UNIVERSITAT DE BARCELONA

Fetal programming in assisted reproductive technologies

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PhD THESIS

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Fetal programming in assisted reproductive technologies

Submitted by

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Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.
Marie Curie

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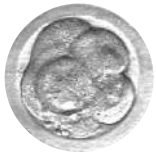

DECLARATION

Barcelona, September 1st, 2022

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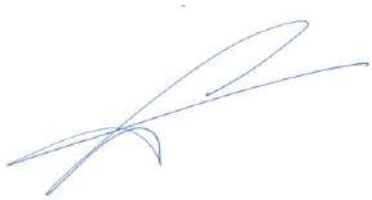
We declare that **María Laura Boutet** has conducted under our supervision the studies presented in the thesis **"Fetal programming in assisted reproductive technologies"**. The present thesis has been structured following the normative for PhD thesis as a compendium of publications to obtain the degree of **International Doctor in Medicine**, and the mentioned studies are ready to be presented to the Tribunal.



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PRESENTATION

The present thesis has been structured following the normative for PhD thesis, as a compendium of publications, to obtain the degree of International Doctor in Medicine. The Comissió de Doctorat de la Facultat de Medicina de la Universitat de Barcelona approved it on September 21, 2022. The studies included in the thesis belong to the same research line leading to five papers already published, submitted to international journals for publication, or in preparation.

1. Boutet ML, Casals G, Valenzuela-Alcaraz B, García-Otero L, Crovetto F, Cívico MS, Borrás A, Manau D, Gratacós E, Crispi F. **Cardiac remodeling in fetuses conceived by ARTs: fresh versus frozen embryo transfer**. Hum Reprod, Vol 36, No.10, pp 2697-2708, 2021.
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   *Status***:** In preparation

4. Boutet ML, Eixarch E, Ahumada-Droguett P, Nakaki A, Crovetto F, Cívico MS, Borrás A, Manau D, Gratacós E, Crispi F, Casals G. **Fetal neurosonography and infant neurobehavior in assisted reproductive technologies following fresh and frozen embryo transfer.** Ultrasound Obstet Gynecol. 2022 Apr 25. doi: 10.1002/uog.24920.
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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

2D	Two dimensional
A1M	α1-microglobulin
ADHD	Attention deficit hyperactivity disorder
ART	Assisted reproductive technologies
ASD	Autism spectrum disorder
AC	Abdominal circumference
BP	Blood pressure
BPD	Biparietal diameter
BMI	Body mass index
CPR	Cerebroplacental ratio
CRL	Crown-rump length
DV	Ductus venosus
EDTA	Ethylenediaminetetraacetic acid
EFW	Estimated fetal weight
ELISA	Enzyme-Linked ImmunoSorbent Assay
ET	Ejection time
FGR	Fetal growth restriction
FSHr	Recombinant follicle stimulating hormone
FL	Femur length
GA	Gestational age
HbF	Fetal hemoglobin
HC	Head circumference
hCG	Human chorionic gonadotropin
hMG	Human menopausal gonadotropin
ICC	Intraclass correlation coefficient
ICT	Isovolumic contraction time
IRT	Isovolumic relaxation time
IVF	*In vitro* fertilization
LBW	Low birth weight
LHr	Recombinant luteinizing hormone
LGA	Large for gestational age
MAPSE	Mitral ring displacement
MCA	Middle cerebral artery

MPI	Myocardial performance index
NC-IVF	Natural cycle IVF
NICU	Neonatal intensive care unit
OS	Ovarian stimulation
PCOS	Polycystic ovarian syndrome
PI	Pulsatility index
PTB	Preterm birth
ROS	Reactive oxygen species
SC	Spontaneously conceived
SD	Standard deviation
SGA	Small for gestational age
TAPSE	Tricuspid ring displacement
TDI	Tissue Doppler Imaging
TTP	Time-to-pregnancy
UA	Umbilical artery
US	Ultrasound
UtA	Uterine artery

Summary

SUMMARY

Background: Pregnancies conceived by assisted reproductive technologies (ART) represent up to 7.9% of births in European countries and this tendency seems to be increasing, with infertility affecting almost 25 million citizens in the European Union. Concerns about safety aspects of ART have been rising, especially after some studies found an association between these technologies and poorer perinatal outcomes, fetal cardiac remodeling, and suboptimal neurodevelopment.

Hypothesis: Different ART procedures are associated with distinct patterns of fetal cardiac and brain development, as well as different preeclampsia risk profiles shown by preeclampsia-related biomarkers.

Objectives: To describe the pattern of fetal cardiac and neurodevelopmental profiles, and the behavior of preeclampsia-related biomarkers, associated to different ART strategies in order to assess the offspring's health related to each technique.

Methodology: A prospective cohort study of singleton pregnancies exposed and not exposed to different ART techniques was conducted. A total of 683 pregnancies were recruited, including spontaneous pregnancies from fertile (n=199) and subfertile couples (n=100), Natural-Cycle (unstimulated) in vitro fertilization (IVF) cycles (n=34), conventional (stimulated) IVF cycles with fresh (n=185) and frozen (n=165) embryo transfer. Fetal chromosomal abnormalities or structural malformations and suspected or confirmed intrauterine infections were considered exclusion criteria. A fetal ultrasound evaluation at third trimester of pregnancy and a set of maternal and cord blood samples were collected at birth. The main outcomes were the presence and pattern of fetal cardiac remodeling and dysfunction and fetal neurodevelopment assessed by ultrasound between 27 and 35 weeks of gestational age, and the preeclampsia-related biomarkers´ profile at birth in maternal and cord blood. Three cohort studies and two nested case-control studies were conducted. The association between different types of ART and offspring´s outcomes have been modelled considering several covariates such as maternal age, parity, ethnicity, birthweight centile, prematurity, preeclampsia, socioeconomic factors, medical and fertility background, offspring´s age, weight, sex, and breastfeeding.

Results: The previously published findings of cardiac remodeling and dysfunction in fetuses from ART have been replicated in this research project. Cardiac features in fetuses from ART seem to be associated with the IVF laboratory procedure itself, not with the underlying subfertility nor the ovarian stimulation. Moreover, more pronounced features were demonstrated in stimulated cycles with fresh embryo transfer as compared to the frozen embryo transfer population.

Fetuses from ART also showed a different pattern of brain cortical folding during the third trimester of intrauterine life, associated to a poorer neurodevelopmental performance at 12 months of corrected age, with more pronounced changes in stimulated cycles with fresh embryo transfer as compared to the frozen embryo transfer group.

Finally, we report more altered levels of the scavengers hemopexin and α_1-microglobulin in the group lacking corpus luteum at the time of conception (embryo transfer in programmed cycles), supporting the hypothesis that the corpus luteum activity may influence the perinatal outcomes through lower levels of oxidative stress.

Conclusions: (1) Fetuses from both fresh and frozen embryo transfer cycles present signs of cardiac remodeling and suboptimal function, with more pronounced changes after fresh as compared to frozen embryo transfer. (2) Fetuses conceived spontaneously by subfertile couples do not show signs of cardiac remodeling and dysfunction as those observed in IVF fetuses, and their cardiac structural and functional features are similar to those of SC fetuses from fertile couples. (3) Fetuses from ART show signs of cardiac remodeling as compared to SC fetuses from fertile couples, even in Natural-Cycle IVF (NC-IVF) cycles (without a previous ovarian stimulation). (4) Fetuses conceived by ART show a distinctive pattern of brain cortical development and suboptimal infant neurodevelopment, with more pronounced changes in fresh embryo transfer offspring. (5) Pregnancies lacking corpus luteum at the time of conception associate more altered heme scavenger´s concentrations in preeclampsia, suggesting that the corpus luteum activity could influence perinatal outcomes through lower levels of oxidative stress. (6) These results underscore the importance of future studies assessing adverse perinatal outcomes and long-term cardiovascular and neurological health in offspring conceived by ART.

Keywords: α1-microglobulin, ART, brain cortical folding, cardiac remodeling, cord blood, corpus luteum, fetal echocardiography, fetal neurosonography, heme scavengers, hemopexin, IVF, maternal blood, mode of conception, neurodevelopment, preeclampsia, pregnancy, subfertility.

1. Introduction

1. Introduction

1.1 Assisted reproductive technologies

The continuous development of assisted reproductive technologies (ART) to overcome the setback of infertility has been accelerating over the last four decades, since the irruption of *in vitro* fecundation (IVF), first performed in 1978. The introduction of the intracytoplasmic sperm injection (ICSI) in 1992 for the management of severe male infertility, the possibility of cryopreservation of surplus embryos and oocytes, and the preimplantation genetic testing (PGT) are examples of an evolution that currently allows new demands to be addressed. Currently, these requests are as relevant as avoiding hereditary diseases in offspring, reducing the rates of complications such as ovarian hyperstimulation syndrome, enabling the elective single embryo transfer strategy to prevent multiple pregnancies, attending the trend towards later parenthood, or saving the reproductive potential of individuals facing oncological therapies.

Nowadays, pregnancies conceived by means of ART represent up to 7.9% of the live births in European countries (Wyns et al., 2021) and this tendency seems to be increasing worldwide (de Geyter et al., 2020), since infertility affects nearly 10% of women at some stage in their reproductive lives remaining a highly prevalent global condition (Mascarenhas et al., 2012).

1.2 Perinatal outcomes in ART

Interest about safety aspects of ART have been increasing, especially since some epidemiological studies began to find an association between these technologies and poorer perinatal outcomes. The high proportion of multiple pregnancies still accounts for the overall increased morbidity and mortality of children born after ART (Qin et al., 2016). Additionally, the vanishing twin phenomenon, most frequently seen in viable singleton pregnancies after a double embryo transfer strategy, worsens the gestation prognosis (Pinborg et al., 2007). Nevertheless, compared to natural conceptions, there is strong evidence that singletons conceived by ART in absence of a vanishing twin are also at higher risk of preterm birth (PTB) and placenta-related outcomes as fetal growth restriction (FGR), low birth weight (LBW), placenta previa and placental abruption (Cavoretto et al., 2018; Pandey et al., 2012; Qin et al.,

2017). The risk of adverse perinatal outcome in this population remained significantly higher in most of the studies even after adjustment for maternal age and parity (Berntsen et al., 2019).

1.2.1 Potential factors related to adverse perinatal outcomes in fetuses from ART

This increased risk is probably multifactorial. It may be related to the treatment, with ovarian stimulation (OS) and laboratory procedures being possible contributing factors. Subfertility itself (without fertility treatment involved) has also been associated with adverse perinatal outcomes (Litzky and Marsit, 2019; Pinborg et al., 2013; Valenzuela-Alcaraz et al., 2017; Vannuccini et al., 2016), suggesting that parental underlying factors related to infertility could also contribute to perinatal results in the IVF population.

When analyzing the potential effect of OS by comparing perinatal outcomes in IVF in natural cycle (NC-IVF) with conventional IVF stimulated cycles no consistent results were found, probably due to the retrospective nature of the studies, which usually prevents an adequate adjustment for covariates (Mak et al., 2016; Pelinck et al., 2010; Sunkara et al., 2016). On the other hand, whether the impact of OS on perinatal outcomes would be through an effect on the endometrium (altering the implantation environment), a direct effect on the oocyte / embryo or both, is unknown, difficult to distinguish and should be explored further. The pathways leading to restricted vascular development in thin endometrial tissues might also affect placental development and function, promoting FGR, but even though an association between endometrial thickness and birthweight has been reported (Liao et al., 2022), it should be further explored in light of other potential contributing factors. Moreover, although higher estradiol levels at human chorionic gonadotropin (hCG) trigger have also been associated to adverse perinatal outcomes related to placentation (Pereira et al., 2015; Royster et al., 2016), reassuring results did not report an association between the number of retrieved oocytes and adverse perinatal outcomes (Bardhi et al., 2020; Magnusson et al., 2018; Sazonova et al., 2011). Hence, OS influence on pregnancy should be addressed from different perspectives and studied further.

Laboratory procedures might also influence embryonic and fetal development. Pregnancies following ART show a range of adverse outcomes that vary by treatment modality. The risk of FGR, LBW and PTB is significatively reduced in pregnancies after frozen embryo transfer (Maheshwari et al., 2018; Spangmose et al., 2020), but these pregnancies are associated with a higher risk of macrosomia and being large for gestational age (LGA) (Berntsen and Pinborg, 2018; Terho et al., 2021). Overall, the risk of being LGA in frozen embryo transfer singletons even exceeds the risk of being LGA in SC newborns. Macrosomia itself has been reported as a risk factor for other adverse perinatal results, such as shoulder dystocia, hypoglycemia, respiratory distress, neonatal metabolic disturbances at birth, perinatal mortality and to also perineal lacerations, Cesarean section and postpartum hemorrhage (Beta et al., 2019). The reasons for differential outcomes in frozen as compared to fresh embryo transfer cycles are still unclear. It has been suggested that the fresh embryo transfer on the third or the fifth day after fertilization implies a degree of asynchrony between the embryonic developmental stage and the endometrial receptivity due to the high levels of estrogen and progesterone after OS, which could affect the intrauterine environment and therefore the implantation process (Shapiro et al., 2011). Another reason could be the freezing and thawing procedures, which may filter out weaker embryos, selecting good quality embryos to survive, with better fetal growth trajectories (Berntsen et al., 2019). An alternative explanation might be the impact of cryoprotectants and the embryo culture (medium composition and culture conditions). The IVF embryo culture would promote *in utero* overgrowth, an effect that would be inhibited by altered steroid environment in fresh embryo transfer cycles (Pinborg et al., 2013). When pregnancy outcomes were compared in pairs of consecutive singleton siblings born after fresh and frozen embryo transfer, the significantly higher mean birthweight in frozen embryo transfer singletons persisted, even after adjustment for maternal age and birth order (Henningsen et al., 2011). Another dimension of the frozen embryo transfer cycles is the possibility to replace the embryos in programmed versus natural cycles, that is, in supplemented cycles with exogenous estradiol and

progesterone (avoiding the development of a corpus luteum) or scheduled according to spontaneous or induced ovulation (with the subsequent development of a corpus luteum). Over the last decade, multiple studies have reported an increased risk for adverse pregnancy outcomes such as hypertensive disorders of pregnancy after frozen embryo transfer (Maheshwari et al., 2018; Opdahl et al., 2015; Sazonova et al., 2012; Sha et al., 2018). From 2019 on, several publications indicated that this increased risk, along with postpartum hemorrhage, post-term birth and macrosomia, was attributable to the programmed embryo transfer cycles, which preclude the corpus luteum development (Ernstad et al., 2019; Saito et al., 2019; Wang et al., 2020; Waschkies et al., 2021; Zong et al., 2020). The corpus luteum is considered a main regulator of the endometrial function, promoting decidualization in the secretory phase and early pregnancy, and its absence has been proposed as an etiologic factor of the impaired maternal cardiovascular adaptations seen during early pregnancy in preeclamptic gestations (Conrad et al., 2021). Both endometrium and maternal cardiovascular system are known targets of at least one corpus luteum product that is not replaced in programmed cycle protocols, the relaxin (Conrad, 2020).

Finally, subfertility together with other parental underlying factors seem to contribute to the perinatal results as well. Patients with reproductive disorders often present multiple risk factors contributing to adverse perinatal outcomes, beginning with advanced maternal age (Vannuccini et al., 2016). Hormonal, inflammatory and metabolic alterations of the ovaries, endometrium, myometrium and uterine cervix underlie the poor obstetric outcomes observed in patients with PCOS, endometriosis, uterine myomas and/or unexplained infertility, probably impairing placentation (Vannuccini et al., 2016). Inflammatory mechanisms are predominant in endometriosis and, whereas metabolic dysfunction prevails in PCOS, a low-grade chronic inflammatory status is also associated to this syndrome (Vannuccini et al., 2016). Advanced paternal age has been statistically associated to a higher risk of adverse pregnancy outcomes such as miscarriage and PTB, congenital defects, and other postnatal disorders (autism spectrum disorder (ASD), schizophrenia, bipolar disorder, childhood

leukemia) (Sharma et al., 2015). In cases of severe male infertility, a modestly increased risk of chromosomal anomalies and impaired reproductive health for male offspring has been reported (Berntsen et al., 2019). A large ART singleton cohort study from the Netherlands reported that birth weight was independently associated to maternal hypertensive disease, ethnicity, nulliparity, infertility length, and PTB was independently associated to ethnicity, nulliparity and cause of infertility, being less common in patients with male factor as treatment indication (Pontesilli et al., 2015). The Danish sibling population-based registry study also compared the perinatal outcome of pairs of singleton siblings SC and from ART to evaluate the effect of different mode of conception controlling by parental characteristics. SC were on average heavier and showed lower risk of PTB than their ART siblings (Henningsen et al., 2011). According to the literature, epigenetics (Berntsen et al., 2019), oxidative stress (Agarwal et al., 2022), and mitochondrial dysfunction (Kristensen et al., 2017) are some of the proposed mechanisms through which the different suggested etiological factors could influence the ART offspring´s outcomes.

Specific epigenetic profiles have been described in ART placentas (Choux et al., 2018; Marjonen et al., 2018) and offspring (El Hajj et al., 2017; Novakovic et al., n.d.; Song et al., 2015; Whitelaw et al., 2014). However, only one study has explored and reported the epigenetic effects of parental infertility in singleton SC children (Barberet et al., 2022). It is likely that these effects are also present after ART and constitute an epigenetic risk for children, although their clinical significance is not well understood yet.

1.2.2 Safety in ART: Trends over time

Nowadays, there is a broad range of treatment strategies to achieve respectable success rates in ART minimizing the number of complications during the procedures. The set of techniques available is constantly evolving and continuously offering better results in terms of efficacy and safety. A registry study reviewing perinatal outcomes through the past two decades in a multinational Nordic cohort shows an improvement of obstetric results over time, probably

related to better clinical and laboratory procedures, cryopreservation and the push towards elective single embryo transfer (Henningsen et al., 2015). However, this trend over time can also be attributable to other factors such as a shift towards less severe reproductive disease in the couples undergoing ART.

Prospective studies designed to assess perinatal outcomes of ART and SC pregnancies adjusted for cause and length of infertility could make an important contribution to clarify the origin of adverse perinatal outcomes in the ART population. This increased risk could be related to OS, laboratory procedures or gamete/embryo cryopreservation and, a rational way to analyze this should involve well-phenotypied prospective cohorts with certain homogeneity in treatment-related variables such as OS protocols, IVF or ICSI techniques, embryo culture characteristics (length, culture medium composition, oxygen tension exposure), cryopreservation techniques and embryo transfer strategies.

1.3 Fetal adaptations in conception by ART

So far, there is extensive literature assessing the quality and efficacy of the different ART techniques mostly based on pregnancy rates, live-birth rates, and perinatal results. However, evidence indicates that prenatal environmental insults affecting embryonic or fetal development trajectories during critical periods can lead to fetal adaptations to survive, which subsequently may result in an altered postnatal physiology (Demicheva and Crispi, 2014).

1.3.1 Fetal cardiovascular remodeling

A wide variety of conditions during prenatal life generate an adaptative fetal response in which the central target organ is the fetal heart. As in postnatal life, in the early stages of an insult, the heart remodels changing its structure, and if the insult persists, a subclinical period of cardiac dysfunction takes place before heart failure occurs (Crispi et al., 2020a). Congenital heart diseases, fetal growth restriction, preeclampsia, diabetes, exposure to antiretroviral drugs and also conception by means of ART have been reported to be associated with cardiac remodeling and suboptimal function during

the fetal life (Crispi et al., 2010; García-Otero et al., 2016; Valenzuela-Alcaraz et al., 2013; Youssef et al., 2020b), and this phenomenon might have long-lasting consequences on health (Crispi et al., 2018; Fatima Crispi et al., 2021; Fàtima Crispi et al., 2021; García-Otero et al., 2021; Ortigosa et al., 2016; Sarvari et al., 2017; Valenzuela-Alcaraz et al., 2019). Furthermore, early preventive approaches such as breastfeeding and dietary supplementation constitute promising strategies for improving the long-term cardiovascular health of FGR individuals (Yzydorczyk et al., 2017). Particularly in reference to cardiovascular remodeling in FGR, breastfeeding and healthy-fat dietary intake have shown an independent beneficial effect in children (Rodriguez-Lopez et al., 2016).

Our research group reported for the first time the presence of cardiac and vascular remodeling in IVF fetuses in comparison with SC, and also demonstrated that these changes persisted postnatally (Valenzuela-Alcaraz et al., 2013). The ART subjects, mainly from a fresh embryo transfer cohort, had more globular hearts, with hypertrophic ventricles and larger atria leading to decreased longitudinal motion and impaired relaxation, and these changes remained significant in the same singleton cohort at 3 years of postnatal age (Valenzuela-Alcaraz et al., 2019). These changes were also observed in twin pregnancies achieved by ART (Valenzuela-Alcaraz et al., 2018), and appear to be qualitatively different and therefore independent from FGR (Valenzuela-Alcaraz et al., 2016), a condition known to induce fetal cardiac remodeling (Crispi et al., 2010). Both cardiac and vascular remodeling are risk factors for future cardiovascular disease, which justify long-term follow-up research studies on these children. As cardiovascular remodeling and dysfunction have been only described in a cohort of conventional stimulated IVF with fresh embryo transfer as compared to SC fetuses from fertile couples (with a time-to-pregnancy (TTP) below 12 months), the effect of IVF variants that involve embryo cryopreservation or NC-IVF still remains unknown. Studies on fetal cardiac performance of fetuses SC from subfertile couples (with a TTP above 12 months) are also lacking. Thus, this PhD was aiming to study cardiac structure and function in fetuses conceived by IVF with frozen

embryo transfer (**Study 1**), SC from subfertile couples (**Study 2**) and conceived by NC-IVF (**Study 3**).

1.3.2 Fetal brain reorganization

The central nervous system is in constant development throughout the fetal period and later, being especially exposed and vulnerable to different insults at any gestational age.

Although overall the postnatal reports present numerous limitations, several studies have described an increased neurodevelopmental risk for the ART subjects. Register-based studies in Sweden and Denmark and other countries as Israel and Australia informed an increased risk of developing neurological sequelae, particularly cerebral palsy, epilepsy and developmental disturbance (Ericson et al., 2002; Goldsmith et al., 2018; Hvidtjørn et al., 2010; Levin et al., 2019; Lidegaard et al., 2005; Strömberg et al., 2002; Zhu et al., 2009). The long-term neurological morbidity indicating brain damage was partially explained by an excess of PTB ad multiple births in most of these studies. However, in a singleton Danish national population-based controlled follow-up study acknowledging differences between fresh and frozen embryo transfer, higher cerebral palsy rates were found in IVF children, particularly in the fresh embryo transfer populations (Pinborg et al., 2010).

Neuropsychiatric disorders affecting social and speech development, including ASD and attention deficit hyperactivity disorder (ADHD) have also been described in ART singletons (Källén et al., 2011; Kissin et al., 2015; Rönö et al., 2022; Sandin et al., 2013; Svahn et al., 2015). Conversely, in a register-based study from USA adjusted by relevant variables, it was concluded that the association between ART and ASD was primarily explained by perinatal outcomes and multiplicity (Fountain et al., 2015).

Several studies have also attempted to demonstrate differences in cognitive development between SC and ART children, with inconsistent results.

Finally, there are scarce investigations on prenatal neurodevelopment in ART pregnancies (Husen et al., 2021; Yin et al., 2015), with the advantage of avoiding the influence of late perinatal outcomes and

postnatal environment. None of them have assessed brain cortical folding or include postnatal neurodevelopment data.

These findings highlight the need for further prenatal studies, to confirm these associations and clarify whether they are related to the perinatal complications, the parental underlying infertility, or the ART procedures. This PhD aimed to prenatally study neurodevelopment in fetuses conceived by ART (**Study 4**).

1.4 Free fetal hemoglobin and heme scavengers´ role in preeclampsia

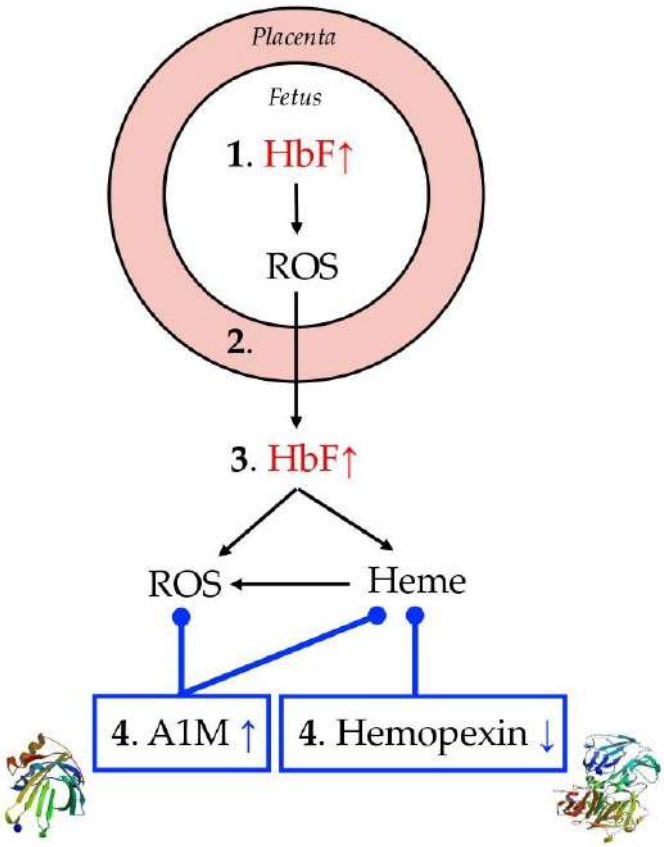
Free fetal hemoglobin (HbF) was discovered in a preeclampsia placental microarray study (Centlow et al., 2008). It was then further evaluated as a maternal plasma biomarker predicting preeclampsia in the first trimester (Anderson et al., 2016), shown to correlate with severity in term preeclamptic pregnancies (Anderson et al., 2018) and FGR (Brook et al., 2018). Increased levels of this molecule are synthesized and accumulated in preeclamptic placentas (Centlow et al., 2008). Free HbF causes oxidative stress and placental tissue damage, which consequently leads to a leakage of it over the blood-placenta barrier into the maternal circulation (Anderson et al., 2011; Gram et al., 2015; May et al., 2011). Actually, increased concentrations of free HbF have been shown in maternal plasma in both early and late onset preeclampsia (Anderson et al., 2016; Olsson et al., 2010), suggesting it to be an etiological factor of this disorder (Gram et al., 2015).

