growth factors, such as PlGF, in endothelial cells (Mueller et al., 2000; Zhang et al., 2010). Interference with this process, for instance caused by increased levels of the soluble fms-like tyrosine kinase 1 (sFLT1), an antagonist of VEGF and PlGF, has long been associated with vasoconstriction and higher risk of developing pre-eclampsia (Zeisler et al., 2016). Recent reports stemming from the seminal model of estrogen regulation of early pregnancy in baboons not only confirmed the previous observation that supraphysiological E2 levels inhibit VEGF expression in the trophoblast thus impairing uterine artery remodeling, which could be restored by exogenous VEGF, but also identified sFLT-1 increase as the mechanism linking E2 overdose and reduced availability of VEGF (Babischkin et al., 2019; Aberdeen et al., 2022). In spite of disease heterogeneity and the complex pathogenesis, vascular remodeling and angiogenesis at the materno-fetal interface provide an example of how opposed settings, i.e. low versus high estrogens, can lead to the same outcome via an apparently common mechanism (Fig. 3). However, additional mechanisms may explain the link between the observed low circulating E2 levels and impaired angiogenesis and placenta development in pre-eclampsia. In fact, estrogens can alter blood pressure by regulating the expression of the enzyme HSD11B2 (hydroxysteroid 11-beta dehydrogenase 2) that converts cortisol, a vasoconstrictor, into cortisone (Baggia et al., 1990). In pre-eclampsia, elevated fetal cortisol caused by low HSD11B2 expression in the placenta not only affects blood pressure but also prevents an increase of estrogen production by limiting the synthesis of the precursor DHEA in the fetal adrenal glands, which has a central role in supporting the local production of estrogens in the primate placenta (see above). Additionally, E2 administered *in vitro* to human first-trimester villous explant cultures promoted trophoblast differentiation into an invasive phenotype

Table 2. Studies evaluating E2 levels and its implications during the first trimester in ART-conceived pregnancies.

Study (year)	Sample size	Age of patients	Way of conception	Results
Vanderlelie *et al.* (2003)	310 patients	33 ± 3 years	IVF/ET	Higher E2 levels in conceiving cycles than non-conceiving cycles
Kumbak *et al.* (2006)	2035 IVF cycles	31 ± 5 years	IVF/ET	Higher E2 levels in conceiving cycles than non-conceiving cycles
Sonntag *et al.* (2013)	71 IVF cycles	Not mentioned	IVF/ET	Higher E2 levels in conceiving cycles than non-conceiving cycles
Melnick *et al.* (2016)	5471 IVF cycles	37 ± 4.5 years	IVF/ET	Higher LBR for E2 50–100 pg/ml on 28th cycle day (14th day after ET)
Póvoa *et al.* (2018)	50 singleton pregnancies 47 dichorionic twin pregnancies	23–40 years	IVF/ET	Higher E2 levels in multiple pregnancies than in singleton ones
Hu *et al.* (2014)	190 fresh IVF/ET pregnancies 86 FET pregnancies 192 SC pregnancies		IVF/FET vs FET vs SC	Higher E2 levels in early pregnancies after COH than EP protocols. Higher risk of LBW in fresh IVF/ET conceived pregnancies
Conrad *et al.* (2019)	65 patients	23–46 years	IVF/ET vs FET	Higher E2 levels in early pregnancies after COH than EP protocols
Sun *et al.* (2019)	409 patients 208 SC pregnancies 201 IVF pregnancies	40 ± 3 years	IVF/ET vs SC	Higher E2 levels in ART-conceived pregnancies
Mandia *et al.* (2020)	55 oocyte donation pregnancies, 48 autologous IVF pregnancies 122 SC pregnancies	27–48 years	OD vs IVF vs SC	Lower E2 levels in OD than in SC or autologous IVF

FET, frozen embryo-transfer; IVF/ET, *in vitro* fertilization/embryo-transfer; SC, spontaneous conception; COH, controlled ovarian hyperstimulation; EP, estro-progestinic; E2, estradiol; LBR, live birth rate; LBW, low birth weight; OD, oocyte donation.

in a hypoxia-inducible factor-1α dependent manner (Cho *et al.*, 2018). The authors speculate that inappropriately elevated E2 levels could lead to the 'shallow' invasion of trophoblast into the spiral arteries and the uterine wall, contributing to complications associated with abnormal trophoblast invasion, as previously observed in pre-eclampsia (Cho *et al.*, 2018). On the other hand, an elevated E2 level was able to induce apoptosis in the first-trimester human cytotrophoblast cell line HTR-8, highlighting a potential role in infertility and placental insufficiency (Patel *et al.*, 2015).