Hemopexin and α_1-microglobulin (A1M) are two scavenger proteins part of the defense mechanisms developed to protect the body against the toxicity of free HbF and its degradation metabolites heme and free iron (Chiabrando et al., 2011). Hemopexin is a circulating plasma glycoprotein predominantly synthesized in the liver that acts as an acute phase reactant that binds free heme (Tolosano et al., 2010), generating an hemopexin-heme complex that is internalised and cleared by macrophages and hepatocytes through a LDL receptor-mediated process (Mehta and Reddy, 2015). In humans, elevated plasma levels of heme and free HbF were associated with a decrease in hemopexin concentration suggesting consumption of this molecule, with levels correlating with maternal blood pressure (Anderson et al., 2018; Gram et al., 2015). Hemopexin has serine protease activity (Bakker et al., 2007, 2005)

and has also been suggested to be a regulator of the vascular responsiveness to angiotensin II (Bakker et al., 2009) and to prevent endothelial damage in a mouse heme-overload model (Vinchi et al., 2008). On the other hand, A1M is a tissue-protective lipocalin protein that binds heme and free radicals and has enzymatic reducing capacity. It has been shown to be up-regulated in preeclampsia (Anderson et al., 2011) as well as having therapeutic properties in several preeclampsia animal models (Erlandsson et al., 2016; Gunnarsson et al., 2017; Wester-Rosenlöf et al., 2014). **Figure 1** illustrates the behaviour of HbF and the scavenger proteins hemopexin and A1M in preeclamptic pregnancies.

Figure 1 The role of HbF in preeclampsia (adapted from Gram et al., 2015)

1. Increased levels of HbF leading to the generation of reactive oxygen species (ROS)
2. Damage to placental barrier that causes the leakage of factors into maternal circulation
3. Increasing levels of HbF in maternal circulation
4. Activation of the scavenger system including depletion of hemopexin and up-regulation of α_1-microglobulin (A1M)



In summary, increased concentrations of HbF have been detected in maternal blood in both early and late onset preeclampsia suggesting it to be an important etiological factor. In preeclamptic pregnancies, maternal plasma levels of hemopexin are expected to decrease and concentrations of A1M are expected to increase, indicating higher levels of oxidative stress in the intrauterine environment. The role of the HbF degradation pathways would be an interesting mechanism to study in IVF pregnancies, particularly those

undergoing embryo transfer in programmed cycles, which are associated to an increased risk of preeclampsia. This PhD aimed to better understand the role of these preeclampsia-related scavenger proteins according to the presence or absence of corpus luteum at the time of conception (**Study 5**).

1.5 Rationale and clinical relevance of the present research study

Evidence indicates that ART singleton subjects are at higher risk of adverse perinatal outcomes, cardiac remodeling with suboptimal cardiac function, and shows inconsistent neurodevelopmental outcomes in children and adolescents. These findings have a potential impact on long-term health. Thus, it is important to study the safety profile of each ART treatment modality.

This study was also designed to unravel the influences of the IVF laboratory procedures, subfertility-related factors, and OS on pregnancy and offspring´s outcomes. While assessing the effects of ART on pregnancy and offspring´s outcomes, NC-IVF can serve as a control group to evaluate the influence of OS. Alternatively, considering the impact of the IVF procedures (oocyte-retrieval, IVF/ICSI, embryo culture medium and conditions, eventual cryopreservation, and embryo transfer) on these outcomes, SC pregnancies in subfertile patients would allow to study the influence of the infertility factors leaving behind the possible effects of ART. **Figure 2** illustrates the relationship between the factors above mentioned and the strategies offered by medically assisted reproduction.

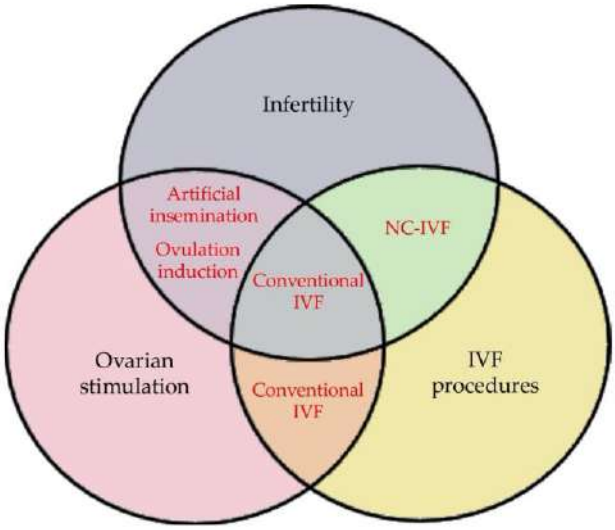


Figure 2 Influence of infertility background, ovarian stimulation, and IVF laboratory procedures on different treatment strategies in medically assisted reproduction.

Our main aim was to assess patterns of cardiac remodeling, neurodevelopment, and adverse perinatal outcomes attributable to the different treatments, to allow the identification of the safest strategy in terms of maternal and offspring´s health. Therefore, **Studies 1 to 3** represented an effort to stablish associations between OS, infertility *per se*, laboratory procedures and fetal cardiac structure and function. **Study 4** investigated fetal brain cortical development in fetuses from conventional stimulated IVF with fresh or frozen embryo transfer and its association with early neurodevelopment. Finally, **Study 5** explored heme-scavengers in maternal and fetal samples from normotensive and preeclamptic pregnancies in IVF treatments in presence or absence of corpus luteum.

2. Hypotheses

2. Hypotheses

2.1 Main hypothesis

Different ART procedures are associated with distinct patterns of fetal cardiac and brain development, as well as different preeclampsia risk profiles shown by preeclampsia-related biomarkers.

2.2 Specific hypotheses

1. Fetuses from IVF with frozen embryo transfer present differences in cardiac performance as compared to fetuses from IVF with fresh embryo transfer.

2. SC fetuses from subfertile couples do not present signs of cardiac remodeling and suboptimal function as those observed after IVF treatments.

3. Fetuses from NC-IVF cycles (without controlled OS) present differences in cardiac structure and function as compared to fetuses from conventional (stimulated) IVF cycles.

4. ART procedures are associated with a distinct fetal and infant neurodevelopment as compared to spontaneous conceptions, with differences between fresh and frozen embryo transfer offspring.

5. Maternal and fetal plasma levels of preeclampsia-related scavenger proteins vary according to the presence or absence of corpus luteum in gestations with preeclampsia.

3. Objectives

3. Objectives

3.1 Main objective

To describe the fetal cardiac and neurodevelopmental profiles, and the behavior of preeclampsia-related biomarkers, associated to different ART strategies in order to identify the safest technique in terms of the ART offspring´s health.

3.2 Specific objectives

1. To evaluate cardiac structure and function by echocardiography in fetuses from IVF with frozen embryo transfer and to compare it with that of IVF fetuses obtained after fresh embryo transfer.

2. To evaluate cardiac structure and function by echocardiography in fetuses from ART and to compare it with that of SC from fertile and subfertile couples.

3. To evaluate fetal cardiac structure and function by echocardiography in pregnancies from NC-IVF cycles and to compare it with that of conventional (stimulated) IVF treatments.

4. To explore and compare cortical brain development by neurosonography in third-trimester fetuses conceived by IVF with frozen versus fresh embryo transfer and SC, and to investigate its association with postnatal neurobehaviour at 12 months of age.

5. To assess the scavenger proteins hemopexin and α_1-microglobulin concentrations in maternal and fetal blood in SC pregnancies and in two different IVF populations (exposed and not exposed to the corpus luteum activity), and to analyze these results according to the occurrence of preeclampsia.

4. Materials and methods

4. Materials and methods

4.1 Study design

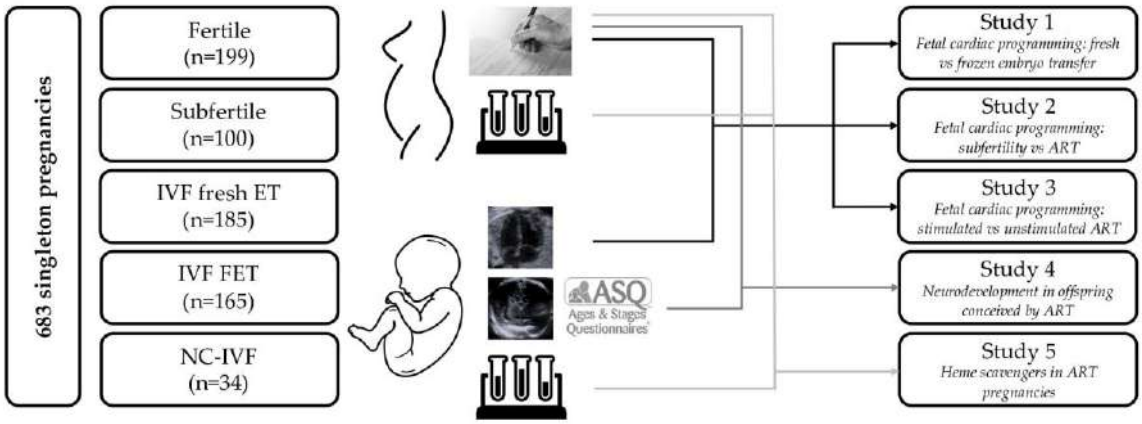
This research project was executed through the prospective recruitment of pregnancies exposed and not exposed to different ART techniques. Pregnancies were enrolled consequently between 2017 and 2021 based on a referral basis, through clinical records.

Pregnancies achieved by ART and SC from subfertile couples (with a time-to-pregnancy over 12 moths) included in this cohort were enrolled in the Assisted Reproduction Unit of the Hospital Clínic de Barcelona (Barcelona, Spain) during their first trimester, at pregnancy diagnosis.

SC pregnancies from fertile couples (with a time-to-pregnancy below 12 moths) who attended the BCNatal Barcelona Center for Maternal Fetal and Neonatal Medicine (Hospital Clínic and Hospital Sant Joan de Déu Barcelona, Spain) were enrolled during their third trimester, being matched with the ART pregnancies by maternal age (± 1 year).

Eligible patients were fully informed about the purpose of the study and the voluntary nature of their participation. The general scheme of the studies presented in this thesis is summarized in **Figure 3.**

Figure 3 Methodological design of studies included in the thesis



Study subjects

Inclusion criteria

- Acceptance to participate in the study
- Maternal age above 18 years at recruitment

Exclusion criteria

- Pregnancies obtained by medically assisted procedures different than IVF (e.g., ovarian induction, intrauterine insemination)
- Multiple gestations
- Fetal anomalies including chromosomal abnormalities or structural malformations detected by ultrasound
- Confirmed or suspected intrauterine infection
- Mental retardation or other maternal mental disorders that impose doubts regarding the true patient´s willingness to participate in the study

Specific populations included in the different studies

Study 1: *Fetal cardiac programming: fresh versus frozen embryo transfer*
A total of 300 pregnancies were included in this cohort study, classified as:

- SC from fertile couples (n=100)
- ART with frozen embryo transfer (n=100)
- ART with fresh embryo transfer (n=100)

Study 2: *Fetal cardiac programming: subfertility versus ART*
A total of 289 pregnancies were included in this cohort study, classified as:

- SC from fertile couples (n=96)
- SC from subfertile couples (n=97)
- ART with fresh embryo transfer (n=96)

Study 3: *Fetal cardiac programming: stimulated versus unstimulated ART*
A total of 102 pregnancies were included in this nested case-control study, classified as:

- SC from fertile couples (n=34)
- ART in natural cycle IVF (NC-IVF) (n=34)
- ART in stimulated cycle with fresh embryo transfer (n=34)

Study 4: *Neurodevelopment in offspring conceived by ART*
A total of 210 pregnancies were included in this cohort study, classified as:

- SC from fertile couples (n=70)
- ART with frozen embryo transfer (n=70)
- ART with fresh embryo transfer (n=70)

Study 5: *Heme scavengers in ART pregnancies*
This is a nested case-control study within a larger cohort. Only the pregnancies followed and delivered in our center with available plasma samples were considered eligible. Exclusion criteria were preimplantation genetic testing, fetal malformations or chromosomal anomalies, intrauterine infection during pregnancy and normotensive fetal growth restricted fetuses).
A total of 160 pregnancies were included in this nested case-control study, classified as:

- SC from fertile couples (n=54)
  - Normotensive (n=31)
  - Preeclampsia (n=14)
  - Severe preeclampsia (n=9)
- ART with corpus luteum at embryo transfer (n=50)
  - Normotensive (n=38)
  - Preeclampsia (n=7)
  - Severe preeclampsia (n=5)
- ART without corpus luteum at embryo transfer (n=56)
  - Normotensive (n=31)
  - Preeclampsia (n=16)
  - Severe preeclampsia (n=9)

4.2 Interventions

All participants underwent the same study protocol, including collection of baseline and perinatal characteristics, and third trimester ultrasound with fetal biometry, feto-placental Doppler, fetal echocardiography and fetal neurosonography assessment.

Figure 4 shows a summary of the assessment plan followed in this research project. All patients recruited received an assessment plan that included the following activities:

1. **Recruitment and pregnancy assessment**

- Signature of the informed consent form
- General data collection
- Feto-placental ultrasonographic assessment including fetal growth, feto-placental Doppler, fetal echocardiography and neurosonography

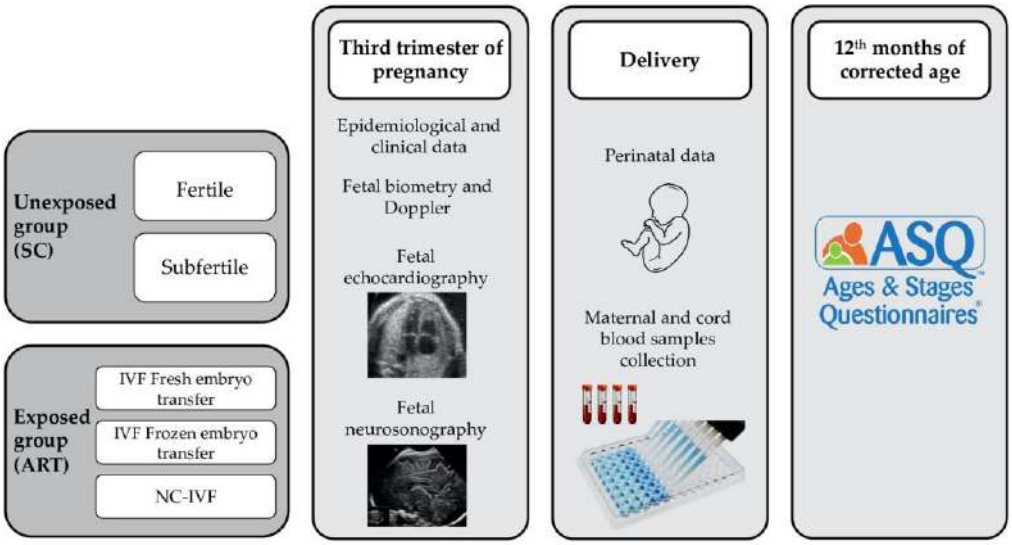
2. **Biosampling and data collection at delivery**

- Maternal and cord blood samples collection and storage
- Obstetric outcomes´ collection

3. Postnatal follow-up

- Data collection at 12 months of corrected age

Figure 4 Research assessment plan.



4.2.1 General data collection

Epidemiological data

Parental demographics including date of birth, ethnicity, pregestational height, weight, and body mass index (BMI), socioeconomic status (highest academic level achieved, employment status) was collected by direct patient´s interview and review of medical records.

Parental clinical and obstetric history and ART outcomes

Clinical, ART-related, and obstetric history variables were also collected by direct patient´s interview and review of medical records. They include the presence of parental chronic diseases, previous surgical procedures, usual medication, smoking status, illicit substance abuse (heroin, cocaine or cannabis), parity, obstetric history, TTP and mode of conception. ART-related parameters included etiology and length of infertility, diagnosis of polycystic ovarian syndrome (PCOS) according to Rotterdam criteria (Fauser, 2004), ovarian reserve tests (anti-Mullerian hormone, antral follicle count), number of previous unsuccessful ART cycles, ovarian stimulation data (type of aGnRH and

gonadotropin, duration in days, total dose of gonadotropins in international units), number of oocytes retrieved, fertilization technique (FIV, ICSI), number of embryos obtained, type of embryo transfer (fresh or frozen), protocol of endometrial preparation at embryo transfer, embryonic stage at embryo transfer, number of embryos transferred, vanishing twin phenomenon and selective embryonic reduction.

4.2.2 Fetoplacental ultrasonographic examination

All pregnancies underwent an ultrasonographic examination at 27–35 weeks of gestation using a Siemens Sonoline Antares (Siemens Medical Systems, Malvern, PA, USA), a Voluson 730 Expert (GE Medical Systems, Milwaukee, Wisconsin, USA) and/or a Voluson S10 ultrasound system (GE Healthcare, Zipf, Austria) with 2-5 MHz or 6–4MHz linear curved-array and 2–10MHz phased-array probes, including the estimation of fetal weight, and the performance of feto-placental Doppler, fetal echocardiography and fetal neurosonography. US acquisitions were obtained by four maternal–fetal medicine specialists skilled in fetal ultrasound blinded to the mode of conception. The images were recorded as clips and anonymized. Finally, all the measurements were performed offline by a very well-trained single investigator, also blinded to the mode of conception, to avoid bias.

4.2.1.1 Fetal biometrics and feto-placental Doppler

Fetal weight was estimated by using the Hadlock formula (Hadlock et al., 1985), based in a composite sonographic measurement of abdominal circumference (AC), head circumference (HC), biparietal diameter (BPD), and femur length (FL). Weight centile was calculated according to local reference curves (Figueras et al., 2008).

Feto-placental Doppler assessment included the measurement of pulsatility index (PI) of the following vessels:

- ***Uterine arteries (UtA) PI***: was obtained transabdominally by placing the probe on the lower quadrant of the abdomen, angled medially, with color Doppler imaging used to identify the

apparent crossover of the UtA with the external iliac artery, and obtaining the measurement 1 cm distal to the crossover point (Bhide et al., 2013). UtA PI from both sides was obtained and mean UtA PI was calculated according to previously reported protocol (Gómez et al., 2008). Normal UtA was considered as a PI below the 95th centile, and Z-scores were calculated following previous publications (Gómez et al., 2008).

- ***Umbilical artery (UA) PI***: was obtained by placing the Doppler gate in one of the UA from a free-floating cord loop (Bhide et al., 2013). Normal UA was considered as a PI below the 95th centile, and Z-scores were calculated based on previous publications (Arduini and Rizzo, 1990).
- ***Middle cerebral artery (MCA) PI***: was obtained in a transversal view of the fetal head, at the level of its origin from the circle of Willis (Bhide et al., 2013). Normal MCA was considered as a PI above the 5th centile, and Z-scores were calculated following previous publications (Baschat and Gembruch, 2003). The *cerebroplacental ratio (CPR)* was calculated as middle cerebral artery PI divided by umbilical artery PI. Normal MCA PI and CPR were considered as above the 5th centile. Values below this cut-off indicate cerebral blood flow redistribution (Baschat and Gembruch, 2003).
- ***Ductus venosus (DV) PI***: was measured in a transverse section of the fetal abdomen, placing the Doppler gate at the isthmic portion (Bhide et al., 2013). Normal DV was considered as a PI below the 95th centile, and Z-scores were calculated following previous publications (Hecher et al., 1994; Kessler et al., 2006).

Pulsed Doppler parameters were obtained automatically from at least three consecutive waveforms, with an insonation angle as close to 0° as possible (Bhide et al., 2013). All estimations were done in the absence of fetal movements and, if required, with the mother in voluntary suspended ventilation. **Figure 5** summarizes the feto placental parameters included in the sonographic assessment of the study protocol.

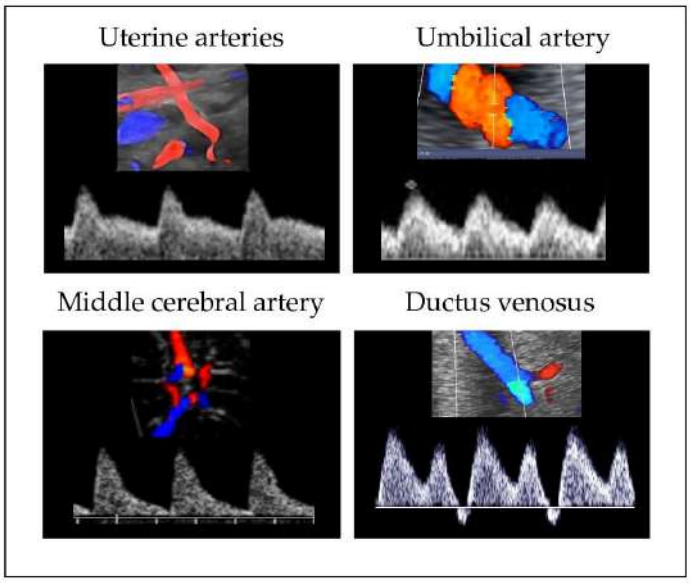


Figure 5 Feto-placental assessment in the study protocol.

4.2.1.2 Fetal echocardiography

To assess structural heart integrity, to exclude cardiac defects, and to evaluate cardiac morphometry and function a comprehensive two-dimensional (2D), M-mode, and Doppler echocardiographic examination was performed following standard protocols (Carvalho et al., 2013; Crispi et al., 2013; García-Otero et al., 2019). **Figures 6 to 13** display the fetal cardiac assessment in the current protocol. Cardiac, thoracic, and ventricular diameters were measured on 2-dimensional (2D) images from an apical or basal four-chamber view at end diastole.

The echocardiography included the evaluation of the following cardiac structure parameters:

- ***Cardiothoracic ratio***: was calculated by dividing the heart area by the thoracic area (Paladini et al., 1990).

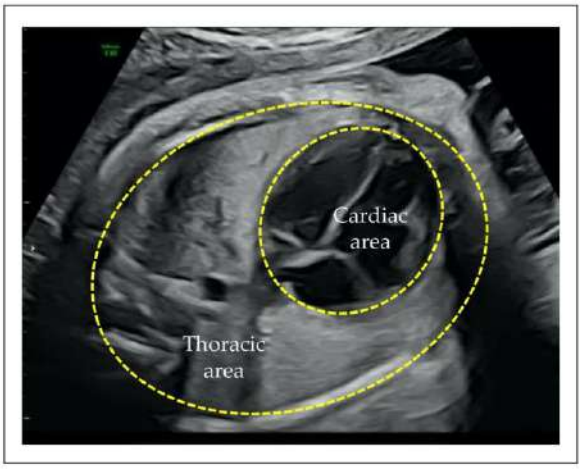
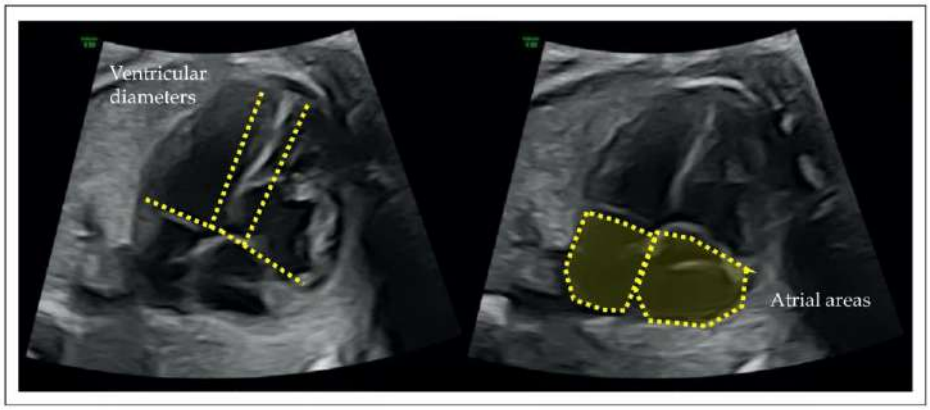


Figure 6 Delineation of cardiac and thoracic areas.

- ***Left and right ventricular sphericity indices***: were calculated by dividing the longitudinal by basal-transverse ventricular diameters (Schneider et al., 2005).
- ***Atrial areas***: were measured at maximum distension from an apical or basal four-chamber view, at end-systole, defined by the frame preceding the atrioventricular valves opening (Lang et al., 2015; Lopez et al., 2010). Atria-to-heart ratios were calculated as atrial area * 100 / heart area.

Figure 7 Delineation of basal and longitudinal ventricular diameters and atrial areas.



- ***Myocardial walls thickness***: were measured on 2-dimensional images from a transverse four-chamber view at end-diastole. Relative wall thickness was calculated as septal plus left wall thickness divided by left ventricular end-diastolic diameter (Foppa et al., 2005).

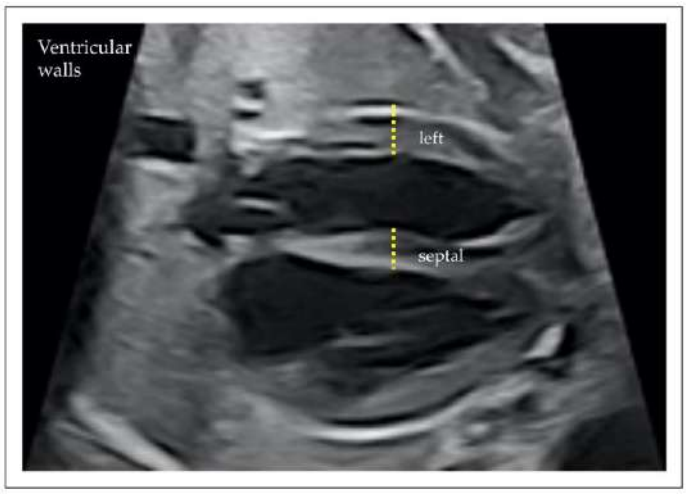


Figure 8 Delineation of left and septal myocardial walls thickness.

Cardiac function assessment involved the following parameters:

- ***Left ventricular ejection fraction (%)***: was obtained from M-mode transverse four-chamber view using the Teichholz's formula (Teichholz et al., 1976).

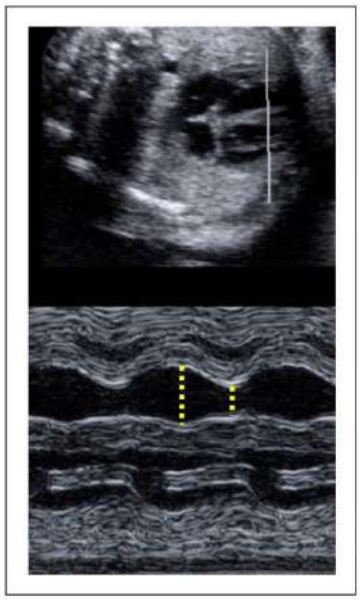
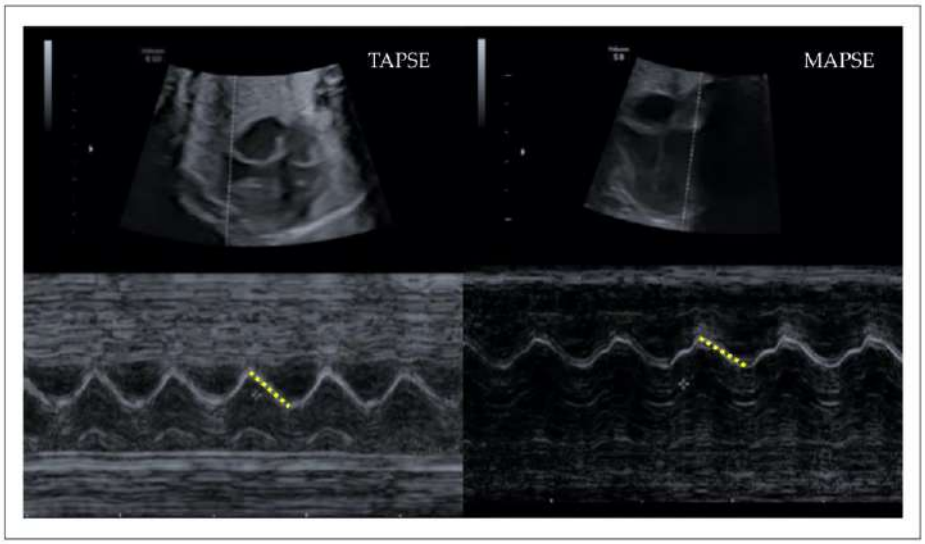


Figure 9 Delineation of left ventricular end diastolic and end systolic diameters

- ***Tricuspid / Mitral ring displacements (TAPSE/ MAPSE)***: were assessed by M-mode from an apical or basal four-chamber view (Germanakis et al., 2012).

Figure 10 Delineation of tricuspid and mitral ring displacements.



- ***Tissue Doppler Imaging (TDI)***: was applied in the spectral Doppler mode to record systolic (S´), early diastolic (E´), and

atrial contraction (A´) peak velocities at tricuspid and mitral annuli from an apical or basal four-chamber view and measured in real time.

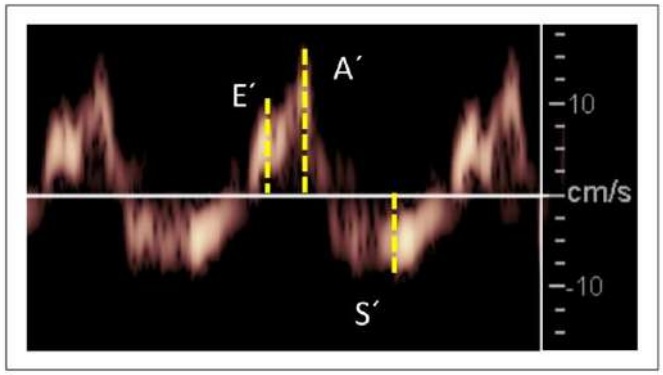


Figure 11 Measurements of E´, A´, and S´ wave velocities.

- ***Tricuspid and mitral early (E) and late (A) ventricular filling***: were obtained by Doppler, then E/A ratios were calculated.

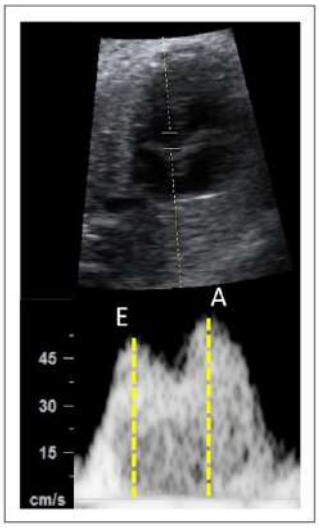


Figure 12 Measurements of E and A wave velocities.

- ***Left myocardial performance index (MPI)***: was obtained from a single Doppler spectrum in a cross-sectional image of the fetal thorax, placing the Doppler sample volume on the medial wall of the ascending aorta, including the leaflets of the aortic and mitral valves. The clicks of the valves registered at the spectral Doppler were used as landmarks to calculate three time periods: isovolumic contraction time (ICT), from the closure of the mitral valve to the opening of the aortic valve, ejection time (ET) from the opening to the closure of the aortic valve, and isovolumic relaxation time (IRT) from the closure of the aortic valve to the opening of the mitral valve. The left MPI was calculated as (ICT

+ IRT)/ET (Hernandez-Andrade et al., 2005). MPI z-scores were then calculated following previous publications (Cruz-Martínez et al., 2012).

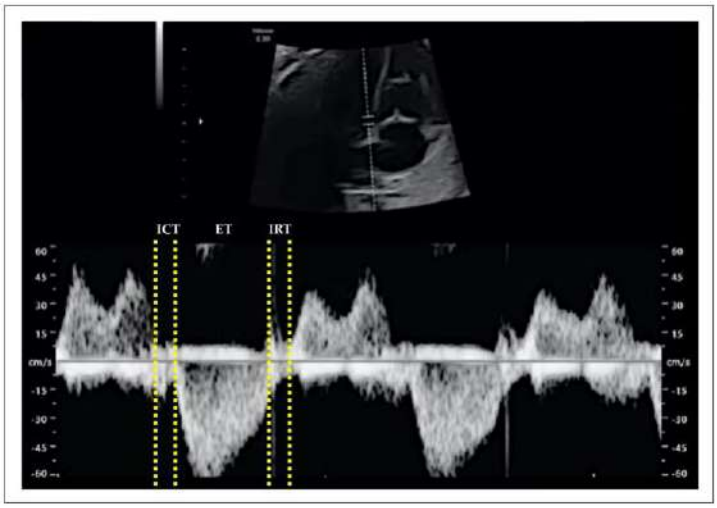


Figure 13 Measurements of isovolumic contraction time (ICT), ejection time (ET), and isovolumic relaxation time (IRT).

4.2.1.3 Fetal neurosonography

Neurosonographic acquisition

A detailed fetal 2D neurosonography was performed during the third trimester using either a Voluson 730 Expert (GE Medical Systems, Milwaukee, Wisconsin, USA) or a Voluson S10 ultrasound system (GE Healthcare, Zipf, Austria). To confirm structural brain normality, a complete neurosonographic examination was performed. A standardized protocol according to the International Society of Ultrasound in Obstetrics and Gynecology (ISUOG) guidelines (Paladini et al., 2007), including axial (transventricular and transthalamic), coronal (transthalamic, transcaudate and transcerebellar), and sagittal (midsagittal and parasagittal) planes was followed. Axial planes were obtained in all cases by transabdominal approach. On the other hand, coronal views were obtained by transvaginal approach in case of cephalic position and transabdominally in breech position fetuses.

Pregnancies with poor image quality for delineation of measurements according to our standards (due to fetal presentation, patient´s intolerance to the transvaginal approach, presence of severe endometriosis, abdominopelvic surgeries

antecedents, high maternal body mass index and placenta previa diagnosis) were excluded.

Image processing, measurements, and cortical folding assessment

All images were recorded as clips and anonymized. Measurements were performed offline using the OsiriX MD 12.0 imaging software (Pixmeo SARL, Geneva, Switzerland) by a single experienced examiner blinded to study groups. In axial and coronal views a straight line projecting the interhemispheric fissure was traced in every plane of interest to provide rigorous perpendicular measurements to the midline. Brain structures´ measurements were done following previously published methodology (Alonso et al., 2010; Egaña-Ugrinovic et al., 2013; Hahner et al., 2018; Paules et al., 2021). Briefly, fissures and sulci depths were measured in millimeters and then values were normalized by head dimension by dividing them by BPD and multiplying by 100. **Figure 15** displays the neurosonographic measurements performed, including fissures/sulci depths and morphology.

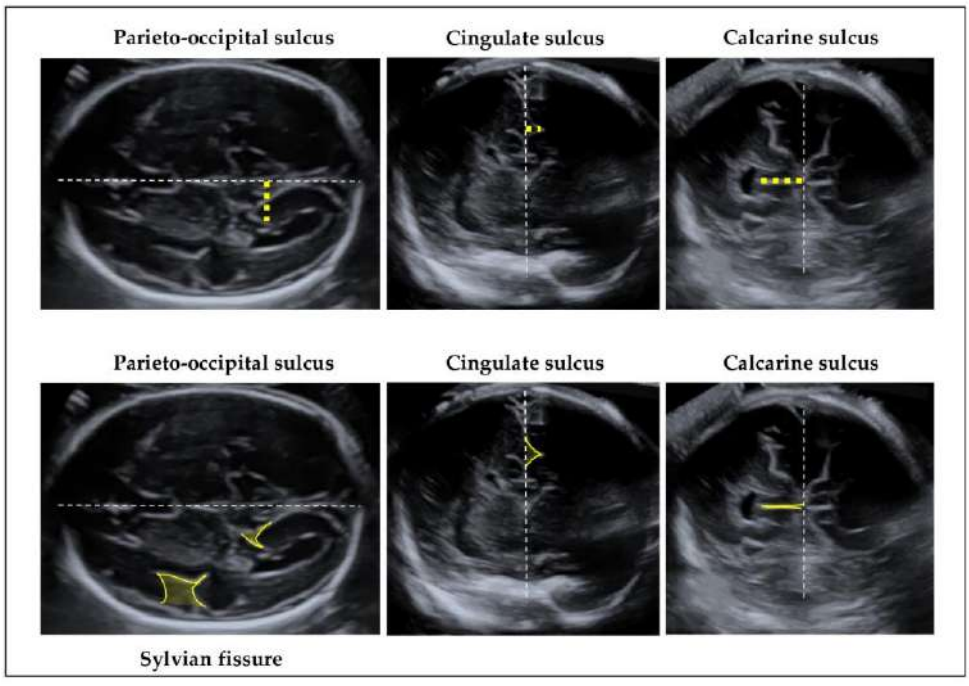


Figure 15 Fetal neurosographic assessment in the study protocol. The first row illustrates the measurements of fissures/sulci depths, and the second row exemplifies the morphologic assessment of the fissures/sulci studied. The interhemispheric fissure is represented by the dashed line.

The neurosonographic assessment included the following sulci depths:

- ***Parieto-occipital sulcus depth***: was evaluated in a slightly cranial plane above the transventricular plane, where the full depth or triangle shape of the sulcus could be displayed. The measurement was made drawing a perpendicular line from midline to the sulcus apex excluding the cortex.
- ***Cingulate sulcus depth***: was measured in the coronal transthalamic plane tracing a perpendicular line from the interhemispheric midline to the apex of the sulcus excluding the cortex.
- ***Calcarine sulcus depth***: was measured in the coronal view at the transcerebellar plane, drawing a perpendicular line from midline to the apex of the sulcus excluding the cortex.

The morphologic assessment of cortical fissures/sulci was performed according to previously described methodology (Pistorius et al., 2010). The degree of cortical development of Sylvian fissure, parieto-occipital, cingulate and calcarine sulci was evaluated, and grading scores in a scale from 0 (no maturation) to 5 (maximum degree of maturation) were assigned to each case.
Our research group has reported a good reproducibility for these neurosonographic parameters in a previous publication (Hahner et al., 2018). For the sulci depth measurements, an intraclass correlation coefficient (ICC) between 0.685 and 0.971 intraobserver, and between 0.773 and 0.917 interobserver were published. The Cohen´s kappa coefficient calculated for the sulci grading scores was between 0.894 y 0.955 for intraobserver, and between 0.765 and 0.906 for interobserver variability.

4.2.3 Perinatal outcomes´ collection

Gestational age was calculated according to first trimester crown-rump length (CRL) measurement on first-trimester ultrasound (Robinson, 1973). Small for gestational age (SGA) and large for gestational age

(LGA) were defined respectively as birth weight below 10th centile or above the 90th centile according to local standards (Figueras et al., 2008). Upon delivery, pregnancy and perinatal outcomes were recorded.

Perinatal exposure to aspirin, corticosteroids, psychopharmaceutical drugs, gestational age at delivery, labor induction, mode of delivery, pregnancy complications and neonatal variables at birth (sex, birthweight, birthweight centile, Apgar score, Umbilical artery pH, admission to Neonatal Intensive Care Unit (NICU), perinatal morbidity and mortality. Preterm birth was defined as delivery prior to the 37th week of gestation. Preeclampsia was defined by new onset of hypertension of ≥ 140 mmHg systolic blood pressure and/or ≥ 90 diastolic blood pressure, on two occasions at least four hours apart, after 20 week´s gestation, together with proteinuria (≥ 300 mg proteins in 24 hours urine or protein/creatinine ratio ≥ 0.3), or -in the absence of proteinuria- new onset of maternal thrombocytopenia, renal insufficiency, liver dysfunction, pulmonary edema or neurological features ("Gestational Hypertension and Preeclampsia: ACOG Practice Bulletin, Number 222", 2020). Gestational diabetes was defined as a glucose intolerance with onset or first recognition during pregnancy, and diagnosed by means of a pathologic oral glucose tolerance test (usually indicated after an altered fasting glucose determination or an altered glucose challenge test from the second trimester on), according to the National Diabetes Data Group (NDDG) (National Diabetes Data Group, 1997).

After birth, major neonatal morbidity was defined by the presence of at least one of the following: bronchopulmonary dysplasia, intraventricular hemorrhage, periventricular leukomalacia, retinopathy, necrotizing enterocolitis, persistent ductus arteriosus, or sepsis. In the absence of major neonatal morbidity, the presence of at least one of the following: respiratory distress, hyperbilirubinemia, or anemia defined minor neonatal morbidity. Perinatal mortality was defined by either intrauterine fetal death after 22 weeks of pregnancy or neonatal death within the first 28 days of life. For these postnatal outcomes, the distribution of cases below 2 SD of the SC population in the whole sample was calculated by domain.

4.2.4 Biological samples´ collection, storage, and transfer

Paired maternal and cord blood samples were collected at delivery into 10 ml EDTA treated tubes. Maternal blood was obtained at hospital admission, drawn from an indwelling cannula in the brachial vein using the Vacutainer system. Cord blood was obtained umbilical vein after cord clamping immediately after delivery. In both cases, serum, plasma and buffy coat were separated by centrifugation at 2000 g for 10 minutes al 4°C and stored immediately at -80°C.

4.2.5 Heme scavengers' assessment on paired maternal and cord blood at delivery

Hemopexin and α_1-microglobulin concentrations were determined in maternal and cord plasma by Enzyme-Linked ImmunoSorbent Assay (ELISA). This assessment was carried out in the laboratories of clinical sciences department of Lund University, Sweden.

Hemopexin concentrations

Hemopexin concentrations were determined using a Human Hemopexin ELISA Kit (GWB-4B6D1A) from Genway Biotech Inc (San Diego, CA, USA). The analysis was performed according to the manufacturer's instructions. Standards and unknown plasma samples were run in duplicates, and their absorbance was read at 450 nm using a GloMax Discover microplate reader (Promega, Madison, WI, USA). For every plate, a standard curve was used to quantify hemopexin concentrations in each sample.

A1M concentrations

An in-house developed sandwich ELISA was used for the quantification of A1M. Standards and unknown plasma samples were run in duplicates and incubated for 1 hour at room temperature, darkness and rotational shaking. Horseradish peroxidase-conjugated detection antibody (mouse monoclonal, clone 57.10) was added and incubated for 1 h at room temperature, darkness, and rotational shaking. Finally, a ready-to-use 3,3´,5,5´-Tetramethylbenzidine, TMB (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) substrate solution was added and incubated in darkness without

shaking. The reaction was stopped after 15 min using 1 M sulphuric acid, and the absorbance was read at 450 nm using a GloMax Discover microplate reader (Promega, Madison, WI, USA). Both anti-A1M and horseradish peroxidase-conjugated detection antibodies were produced in-house by Agrisera AB (Vännäs, Sweden) by immunization with human urinary A1M prepared as previously described (*Berggård et al., 2008*). A standard curve for every plate was used to quantify A1M concentration in each sample.

4.2.6 Postnatal data collection

Postnatal neurobehaviour was assessed at a mean age of 12 ± 1 month of corrected age by means of a Spanish version of the ASQ (2nd edition), a first-level comprehensive screening program widely used to determine children's performance compared with standards taken from typically developing children of the same age, and validated for its application by both parents and primary caregivers (Bricker et al., 1999; Sarmiento Camposa et al., 2011). The ASQ screening system involves several questionnaires designed for use from 4 to 60 months, providing information on five different areas at each age: communication, personal-social, problem-solving, gross-motor and fine-motor skills. In most cases, these questionnaires accurately identify infants or young children who are in need of further evaluation to determine whether they require early intervention services. Parents completed the questionnaires and emailed them to the researchers, who validated the tests.

4.3 Data management and quality control of data

By signing the informed consent form, participants agreed on the handling of their and their newborn´s personal information. The principal investigator ensured the privacy of patients and data confidentiality according to the Spanish Organic Law 3/2018 on Protection of personal data and guarantee of digital rights in conformation with European regulations.

Subjects who participated in the study have the right to access, correct, delete, oppose, and the right to restriction of processing and the right of portability of data. The subjects were not identified by name in any publicly available, written, or oral reports. Paper records and case report forms are maintained in locked cabinets, rooms, or in computer files protected by digital passwords. Only authorized personnel (the principal investigator and his team) have access to the data. When data was shared with a third-party center, it was codified, eliminating any information that could identify the patients.

Regarding the quality control of US scans, before the enrolment period several pilot ultrasound scans were performed to standardize the technique, and image views and strict scanning and recording protocols were established. Ultrasound data collection, imaging acquisition and measurement were monitored periodically by the study supervisor using a random selection of ultrasound scans.

4.4 Samples management

The biological samples collected during this research project were treated according to the Spanish Law 14/2007 on biomedical research and the Royal Decree 1716/2011, which establishes the basic requirements for the biobanks in purposes of biomedical investigation, on how to deal with human biologic samples, and regulates the function and organization of the national register of biomedical investigation biobanks.

Human specimens were codified and stored in locked freezers at Biobank HCB-IDIBAPS, registered in the health institute Carlos III under the number B.0000575. Only the principal investigator and listed co-investigators are able to make the linkage between coded specimens and human data.

The residual samples remain stored in the biobank after the experiments, codified and under authorized consent of the patients.

4.5 Statistical analyses

Statistical analyses were conducted by means of STATA software (StataCorp LLC, Texas, USA) versions 15 and 16. The study outcomes were the parameters of fetal cardiac and neurosonographic assessment, infant neurobehavioral test and plasma heme scavengers' concentrations in maternal and fetal samples at delivery. The independent variable of interest was the mode of conception (spontaneous from fertile or subfertile couples or by different ART techniques). Data were presented as continuous or categorical variables, expressed as mean (± standard deviation (SD)), median (interquartile range), or number (percentage), as appropriate. Normal distribution of continuous variables was checked using the Shapiro-Wilk test and histograms. Comparisons among the study groups were assessed by Pearson χ^2 or Fisher's exact test for categorical variables. Differences in continuous variables were evaluated by t-test or ANOVA, U Mann Whitney or Kruskal-Wallis with Bonferroni correction, after checking the fulfilment of each test assumptions. Differences between the study groups were assessed by using multiple regression analyses to adjust by relevant covariates. All reported p-values are two-sided. The level of significance was set at 0.05 for all statistical tests. Additional details form each particular study can be find in the following paragraphs.

4.5.1 **Study 1**: *Fetal cardiac programming: fresh versus frozen embryo transfer*

Based on previously reported features in pregnancies achieved by ART (Valenzuela-Alcaraz et al., 2013), sample size was calculated to enable us to observe a difference of 20% in the right atrial area, and a difference of 5 mm in MAPSE between the group of ART with frozen embryo transfer and SC fetuses, with 90% power and a 5% type I risk. Basal mean and within-group SD were estimated according to previously published data in fetuses (García-Otero et al., 2019; Gardiner et al., 2006), resulting in a required sample of 32 individuals in each group for the right atrial area and 74 individuals for MAPSE. Therefore, a sample size of 100 individuals in each group was designed.

In addition to the univariate analysis, a multiple regression analysis adjusting by maternal age, nulliparity, birthweight centile, preterm delivery and preeclampsia occurrence was performed. Regarding the results, we have reported the mean/median absolute values in the main document, but we have additionally expressed the main results as

confidence intervals of the adjusted mean differences in *Suplemmentary data* to better show the effect size estimations. Furthermore, p-value for linear trend was calculated by means of the Jonckheere-Terpstra test to show the magnitude of the differences in each IVF group as compared to the SC. Finally, a sub-analysis excluding the vanishing twin phenomenon cases was also performed to avoid any potential bias.

4.5.2 Study 2: *Fetal cardiac programming: subfertility versus ART*

A sample size of 32 fetuses in each group was estimated to detect a difference of 20% in right atrial area, and a sample size of 74 fetuses in each group was calculated to detect a difference of 5 mm in MAPSE between the SC and ART groups, with 90% power and a 95% confidence interval, based on previous publications (García-Otero et al., 2019; Gardiner et al., 2006; Valenzuela-Alcaraz et al., 2013). Conservatively, we decided to include at least 80 individuals per group.

In addition to the univariate analysis, a multiple regression analysis adjusting by nulliparity, birthweight centile, gestational and estimated fetal weight (EFW) at scan. In order to avoid bias, a sub-analysis excluding the vanishing twin phenomenon cases was also performed in this study.

4.5.3 Study 3: *Fetal cardiac programming: stimulated versus natural-cycle ART*

Due to the smaller sample size of the natural cycle IVF population in our cohort, this was designed as a case-control study in which each natural cycle IVF pregnancy was matched with a SC from a fertile couple and a stimulated IVF cycle with fresh embryo transfer by 5 different variables: maternal age (± 1 year), ethnicity (Caucasian or not), nulliparity (nullipara or not), gestational age (± 2 weeks) at scan and prematurity (preterm or not). In addition to the univariate analysis, a multiple regression analysis adjusting by gestational age and EFW at scan, and birthweight centile was performed.

4.5.4 Study 4: *Neurodevelopment in offspring conceived by ART*

In this study neurodevelopment was assessed at third trimester of gestation and at 12 months of corrected age.

Due to the lack of studies on cortical folding in ART fetuses, sample size was calculated considering a previous publication that acknowledged differences between fetuses with ventriculomegaly and controls. Sample sizes of 36 and 63 patients per group were estimated for detecting a 15% difference in calcarine and parieto-occipital sulci depths, respectively. Accordingly, the study design contemplated a recruitment of at least 70 patients per exposure group.

For fetal neurosonographic comparisons among groups, in addition to the univariate analysis, a multiple regression analysis was performed, and comparisons were adjusted by confounding factors including maternal age, ethnicity, nulliparity, fetal sex, gestational age and EFW centile at ultrasound scan. A sub-analysis excluding the pregnancies following embryo transfers in blastocyst stage was performed, in order to exclude any selection bias as between both IVF populations.

For the infant assessment, z-scores were calculated using the SC population as a reference group and, to compare the groups, the results were adjusted by maternal age, ethnicity, nulliparity, educational level, employment status, breastfeeding, infant´s sex, gestational age and age at postnatal evaluation.

Finally, Pearson´s correlations between the neurosonographic features and the infants´ scores were performed to examine the potential association between prenatal and postnatal findings.

4.5.5 **Study 5**: *Heme scavengers in ART pregnancies*

Based on previous publications (Gram et al., 2015; Youssef et al., 2020a), a sample size of 46 patients per group was calculated by expecting differences of 2.5 SD in A1M concentrations between the normotensive and the preeclampsia cases in a proportion 3:1, for a confidence interval width no larger than 4 μg/ml with a 90% probability of achieving the target confidence interval width, and a two-sided 95% confidence interval.

Regarding the analysis, in addition to the univariate evaluation, multiple regression analysis was performed to adjust the results for relevant confounding factors, including maternal age, ethnicity, oocyte donation, frozen embryo transfer cycle, prematurity, gestational age and weight centile at delivery.

4.6 Ethical aspects

The hospital Ethics Committee approved the study protocol (HCB/2017/0714) and all participants provided their written informed consent. This was an observational study, in which safe and non-invasive methods were used in response to scientific gaps of knowledge related to safety in ART offspring, following standards from the Nuremberg Code and the Helsinki Declaration for Medical Research involving Human Subjects (World Medical Association, 2013). Confidentiality and anonymity have been ensured. Data were used only for scientific purposes, including publications and/or scientific-academic meetings. Ethical approval documentation can be consulted in *Annexes*.

All patients were invited to participate during clinical assistance in the recruitment centers (Assisted Reproduction and Maternal-Fetal Medicine Units). At enrollment both parents were fully informed about the purpose, methods, interventions and intended possible uses of the research, including the duration of clinical assessment. In case of acceptance, two written consents were taken: one of them regarding the research project and the other one related to future uses of the biological samples to be collected.

5. Studies

5. Studies

Study 1

Cardiac remodeling in fetuses conceived by ARTs: fresh versus frozen embryo transfer

Boutet ML, Casals G, Valenzuela-Alcaraz B, García-Otero L, Crovetto F, Cívico MS, Borrás A, Manau D, Gratacós E, Crispi F.
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human reproduction **ORIGINAL ARTICLE** ***Infertility***

Cardiac remodeling in fetuses conceived by ARTs: fresh versus frozen embryo transfer

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STUDY QUESTION: Do fetuses from frozen embryo transfer (FET) present signs of cardiac remodeling and suboptimal function similar to those observed in fetuses from fresh embryo transfer (ET)?

SUMMARY ANSWER: Fetuses from both fresh ET and FET present signs of fetal cardiac remodeling and suboptimal function, with more pronounced changes after fresh ET as compared to FET.

WHAT IS KNOWN ALREADY: Our group and others have previously demonstrated that fetuses and children conceived by ARTs present cardiac remodeling and suboptimal function. These fetuses show dilated atria, more globular and thicker ventricles, reduced longitudinal motion, and impaired relaxation. Cardiac changes were already present *in utero* and persisted after birth. Most of the ART fetuses included in previous publications were from fresh ET. However, singletons from FET have different perinatal outcomes compared to those from fresh ET. There are no previous studies comparing cardiac morphology and function between fetuses following fresh and FET.