Although the local immune-regulatory activity of sex hormones in the endometrium has been mainly investigated in the context of inflammatory and infectious diseases (Wira *et al.*, 2015), with E2 being regarded as protective in contrast to progesterone, the role of immune cells in endometrial tissue remodeling during early pregnancy represents a good example of the complexity of estrogen effects. Estrogens are sensed by a number of immune cells, such as NK cells, myeloid cells, glial cells, T and B lymphocytes (Medina *et al.*, 2001). Through multiple receptors differentially expressed according to the cell type and differentiation stage, estrogens cover a wide range of immune processes, by regulating cell proliferation, activation, differentiation, and the response to other immunomodulatory hormones and cytokines, such as the GnRH (Igarashi *et al.*, 2001; Tanriverdi *et al.*, 2003; Lang, 2004; Shao *et al.*, 2013). The uNK cells are a peculiar subset of non-cyto-toxic NK cells that accounts for the majority of immune cells present in the endometrium, typically of large primates including humans. They proliferate and differentiate according to the menstrual cycle or pregnancy stage in response to both estrogens and progesterone, among other factors (Kalkunte *et al.* 2008). In particular, the estrogens synthesized by decidualized stromal cells promote the secretion of angiogenic and vascular-remodeling factors by uNK cells, such as the chemokine (C–C motif) ligand 2 (CCL2) and VEGF (Gibson *et al.*, 2015; Wang *et al.*, 2021). It has been proposed that uNK cells *in vivo* require estrogen priming to reach their mature and functional stage (Borzychowski *et al.*, 2003), but whether estrogens have a direct receptor-mediated activity on uNK cells or act via paracrine signaling remains controversial (Borzychowski *et al.*, 2003; Gaynor and Colucci, 2017). Likewise, the association between disorders of pregnancy entailing impaired tissue remodeling, such as pre-eclamspia, and alterations in uNK cell number and function (Moffett and Colucci, 2015), as well as their predictive value of pregnancy outcome in ART need further investigation (Tuckerman *et al.*, 2007; Chen, 2019; Donoghue *et al.*, 2019).

In addition to tissue remodeling, the main task of endometrial/decidual immune cells is to shield the semi-allogenic embryo from maternal immunity and at the same time provide protection against potential invading pathogens (Robinson and Klein, 2012; Schumacher *et al.*, 2014). The fulfilment of these important tasks is likely achieved by estrogens, although our understanding of their immune-regulatory action relies on systemic correlates of inflammation, rather than localized at the maternal–fetal interface in human pregnancy (Straub, 2007; Vallvé-Juanico *et al.*, 2019). If the general consensus is that an overall shift from inflammatory responses is needed for a successful pregnancy, the traditional paradigm of a switch occurring among peripheral CD4 T helper (Th) cells from pro-inflammatory Th1 to Th2 cells, supporting cell-mediated versus humoral immunity, has been revised with the inclusion of other relevant immune subsets over the years (Marzi *et al.*, 1996). For instance, the expansion of another pro-inflammatory subset, namely Th17 cells, as well as an altered balance between these and tolerogenic Treg lymphocytes, was previously associated with implantation