STUDY DESIGN, SIZE, DURATION: This is a prospective cohort study of 300 singleton pregnancies recruited from 2017 to 2020, including 100 spontaneously conceived (SC) pregnancies, 100 fetuses conceived by IVF with FET, and 100 fetuses conceived by IVF with fresh ET. Fetal structural and functional echocardiography was performed in all pregnancies.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Pregnancies conceived by IVF were recruited from a single assisted reproduction center, ensuring homogeneity in IVF stimulation protocols, endometrial preparation for FET, laboratory procedures, and embryo culture conditions. SC pregnancies from fertile couples were selected from the general population and matched to IVF pregnancies by maternal age. Epidemiological and perinatal outcomes were collected in all cases. Fetal echocardiography was performed at 28–33 weeks of pregnancy to assess cardiac structure and function in all pregnancies. All echocardiographic comparisons were adjusted by maternal age, nulliparity, birthweight centile, preeclampsia, and prematurity.

MAIN RESULTS AND THE ROLE OF CHANCE: Parental age, ethnicity, body mass index and smoking were similar among the study groups. Median gestational age at echocardiography and estimated fetal weight were similar in all study groups. Both fresh ET and FET groups showed similar fetal echocardiographic changes, with more pronounced features in the fresh ET as compared to FET pregnancies. Fetuses conceived by IVF showed larger atria (right atria-to-heart ratio: fresh ET mean 18.1% (SD 3.2) vs FET 18.0% (3.9) vs SC 17.3% (3.2); linear tendency *P*-value <0.001), more globular ventricles (right ventricular sphericity index: fresh ET 1.62 (0.29) vs FET 1.61 (0.25) vs SC 1.68 (0.26); <0.001) and thicker myocardial walls (relative wall thickness: fresh ET 0.79 (0.21) vs FET 0.74 (0.22) vs SC 0.65 (0.25); <0.001) as compared to SC pregnancies. Both fresh ET and FET groups also had signs of suboptimal systolic and diastolic function, with reduced tricuspid annular systolic peak velocity (fresh ET 7.17 cm/s (1.22) vs FET 7.41 cm/s (1.19) vs SC 7.58 cm/s (1.32); <0.001) and increased left myocardial performance index (fresh ET 0.53 (0.08) vs FET 0.53 (0.08) vs SC 0.50 (0.09); <0.001) as compared to SC pregnancies.

LIMITATIONS, REASONS FOR CAUTION: The cardiac changes reported here are subclinical, with most cardiovascular indexes lying within normal ranges. Although echocardiographic changes are recognized as potential cardiovascular risk factors, their association with the

long-term cardiovascular disease remains to be proven. The observed milder fetal cardiac features in FET fetuses cannot condition the choice of this technique and must be considered together with the global perinatal results related to these gestations.

WIDER IMPLICATIONS OF THE FINDINGS: The identification of cardiac remodeling in fetuses conceived by IVF with fresh ET and FET represents an opportunity for early detection. Future studies are warranted to study the potential long-term consequences of these findings.

STUDY FUNDING/COMPETING INTEREST(S): This project has been partially funded with support from the Erasmus + Programme of the European Union (Framework Agreement number: 2013-0040). This publication reflects the views only of the author, and the Commission cannot be held responsible for any use, which may be made of the information contained therein. Additionally, the research leading to these results has received funding from 'la Caixa' Foundation under grant agreement LCF/PR/GN18/10310003, the Instituto de Salud Carlos III (PI15/00130, PI17/00675, PI18/00073) integrated into the Plan Nacional de I+D+I and cofinanced by ISCIII-Subdirección General de Evaluación and Fondo Europeo de Desarrollo Regional (FEDER) 'Una manera de hacer Europa', Cerebra Foundation for the Brain Injured Child (Carmarthen, Wales, UK) and AGAUR 2017 SGR grant n° 1531. The authors declare no conflicts of interest.

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Key words: IVF / pregnancy / cardiac remodeling / frozen embryo transfer / fetal heart

Introduction

The health of children born after ART and the safety of specific ART procedures are of great concern to public health as up to 7.7% of the births in European countries are conceived by ART (Wyns *et al.*, 2020). These pregnancies present an increased risk of adverse perinatal outcomes and also a potentially increased cardiometabolic risk later in life (Berntsen *et al.*, 2019). The assessment of cardiovascular health in ART offspring has gained attention in recent years (Guo *et al.*, 2017; Berntsen *et al.*, 2019).

In previous investigations, we and others have shown that fetuses and children conceived by ART present cardiovascular remodeling and suboptimal function (Ceelen *et al.*, 2008; Scherrer *et al.*, 2012; Valenzuela-Alcaraz *et al.*, 2013, 2019; Zhou *et al.*, 2014; Liu *et al.*, 2015; von Arx *et al.*, 2015; Meister *et al.*, 2018; Forton *et al.*, 2019). Fetuses conceived by means of IVF show more globular hearts, dilated atria, reduced longitudinal motion, and impaired relaxation. These studies mainly included fetuses conceived by IVF with fresh embryo transfer (ET). The cardiac features are already present in fetal life and persist after delivery (Valenzuela-Alcaraz *et al.*, 2013, 2019). Although the underlying cause of these cardiac changes remains unclear, the suggested mechanisms may be related to intrinsic maternal or paternal factors associated with infertility, manipulation of gametes and early embryos, culture conditions, and/or the increased risk of fetal insults in comparison with spontaneously conceived (SC) fetuses (Scherrer *et al.*, 2015), such as higher rates of prematurity, abnormal fetal, growth and preeclampsia (PE).

Since the first human pregnancy following frozen embryo transfer (FET) was reported in 1983, there has been a marked increase in FET in IVF cycles. In recent years, there has been a controversial shift to a 'freeze-all' strategy against the conventional fresh ET policy in an increasing number of IVF cycles (Berntsen *et al.*, 2019). Therefore, it is crucial to explore the influence of different ART methods on the health of fetuses and children, especially those born after FET, since their number is currently rising. Some publications have demonstrated that FET results in similar or even higher live birth rates than fresh ET (Chen *et al.*, 2016; Shi *et al.*, 2018). Systematic reviews and meta-analyses initially suggested that perinatal outcomes were better in pregnancies obtained following FET as compared with fresh ET (Pinborg *et al.*, 2003; Wennerholm *et al.*, 2009; Pandey *et al.*, 2012; Zhao *et al.*, 2016), although the updated meta-analysis by Maheshwari *et al.* (2018) confirmed the reduction in preterm birth (PTB), low birthweight and small for gestational age (SGA) rates but also showed an increased risk of large for gestational age (LGA), macrosomia and PE in FET pregnancies compared to fresh ET.

Most of the fetuses and children included in the previous cardiovascular studies were after fresh ET (Scherrer *et al.*, 2012; Valenzuela-Alcaraz *et al.*, 2013, 2019; Zhou *et al.*, 2014) or the technique was not specified (Liu *et al.*, 2015; von Arx *et al.*, 2015), and there are no available investigations comparing cardiac morphology and function between fetuses or children following FET versus fresh ET. The aim of the present study was to evaluate whether cardiac remodeling is also present in the third trimester of pregnancy in fetuses after FET and to compare it with fetuses following fresh ET.

Materials and methods

Study design, size, duration, and setting

This was a prospective observational study of 300 singleton pregnancies conceived by IVF and SC, at the Barcelona Center for Maternal Fetal and Neonatal Medicine (BCNatal, Barcelona, Spain) from April 2017 to March 2020. Fetal cardiac morphometry (right atrial area) and function (mitral ring displacement) were used to calculate sample size based on the previously reported changes in pregnancies conceived by ART (Valenzuela-Alcaraz *et al.*, 2013). The sample size was calculated to enable us to observe a difference of 20% in the right atrial area and of 5 mm in mitral ring displacement between the group of FET and SC fetuses, with 90% power and a 5% type I risk. Basal mean and within-group standard deviations were estimated according to previously published data in fetuses (Gardiner *et al.*, 2006; García-Otero *et al.*, 2020), which resulted in a required sample of 32 individuals in each group for the right atrial area and 74 individuals in each group for mitral ring displacement. Conservatively, a sample size of 100 individuals in each group was designed.

Participants and materials

We included 300 singleton pregnancies: 100 were SC from fertile couples, 100 were from FET, and 100 were from fresh ET. Oocyte donation cycles, preimplantation genetic diagnosis cycles, fetal malformations (including congenital heart disease), and fetal chromosomal anomalies were considered exclusion criteria. Figure 1 shows a flow diagram of the recruitment.

All IVF pregnancies were recruited from a single center (Assisted Reproduction Unit, Hospital Clínic de Barcelona), warranting high homogeneity in ovarian stimulation and FET endometrial preparation protocols, laboratory procedures, and embryo culture conditions between the study groups. They were identified as eligible by the time of pregnancy diagnosis at our Assisted Reproduction Unit and invited to participate if they were planning to give birth at Barcelona Metropolitan Area, in order to guarantee an optimal follow-up.

Eligible SC singleton pregnancies from fertile couples (with a time-to-pregnancy no longer than 12 months) who attended our Maternal–Fetal Unit in the third trimester were invited to participate, being matched for maternal age (±1 year) with the IVF cases.

Enrollment stopped at 100 patients per group according to our prior sample-size calculation and all ultrasounds (USs) performed were included in the analysis. Reasons for not attending the third-trimester US were miscarriage, diagnosis of fetal anomaly, preterm delivery before the scan, move to another city, or withdrawn consent.

Ovarian stimulation protocols

The ovarian stimulation protocol for IVF and the gonadotropin doses were chosen according to age and ovarian reserve markers. Either long agonist or antagonist protocols were used. Ovarian stimulation was achieved with daily doses from 150 to 300 IU of FSHr (Gonal-F; Merck-Serono S.A., Madrid, Spain) alone or with the addition of 75 IU of LHr (Luveris; Merck-Serono S.A., Madrid, Spain) or HMG (Menopur, Ferring SA. Madrid, Spain). The hCG administration (Ovitrelle 250 mg s.c., Serono S.A.) was indicated in the presence of two or more follicles ≥18 mm of diameter, with ≥4 follicles measuring ≥14 mm in association with a consistent rise in serum estradiol concentration. US-guided transvaginal oocyte retrieval was performed 36 h after hCG administration.

Embryo culture and cryopreservation protocols

Embryo culture was carried out in microdrops of Global Media (Life Global, CooperSurgical Målov, Denmark) under mineral oil at 37°C in an atmosphere of 6.5% CO_2 and 7% O_2. Embryo quality was assessed according to the ASEBIR criteria (Balaban *et al.*, 2011). The quality of blastocysts was assessed according to the criteria of Gardner and Schoolcraft (Gardner *et al.*, 2000).

Vitrification and warming protocols of both cleavage embryos and blastocysts were performed using commercially available kits (Kitazato, Tokyo, Japan) according to the method described by Kuwayama (2007). After warming, embryos were cultured in a Global medium containing 10% protein substitute supplement (Life Global, CooperSurgical, Målov, Denmark) until ET. Cleavage embryos with at least 50% of their cells intact immediately after warming and further development after a 24 h culture period were considered as surviving embryos and transferred. Surviving blastocysts were defined as re-expanded or starting to re-expand within 2 h after warming.

ET protocols

FET was performed in all cases under endometrial preparation with transdermal estrogens (Evopad 50 mcg; Janssen, Toledo, Spain) with three patches replaced every 72 h, or oral estradiol valerate (Progynova 2 mg every 8 h; Bayer, Barcelona, Spain). Estrogen was started the first day of the cycle and US monitoring was performed after 12–15 days of treatment. Vaginal natural progesterone (200 mg every 8 h) was added when the endometrial thickness was ≥7 mm by US (Progeffik; Effik, Alcobendas, Spain; or Utrogestan; SEID, Barcelona, Spain). The first day of progesterone treatment was considered Day 0, cleavage embryos were thawed on Day 3 and transferred on Day 4, and blastocyst embryos were thawed and transferred on Day 5. Supplementation with estrogens and progesterone was performed until the 12th week of pregnancy. FET in natural cycles was not included in the present investigation to achieve more homogeneity in this group. Programmed cycles were the first therapeutic option at our center at the beginning of the present study.

In fresh ET cases, vaginal natural progesterone was started the morning after the oocyte retrieval with a dose of 200 mg every

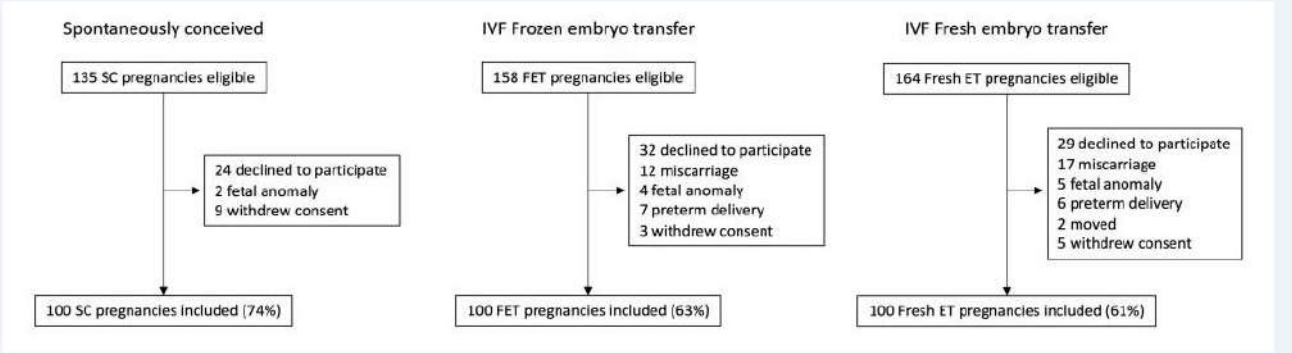

Figure 1. Flow diagram of the study populations. SC, spontaneously conceived; FET, frozen embryo transfer; ET, embryo transfer.

8 h. Cleavage embryos were transferred on Day 3 and blastocysts on Day 5.

Pregnancy was diagnosed by a positive serum β-hCG test 12 days after ET and a transvaginal ultrasonography was performed in all pregnancies at 5–6 weeks of gestation.

Data collection and study protocol

All participants underwent the same study protocol involving collection of baseline and perinatal characteristics, and third-trimester US including fetal biometry, feto-placental Doppler assessment, and fetal echocardiography.

Maternal epidemiological and obstetric parameters were collected by interview and review of medical records, including parental age, body mass index, ethnicity, socioeconomic status (illiterate or only primary educational level were considered low socioeconomic status), chronic diseases (e.g. hypertension, diabetes mellitus), parity, obstetric history, mode of conception, smoking status and illicit substance abuse (heroin, cocaine or cannabis) during pregnancy.

IVF-related parameters included etiology and length of infertility, diagnosis of polycystic ovarian syndrome (PCOS), number of previous failed ART cycles, ovarian stimulation data, number of oocytes retrieved, number of embryos obtained and transferred, endometrial preparation protocol, use of cryopreserved or fresh gametes, embryonic stage at the time of ET, vanishing twin detection or selective embryonic reduction.

Upon delivery, pregnancy and perinatal outcomes were recorded and included gestational age at delivery, mode of delivery, birth weight, Apgar score, umbilical artery pH, neonatal admission to ICU, perinatal morbidity and mortality, and the presence of pregnancy complications such as gestational diabetes, pregnancy-induced hypertension/PE/eclampsia, HELLP syndrome, cholestasis, placenta previa, placental abruption and PTB (<37 weeks of gestation). Gestational age was calculated according to first-trimester crown-rump length (Robinson, 1973). SGA and LGA were defined, respectively, as birth weight below 10th centile and above the 90th centile according to local standards (Figueras *et al.*, 2008).

PE was defined by new onset of hypertension of more than 140 mmHg systolic blood pressure or more than 90 diastolic blood pressure together with more than 300 mg proteins in 24 h urine (Walker, 2000). PE cases were classified as early (gestational age under 34 weeks at clinical onset) or late onset (34 weeks or over) (Valensise *et al.*, 2008).

Major neonatal morbidity was defined by the presence of bronchopulmonary dysplasia, necrotizing enterocolitis, intraventricular hemorrhage, periventricular leukomalacia, retinopathy, persistent ductus arteriosus, or sepsis. Minor neonatal morbidity was defined by the presence of respiratory distress, hyperbilirubinemia, or anemia. Perinatal mortality was defined by either intrauterine fetal death after 22 weeks of pregnancy or neonatal death within the first 28 days of life.

Fetal US

All pregnancies underwent ultrasonographic examination at 28–33 weeks of gestation using a Siemens Sonoline Antares (Siemens Medical Systems, Malvern, PA, USA) and or a Voluson 730 Expert (GE Medical Systems, Milwaukee, WI, USA) with 6–4 MHz linear curved-array and 2–10 MHz phased-array probes, including estimated fetal weight, feto-placental Doppler, and fetal echocardiography. US acquisitions were obtained by four maternal–fetal medicine specialists skilled in fetal echocardiography blinded to the mode of conception; the images were recorded as clips and anonymized, and all the measurements were performed offline by a single investigator to avoid bias.

Fetal biometry and feto-placental Doppler

Fetal weight was estimated by measuring the abdominal circumference, head circumference, biparietal diameter, and femur length, following the Hadlock formula (Hadlock *et al.*, 1985) and centile calculations, based on local reference curves (Figueras *et al.*, 2008). Feto-placental Doppler assessment included the measurement of pulsatility index of umbilical artery, middle cerebral artery, and ductus venosus (Arduini and Rizzo, 1990; Hecher *et al.*, 1994; Bhide *et al.*, 2013). Cerebroplacental ratio was calculated as middle cerebral artery pulsatility index (PI) divided by umbilical artery PI (Baschat and Gembruch, 2003). All estimations were done in the absence of fetal movements and, when required, with the mother in voluntary suspended ventilation.

Fetal echocardiography

A comprehensive two-dimensional, M-mode, and Doppler echocardiographic examination was performed to assess structural heart integrity, to rule out cardiac defects, and to evaluate cardiac morphometry and function following standard protocols (Carvalho *et al.*, 2013; Crispi *et al.*, 2013; García-Otero *et al.*, 2020). Cardiac, thoracic, and ventricular areas and diameters were measured on 2-dimensional images from an apical or basal four-chamber view at end diastole. Ventricular sphericity indices were calculated by dividing the longitudinal by basal-transverse ventricular diameters. Atrial areas were measured at maximum distension from an apical or basal four-chamber view. Atria-to-heart ratios were calculated as atrial area * 100/heart area. Myocardial wall thicknesses were measured on 2-dimensional images from a transverse four-chamber view. Relative wall thickness was calculated as septal plus left wall thickness divided by left ventricular end-diastolic diameter (Foppa *et al.*, 2005). Left ventricular ejection fraction (%) was obtained from M-mode transverse four-chamber view using the Teichholz's formula. Mitral/tricuspid ring displacements (MAPSE/TAPSE) were assessed by M-mode from an apical or basal four-chamber view. Tissue Doppler Imaging was applied in the spectral Doppler mode to record systolic (S'), early diastolic (E'), and atrial contraction (A') peak velocities at mitral and tricuspid annuli from an apical or basal four-chamber view and measured in real time. Mitral and tricuspid early (E) and late (A) ventricular filling were obtained by Doppler, and E/A ratios were calculated. The left myocardial performance index was obtained from a single Doppler spectrum in a cross-sectional image of the fetal thorax, calculated as (ICT + IRT)/ET (Hernandez-Andrade *et al.*, 2005).

Statistical analysis

Data were analyzed by means of the statistical software STATA 15.1 (StataCorp LLC, College Station, TX, USA). The study outcome was fetal cardiovascular assessment. The independent variable of interest was the mode of conception (spontaneous, FET, or fresh ET). Descriptive statistics and results were expressed as mean (± standard deviation), median (interquartile range), or number (percentage), as

appropriate. The normal distribution of continuous variables was checked using the Shapiro–Wilk test and histograms. Comparisons among the study groups were assessed by ANOVA or Kruskal–Wallis with Bonferroni correction, after checking the fulfillment of each test assumptions. Differences between groups on feto-placental data and cardiovascular parameters were assessed by using multiple regression analyses to adjust by maternal age, nulliparity, birthweight centile, PE, and preterm delivery. Additionally, *P*-value for the linear trend was calculated by means of the Jonckheere–Terpstra test. All reported *P*-values are two-sided. The level of significance was set at 0.05 for all the statistical tests.

Ethical approval

This clinical study was conducted according to the Declaration of Helsinki for Medical Research involving Human Subjects (World Medical Association, 2013); the study protocol was approved by the local ethics committee (HCB/2017/0714). All patients agreeing to participate provided their written informed consent.

Results

Baseline and perinatal characteristics of the study population

Baseline characteristics of the study groups are shown in Table I. The study groups were similar in terms of parental baseline characteristics except for a higher rate of nulliparity in the IVF groups and an increased rate of PCOS in the FET group as compared to the fresh ET and SC pregnancies. Infertility causes and the number and stage of embryos transferred were similar between the IVF study groups. A freeze-all strategy was applied in 46% of included FET cycles.

IVF patients had two US scans prior to their 11–13 weeks scan, but this early assessment was lacking in most SC pregnancies, who in general had their first US check-up during or after the 11th week. Taking that early data into account, the vanishing twin phenomenon rate was 9% for FET and 11% for fresh ET. As far as we know, there were no vanishing twin cases within the SC group, but we acknowledge that this characteristic could have been underestimated in this population due to the timing of the first US performed.

Perinatal outcomes are shown in Table II. FET pregnancies had a higher occurrence of pregnancy complications, such as gestational diabetes as compared to SC pregnancies and PE as compared to fresh ET pregnancies. Delivery and neonatal characteristics were similar among the study groups except for a higher rate of induction of labor and cesarean section in the FET population as compared to the fresh ET and SC groups and a higher rate of minor morbidity in the FET neonates as compared to the SC group. There was one single case of perinatal mortality in the study population, corresponding to a stillbirth of unknown cause at 39 weeks of gestation in the FET group.

Fetal cardiac results

Gestational age at assessment, estimated fetal weight and feto-placental Doppler were similar between groups (Table III).

Both FET and fresh ET groups showed significant signs of fetal cardiac remodeling and suboptimal function, with more pronounced changes in the fresh ET as compared to the FET (Table IV and Fig. 2). Both groups of fetuses conceived by ART showed larger hearts and atria, increased myocardial thickness and more spherical ventricles as compared to SC pregnancies. Cardiac morphometric changes were more pronounced in the fresh ET group as compared to FET.

Fetuses conceived by ART showed reduced left ejection and shortening fraction. Both fresh ET and FET groups also presented signs of reduced myocardial motility (ring displacement and annular S′ peak wave velocities) as compared with SC subjects, with a significant *P*-value for linear trend toward lower values in the fresh ET group compared to the FET population. Both IVF groups also presented signs of suboptimal diastolic function with reduced diastolic (E′ and A′) peak wave velocities in mitral and tricuspid annulus as compared to SC pregnancies. Heart rate and left myocardial performance index were significantly increased in both FET and fresh ET groups as compared to the SC group. Suboptimal cardiac function was also more pronounced in the fresh ET group with significant *P*-values for linear trend toward worse results for fresh ET as compared to FET.

Adjusted mean differences for all of the cardiac parameters assessed are shown in Supplementary Table SI.

Discussion

We report significant fetal cardiac morphometric and functional changes in FET. These prenatal changes are similar to those previously reported for fresh ET, albeit of a milder magnitude.

This study describes a similar pattern of cardiac remodeling and suboptimal function in fetuses conceived by IVF both with FET and fresh ET. These fetuses showed larger atria, thicker and more spherical ventricles, and signs of both suboptimal systolic and diastolic function. A similar pattern could be observed in both groups of ART, although fetuses from FET displayed milder changes as compared to those from fresh ET. The fetal cardiac pattern here described is consistent with previous cardiovascular reports in fetuses and children conceived by ART. We had previously demonstrated more globular and thicker hearts with dilated atria and suboptimal function in a cohort of 100 fetuses conceived by ART of which 90% were from IVF with fresh ET (Valenzuela-Alcaraz *et al.*, 2013). These changes were present both in singleton and twins (Valenzuela-Alcaraz *et al.*, 2018) and persisted postnatally up to 3 years of age (Valenzuela-Alcaraz *et al.*, 2013, 2019). The present data are also consistent with numerous studies reporting increased blood pressure and pulmonary artery pressure (Ceelen *et al.*, 2008; Scherrer *et al.*, 2012; von Arx *et al.*, 2015; Meister *et al.*, 2018; Forton *et al.*, 2019) and suboptimal cardiac function (Zhou *et al.*, 2014; Liu *et al.*, 2015; von Arx *et al.*, 2015) in children and adolescents conceived by ART (Guo *et al.*, 2017). The underlying pathophysiology of the observed cardiac features is not clear, but cardiac changes observed in ART offspring could be explained by an increase in vascular stiffness (Mayet and Hughes, 2003; Scherrer *et al.*, 2015) leading to cardiac pressure overload. The fetal heart usually adapts to pressure overload by hypertrophying the ventricles, dilating the atria, and impairing function (Crispi *et al.*, 2020). Overall, our data are in line with the previously reported cardiovascular changes in ART offspring. The novelty of the present study is the inclusion of a large separate cohort of fetuses conceived by IVF with

Table I Baseline and infertility characteristics of the study groups.

	SC (n = 100)	FET (n = 100)	Fresh ET (n = 100)
Maternal characteristics			
Age (year)	36.0 (33.8–38.5)	36.3 (33.9–38.8)	36.5 (34.5–38.6)
Body mass index (kg/m^2)	22.5 (20.5–24.7)	23.4 (20.6–25.7)	23.4 (21.2–26.0)
Smoking habit (%)	18	15	17
Caucasian (%)	81	81	86
Nulliparity (%)	54	74*	79*
Polycystic ovarian syndrome (%)	4	33*†	10
Paternal characteristics			
Age (year)	37.5 (34.2–41.5)	38.6 (35.4–41.6)	38.3 (36.7–40.8)
Body mass index (kg/m^2)	25.1 (23.4–27.1)	25.8 (23.6–28.4)	25.6 (23.9–28.0)
Smoking habit (%)	20	33	30
Caucasian (%)	74	79	87
Infertility and ART characteristics			
Infertility cause (%)			
Unexplained infertility		31	36
Endometriosis		8	13
Tubal factor		10	11
Male factor		56	44
Previous failed ET cycles (N)		0.7 (±0.9)	0.5 (±0.9)
Oocytes retrieved (N)		13 (10–17)†	8 (6–10)
Embryos obtained (N)**		9 (6–11)†	5 (3–6)
Transferred embryos (N)		1.8 (±0.6)	1.9 (±0.4)
ET at cleavage stage (%)		72	82

Data are mean (± SD), median (interquartile range), or percentage (%), as appropriate.
*$P < 0.05$ as compared to SC.
†$P < 0.05$ as compared to fresh ET.
**Two pronuclear stages.
SC, spontaneously conceived pregnancies; FET, frozen embryo transfer; ET, embryo transfer.

FET enabling us to describe the cardiac changes in this subgroup identifying a similar but milder pattern.

The mechanisms driving fetal cardiac changes in ART fetuses remain to be elucidated. Several potential factors have been postulated as inductors of perinatal environmental changes mediated by mechanic or epigenetic mechanisms: parental factors, infertility *per se*, ovarian stimulation and its impact on the oocyte and the endometrium, embryo culture conditions, and laboratory techniques (Berntsen *et al.*, 2019). The milder cardiac features observed in FET could be partially explained by the laboratory procedures involved in FET compared to fresh ET. In FET, the vitrification process involves an early supplementary manipulation of embryos, which are exposed to high concentrations of cryoprotectants and a later process of thawing and culture. This process has raised concerns about possible negative health effects for the offspring but could also be considered an embryo quality selection process (or a selection process of evolutive embryos). On the other hand, these embryos would be replaced in an endometrium not exposed to the effects of ovarian stimulation, in more physiological conditions. In order to optimize the homogeneity of the FET group, our study only included cases of FET in programmed cycles, involving hypothalamic-pituitary suppression after the administration of estrogen and progesterone to prepare the endometrium, which prevents the development of the corpus luteum. FET in a natural cycle is performed in normo-ovulatory women, while in anovulatory patients the most common strategy is FET with endometrial preparation (Mackens *et al.*, 2017). Pregnancies resulting from FET in programmed cycles have an increased risk of PE, probably attributable to the release of vasoactive factors from the corpus luteum such as relaxin (Conrad *et al.*, 2019; Ernstad *et al.*, 2019; von Versen-Höynck *et al.*, 2019). These baseline characteristics could be associated with differences in perinatal outcomes and potentially influence the fetal cardiovascular parameters. In fact, the coexistence of pregnancy complications is another factor that could influence fetal cardiac morphology and function. While fresh ET has been extensively associated with SGA, FET subjects show higher rates of LGA and macrosomy (Berntsen and Pinborg, 2018; Litzky *et al.*, 2018; Ernstad *et al.*, 2019; Pinborg, 2019). First-trimester placental volume assessed by 3-dimensional US is reduced in IVF pregnancies and is significantly lower in fresh ET pregnancies as compared to FET (Rizzo *et al.*, 2016). However, FET gestations are more likely to develop PE (Sazonova *et al.*, 2012; Opdahl *et al.*, 2015; Maheshwari

Table II Perinatal characteristics of the study groups.

	SC (n = 100)	FET (n = 100)	Fresh ET (n = 100)
Pregnancy complications			
Vanishing twin phenomenon (%)	0	9	11
Preterm delivery (%)	5	9	5
Spontaneous preterm delivery (%)	2	7	5
Iatrogenic preterm delivery (%)	3	2	0
Preeclampsia (%)	4	11†	3
Early preeclampsia (%)	0	4	1
Late preeclampsia (%)	4	7	2
Gestational diabetes (%)	3	13*	8
Placenta previa (%)	1	3	3
Abruptio placentæ (%)	0	1	0
Prenatal corticoid exposure (%)	3	5	6
Prenatal aspirin exposure (%)	9	9	7
Delivery data			
Gestational age at birth (weeks, days)	40.0 (39.0–40.5)	39.4 (38.4–40.4)	39.5 (38.5–40.5)
Birthweight (grams)	3290 (3030–3500)	3270 (2915–3620)	3215 (2890–3610)
Female (%)	53	45	40
Birthweight centile	40 (22–66)	41 (18–76)	36 (16–66)
Small-for-gestational age (%)	13	15	17
Large-for-gestational age (%)	6	15	8
Induction of labor (%)	30	54*†	40
Cesarean section (%)	26	48*†	30
Neonatal outcome			
Admission to NICU (%)	5	7	5
Days at NICU (days)	4 (2–6)	9 (4–23)	6 (3–15)
Neonatal morbidity (%)	5	18*	9
Minor neonatal morbidity	4	17*	8
Major neonatal morbidity	1	1	1
Neontal mortality (%)	0	0	0

Data are median (interquartile range) or percentage (%).
*$P < 0.05$ as compared to spontaneously conceived.
†$P < 0.05$ as compared to fresh ET.
SC, spontaneously conceived pregnancies; FET, frozen embryo transfer; ET, embryo transfer; NICU, neonatal intensive care unit.

Table III Feto-placental data at scan.

	SC (n = 100)	FET (n = 100)	Fresh ET (n = 100)	P-value
Gestational age (weeks, days)	30,0 (28,5–33,6)	31,1 (29,1–32,6)	30,3 (29,4–31,5)	0.645
Estimated fetal weight (g)	1508 (1314–2028)	1652 (1415–1968)	1570 (1424–1895)	0.472
Estimated fetal weight centile	48 (22–80)	59 (18–79)	56 (22–75)	0.919
Cerebroplacental ratio (z-score)	−0.24 (±1.07)	−0.09 (±0.97)	0.03 (±1.09)	0.223
Ductus venosus PI (z-score)	−0.49 (−1.15–0.09)	−0.80 (−1.43–0.12)	−0.53 (−1.06–0.14)	0.329

Data are mean (± SD) or median (interquartile range).
SC, spontaneously conceived pregnancies; FET, frozen embryo transfer; ET, embryo transfer; PI, pulsatility index.

Table IV Fetal cardiac assessment of the study groups.

	SC (n = 100)	FET (n = 100)	Fresh ET (n = 100)	Crude P-value	Adjusted P-value¥	Linear trend P-value¥
Fetal echocardiography						
Fetal cardiac morphometry§						
Cardio-thoracic Ratio	0.27 ± 0.05	0.29 ± 0.05*	0.29 ± 0.05*	0.044	<0.001	<0.001
Left atrium/heart area ratio	15.39 ± 3.06	15.86 ± 3.84*	16.63 ± 3.66*‡	0.069	<0.001	<0.001
Right atrium/heart area ratio	17.29 ± 3.19	17.95 ± 3.86*	18.12 ± 3.18*‡	0.226	<0.001	<0.001
Left ventricular sphericity index	1.87 ± 0.29	1.82 ± 0.28*†	1.83 ± 0.29*	0.438	<0.001	<0.001
Right ventricular sphericity index	1.68 ± 0.26	1.61 ± 0.25*†	1.62 ± 0.29*	0.219	<0.001	<0.001
Left-free wall thickness (mm)	3.33 ± 0.87	3.86 ± 0.83*	3.97 ± 0.80*‡	<0.001	<0.001	<0.001
Septal wall thickness (mm)	3.07 ± 0.89	3.37 ± 0.64*	3.36 ± 0.70*	0.008	0.003	0.001
Relative wall thickness	0.65 ± 0.25	0.74 ± 0.22*	0.79 ± 0.21*‡	0.001	<0.001	<0.001
Systolic function						
Left ejection fraction (%)	63.73 ± 9.98	62.79 ± 11.36*	61.61 ± 11.41*‡	0.434	<0.001	<0.001
Left shortening fraction (%)	37.24 ± 8.10	36.53 ± 8.93*	35.67 ± 9.22*‡	0.468	<0.001	<0.001
Mitral ring displacement (mm)	5.33 ± 1.07	5.31 ± 0.89	5.16 ± 0.91*‡	0.424	<0.001	<0.001
Tricuspid ring displacement (mm)	7.14 ± 1.32	7.27 ± 1.50*	7.02 ± 1.13*‡	0.400	<0.001	0.003
Mitral S′ (cm/s)	6.72 ± 1.09	6.44 ± 1.17*	6.47 ± 0.97*	0.166	<0.001	<0.001
Tricuspid S′ (cm/s)	7.58 ± 1.32	7.41 ± 1.19*	7.17 ± 1.22*‡	0.091	<0.001	<0.001
Diastolic function						
Mitral E/A ratio	0.76 ± 0.13	0.77 ± 0.11*	0.78 ± 0.11*‡	0.507	<0.001	<0.001
Mitral E′ (cm/s)	7.39 ± 1.32	6.92 ± 0.98*†	7.32 ± 1.34	0.040	<0.001	0.338
Mitral A′ (cm/s)	9.88 ± 2.36	8.92 ± 1.99*†	9.37 ± 2.21*	0.022	<0.001	<0.001
Tricuspid E/A ratio	0.79 ± 0.16	0.77 ± 0.13*†	0.78 ± 0.14*	0.670	<0.001	0.002
Tricuspid E′ (cm/s)	8.33 ± 1.42	8.07 ± 1.63*	8.08 ± 1.48*	0.434	<0.001	<0.001
Tricuspid A′ (cm/s)	10.98 ± 1.82	10.76 ± 2.10*	10.69 ± 2.09*	0.599	<0.001	<0.001
Heart rate and timing						
Heart rate (bpm)	141 ± 8	141 ± 10	142 ± 8*‡	0.330	<0.001	<0.001
Left myocardial performance index	0.50 ± 0.09	0.53 ± 0.08*	0.53 ± 0.08*‡	0.028	<0.001	<0.001

Data are mean ± SD.
§Cardiac morphometry results measured at end diastole, except for atrial areas.
¥Comparisons adjusted by linear regression analyses for maternal age, nulliparity, birthweight centile, preeclampsia, and preterm birth.
*$P < 0.05$ as compared with spontaneously conceived.
†$P < 0.05$ as compared to fresh ET.
‡$P < 0.05$ as compared to FET.
SC, spontaneously conceived pregnancies; FET, frozen embryo transfer; ET, embryo transfer.

et al., 2018; Roque et al., 2018; Sha et al., 2018; Ernstad et al., 2019; Hwang et al., 2019). A recent cumulative meta-analysis (Maheshwari et al., 2018) revealed that fresh ET singletons show a higher relative risk of PTB and SGA, whereas FET face an increased risk of hypertensive disorders of pregnancy and LGA. Being born SGA, preterm or under PE can be associated with fetal cardiac remodeling *per se* (Crispi et al., 2020). Our group has previously evaluated the potential interaction of ART and SGA, identifying a distinct fetal cardiac pattern in each condition, suggesting that they are independent causes of cardiac programming (Valenzuela-Alcaraz et al., 2017). However, although all cardiovascular comparisons were adjusted by birthweight centile, prematurity and PE, we acknowledge that the differential coexistence of pregnancy complications associated with ART may also account for the different degree of the cardiac changes observed in FET versus fresh ET. With regards to the vanishing twin phenomenon, which is also related to impaired perinatal results, adjusted mean differences for the fetal cardiac parameters assessed when excluding this condition replicate the above-mentioned results and are shown in Supplementary Table SII.

Strengths and limitations

This paper has some strengths and limitations that merit comment. This is a very well-phenotyped prospective cohort study recruited from conception up to birth, including complete fetal and perinatal data, with all IVF pregnancies enrolled from a single center. We used a well-defined and strict methodology for measuring fetal cardiac dimensions and function in order to achieve the optimal accuracy and reproducibility (Crispi et al., 2013; García-Otero et al., 2020).

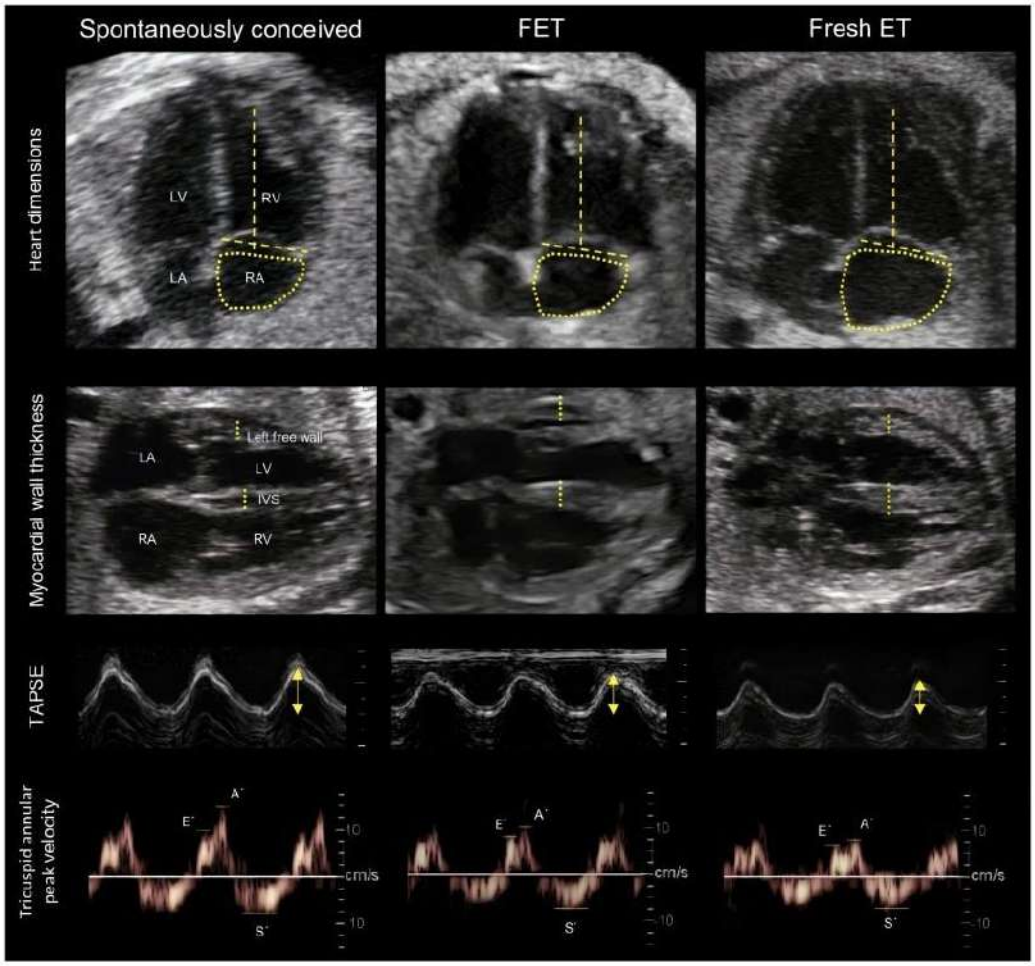

Figure 2. Illustrative echocardiographic images in fetuses spontaneously conceived, and those conceived by ARTs with frozen embryo transfer (FET) and fresh embryo transfer (ET). Top: two-dimensional apical and transverse four-chamber views at end diastole illustrating the more prominent right atria, spherical ventricles, and thicker myocardial walls. Bottom: M-mode and tissue Doppler waves illustrating the linear trend toward a decrease in tricuspid ring displacement (TAPSE) and early diastolic (E '), late diastolic (A ') and systolic (S ') tricuspid annular peak velocities from fetuses after FET and fresh ET as compared to those spontaneously conceived. RA, right atrium; LA, left atrium; RA, right ventricle; LV, left ventricle; IVS, interventricular septum.

As limitations, we first acknowledge that research into fetal outcomes in ART faces difficulty in unraveling the contribution of infertility *per se* from the ART procedure. In the current study, although main infertility causes were equally distributed in both ART groups, we have compared IVF populations with spontaneous conceived fetuses from fertile couples. If parental factors such as infertility influence the offspring's health, then fetuses conceived naturally by subfertile parents (with a time-to-pregnancy of more than 1 year) may constitute a more appropriate control group. However, subfertility is a condition often underreported in our center's clinical records and consequently, we found it more difficult to identify and recruit these patients. We also acknowledge that the fetal outcome could be influenced by the vanishing twin phenomenon, so a subanalysis was performed after excluding these cases, obtaining similar echocardiographic results in the study groups (Supplementary Table SII). Regarding the maternal baseline characteristics, we must consider the higher rate of nulliparity in the IVF groups as compared to the SC. Although analyses were adjusted by parity, we acknowledge that parity could not be fully discarded as a potential confounder. There is also a higher rate of PCOS in the FET population as compared to the fresh ET and fertile groups. PCOS patients are more likely to undergo the elective cryopreservation of all embryos because of their risk of developing ovarian hyperstimulation syndrome (freeze-all strategy), and FET is usually performed with endometrial preparation because they are anovulatory patients, so these factors would explain the higher rate of PCOS in FET group. However, milder and ovulatory PCOS phenotypes could have been underreported within the fertile group. Nevertheless, the results are replicated when including PCOS among the adjustment

variables in our dataset. In addition, this study only included cases of FET in programmed cycles, as this was the most common approach for FET at the beginning of the study, in order to optimize the homogeneity of the FET group. Hence, these results may not be extrapolated to patients undergoing FET in natural cycles and further studies are needed to investigate fetal cardiovascular features in these pregnancies. We also acknowledge a potential selection bias as approximately 20% per group refused to participate when invited (Fig. 1). In any case, the baseline characteristics of all invited and not included patients were similar to the included population (Supplementary Table SIII). Although the mass-significance effect has been attenuated by applying Bonferroni corrections to each comparison, we cannot exclude that some could be due to chance. Finally, it is important to highlight that the cardiac changes reported here are subclinical, with most cardiovascular indexes lying within normal ranges. The long-term health implications remain unclear: although these features are recognized as potential cardiovascular risk factors, their association with the adult cardiovascular disease remains to be proven.

Clinical relevance and conclusions

Our findings provide additional evidence for the existence of fetal cardiovascular programming in both fresh and FET IVF pregnancies. Given the young age of the ART population, we do not know yet how these cardiovascular features will evolve nor their potential long-term consequences. Therefore, future follow-up studies are warranted to assess its potential impact on future cardiovascular health. If they persisted, these prenatal changes may condition higher susceptibility for cardiovascular disease later in life, which might benefit from early preventive measures.

The milder features found in FET fetuses cannot condition the choice of technique *per se* and must be considered together with the global perinatal results related to these gestations. However, it shows the importance of increasing our knowledge on fetal cardiovascular health in ART so as to determine which technique offers more safety. Future research is warranted to better elucidate the mechanisms underlying fetal cardiac adaptation in subfertile populations and with different assisted reproduction conditions and procedures.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

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

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Authors' roles

Study design: M.L.B., G.C., F.C., and E.G.; patient's enrolment: M.L.B., G.C., B.V.-A., F.C. A.B., and D.M.; data collection: M.L.B., G.C., B.V.-A., F.C., and M.S.C.; US acquisitions: M.L.B., B.V.-A., F.C., and F.C.; US offline measurements: M.L.B.; data analysis: M.L.B., B.V.-A., G.C., and F.C.; interpretation of data and critical discussion: all authors; manuscript drafting: M.L.B., G.C., and F.C.; and critical revision of the manuscript: all authors.

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Conflict of interest

The authors have no conflicts of interest to declare.

References

Arduini D, Rizzo G. Normal values of pulsatility index from fetal vessels: a cross-sectional study on 1556 healthy fetuses. *J Perinat Med* 1990;**18**:165–172.

Arx RV, Allemann Y, Sartori C, Rexhaj E, Cerny D, Marchi S. D, Soria R, Germond M, Scherrer U, Rimoldi SF. Right ventricular dysfunction in children and adolescents conceived by assisted reproductive technologies. *J Appl Physiol (1985)* 2015;**118**:1200–1206.

Balaban B, Brison D, Calderón G, Catt J, Conaghan J, Cowan L, Ebner T, Gardner D, Hardarson T, Lundin K. *et al.* Istanbul consensus workshop on embryo assessment: Proceedings of an expert meeting. *Reprod Biomed Online* 2011;**22**:632–646.

Baschat AA, Gembruch U. The cerebroplacental Doppler ratio revisited. *Ultrasound Obstet Gynecol* 2003;**21**:124–127.

Berntsen S, Pinborg A. Large for gestational age and macrosomia in singletons born after frozen/thawed embryo transfer (FET) in assisted reproductive technology (ART). *Birth Defects Res* 2018;**110**:630–643.

Berntsen S, Söderström-Anttila V, Wennerholm U-B, Laivuori H, Loft A, Oldereid NB, Bente Romundstad L, Bergh C, Pinborg A. The health of children conceived by ART: "the chicken or the egg?". *Hum Reprod Update* 2019;**25**:137–158.

Bhide A, Acharya G, Bilardo CM, Brezinka C, Cafici D, Hernandez-Andrade E, Kalache K, Kingdom J, Kiserud T, Lee W. *et al.* ISUOG

practice guidelines: use of Doppler ultrasonography in obstetrics. *Ultrasound Obstet Gynecol* 2013;**41**:233–239.

Carvalho J, Allan L, Chaoui R, Copel J, DeVore G, Hecher K, Lee W, Munoz H, Paladini D, Tutschek B. *et al.* ISUOG Practice Guidelines (updated): sonographic screening examination of the fetal heart. *Ultrasound Obstet Gynecol* 2013;**41**:348–359.

Ceelen M, Weissenbruch MM, Van Vermeiden JPW, Leeuwen FE, Van Delemarre-Van De Waal, HA. Cardiometabolic differences in children born after in vitro fertilization: follow-up study. *J Clin Endocrinol Metab* 2008;**93**:1682–1688.

Chen Z-J, Shi Y, Sun Y, Zhang B, Liang X, Cao Y, Yang J, Liu J, Wei D, Weng N *et al.* Fresh versus frozen embryos for infertility in the polycystic ovary syndrome. *N Engl J Med* 2016;**375**:523–533.

Conrad KP, Graham GM, Chi Y-Y, Zhai X, Li M, Stan Williams R, Rhoton-Vlasak A, Segal MS, Wood CE, Keller-Wood M. Potential influence of the corpus luteum on circulating reproductive and volume regulatory hormones, angiogenic and immunoregulatory factors in pregnant women. *Am J Physiol Endocrinol Metab* 2019;**317**: e677–e685.

Crispi F, Sepúlveda-Martínez Á, Crovetto F, Gómez O, Bijnens B, Gratacós E. Main patterns of fetal cardiac remodeling. *Fetal Diagn Ther* 2020;**47**:337–344.

Crispi F, Valenzuela-Alcaraz B, Cruz-Lemini M, Gratacós E. Ultrasound assessment of fetal cardiac function. *Australas J Ultrasound Med* 2013;**16**:158–167.

Ernstad EG, Wennerholm U-B, Khatibi A, Petzold M, Bergh C. Neonatal and maternal outcome after frozen embryo transfer: increased risks in programmed cycles. *Am J Obstet Gynecol* 2019; **221**:126.e1–126.e18.

Figueras F, Meler E, Iraola A, Eixarch E, Coll O, Figueras J, Francis A, Gratacos E, Gardosi J. Customized birthweight standards for a Spanish population. *Eur J Obstet Gynecol Reprod Biol* 2008;**136**: 20–24.

Foppa M, Duncan BB, Rohde LEP. Echocardiography-based left ventricular mass estimation. How should we define hypertrophy? *Cardiovasc Ultrasound* 2005;**3**:17.

Forton K, Motoji Y, Pezzuto B, Caravita S, Delbaere A, Naeije R, Faoro V. Decreased pulmonary vascular distensibility in adolescents conceived by in vitro fertilization. *Hum Reprod* 2019;**34**: 1799–1808.

García-Otero L, Gómez O, Rodriguez-López M, Torres X, Soveral I, Sepúlveda-Martínez Á, Guirado L, Valenzuela-Alcaraz B, López M, Martínez JM *et al.* Nomograms of fetal cardiac dimensions at 18–41 weeks of gestation. *Fetal Diagn Ther* 2020;**47**:387–312.

Gardiner HM, Pasquini L, Wolfenden J, Barlow A, Li W, Kulinskaya E, Henein M. Myocardial tissue Doppler and long axis function in the fetal heart. *Int J Cardiol* 2006;**113**:39–47.

Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertil Steril* 2000;**73**:1155–1158.

Guo XY, Liu XM, Jin L, Wang TT, Ullah K, Sheng JZ, Huang HF. Cardiovascular and metabolic profiles of offspring conceived by assisted reproductive technologies: a systematic review and meta-analysis. *Fertil Steril* 2017;**107**:622–631.e5.

Hadlock FP, Harrist RB, Sharman RS, Deter RL, Park SK. Estimation of fetal weight with the use of head, body, and femur measurements—a prospective study. *Am J Obstet Gynecol* 1985; **151**:333–337.

Hecher K, Campbell S, Snijders R, Nicolaides K. Reference ranges for fetal venous and atrioventricular blood flow parameters. *Ultrasound Obstet Gynecol* 1994;**4**:381–390.

Hernandez-Andrade E, López-Tenorio J, Figueroa-Diesel H, Sanin-Blair J, Carreras E, Cabero L, Gratacos E. A modified myocardial performance (Tei) index based on the use of valve clicks improves reproducibility of fetal left cardiac function assessment. *Ultrasound Obstet Gynecol* 2005;**26**:227–232.

Hwang SS, Dukhovny D, Gopal D, Cabral H, Diop H, Coddington CC, Stern JE. Health outcomes for Massachusetts infants after fresh versus frozen embryo transfer. *Fertil Steril* 2019;**112**:900–907.

Kuwayama M. Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method. *Theriogenology* 2007;**67**:73–80.

Litzky JF, Boulet SL, Esfandiari N, Zhang Y, Kissin DM, Theiler RN, Marsit CJ. Effect of frozen/thawed embryo transfer on birthweight, macrosomia, and low birthweight rates in US singleton infants. *Am J Obstet Gynecol* 2018;**218**:433.e1–433.e10.

Liu H, Zhang Y, Gu HT, Feng QL, Liu JY, Zhou J, Yan F. Association between assisted reproductive technology and cardiac alteration at age 5 years. *JAMA Pediatr* 2015;**169**:603–605.

Mackens S, Santos-Ribeiro S, Vijver A, Van De Racca A, Landuyt L, Van Tournaye H, Blockeel C. Frozen embryo transfer: a review on the optimal endometrial preparation and timing. *Hum Reprod* 2017;**32**:2234–2242.

Maheshwari A, Pandey S, Raja EA, Shetty A, Hamilton M, Bhattacharya S. Is frozen embryo transfer better for mothers and babies? Can cumulative meta-analysis provide a definitive answer? *Hum Reprod Update* 2018;**24**:35–58.

Mayet J, Hughes A. Pathophysiology in hypertension. *Heart* 2003;**89**: 1104–1109.