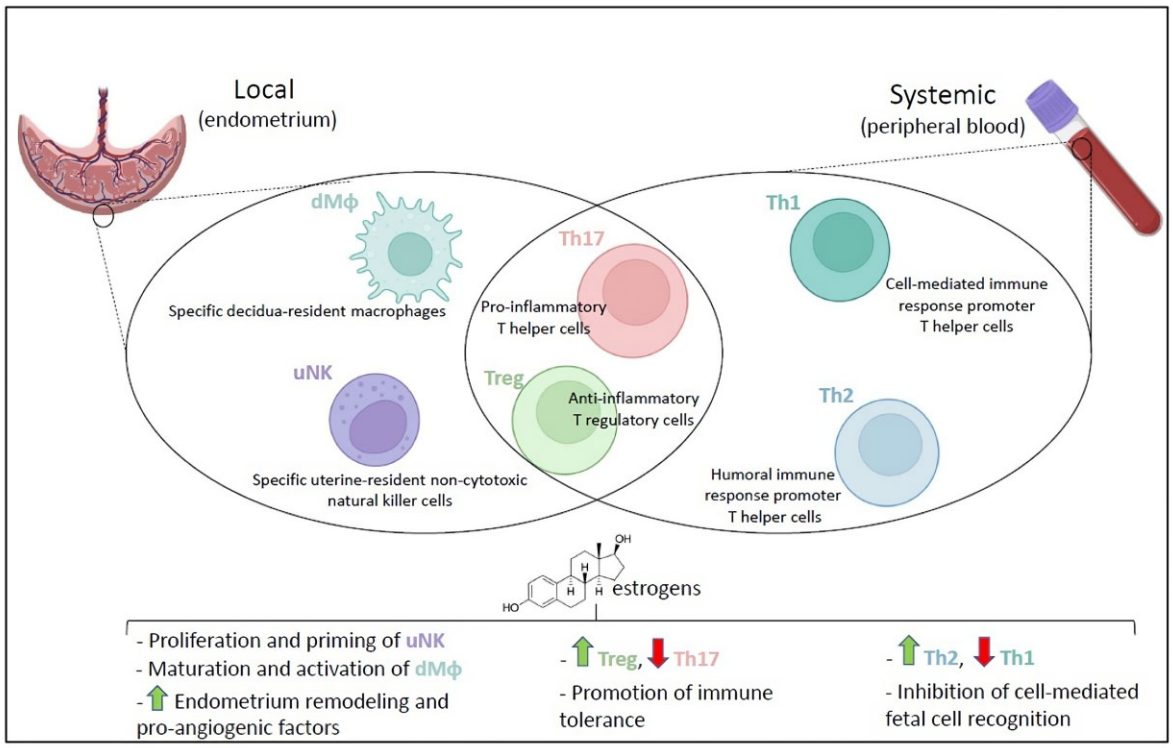


Figure 4. Synoptic representation of the main effects of estrogens on immune cells, both locally in syncytiotrophoblast/placenta and systemically in the peripheral blood. uNK, uterine natural killer; Treg, T-regulatory lymphocytes.

failure, recurrent pregnancy loss, preterm birth, intrauterine fetal growth restriction, and pre-eclampsia (Harrington *et al.*, 2005; Santner-Nanan *et al.*, 2009; Lee *et al.*, 2011, 2012). Estrogens regulate both Th17 and Treg phenotype, activation, and localization (Laurence and O'Shea, 2007; Polanczyk *et al.*, 2007; Tai *et al.*, 2008; Javadian *et al.*, 2014; Andersson *et al.*, 2015; Fuseini *et al.*, 2019). Treg cells are a specialized subset of T cells that provide an immune-tolerant environment for implantation and fetal development by inhibiting alloreactive immune responses of the maternal immune system against the fetus, also through secretion of cytokines such as IL-10 and transforming growth factor-β (Arruvito *et al.*, 2007; Tai *et al.*, 2008). These cytokines contribute to maintain a tolerogenic environment and their production is further supported by other cell types including antigen presenting cells, such as macrophages and dendritic cells under estrogen regulation, as shown, for example, in *ex vivo*-treated human chorionic villi and decidua (Wang *et al.*, 2020a) (Fig. 4).

Discussion

Most evidence available to date endorses a relation between periconceptional estrogens levels and pregnancy outcomes, both in terms of LBR and pregnancy complications. In IVF/ET protocols, either extremely low or extremely elevated E2 blood titers appear inadequate to support a pregnancy. In the case of low E2, the lack of appropriate hormonal support may be responsible for reduced pregnancy and implantation rates. For elevated E2, gonadotrophin-stimulated multi-follicular development and production of supraphysiological levels of sex steroid hormones immediately before embryo implantation may represent an independent risk factor of developing LBW and other disorders of abnormal placentation, such as pre-eclampsia. We speculate that a reduced extravillous trophoblast invasion of the decidua owing to a premature and excessive exposure of the endometrium to E2, as modelled in early baboon pregnancy, might be one of the causes underlying such observations.