Meister TA, Rimoldi SF, Soria R, von AR, Messerli FH, Sartori C, Scherrer U, Rexhaj E. Association of assisted reproductive technologies with arterial hypertension during adolescence. *J Am Coll Cardiol* 2018;**72**:1267–1274.

Opdahl S, Henningsen AA, Tiitinen A, Bergh C, Pinborg A, Romundstad PR, Wennerholm UB, Gissler M, Skjærven R, Romundstad LB. Risk of hypertensive disorders in pregnancies following assisted reproductive technology: a cohort study from the CoNARTaS group. *Hum Reprod* 2015;**30**:1724–1731.

Pandey S, Shetty A, Hamilton M, Bhattacharya S, Maheshwari A. Obstetric and perinatal outcomes in singleton pregnancies resulting from IVF/ICSI: a systematic review and meta-analysis. *Hum Reprod Update* 2012;**18**:485–503.

Pinborg A. Short- and long-term outcomes in children born after assisted reproductive technology. *BJOG* 2019;**126**:145–148.

Pinborg A, Loft A, Schmidt L, Andersen AN. Morbidity in a Danish National cohort of 472 IVF/ICSI twins, 1132 non-IVF/ICSI twins and 634 IVF/ICSI singletons: health-related and social implications for the children and their families. *Hum Reprod* 2003;**18**: 1234–1243.

Rizzo G, Aiello E, Pietrolucci ME, Arduini D. Are there differences in placental volume and uterine artery Doppler in pregnancies resulting from the transfer of fresh versus frozen-thawed embryos through in vitro fertilization. *Reprod Sci* 2016;**23**:1381–1386.

Robinson HP. Sonar measurement of fetal crown-rump length as means of assessing maturity in first trimester of pregnancy. *Br Med J* 1973;**4**:28–31.

Roque M, Valle M, Sampaio M, Geber S. Obstetric outcomes after fresh versus frozen-thawed embryo transfers: a systematic review and meta-analysis. *J Bras Reprod Assist* 2018;**22**:253–260.

Sazonova A, Källen K, Thurin-Kjellberg A, Wennerholm U-B, Bergh C. Obstetric outcome in singletons after in vitro fertilization with cryopreserved/thawed embryos. *Hum Reprod* 2012;**27**: 1343–1350.

Scherrer U, Rexhaj E, Allemann Y, Sartori C, Rimoldi SF. Cardiovascular dysfunction in children conceived by assisted reproductive technologies. *Eur Heart J* 2015;**36**:1583–1589.

Scherrer U, Rimoldi SF, Rexhaj E, Stuber T, Duplain H, Garcin S, de Marchi SF, Nicod P, Germond M, Allemann Y. *et al.* Systemic and pulmonary vascular dysfunction in children conceived by assisted reproductive technologies. *Circulation* 2012;**125**:1890–1896.

Sha T, Yin X, Cheng W, Massey IY. Pregnancy-related complications and perinatal outcomes resulting from transfer of cryopreserved versus fresh embryos in vitro fertilization: a meta-analysis. *Fertil Steril* 2018;**109**:330–342.e9.

Shi Y, Sun Y, Hao C, Zhang H, Wei D, Zhang Y, Zhu Y, Deng X, Qi X, Li H *et al.* Transfer of fresh versus frozen embryos in ovulatory women. *N Engl J Med* 2018;**378**:126–136.

Valensise H, Vasapollo B, Gagliardi G, Novelli GP. Early and Late preeclampsia: two different maternal hemodynamic states in the latent phase of the disease. *Hypertension* 2008;**52**:873–880.

Valenzuela-Alcaraz B, Crispi F, Bijnens B, Cruz-Lemini M, Creus M, Sitges M, Bartrons J, Civico S, Balasch J, Gratacós E. Assisted reproductive technologies are associated with cardiovascular remodeling in utero that persists postnatally. *Circulation* 2013;**128**: 1442–1450.

Valenzuela-Alcaraz B, Crispi F, Cruz-Lemini M, Bijnens B, García-Otero L, Sitges M, Balasch J, Gratacós E. Differential effect of assisted reproductive technology and small-for-gestational age on fetal cardiac remodeling. *Ultrasound Obstet Gynecol* 2017;**50**:63–70.

Valenzuela-Alcaraz B, Cruz-Lemini M, Rodríguez-López M, Goncé A, García-Otero L, Ayuso H, Sitges M, Bijnens B, Balasch J, Gratacós E. *et al.* Fetal cardiac remodeling in twin pregnancy conceived by assisted reproductive technology. *Ultrasound Obstet Gynecol* 2018; **51**:94–100.

Valenzuela-Alcaraz B, Serafini A, Sepulveda-Martínez A, Casals G, Rodríguez-López M, Garcia-Otero L, Cruz-Lemini M, Bijnens B, Sitges M, Balasch J. *et al.* Postnatal persistence of fetal cardiovascular remodelling associated with assisted reproductive technologies: a cohort study. *BJOG* 2019;**126**:291–298.

von Versen-Höynck F, Narasimhan P, Selamet Tierney ES, Martinez N, Conrad KP, Baker VL, Winn VD. Absent or excessive corpus luteum number is associated with altered maternal vascular health in early pregnancy. *Hypertension* 2019;**73**:680–690.

Walker JJ. Pre-eclampsia. *Lancet* 2000;**356**:1260–1265.

Wennerholm U-B, Derström, S Anttila, M Bergh, V Aittomäki, C Hazekamp, K Nygren, J Selbing, K-G Loft, A. Children born after cryopreservation of embryos or oocytes: a systematic review of outcome data. *Hum Reprod* 2009;**24**:2158–2172.

World Medical Association. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. *JAMA* 2013;**310**:2191–2194.

Wyns C, Bergh C, Calhaz-Jorge C, Geyter C, De Kupka MS, Motrenko T, Rugescu I, Smeenk J, Tandler-Schneider A, Vidakovic S. *et al.*; The European IVF-monitoring Consortium (EIM)‡ for the European Society of Human Reproduction and Embryology (ESHRE) . ART in Europe, 2016: results generated from European registries by ESHRE. *Hum Reprod Open* 2020;**2020**:1–17.

Zhao J, Xu B, Zhang Q, Li YP. Which one has a better obstetric and perinatal outcome in singleton pregnancy, IVF/ICSI or FET?: a systematic review and meta-analysis. *Reprod Biol Endocrinol* 2016; **14**:51.

Zhou J, Liu H, Gu HT, Cui YG, Zhao NN, Chen J, Gao L, Zhang Y, Liu JY. Association of cardiac development with assisted reproductive technology in childhood: a prospective single-blind pilot study. *Cell Physiol Biochem* 2014;**34**:988–1000.

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human reproduction **SUPPLEMENTARY DATA**

Supplementary Table SI Adjusted mean differences of fetal cardiac parameters by mode of conception.

	SC vs FET		SC vs Fresh ET		FET vs Fresh ET	
	Adjusted mean difference* (95% CI)	*P*-value	Adjusted mean difference* (95% CI)	*P*-value	Adjusted mean difference* (95% CI)	*P*-value
Fetal echocardiography						
Fetal cardiac morphometry§						
Cardio-thoracic Ratio	0.02 (0.01–0.02)	<0.001	0.01 (0.01–0.02)	<0.001	0	0.216
Left atrium/heart area ratio	0.47 (0.23–0.71)	<0.001	1.22 (0.98–1.46)	<0.001	0.75 (0.51–0.98)	<0.001
Right atrium/heart area ratio	0.71 (0.47–0.95)	<0.001	0.87 (0.63–1.10)	<0.001	0.16 (−0.08–0.40)	0.329
Left ventricular sphericity index	−0.05 (−0.05 to −0.04)	<0.001	−0.04 (−0.05 to −0.04)	<0.001	0.01 (0–0.01)	0.014
Right ventricular sphericity index	−0.06 (−0.08 to −0.05)	<0.001	−0.05 (−0.06 to −0.04)	<0.001	0.01 (0.01–0.03)	0.050
Left-free wall thickness (mm)	0.51 (0.48–0.54)	<0.001	0.63 (0.60–0.66)	<0.001	0.12 (0.09–0.15)	<0.001
Septal wall thickness (mm)	0.31 (0.01–0.60)	0.040	0.38 (0.09–0.68)	0.006	0.08 (−0.22–0.37)	1.000
Relative wall thickness	0.09 (0.08–0.10)	<0.001	0.14 (0.13–0.15)	<0.001	0.05 (0.04–0.06)	<0.001
Systolic function						
Left ejection fraction (%)	0.98 (−1.56 to −0.41)	<0.001	−2.11 (−2.68 to −1.53)	<0.001	−1.12 (−1.70 to −0.55)	<0.001
Left shortening fraction (%)	−0.73 (−1.15 to −0.31)	<0.001	−1.56 (−1.98 to −1.14)	<0.001	−0.83 (−1.25 to −0.41)	<0.001
Mitral ring displacement (mm)	−0.02 (−0.07–0.03)	1.000	−0.17 (−0.22 to −0.12)	<0.001	−0.15 (−0.20 to −0.10)	<0.001
Tricuspid ring displacement (mm)	0.13 (0.02–0.24)	0.011	−0.12 (−0.23 to −0.02)	0.016	−0.26 (−0.36 to −0.15)	<0.001
Mitral S′ (cm/s)	−0.28 (−0.36 to −0.21)	<0.001	−0.25 (−0.33 to −0.17)	<0.001	0.03 (−0.04–0.11)	0.923
Tricuspid S′ (cm/s)	−0.17 (−0.21 to −0.13)	<0.001	−0.41 (−0.45 to −0.37)	<0.001	−0.23 (−0.27 to −0.19)	<0.001
Diastolic function						
Mitral E/A ratio	0.01 (0–0.02)	<0.001	0.02 (0.01–0.03)	<0.001	0.01 (0–0.02)	<0.001
Mitral E′ (cm/s)	−0.47 (−0.56 to −0.39)	<0.001	−0.06 (−0.14–0.03)	0.318	0.42 (0.33–0.50)	<0.001
Mitral A′ (cm/s)	−0.98 (−1.15 to −0.80)	<0.001	−0.49 (−0.66 to −0.31)	<0.001	0.49 (0.32–0.66)	<0.001
Tricuspid E/A ratio	−0.02 (−0.03 to −0.01)	<0.001	−0.01 (−0.02–0)	0.003	0.01 (0–0.02)	0.019
Tricuspid E′ (cm/s)	−0.26 (−0.35 to −0.16)	<0.001	−0.25 (−0.34 to −0.15)	<0.001	0.01 (−0.09–0.10)	1.000
Tricuspid A′ (cm/s)	−0.24 (−0.39 to −0.08)	0.001	−0.27 (−0.43 to −0.12)	<0.001	−0.04 (−0.19–0.12)	1.000
Heart rate and timing						
Heart rate (bpm)	0.12 (−0.31–0.55)	1.000	1.70 (1.27–2.13)	<0.001	1.58 (1.15–2.01)	<0.001
Left myocardial performance index	0.03 (0.03–0.03)	<0.001	0.06 (0.06–0.06)	<0.001	0.03 (0.03 – 0.03)	<0.001

*Adjusted by linear regression analyses for maternal age, nulliparity, birthweight centile, preeclampsia, and preterm birth.
§Cardiac morphometry results measured at end diastole, except for atrial areas.
SC, spontaneously conceived pregnancies; FET, frozen embryo transfer; ET, embryo transfer.

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human reproduction **SUPPLEMENTARY DATA**

Supplementary Table SII Adjusted mean differences of fetal cardiac parameters by mode of conception excluding the vanishing twin phenomenon cases.

	SC vs FET		SC vs Fresh ET		FET vs Fresh ET	
	Adjusted mean difference* (95% CI)	*P*-value	Adjusted mean difference* (95% CI)	*P*-value	Adjusted mean difference* (95% CI)	*P*-value
Fetal echocardiography						
Fetal cardiac morphometry§						
Cardio-thoracic Ratio	0.02 (0.01–0.02)	<0.001	0.01 (0.01–0.02)	<0.001	0 (−0.01–0)	0.025
Left atrium/heart area ratio	0.47 (0.20–0.73)	<0.001	1.21 (0.95–1.48)	<0.001	0.75 (0.48–1.02)	<0.001
Right atrium/heart area ratio	0.72 (0.47–0.97)	<0.001	0.98 (0.73–1.23)	<0.001	0.26 (0.1–0.51)	0.044
Left ventricular sphericity index	−0.04 (−0.05 to −0.04)	<0.001	−0.05 (−0.06 to −0.05)	<0.001	−0.01 (−0.02 to −0.01)	<0.001
Right ventricular sphericity index	−0.06 (−0.07 to −0.05)	<0.001	−0.05 (−0.06 to −0.04)	<0.001	0.01 (0–0.03)	0.105
Left-free wall thickness (mm)	0.55 (0.51–0.59)	<0.001	0.63 (0.59–0.67)	<0.001	0.08 (0.04–0.12)	<0.001
Septal wall thickness (mm)	0.32 (0.28–0.35)	<0.001	0.27 (0.23–0.30)	<0.001	−0.05 (−0.09 to −0.01)	0.004
Relative wall thickness	0.10 (0.09–0.11)	<0.001	0.15 (0.14–0.15)	<0.001	0.05 (0.04–0.05)	<0.001
Systolic function						
Left ejection fraction (%)	−0.77 (−1.36 to −0.18)	0.006	−1.96 (−2.56 to −1.37)	<0.001	−1.20 (−1.80 to −0.59)	<0.001
Left shortening fraction (%)	−0.51 (−0.94 to −0.09)	0.012	−1.45 (−1.88 to −1.01)	<0.001	−0.93 (−1.37 to −0.49)	<0.001
Mitral ring displacement (mm)	0 (−0.04–0.05)	1.000	−0.19 (−0.24 to −0.15)	<0.001	−0.20 (−0.24 to −0.15)	<0.001
Tricuspid ring displacement (mm)	0.19 (0.08–0.29)	<0.001	−0.12 (−0.22 to −0.01)	0.027	−0.30 (−0.41 to −0.19)	<0.001
Mitral S′ (cm/s)	−0.25 (−0.33 to −0.17)	<0.001	−0.26 (−0.34 to −0.17)	<0.001	0.04 (−0.09–0.08)	1.000
Tricuspid S′ (cm/s)	−0.17 (−0.21 to −0.14)	<0.001	−0.40 (−0.44 to −0.36)	<0.001	−0.23 (−0.27 to −0.19)	<0.001
Diastolic function						
Mitral E/A ratio	0.01 (0.01–0.02)	<0.001	0.02 (0.01–0.03)	<0.001	0.01 (0–0.01)	0.039
Mitral E′ (cm/s)	−0.47 (−0.57 to −0.38)	<0.001	−0.04 (−0.14–0.06)	0.975	0.43 (0.33–0.53)	<0.001
Mitral A′ (cm/s)	−0.85 (−1.05 to −0.66)	<0.001	−0.45 (−0.65 to −0.25)	<0.001	0.41 (0.20–0.61)	<0.001
Tricuspid E/A ratio	−0.01 (−0.02–0)	0.002	−0.01 (−0.02–0)	0.021	0 (−0.01–0.01)	1.000
Tricuspid E′ (cm/s)	−0.15 (−0.25 to −0.04)	0.002	−0.25 (−0.35 to −0.14)	<0.001	−0.10 (−0.21–0.01)	0.071
Tricuspid A′ (cm/s)	−0.12 (−0.28–0.04)	0.198	−0.25 (−0.41 to −0.08)	0.001	−0.12 (−0.29–0.04)	0.220
Heart rate and timing						
Heart rate (bpm)	0.03 (−0.50–0.55)	1.000	1.34 (0.81–1.87)	<0.001	1.31 (0.77–1.85)	<0.001
Left myocardial performance index	0.03 (0.03–0.03)	<0.001	0.06 (0.06–0.06)	<0.001	0.03 (0.03–0.03)	<0.001

*Adjusted by linear regression analyses for maternal age, nulliparity, birthweight centile, preeclampsia, and preterm birth.
§Cardiac morphometry results measured at end diastole, except for atrial areas.
SC, spontaneously conceived pregnancies; FET, frozen embryo transfer; ET, embryo transfer.

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human reproduction **SUPPLEMENTARY DATA**

Supplementary Table SIII Baseline and infertility characteristics of the invited and not included population.

	SC (n = 35)	FET (n = 58)	Fresh ET (n = 64)
Maternal characteristics			
Age (year)	37.4 (35.4–40.1)	37.4 (34.7–38.8)	36.3 (34.3–38.7)
Body mass index (kg/m^2)	23.2 (19.8–25.7)	23.7 (20.6–26.2)	23.6 (20.9–28.8)
Smoking habit (%)	25	10	14
Caucasian (%)	77	84	85
Nulliparity (%)	66	89*	89*
Polycystic ovarian syndrome (%)	5	32*†	19*
Paternal characteristics			
Age (year)	38.1 (±6.5)	38.0 (±4.0)	39.2 (±4.3)
Body mass index (kg/m^2)	26.1 (24.8–29.4)	25.0 (23.5–26.9)	25.5 (23.9–29.5)
Smoking habit (%)	38	25	32
Caucasian (%)	75	84	86
Infertility and ART characteristics			
Infertility cause (%)			
Unexplained infertility		40	39
Endometriosis		17	13
Tubal factor		6	10
Male factor		41	43
Previous failed ET cycles (N)		0.4 (±0.6)	0.4 (±0.7)
Oocytes retrieved (N)		14 (10–18)†	8 (7–10)
Embryos obtained (N)**		9 (7–11)†	6 (3–8)
Transferred embryos (N)		1.4 (±0.5)	1.8 (±0.5)
ET at cleavage stage (%)		71	80

These clinical data were retrieved after invited and not included patients provided their written informed consent. Data are mean (± SD), median (interquartile range), or percentage (%), as appropriate.
*$P < 0.05$ as compared to SC.
†$P < 0.05$ as compared to fresh ET.
**Two pronuclear stage.
SC, spontaneously conceived pregnancies; FET, frozen embryo transfer; ET, embryo transfer.

Study 2

Subfertility versus assisted reproductive technologies: unravelling the origins of fetal cardiac programming

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Subfertility versus assisted reproductive technologies: unravelling the origins of fetal cardiac programming

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Keywords:	SUBFERTILITY, IVF/ICSI OUTCOME, PREGNANCY, CARDIAC REMODELING, FETAL HEART
Subject Section:	Infertility

Subfertility versus assisted reproductive technologies: unravelling the origins of fetal cardiac programming

Running title: "Fetal cardiac programming: subfertility versus ART"

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Abstract

Study question: Do spontaneously conceived (SC) fetuses from subfertile couples present signs of cardiac remodeling as those observed after in vitro fertilisation (IVF) treatments?

Summary answer: SC fetuses from subfertile couples show a similar cardiac structure and function to those of SC from fertile couples.

What is known already: Fetuses and children from IVF associate cardiac remodeling and suboptimal function, including dilated atria, more globular and thicker ventricles, reduced longitudinal motion and impaired relaxation *in utero* and after birth. Fetal cardiac changes have been demonstrated both after fresh and frozen embryo transfer. The SC fetuses used as 'controls' in our previous publications were conceived by fertile couples thus making it difficult to assess the contribution of infertility *per se* from the IVF procedures on cardiac programming. There are no previous publications investigating the independent cardiac effects of infertility in SC fetuses from subfertile couples (with time-to-pregnancy (TTP) over 12 months).

Study design, size, duration: Prospective cohort study of 289 singleton pregnancies recruited from 2019 to 2021, including 96 SC pregnancies from fertile couples (TTP less than 12 months), 97 SC from subfertile couples (TTP over 12 months) and 96 from IVF after fresh ET. Fetal echocardiography was performed in all pregnancies. Epidemiological data and perinatal outcomes were collected in all pregnancies.

Participants/materials, setting, methods: IVF pregnancies and SC from subfertile couples were identified as eligible at pregnancy diagnosis. Eligible SC pregnancies from fertile couples who attended our Maternal-Fetal Unit were invited to participate at third trimester, being matched to the other groups by maternal age. Fetal echocardiography was performed at 29-34 weeks of pregnancy to assess cardiac structure and function and results were adjusted by nulliparity, birthweight centile, gestational age and estimated fetal weight at scan.

Main results and the role of chance: Parental age, ethnicity, body mass index and smoking exposure, median gestational age and estimated fetal weight were similar in all study groups. There were no significant differences in infertility duration or aetiology between the subfertile and the IVF populations (TTP: subfertile median 35 months [IQR 20-48] versus IVF: 47 [25-61]; p-value=0.051). While both fertile and subfertile SC groups presented similar fetal cardiac results, IVF fetuses showed larger atria (right atria-to-heart ratio: IVF mean 18.9% [SD 3.4] versus subfertile 17.8% [3.5] versus fertile 17.6% [3.3]; adjusted P-value<0.001), more globular ventricles (right ventricular sphericity index: IVF 1.56 [0.25] versus subfertile 1.72 [0.26] versus fertile 1.72 [0.26]; <0.001), and

thicker myocardial walls (relative wall thickness: IVF 0.86 [0.22] versus subfertile 0.64 [0.13] versus fertile 0.64 [0.18]; <0.001). Whereas SC fetuses from fertile and subfertile couples had preserved cardiac function, IVF fetuses showed signs of suboptimal systolic and diastolic function with reduced tricuspid ring displacement (IVF 7.26 mm [1.07] versus subfertile 8.04 mm [1.18] versus fertile 7.89 mm [1.51]; <0.001) and increased left myocardial performance index (IVF 0.49 [0.08] versus subfertile 0.45 [0.09] versus fertile 0.45 [0.10]; <0.001). A sub-analysis including only unexplained infertility cases in subfertile SC and IVF groups showed similar results.

Limitations, reasons for caution: The fetal cardiac changes reported here are subclinical, being most of the cardiovascular parameters within normal ranges. Although echocardiographic changes are recognized as potential cardiovascular risk factors, their association with long-term cardiovascular disease remains to be demonstrated.

Wider implications of the findings: Subfertility *per se* does not seem to be associated to fetal cardiac remodeling, which has been previously described in IVF fetuses. Future studies are warranted to further investigate other factors related to the observed fetal cardiac changes associated to assisted reproductive technologies (ART).

Author keywords: subfertility - IVF - pregnancy - cardiac remodeling - fetal heart

Introduction

Up to 7.9% of the live births in European countries result from pregnancies obtained by assisted reproductive technologies (ART) (Wyns *et al.*, 2021), underscoring the relevance of the different ART procedures´ safety.

Previous studies have reported cardiovascular morphological and functional changes in foetuses and children conceived by ART (Ceelen *et al.*, 2008; Scherrer *et al.*, 2012; Valenzuela-Alcaraz *et al.*, 2013, 2019; Zhou *et al.*, 2014; Liu *et al.*, 2015; von Arx *et al.*, 2015; Meister *et al.*, 2018; Forton *et al.*, 2019; Boutet *et al.*, 2021). Fetuses from ART present more globular hearts, dilated atria, decreased longitudinal motion and impaired relaxation. These cardiac features are already present *in utero*, persist after birth (Valenzuela-Alcaraz *et al.*, 2013, 2019), and have also been demonstrated to be independent from different gestational conditions such as fetal growth restriction (Valenzuela-Alcaraz *et al.*, 2017) and multiplicity (Valenzuela-Alcaraz *et al.*, 2018). Moreover, these ART-associated cardiac features are observed in fetuses conceived by both fresh or frozen embryo transfer (Boutet *et al.*, 2021).

The underlying causes of ART-associated cardiac findings are still unclear. Proposed mechanisms are related to intrinsic parental factors associated to infertility, ovarian stimulation, manipulation of gametes and early embryos, culture conditions, embryo transfer characteristics and / or the increased risk of perinatal complications in comparison with SC fetuses, as higher rates of fetal growth restriction, prematurity, and preeclampsia (Scherrer *et al.*, 2015). The spontaneously conceived (SC) fetuses included in previous publications investigating cardiac performance in ART were conceived by fertile couples (Valenzuela-Alcaraz *et al.*, 2013; Boutet *et al.*, 2021) or at least do not declare a parental infertility background (Rizzo *et al.*, 2020; Bi *et al.*, 2022) thus making it difficult to assess the contribution of infertility *per se* from the ART procedures on cardiac programming. The aim of the present study is to describe cardiac morphology and function in SC fetuses from fertile and subfertile couples and compare it to those conceived by IVF.

Methods

Study design, size, duration, setting

This is a prospective observational study of 289 singleton pregnancies conceived by IVF and SC, who attended the Barcelona Center for Maternal Fetal and Neonatal Medicine (BCNatal, Barcelona, Spain) from 2019 to 2021. Sample size was calculated based on previously reported features in fetal cardiac morphometry (right atrial area) and function (mitral ring displacement) from IVF subjects (Valenzuela-Alcaraz *et al.*, 2013). Basal mean and within-group standard deviations were estimated according to previously published data in fetuses (Gardiner *et al.*, 2006; García-Otero *et al.*, 2019). To detect a difference of 20% in right atrial area and of 5 mm in mitral ring displacement between the SC and IVF groups, with 90% power and a 5% type I risk, a required sample of 32 fetuses in each group for right atrial area and 74 for mitral ring displacement was estimated. Therefore, a sample size of at least 80 individuals in each group was initially calculated.

Participants and materials

We included 289 singleton pregnancies; 96 of which were SC from fertile couples (with a time-to-pregnancy (TTP) below 12 months), 97 were SC from subfertile couples (with a TTP above 12 months), and 97 were from IVF with fresh embryo transfer. Oocyte donation cycles, fetal malformations and fetal chromosomal anomalies were considered exclusion criteria. A flow diagram of the recruitment is displayed in **Figure 1**.

From 2019 to 2021, eligible SC singleton pregnancies from fertile couples who attended our Maternal-Fetal Unit were invited to participate at third trimester. During the same period, eligible singleton pregnancies from IVF procedures and SC from subfertile couples attending our Assisted Reproduction Unit were identified as eligible at pregnancy diagnosis, and only invited to participate in case they were planning to give birth at Barcelona Metropolitan Area to guarantee an optimal follow-up.

Patient´s enrolment stopped in December of 2021, and all ultrasounds performed during the recruitment period of the current study were included in the analyses. Motives for not attending the third trimester fetal echocardiography were diagnosis of fetal anomaly, miscarriage, preterm delivery before the ultrasound scan, or withdrawn consent.

IVF protocol

The ovarian stimulation protocol for IVF and the gonadotropin doses were chosen according to age and ovarian reserve markers. Either long agonist or antagonist protocols were used. Ovarian stimulation was achieved with daily doses from 150 to 300 IU of FSHr (Gonal-F; Merck-Serono S.A., Madrid, Spain) alone or with the addition of 75 IU of LHr (Luveris; Merck-Serono S.A., Madrid, Spain) or HMG (Menopur, Ferring SA, Madrid, Spain). The hCG administration (Ovitrelle 250 mg s.c., Serono S.A.) was indicated in the presence of two or more follicles ≥18 mm of diameter, with ≥4 follicles measuring ≥14 mm in association with a consistent rise in serum estradiol concentration. Ultrasound (US)-guided transvaginal oocyte retrieval was performed 36 hours after hCG administration. Embryo culture was carried out in microdrops of Global Media (Life Global, CooperSurgical Målov, Denmark) under mineral oil at 37ºC in an atmosphere of 6.5% CO2 and 7% O2. Embryo quality was assessed according to the ASEBIR criteria (Balaban *et al.*, 2011). The quality of blastocysts was assessed according to the criteria of Gardner and Schoolcraft (Gardner *et al.*, 2000).

Vaginal natural progesterone was started the morning after the oocyte retrieval with a dose of 200 mg every 8 hours. Cleavage embryos were transferred on day 3 and blastocysts on day 5. Pregnancy was diagnosed by a positive serum β-hCG test 12 days after embryo transfer and a transvaginal ultrasonography was performed in all pregnancies at 5-6 weeks of gestation.

Data collection and study protocol

All participants underwent the same study protocol, including collection of demographics, clinical and perinatal characteristics, and third trimester ultrasound with fetal biometry, feto-placental Doppler assessment and fetal echocardiography.

Maternal epidemiological and obstetric parameters were collected by questionnaires and review of medical records, involving parental age, body mass index, ethnicity, socioeconomic status (illiterate or only primary educational level were considered low socioeconomic status), chronic diseases (e.g. hypertension, diabetes mellitus), parity, obstetric history, mode of conception, smoking status and illicit substance abuse (heroin, cocaine, or cannabis) during pregnancy.

IVF-related parameters included aetiology and length of infertility, diagnosis of polycystic ovarian syndrome (PCOS) according to Rotterdam criteria (Fauser, 2004), number of previous unsuccessful IVF cycles, ovarian stimulation data (stimulation protocol, gonadotropin dose in international units, duration in days), number of oocytes retrieved, number of embryos obtained and transferred, embryonic stage at ET, vanishing twin detection or selective embryonic reduction.

Upon delivery, pregnancy and perinatal outcomes were recorded including gestational age at delivery, mode of delivery, birth weight, Apgar score, umbilical artery pH, neonatal admission to ICU, perinatal morbidity and mortality, and the presence of pregnancy complications such as gestational diabetes, pregnancy-induced hypertension / preeclampsia / eclampsia, HELLP syndrome, cholestasis, placenta previa, abruptio placentæ and PTB (<37 weeks of gestation). Gestational age was calculated according to first trimester crown-rump length (Robinson, 1973). SGA and LGA were defined respectively as birth weight below 10th centile and above the 90th centile according to local standards (Figueras *et al.*, 2008).

Preeclampsia was defined by new onset of hypertension after 20 week´s gestation, of ≥ 140mmHg systolic blood pressure and/or ≥ 90 mmHg diastolic blood pressure, on two occasions at least four hours apart, together with proteinuria (≥ 300mg proteins in 24-hour urine or protein/creatinine ratio ≥ 0.3) or new onset of maternal thrombocytopenia, renal insufficiency, liver dysfunction, pulmonary edema or neurological features (ACOG Practice Bulletin, Number 222, 2020). Major neonatal morbidity was defined by the presence of at least one of the following: bronchopulmonary dysplasia, necrotizing enterocolitis, intraventricular haemorrhage, periventricular leukomalacia, retinopathy, persistent ductus arteriosus, or sepsis. Minor neonatal morbidity was defined by the presence of at least one of the following, in the absence of major neonatal morbidity criteria: respiratory distress, hyperbilirubinemia, or anaemia. Perinatal mortality was defined by either intrauterine fetal death after 22 weeks of pregnancy or neonatal death within the first 28 days of postnatal life.

Fetal ultrasound

All pregnancies underwent an ultrasonographic examination at 29-34 weeks of gestation using either a Voluson 730 Expert (GE Medical Systems, Milwaukee, Wisconsin, USA) or a Voluson S10 ultrasound system (GE Healthcare, Zipf, Austria) with 2-5 MHz linear curved-array probes, including estimated fetal weight, feto-placental Doppler, and fetal echocardiography. Ultrasound acquisitions were obtained by four maternal-fetal medicine specialists skilled in fetal echocardiography blinded to the mode of conception, the images were recorded as clips and anonymized, and all the measurements were performed off-line by a single investigator to avoid bias.

Fetal biometry and feto-placental Doppler

Fetal weight was estimated following the Hadlock formula (Hadlock *et al.*, 1985) by measuring the abdominal circumference, head circumference, biparietal diameter and femur length. Centile calculations were made based on local reference curves (Figueras *et al.*, 2008). Feto-placental Doppler assessment included the measurement of pulsatility index of maternal uterine arteries, umbilical artery, fetal middle cerebral artery and ductus venosus (Arduini and Rizzo, 1990; Hecher *et al.*, 1994; Bhide *et al.*, 2013). Cerebroplacental ratio was calculated as middle cerebral artery PI divided by umbilical artery PI (Baschat and Gembruch, 2003). Z-scores of the above mentioned Doppler parameters were calculated based on previous studies (Baschat and Gembruch, 2003; Kessler *et al.*, 2006; Gómez *et al.*, 2008). All estimations were done in the absence of fetal movements and with the mother in voluntary suspended ventilation when required.

Fetal echocardiography

A comprehensive two-dimensional, M-mode and Doppler echocardiographic examination was performed to assess structural heart integrity, to rule out cardiac defects, and to evaluate cardiac morphometry and function following standard protocols (Carvalho *et al.*, 2013; Crispi *et al.*, 2013; García-Otero *et al.*, 2019). Cardiac, thoracic, and ventricular areas and diameters were measured on 2D images from an apical or basal four-chamber view at end-diastole. Ventricular sphericity indices were calculated by dividing the longitudinal by basal-transverse ventricular diameters. Atrial areas were measured at maximum distension from an apical or basal four-chamber view. Atria-to-heart ratios were calculated as atrial area * 100 / heart area. Myocardial walls thickness was measured on 2D images from a transverse four-chamber view. Relative wall thickness was calculated as septal plus left wall thickness divided by left ventricular end-diastolic diameter (Foppa *et al.*, 2005). Mitral/tricuspid ring displacements (MAPSE/TAPSE) were assessed by M-mode from an apical or basal four-chamber view. Left myocardial performance index was obtained from a single Doppler spectrum in a cross-sectional image of the fetal thorax, calculated as (ICT + IRT) / ET (Hernandez-Andrade *et al.*, 2005). **Figure 2** illustrates the methodology used for assessing the main echocardiographic parameters.

Statistical analysis

Data were analysed by means of the statistical software STATA 15.1 (StataCorp LLC, College Station, TX, USA). The study outcome was fetal cardiovascular assessment. The independent variable of interest was the mode of conception (IVF or spontaneous pregnancy from fertile or subfertile

219 couples). Descriptive statistics and results were expressed as mean (± standard deviation), median
220 (interquartile range), or number (percentage), as appropriate. Normal distribution of continuous
221 variables was checked using the Shapiro-Wilk test and histograms. Comparisons among the study
222 groups were assessed by ANOVA or Kruskal-Wallis with Bonferroni correction, after checking the
223 fulfilment of each test assumptions. Differences between groups on feto-placental data and
224 cardiovascular parameters were assessed by using multiple regression analyses to adjust by
225 nulliparity, birthweight centile, gestational and estimated fetal weight at scan. All reported p-values
226 are 2 sided. The level of significance was set at 0.05 for all the statistical tests.
227
228 **Ethical approval**
229 This clinical study was conducted according to the Declaration of Helsinki for Medical Research
230 involving Human Subjects (World Medical Association, 2013), the study protocol was approved by
231 the local ethics committee (HCB/2017/0714), and all patients agreeing to participate provided their
232 written informed consent.

Results

Baseline and perinatal characteristics of the study population

The baseline characteristics of the study population are displayed in **Table I**. With the exception of a higher rate of nulliparity in both IVF and subfertile groups as compared to the SC pregnancies from fertile couples, the study groups were similar in terms of parental baseline characteristics. Both IVF and subfertile couples conceiving spontaneously had a comparable distribution of underlying infertility factors. The vanishing twin phenomenon rate was 10.4% (n=10) for the IVF group. No vanishing twin cases were registered within the SC populations, nevertheless this characteristic could have been underestimated in SC pregnancies because of the timing of the first ultrasound performed (IVF pregnancies were scanned at least two times prior to their 11-13 weeks scan, being this early assessment not done in most SC pregnancies).

Perinatal outcomes are shown in **Table II**. All groups had similar rates of pregnancy complications. Delivery and neonatal characteristics were also similar among the study groups.

Fetal cardiac results of the study population

Gestational age, estimated fetal weight, and fetoplacental Doppler were similar between groups at scan (**Table II**).

Fetuses conceived by IVF showed significant signs of fetal cardiac remodeling and suboptimal function as compared to both SC populations (**Table III** and **Figure 3**). Fetuses conceived by IVF presented larger hearts and atria, increased myocardial thickness and more spherical ventricles as compared to SC pregnancies. The IVF group also showed significant reduced myocardial motility (mitral and tricuspid ring displacements), higher heart rate, and increased left myocardial performance index as compared to the SC. There were no significant differences for any of the echocardiographic outcomes between the fertile and subfertile populations. These results were confirmed in a sub-analysis including only pregnancies from couples with unexplained infertility, in which no infertility factor had been identified after a systematic fertility assessment of the couple. The sub-analysis results are displayed in **Table SI** on *Supplementary data*.

Discussion

This is the first study on fetal cardiac performance in ART offspring including a cohort of SC fetuses from subfertile versus fertile couples. We provide further evidence of fetal cardiac remodeling and suboptimal function in fetuses conceived by IVF as compared to those from SC pregnancies. Additionally, we report no significant cardiac morphometric and functional differences between SC fetuses from fertile and subfertile couples, suggesting that the cardiac features described in pregnancies obtained by IVF are not linked to the underlying parental factors or the infertility *per se*.

Cardiac remodeling associated to IVF

Our results support previous evidence that fetuses from IVF showed a distinctive pattern of cardiac remodeling including dilated atria, thicker cardiac walls and more spherical ventricles, together with signs of suboptimal cardiac function as previously described in different cohort studies of IVF *versus* SC fetuses from fertile couples (Valenzuela-Alcaraz *et al.*, 2013; Rizzo *et al.*, 2020; Boutet *et al.*, 2021; Bi *et al.*, 2022). An increase of vascular stiffness could explain the distinctive cardiac features observed in IVF offspring (Mayet and Hughes, 2003; Scherrer *et al.*, 2015). This particular feature explains cardiac pressure overload, being compensated by ventricular hypertrophy, atrial dilation and, consequently, different cardiac function during the intrauterine life (Crispi *et al.*, 2020). Increased blood pressure and carotid wall thickness were demonstrated postnatally in ART offspring since birth (Valenzuela-Alcaraz *et al.*, 2013) and at 3 years of age (Valenzuela-Alcaraz *et al.*, 2019). These data are in line with the findings of pulmonary and systemic vascular dysfunction later in life, specially under hypobaric conditions that induce a right ventricle overload (Ceelen *et al.*, 2008; Scherrer *et al.*, 2012, 2015; von Arx *et al.*, 2015; Meister *et al.*, 2018; Forton *et al.*, 2019). Scherrer et al. demonstrated an impaired flow-mediated vasodilation with increased carotid-femoral pulse wave velocity, carotid intima-media thickness and pulmonary pressure in ART children and adolescents (Scherrer *et al.*, 2012). A 5-year follow-up study of this cohort showed the persistence of higher arterial blood pressure, carotid-femoral pulse wave velocity, carotid intima-media thickness, and lower flow-mediated dilation (Meister *et al.*, 2018). As opposed to these findings, a recent cohort study (Mizrak *et al.*, 2022) conducted on 8 to 9 year-old children found no significant differences between ART and SC on blood pressure, left ventricular ejection fraction, cardiac output and aorta distensibility, a proxy of arterial stiffness, measured by magnetic resonance. These

296 discrepancies could be due to differences in the study design (hypobaric versus normobaric
297 conditions), study populations (different ART protocols) and / or individual study limitations (sample
298 size, selection bias).
299
300 *Potential etiological factors of cardiac features in ART fetuses*
301 The etiological factors promoting fetal cardiac changes in ART fetuses are still unknown. Ovarian
302 stimulation, laboratory manipulation of gametes and early embryos, culture media and conditions,
303 ET characteristics and / or the increased risk of perinatal complications in comparison with SC
304 fetuses are some of the potential factors postulated as inductors of perinatal environmental
305 variations. Parental characteristics and infertility *per se* have also been proposed. Advanced
306 maternal age and nulliparity have proven to be independent risk factors for adverse pregnancy
307 outcomes (Kiserud *et al.*, 2018). Hormonal and / or metabolic disorders, and inflammatory
308 conditions affecting the female genital tract associated to infertility might impair physiological
309 placentation and fetal development processes (Vannuccini *et al.*, 2016). Severe male infertility
310 diagnosis has also been proposed to be associated to a higher risk of birth defects in ICSI cycles
311 (Berntsen *et al.*, 2019). Recently, specific epigenetic changes associated to infertility and probably
312 linked to the pregnancy outcomes (Litzky and Marsit, 2019; Barberet *et al.*, 2022) were identified in
313 subfertile patients exposed and not exposed to medically assisted reproduction, including IVF,
314 ovulation induction and intrauterine insemination procedures. However, further studies should be
315 conducted to associate these epigenetic features with adverse events discarding the effect of
316 different conditions such as depression and anxiety, which may confound these results in patients
317 suffering from unwanted infertility.
318
319 *Fetal cardiac performance in subfertility*
320 Most of the studies on cardiac performance in ART do not specify the characteristics of the non-ART
321 group in terms of parental morbidities and infertility factors. Only some studies include SC children
322 and adolescents from subfertile couples and discuss it (Ceelen *et al.*, 2008; Scherrer *et al.*, 2012;
323 Meister *et al.*, 2018). Although the sample sizes are probably underpowered, the results on SC
324 children from subfertile couples provide no evidence linking parental-related factors to vascular
325 dysfunction (Scherrer *et al.*, 2012). The uniqueness of the present study is the inclusion of a large
326 cohort of SC fetuses from subfertile couples recruited in a tertiary hospital and most of them with a
327 previous indication of ART, with similar infertility background and baseline parental characteristics

to those of the ART cohort, which allows us to compare their cardiac performance during the intrauterine life in a reliable way.

In the current study, cardiac remodeling and suboptimal function are demonstrated for the ART population while fetuses from both SC groups -fertile and subfertile- show consistent similar results on each parameter assessed, not supporting the hypothesis of fetal cardiac features observed in ART being related to underlying infertility factors. SC fetuses from fertile and subfertile couples not only do not present cardiac changes, but both show similar cardiac structural (size, shape and wall thickness) and functional parameters (atrioventricular ring displacements, MPI), even after adjusting for relevant covariables.

Strengths and limitations

Among the strengths, the present study includes a very well-phenotyped prospective cohort from conception to birth, with comprehensive baseline, fetal and perinatal data. All IVF pregnancies included were enrolled from a single centre and their infertility causes and length were comparable among subfertile and IVF groups. To achieve the optimal accuracy and reproducibility, a strict and predetermined methodology for assessing fetal cardiac structure and function was used (Crispi *et al.*, 2013; García-Otero *et al.*, 2019).

As limitations, we mention the lower rate of nulliparity in the SC from fertile couples' group as compared to the other two. Parity constitutes a relevant variable on perinatal results. Although analyses were adjusted by parity, we acknowledge that it could not be fully discarded as a potential confounder. The vanishing twin phenomenon, which in line with previous publications (Pinborg *et al.*, 2005) affects about 10% of the IVF pregnancies in this cohort, could also influence the perinatal results. Therefore, we have performed a sub-analysis after the exclusion of those cases, obtaining the same echocardiographic results (**Table SII** on *Supplementary data*). We have also performed a sub-analysis including only the unexplained infertility cases (**Table SI** on *Supplementary data*), replicating the results. Subfertile couples conceiving spontaneously could have milder infertility factors than the ones undergoing ART treatments. Although we cannot discard this, to prevent this issue, we designed the enrolment circuit in order to effectively recruit patients with previous infertility assessment, most of which already had an indication of ART and were included in our IVF waiting list. In addition, we cannot exclude the mass-significance effect, although it has been attenuated after applying Bonferroni corrections to the comparisons performed. Finally, we would like to address that the cardiac changes reported here are subclinical and most cardiovascular values

and indices lie within standard ranges. The long-term health implications remain unclear: although these features are recognized as potential cardiovascular risk factors, their ultimate association with adult cardiovascular disease remains to be proven.

Clinical relevance and conclusions

In the current study we provide additional evidence for the existence of fetal cardiovascular programming in ART pregnancies. The inclusion of a group of SC fetuses from subfertile couples enables us to dissect the contribution of infertility from the ART treatment, underscoring the effect of the ART procedure on cardiac structure and function. SC fetuses from fertile and subfertile couples showed similar cardiac structure and function.

Future research is warranted to better elucidate the mechanisms underlying fetal cardiac adaptation in ART and to assess its potential impact on future cardiovascular health, in order to increase our knowledge in terms of safety, and thus improve the selection of the appropriate technique for each case. The identification of cardiovascular changes during the prenatal life represents an opportunity for potential preventive measures to ameliorate the future cardiovascular health of ART subjects.

Author's roles

Study design: Boutet ML, Casals G, Crispi F, Manau D, Gratacós E

Patient´s enrolment: Boutet ML, Casals G, Valenzuela-Alcaraz B, Crovetto F, Borràs A, Manau D

Data collection: Boutet ML, Casals G, Valenzuela-Alcaraz B, Crovetto F, Cívico MS

US acquisitions: Boutet ML, Valenzuela-Alcaraz B, Crovetto F, Crispi F

US off-line measurements: Boutet ML

Data analysis: Boutet ML, Valenzuela-Alcaraz B, Casals G, Crispi F

Interpretation of data and critical discussion: All authors

Manuscript drafting: Boutet ML, Casals G, Crispi F

Critical revision of the manuscript: All authors

Data availability

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

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Conflict of interest

The authors have no conflicts of interest to declare.

Reference list

Arduini D, Rizzo G. Normal values of pulsatility index from fetal vessels: A cross-sectional study on 1556 healthy fetuses. *J Perinat Med* 1990;**18**:165–172.

Arx R von, Allemann Y, Sartori C, Rexhaj E, Cerny D, Marchi SF de, Soria R, Germond M, Scherrer U, Rimoldi SF, *et al.* Right ventricular dysfunction in children and adolescents conceived by assisted reproductive technologies. *J Appl Physiol* 2015;**118**:1200–1206.

Balaban B, Brison D, Calderón G, Catt J, Conaghan J, Cowan L, Ebner T, Gardner D, Hardarson T, Lundin K, *et al.* Istanbul consensus workshop on embryo assessment: Proceedings of an expert meeting. *Reprod Biomed Online* 2011;**22**:632–646. Reproductive Healthcare Ltd.

Barberet J, Binquet C, Guilleman M, Romain G, Bruno C, Martinaud A, Ginod P, Cavalieri M, Amblot C, Choux C, *et al.* Does underlying infertility in natural conception modify the epigenetic control of imprinted genes and transposable elements in newborns? *Reprod Biomed Online* 2022;**44**:706–715. Elsevier Ltd.

Baschat AA, Gembruch U. The cerebroplacental Doppler ratio revisited. *Ultrasound Obstet Gynecol*

2003;**21**:124–127.

Berntsen S, Söderström-Anttila V, Wennerholm U-B, Laivuori H, Loft A, Oldereid NB, Bente Romundstad L, Bergh C, Pinborg A. The health of children conceived by ART: "the chicken or the egg?" *Hum Reprod Update* 2019;**25**:137–158.

Bhide A, Acharya G, Bilardo CM, Brezinka C, Cafici D, Hernandez-Andrade E, Kalache K, Kingdom J, Kiserud T, Lee W, *et al.* ISUOG practice guidelines: Use of Doppler ultrasonography in obstetrics. *Ultrasound Obstet Gynecol* 2013;**41**:233–239.

Bi W, Xiao Y, Wang X, Cui L, Song G, Yang Z, Zhang Y, Ren W. The association between assisted reproductive technology and cardiac remodeling in fetuses and early infants: a prospective cohort study. *BMC Med* 2022;**20**:104.

Boutet ML, Casals G, Valenzuela-Alcaraz B, García-Otero L, Crovetto F, Cívico MS, Borrás A, Manau D, Gratacós E, Crispi F. Cardiac remodeling in fetuses conceived by ARTs: fresh versus frozen embryo transfer. *Hum Reprod* 2021;**36**:2697–2708.

Carvalho J, Allan L, Chaoui R, Copel J, DeVore G, Hecher K, Lee W, Munoz H, Paladini D, Tutschek B, *et al.* ISUOG Practice Guidelines (updated): sonographic screening examination of the fetal heart. *Ultrasound Obstet Gynecol* 2013;**41**:348–359.

Ceelen M, Weissenbruch MM Van, Vermeiden JPW, Leeuwen FE Van, Delemarre-Van De Waal HA. Cardiometabolic Differences in Children Born After in Vitro Fertilization: Follow-Up Study. *J Clin Endocrinol Metab* 2008;**93**:1682–1688.

Crispi F, Sepúlveda-Martínez Á, Crovetto F, Gómez O, Bijnens B, Gratacós E. Main Patterns of Fetal Cardiac Remodeling. *Fetal Diagn Ther* 2020;**47**:337–344. S. Karger AG.

Crispi F, Valenzuela-Alcaraz B, Cruz-Lemini M, Gratacós E. Ultrasound assessment of fetal cardiac function. *Australas J Ultrasound Med* 2013;**16**:158–167. Wiley.

Fauser BCJM. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. *Fertil Steril* 2004;**81**:19–25.

Figueras F, Meler E, Iraola A, Eixarch E, Coll O, Figueras J, Francis A, Gratacos E, Gardosi J. Customized birthweight standards for a Spanish population. *Eur J Obstet Gynecol Reprod Biol* 2008;**136**:20–24. Elsevier Ireland Ltd.

Foppa M, Duncan BB, Rohde LEP. Echocardiography-based left ventricular mass estimation. How should we define hypertrophy? *Cardiovasc Ultrasound* 2005;**3**:17.

Forton K, Motoji Y, Pezzuto B, Caravita S, Delbaere A, Naeije R, Faoro V. Decreased pulmonary vascular distensibility in adolescents conceived by in vitro fertilization. *Hum Reprod*

2019;**34**:1799–1808. Advance Access Publication on August.

García-Otero L, Gómez O, Rodriguez-López M, Torres X, Soveral I, Sepúlveda-Martínez Á, Guirado L, Valenzuela-Alcaraz B, López M, Martínez JM, *et al.* Nomograms of Fetal Cardiac Dimensions at 18–41 Weeks of Gestation. *Fetal Diagn Ther* 2019;1–12. S. Karger AG.

Gardiner HM, Pasquini L, Wolfenden J, Barlow A, Li W, Kulinskaya E, Henein M. Myocardial tissue Doppler and long axis function in the fetal heart. *Int J Cardiol* 2006;**113**:39–47.

Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: Towards a single blastocyst transfer. *Fertil Steril* 2000;**73**:1155–1158.

Gestational Hypertension and Preeclampsia: ACOG Practice Bulletin, Number 222. *Obs Gynecol* 2020;**135**:e237–e260.

Gómez O, Figueras F, Fernández S, Bennasar M, Martínez JM, Puerto B, Gratacós E. Reference ranges for uterine artery mean pulsatility index at 11-41 weeks of gestation. *Ultrasound Obstet Gynecol* 2008;**32**:128–132.

Hadlock FP, Harrist RB, Sharman RS, Deter RL, Park SK. Estimation of fetal weight with the use of head, body, and femur measurements-A prospective study. *Am J Obstet Gynecol* 1985;**151**:333–337.

Hecher K, Campbell S, Snijders R, Nicolaides K. Reference ranges for fetal venous and atrioventricular blood flow parameters. *Ultrasound Obstet Gynecol* 1994;**4**:381–390.

Hernandez-Andrade E, López-Tenorio J, Figueroa-Diesel H, Sanin-Blair J, Carreras E, Cabero L, Gratacos E. A modified myocardial performance (Tei) index based on the use of valve clicks improves reproducibility of fetal left cardiac function assessment. *Ultrasound Obstet Gynecol* 2005;**26**:227–232.

Kessler J, Rasmussen S, Hanson M, Kiserud T. Longitudinal reference ranges for ductus venosus flow velocities and waveform indices. *Ultrasound Obstet Gynecol* 2006;**28**:890–898.

Kiserud T, Benachi A, Hecher K, Perez RG, Carvalho J, Piaggio G, Platt LD. The World Health Organization fetal growth charts: concept, findings, interpretation, and application. *Am J Obstet Gynecol* 2018;**218**:S619–S629.

Litzky JF, Marsit CJ. Epigenetically regulated imprinted gene expression associated with IVF and infertility: possible influence of prenatal stress and depression. *J Assist Reprod Genet* 2019;**36**:1299–1313. Journal of Assisted Reproduction and Genetics.

Liu H, Zhang Y, Gu HT, Feng QL, Liu JY, Zhou J, Yan F. Association between assisted reproductive

technology and cardiac alteration at age 5 years. *JAMA Pediatr* 2015;**169**:603–605. American Medical Association.

Mayet J, Hughes A. Pathophysiology in hypertension. *Hear J* 2003;**89**:1104–1109.

Meister TA, Rimoldi SF, Soria R, Arx R von, Messerli FH, Sartori C, Scherrer U, Rexhaj E. Association of Assisted Reproductive Technologies With Arterial Hypertension During Adolescence. *J Am Coll Cardiol* 2018;**72**:1267–1274. Elsevier USA.

Mizrak I, Asserhøj LL, Lund MA V, Kielstrup LR, Greisen G, Clausen TD, Main KM, Jensen RB, Vejlstrup NG, Madsen PL, *et al*. Cardiovascular function in 8- to 9-year-old singletons born after ART with frozen and fresh embryo transfer. *Hum Reprod* 2022;1–12.