A few considerations should be noted. First, this narrative review has limitations owing to the non-systematic nature and the available literature: despite the big sample size of most studies, they all have a retrospective design. In addition, different stimulation protocols, techniques, and procedures were compared between study populations that have different demographic characteristics, thus representing a confounding factor. Second, none of the research groups has highlighted a fixed cutoff to define 'excessive supraphysiological E2 levels', probably owing to both the heterogeneity of patients and the different analytical methods used to measure E2 concentrations. Therefore, we suggest that an E2 concentration above 2500–3000 pg/ml on the day of hCG administration in fresh IVF/ET cycles might be referred as detrimental, i.e. resulting in poor pregnancy outcomes, although no reference value could be identified for FET procedures. We know that high serum E2 levels on the day of hCG administration and in the first weeks of pregnancy during controlled ovarian stimulation are associated with an increased risk for small for GA and pre-eclampsia, which can be overcome by freezing all of the embryos and transferring them after thawing in an unstimulated cycle (Imudia *et al.*, 2012, 2014). It is difficult to set reference values, either upper or lower, because of the great heterogeneity of patients and confounding variables. We could suggest that the upper E2 limit at transfer should not be higher than 2500/3000 pmol/l, while the lower limit not be lower than 200/300 pmol/l, which is the physiological value during embryo implantation.

Moreover, in all studies the estrogen concentrations refer to peripheral blood, while the local concentrations in the female reproductive tract may differ.

How estrogens co-operate with other factors to maintain a fine balance between local tolerance towards the developing fetus and immune responses to invading pathogens and/or commensals

remains elusive. In recent years, the interaction between the host and the local microbiota in the female reproductive tract, as well as in the intestine, has gained more attention also with respect to its potential effects on host metabolism, including estrogen processing, i.e. estrobolome (Salliss *et al.*, 2021). A careful spatial-temporal mapping of the changes in the levels of estrogens and correlates of immunity, locally in the endometrium as well as systemically, may provide a mechanistic insight into the role of estrogen in the physiopathological changes occurring during pregnancy. Although obvious ethical issues restrict sample access and the opportunity to conduct longitudinal studies in women seeking pregnancy, by leveraging transcriptomic analysis of uterine biopsies collected across the menstrual cycle, multiple studies have attempted to unbiasedly identify the molecular determinants of endometrial receptivity (Díaz-Gimeno *et al.*, 2011; Gómez *et al.*, 2015). This approach has been further refined by single-cell and spatial transcriptomics that allows a better resolution in identifying the cell types, as well as their interaction, behind the main changes observed in bulk endometrial biopsies (Wang *et al.*, 2020b; Garcia-Alonso *et al.*, 2021). However, studies in humans do not allow us to accurately discriminate the activity of individual factors of interest to establish a direct unequivocal relationship between observations. To this end, innovative technical solutions in the culture of primary endometrial epithelial cells, which have been historically challenging to maintain for long periods, i.e. organoids, have been employed to study the hormonal regulation of epithelial cells (Turco *et al.*, 2017; Nikolakopoulou and Turco, 2021). By combining advanced genomic analysis of the endometrium and organoid studies, recent work has led to the identification of potential binding sites for ERα across the genome (Hewitt *et al.*, 2022), and to the partial characterization of molecular determinants of the proliferation and differentiation responses in epithelial cells to E2 and progesterone stimulation (Garcia-Alonso *et al.*, 2021). Although organoids provide significant advantages over traditional cell lines and *in vitro* 2D cell systems, they are still far from recapitulating the full complexity of the endometrium, and therefore their potential translational value relies on the inclusion of stromal as well as immune components, in addition to the above-mentioned microbial partners, in a relevant spatial and functional interface (Murphy *et al.*, 2022). Nevertheless, organoids, along with any other cell line that can be established from a patients' endometrium, capture their genetic diversity, which is an indispensable factor for developing personalized interventions (Boretto *et al.*, 2019).

Overall, the scenario depicted by our review for the role of estrogens and their optimal concentration in the early events surrounding reproduction remains incomplete, with some elements of controversy. The urgent necessity to investigate and define reference estrogens levels remains, in order to further ameliorate women's health as well as ART, particularly when considering a steadily increasing demand. Hence, further prospective and experimental studies are highly warranted, also with the goal of identifying the determinants of estrogen response and biomarkers for personalized estrogen-administration regimes in ART.

Data availability

There are no new data associated with this article.

Authors' roles

S.V. conceived the original idea and supervised the project. P.F. and F.C. wrote the manuscript with support from I.A., Z.A., S.C., and M.B. All authors discussed the results and contributed to the final manuscript. P.F and F.C. contributed to the final version of the manuscript.

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Conflict of interest

All authors declare no conflicts of interest.

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