Pinborg A, Lidegaard Ø, Cour Freiesleben N la, Andersen AN. Consequences of vanishing twins in IVF/ICSI pregnancies. *Hum Reprod* 2005;**20**:2821–2829.

Rizzo G, Pietrolucci ME, Mappa I, Bitsadze V, Khizroeva J, Makatsariya A, D'Antonio F. Fetal Cardiac Remodeling Is Affected by the Type of Embryo Transfer in Pregnancies Conceived by in vitro Fertilization: A Prospective Cohort Study. *Fetal Diagn Ther* 2020;**47**:772–778.

Robinson HP. Sonar Measurement of Fetal Crown-Rump Length as Means of Assessing Maturity in First Trimester of Pregnancy. *Br Med J* 1973;**4**:28–31.

Scherrer U, Rexhaj E, Allemann Y, Sartori C, Rimoldi SF. Cardiovascular dysfunction in children conceived by assisted reproductive technologies. *Eur Hear J* 2015;**36**:1583–1589.

Scherrer U, Rimoldi SF, Rexhaj E, Stuber T, Duplain H, Garcin S, Marchi SF De, Nicod P, Germond M, Allemann Y, *et al*. Systemic and pulmonary vascular dysfunction in children conceived by assisted reproductive technologies. *Circulation* 2012;**125**:1890–1896.

Valenzuela-Alcaraz B, Crispi F, Bijnens B, Cruz-Lemini M, Creus M, Sitges M, Bartrons J, Civico S, Balasch J, Gratacós E. Assisted reproductive technologies are associated with cardiovascular remodeling in utero that persists postnatally. *Circulation* 2013;**128**:1442–1450.

Valenzuela-Alcaraz B, Crispi F, Cruz-Lemini M, Bijnens B, García-Otero L, Sitges M, Balasch J, Gratacós E. Differential effect of assisted reproductive technology and small-for-gestational age on fetal cardiac remodeling. *Ultrasound Obstet Gynecol* 2017;**50**:63–70. John Wiley and Sons Ltd.

Valenzuela-Alcaraz B, Cruz-Lemini M, Rodríguez-López M, Goncé A, García-Otero L, Ayuso H, Sitges M, Bijnens B, Balasch J, Gratacós E, *et al*. Fetal cardiac remodeling in twin pregnancy conceived by assisted reproductive technology. *Ultrasound Obstet Gynecol* 2018;**51**:94–100. John Wiley and Sons Ltd.

519 Valenzuela-Alcaraz B, Serafini A, Sepulveda-Martínez A, Casals G, Rodríguez-López M, Garcia-Otero
520 L, Cruz-Lemini M, Bijnens B, Sitges M, Balasch J, *et al*. Postnatal persistence of fetal
521 cardiovascular remodelling associated with assisted reproductive technologies: a cohort
522 study. *BJOG An Int J Obstet Gynaecol* 2019;**126**:291–298. Blackwell Publishing Ltd.
523 Vannuccini S, Clifton VL, Fraser IS, Taylor HS, Critchley H, Giudice LC, Petraglia F. Infertility and
524 reproductive disorders: Impact of hormonal and inflammatory mechanisms on pregnancy
525 outcome. *Hum Reprod Update* 2016;**22**:104–115.
526 World Medical Association. Declaration of Helsinki Ethical Principles for Medical Research
527 Involving Human Subjects. *JAMA* 2013;**310**:2191–2194.
528 Wyns C, Geyter C De, Calhaz-Jorge C, Kupka MS, Motrenko T, Smeenk J, Bergh C, Tandler-
529 Schneider A, Rugescu IA, Vidakovic S, *et al*. ART in Europe, 2017: results generated from
530 European registries by ESHRE. *Hum Reprod Open* 2021;1–17.
531 Zhou J, Liu H, Gu HT, Cui YG, Zhao NN, Chen J, Gao L, Zhang Y, Liu JY. Association of cardiac
532 development with assisted reproductive technology in childhood: A prospective single-blind
533 pilot study. *Cell Physiol Biochem* 2014;**34**:988–1000. S. Karger AG.
534

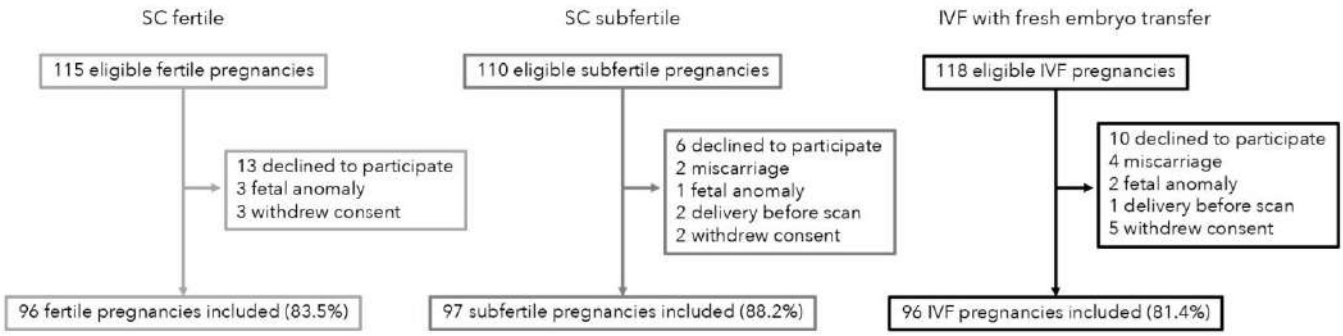

Figure 1
Flowchart of the study populations. SC indicates spontaneously conceived; IVF, in vitro fertilisation.

340x120mm (150 x 150 DPI)

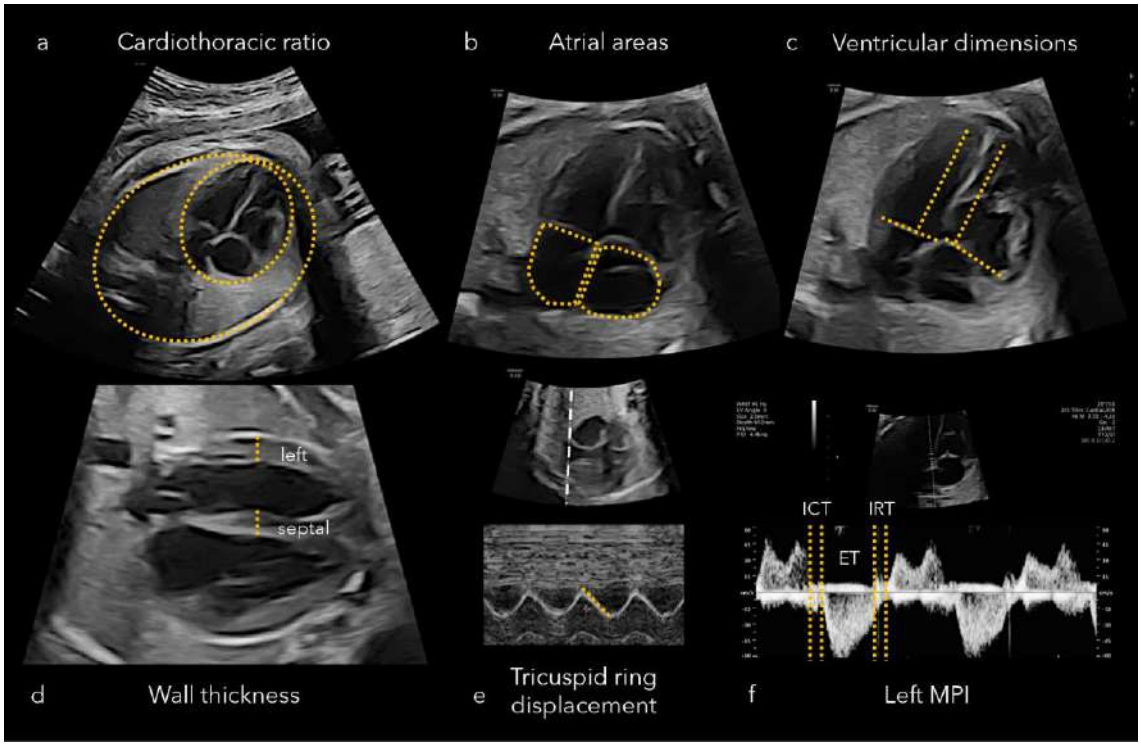

Figure 2
Illustrative echocardiographic measurements: (a) cardiothoracic ratio; (b) left and right atrial areas; (c) left and right ventricular dimensions; (d) left and septal wall thickness; (e) tricuspid ring displacement; (f) left myocardial performance index (MPI), ICT indicates isovolumic contraction time, ET, ejection time, IRT, isovolumic relaxation time.

338x220mm (150 x 150 DPI)

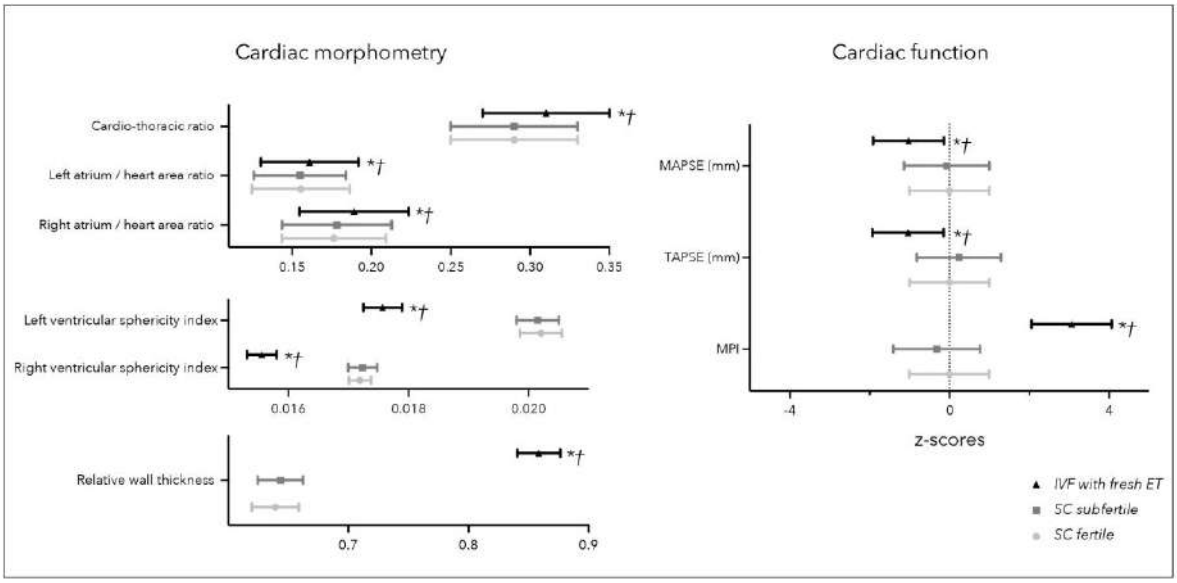

Figure 3
Adjusted cardiac parameters assessed in the study populations displayed as mean ± standard deviation. SC indicates spontaneously conceived; IVF, in vitro fertilisation; MAPSE, mitral annular plane systolic excursion; TAPSE, tricuspid annular plane systolic excursion; MPI, myocardial performance index. Values, z-scores and comparisons are adjusted by linear regression for nulliparity, gestational age and fetal weight at scan, and birthweight centile.
* P<0.05 as compared to SC fertile.
† P<0.05 as compared to SC subfertile.

400x200mm (150 x 150 DPI)

Table I Baseline and infertility characteristics of the study groups.

	SC fertile (n=96)	SC subfertile (n=97)	IVF (n=96)
Maternal characteristics			
Age (y)	37.1 (34.8-38.9)	36.9 (34.3-39.8)	36.6 (34.4-39.0)
Body mass index (kg/m^2)	22.8 (20.4-25.1)	23.3 (20.4-27.9)	23.4 (21.0-27.5)
Caucasian (n(%))	73 (76.0)	74 (76.3)	81 (84.4)
Nulliparity (n(%))	59 (61.5)	**76 (78.4)***	**83 (86.5)***
Polycystic ovarian syndrome (n(%))	8 (8.3)	19 (19.6)	17 (17.7)
Hypertension (n(%))	4 (4.2)	1 (1.0)	2 (2.1)
Cardiovascular disease (n(%))	1 (1.0)	0	2 (2.1)
Kidney disease (n(%))	3 (3.1)	1 (1.0)	0
Thyroid disease (n(%))	9 (9.4)	14 (14.4)	16 (16.7)
Autoimmune disease (n(%))	8 (8.3)	9 (9.3)	5 (5.2)
Pregestational diabetes (n(%))	5 (5.2)	3 (3.1)	1 (1.0)
Psychopharmaceuticals exposure during pregnancy (n(%))	2 (2.1)	0	2 (2.1)
Smoking habit (n(%))	26 (27.1)	30 (30.9)	36 (37.5)
Paternal characteristics			
Age (y)	38.1 ± 5.7	39.7 ± 5.9	39.1 ± 4.3
Body mass index (kg/m^2)	25.3 (23.4-27.0)	25.1 (23.2-27.7)	25.7 (23.7-28.7)
Caucasian (n(%))	70 (72.9)	79 (81.4)	81 (84.4)
Smoking habit (n(%))	15 (5.6)	17 (17.5)	26 (27.1)
Infertility background			
Infertility cause (n(%))			
Unexplained infertility	NA	58 (59.8)	46 (47.9)
Endometriosis	NA	5 (5.2)	13 (13.5)
Tubal factor	NA	3 (3.1)	6 (6.3)
Male factor	NA	31 (32.0)	40 (41.7)

Infertility length (months)	NA	35 (20-48)	47 (25-61)

SC indicates spontaneously conceived pregnancies; IVF, in vitro fertilisation; NA, non-applicable.
Data are mean ± SD, median (interquartile range) or number (percentage), as appropriate.
Statistically significant differences are shown in bold font.
*P<0.05 as compared to SC fertile.

Table II Feto-placental data at scan and perinatal characteristics of the study populations.

	SC fertile (n=96)	SC subfertile (n=97)	IVF (n=96)
Feto-placental data at scan			
Gestational age (weeks, days)	33.5 (30.4-34.2)	33.0 (30.3-34.1)	32.3 (30.2-33.4)
Estimated fetal weight (g)	2026 (1553-2286)	2162 (1615-2429)	1868 (1589-2212)
Mean uterine arteries PI (z-score)	-0.40 (-0.94-0.72)	-0.01 (-0.59-0.74)	-0.06 (-0.75-0.84)
Umbilical artery PI (z-score)	-0.10 ± 0.61	-0.12 ± 0.46	-0.08 ± 0.55
Middle cerebral artery (z-score)	0.01 (-0.90-0.85)	0.02 (-0.64-0.71)	-0.08 (-0.69-0.40)
Cerebroplacental ratio (z-score)	-0.05 (-0.99-0.66)	-0.27 (-0.83-0.61)	-0.34 (-0.98-0.37)
Ductus venosus PI (z-score)	-0.58 ± 0.85	-0.51 ± 0.94	-0.57 ± 0.83
Pregnancy complications (n(%))			
Preterm delivery	4 (4.2)	3 (3.1)	3 (3.1)
Preeclampsia	9 (9.4)	4 (4.1)	6 (6.3)
Gestational diabetes	5 (5.2)	8 (8.2)	12 (12.5)
Placenta previa	1 (1.0)	1 (1.0)	2 (2.1)
Abruptio placentæ	0	1 (1.0)	0
Delivery data			
Gestational age at birth (weeks, days)	39.6 (38.6-40.3)	39.6 (38.6-40.4)	39.6 (39.0-40.5)
Birthweight (grams)	3218 (2971-3425)	3285 (2955-3550)	3245 (2955-3620)
Female (n(%))	45 (46.9)	47 (48.5)	41 (42.7)
Birthweight centile	36 (19-51)	40 (18-73)	47 (15-67)
Small-for-gestational age (n(%))	17 (17.7)	13 (13.4)	15 (15.6)
Large-for-gestational age (n(%))	5 (5.2)	9 (9.3)	7 (7.3)
Induction of labour (n(%))	43 (44.8)	42 (43.3)	39 (40.6)
Caesarean section (n(%))	34 (35.4)	25 (25.8)	36 (37.5)
Neonatal outcome			

Admission to NICU (n(%))	6 (6.3)	4 (4.1)	4 (4.2)
Days at NICU (days)	5 (4-5)	4 (3-16)	3 (3-24)
Neonatal morbidity (n(%))	11 (11.5)	11 (11.5)	6 (6.3)
Minor neonatal morbidity (n(%))	11 (11.5)	10 (10.5)	6 (6.3)
Major neonatal morbidity (n(%))	0	1 (1.0)	0
Neonatal mortality (n(%))	0	0	0

SC indicates spontaneously conceived pregnancies; IVF, in vitro fertilisation; PI, pulsatility index; NA, non-applicable.
Data are median (interquartile range) or number (percentage), as appropriate.

Table III Fetal cardiac assessment of the study groups.

	SC fertile (n=96)	SC subfertile (n=97)	IVF (n=96)	*Crude p-value*	*Adjusted p-value¥*
Fetal echocardiography					
Gestational age at scan (weeks, days)	33.5 (30.4-34.2)	33.0 (30.3-34.1)	32.3 (30.2-33.4)	*0.068*	*NA*
Fetal cardiac morphometry§					
Cardio-thoracic ratio	0.29 ± 0.04	0.29 ± 0.04	**0.31 ± 0.04*†**	***0.003***	***<0.001***
Left atrium / heart area ratio	15.55 ± 3.09	15.50 ± 2.91	**16.11 ± 3.07*†**	*0.324*	***<0.001***
Right atrium / heart area ratio	17.63 ± 3.28	17.82 ± 3.45	**18.91 ± 3.43*†**	***0.027***	***<0.001***
Left ventricular sphericity index	2.02 ± 0.33	2.02 ± 0.27	**1.76 ± 0.25*†**	***<0.001***	***<0.001***
Right ventricular sphericity index	1.72 ± 0.26	1.72 ± 0.26	**1.56 ± 0.25*†**	***<0.001***	***<0.001***
Left-free wall thickness (mm)	3.65 ± 0.69	3.56 ± 0.57	**4.42 ± 0.91*†**	***<0.001***	***<0.001***
Septal wall thickness (mm)	3.43 ± 0.80	3.46 ± 0.48	**4.28 ± 0.73*†**	***<0.001***	***0.001***
Relative wall thickness	0.64 ± 0.18	0.64 ± 0.13	**0.86 ± 0.22*†**	***<0.001***	***<0.001***
Fetal cardiac function					
Mitral ring displacement (mm)	5.89 ± 1.18	5.88 ± 0.98	**5.38 ± 0.96*†**	***0.001***	***<0.001***
Tricuspid ring displacement (mm)	7.89 ± 1.51	8.04 ± 1.18	**7.26 ± 1.07*†**	***<0.001***	***<0.001***
Heart rate (bpm)	137 ± 11	138 ± 13	**139 ± 10*†**	*0.413*	***<0.001***
Left myocardial performance index	0.45 ± 0.10	0.45 ± 0.09	**0.49 ± 0.08*†**	***0.005***	***<0.001***

SC indicates spontaneously conceived pregnancies; IVF, in vitro fertilisation.
§ Cardiac morphometry results measured at end diastole, except for atrial areas.
Data are mean ± SD or median (interquartile range).
¥ Comparisons adjusted by linear regression analyses for nulliparity, gestational age and fetal weight at scan, and birthweight centile.
Statistically significant differences are shown in bold font.
* P<0.05 as compared to SC fertile.
† P<0.05 as compared to SC subfertile.

Table SI Fetal cardiac assessment of the study groups in fertile and unexplained infertility cases.

	SC fertile (n=96)	SC subfertile (n=58)	IVF (n=46)	*Crude p-value*	*Adjusted p-value¥*
Feto-placental data					
Gestational age (weeks, days)	33,5 (30,4-34,2)	33,3 (30,4-34,2)	32,2 (30,1-33,3)	*0.140*	
Estimated fetal weight (g)	2026 (1553-2286)	2171 (1628-2434)	1814 (1591-2188)	*0.196*	
Mean uterine arteries PI (z-score)	-0.40 (-0.94-0.72)	-0.06 (-0.68-0.80)	-0.03 (-0.73-0.92)	*0.356*	
Umbilical artery PI (z-score)	-0.10 (± 0.61)	-0.12 (± 0.44)	-0.01 (± 0.53)	*0.581*	
Middle cerebral artery (z-score)	0.01 (-0.90-0.85)	0.13 (-0.47-0.79)	-0.13 (-1.04-0.27)	*0.106*	
Cerebroplacental ratio (z-score)	-0.05 (-0.99-0.66)	-0.16 (-0.66-0.78)	-0.55 (-1.14-0.28)	*0.164*	
Ductus venosus PI (z-score)	-0.58 (± 0.85)	-0.39 (± 0.90)	-0.64 (± 0.82)	*0.279*	
Fetal echocardiography					
Fetal cardiac morphometry§					
Cardio-thoracic ratio	0.29 ± 0.04	0.29 ± 0.05	**0.32 ± 0.04*†**	***0.002***	***<0.001***
Left atrium / heart area ratio	15.55 ± 3.09	15.63 ± 2.84	**15.83 ± 3.14*†**	*0.883*	***<0.001***
Right atrium / heart area ratio	17.63 ± 3.28	**18.13 ± 3.50***	**18.80 ± 3.52*†**	*0.166*	***<0.001***
Left ventricular sphericity index	2.02 ± 0.33	2.04 ± 0.27	1.75 ± 0.25	***<0.001***	*0.233*
Right ventricular sphericity index	1.72 ± 0.26	1.72 ± 0.27	**1.52 ± 0.22*†**	***<0.001***	***<0.001***
Left-free wall thickness (mm)	3.65 ± 0.69	3.56 ± 0.63	**4.51 ± 0.94*†**	***<0.001***	***<0.001***
Septal wall thickness (mm)	3.43 ± 0.80	3.51 ± 0.48	**4.26 ± 0.71*†**	***<0.001***	***0.001***
Relative wall thickness	0.64 ± 0.18	0.63 ± 0.13	**0.86 ± 0.24*†**	***<0.001***	***<0.001***
Fetal cardiac function					
Mitral ring displacement (mm)	5.89 ± 1.18	5.92 ± 1.01	**5.45 ± 0.85*†**	***0.048***	***<0.001***
Tricuspid ring displacement (mm)	7.89 ± 1.51	8.02 ± 1.18	**7.19 ± 0.96*†**	***0.003***	***<0.001***
Heart rate (bpm)	137 ± 11	137 ± 13	**140 ± 10*†**	*0.449*	***<0.001***
Left myocardial performance index	0.45 ± 0.01	0.45 ± 0.02	**0.50 ± 0.01*†**	***<0.001***	***<0.001***

SC indicates spontaneously conceived pregnancies; IVF, in vitro fertilisation; PI, pulsatility index.

§ Cardiac morphometry results measured at end diastole, except for atrial areas.

Data are mean ± SD or median (interquartile range).

¥ Comparisons adjusted by linear regression analyses for nulliparity, gestational age and fetal weight at scan, and birthweight centile.

*P<0.05 as compared to fertile.

†P<0.05 as compared to subfertile.

Table SII Fetal cardiac assessment of the study groups excluding the cases of vanishing twin phenomenon.

	SC fertile (n=96)	SC subfertile (n=97)	IVF (n=86)	*Crude p-value*	*Adjusted p-value¥*
Feto-placental data					
Gestational age (weeks.days)	32.4 (± 2)	32.2 (± 2)	31.6 (± 2)	*0.177*	
Estimated fetal weight (g)	1975 (± 476)	2023 (± 488)	1895 (± 430)	*0.181*	
Mean uterine arteries PI (z-score)	-0.40 (-0.94-0.72)	-0.01 (-0.59-0.74)	-0.09 (-0.75-0.85)	*0.421*	
Umbilical artery PI (z-score)	-0.10 (± 0.61)	-0.12 (± 0.46)	-0.10 (± 0.55)	*0.971*	
Middle cerebral artery (z-score)	0.01 (-0.90-0.85)	0.16 (-0.64-0.71)	-0.09 (-0.67-0.35)	*0.535*	
Cerebroplacental ratio (z-score)	-0.05 (-0.99-0.66)	-0.27 (-0.83-0.61)	-0.34 (-0.98-0.39)	*0.625*	
Ductus venosus PI (z-score)	-0.58 (± 0.85)	-0.51 (± 0.94)	-0.63 (± 0.80)	*0.637*	
Fetal echocardiography					
Fetal cardiac morphometry§					
Cardio-thoracic ratio	0.29 ± 0.04	0.29 ± 0.05	**0.31 ± 0.04*†**	***0.002***	***<0.001***
Left atrium / heart area ratio	15.55 ± 3.09	15.63 ± 2.91	**16.22 ± 3.13*†**	*0.229*	***<0.001***
Right atrium / heart area ratio	17.63 ± 3.28	17.82 ± 3.45	**19.04 ± 3.28*†**	***0.012***	***<0.001***
Left ventricular sphericity index	2.02 ± 0.33	2.02 ± 0.27	**1.75 ± 0.24*†**	***<0.001***	***<0.001***
Right ventricular sphericity index	1.72 ± 0.26	1.72 ± 0.26	**1.56 ± 0.24*†**	***<0.001***	***<0.001***
Left-free wall thickness (mm)	3.65 ± 0.69	3.56 ± 0.57	**4.39 ± 0.93*†**	***<0.001***	***<0.001***
Septal wall thickness (mm)	3.43 ± 0.80	3.46 ± 0.48	**4.26 ± 0.73*†**	***<0.001***	***0.001***
Relative wall thickness	0.64 ± 0.18	0.64 ± 0.13	**0.85 ± 0.22*†**	***<0.001***	***<0.001***
Fetal cardiac function					
Mitral ring displacement (mm)	5.89 ± 1.18	5.88 ± 0.98	**5.39 ± 0.96*†**	***0.002***	***<0.001***
Tricuspid ring displacement (mm)	7.89 ± 1.51	8.04 ± 1.18	**7.31 ± 1.08*†**	***<0.001***	***<0.001***
Heart rate (bpm)	137 ± 10	137 ± 13	**139 ± 9*†**	*0.481*	***<0.001***
Left myocardial performance index	0.45 ± 0.10	0.45 ± 0.09	**0.49 ± 0.08*†**	***0.004***	***<0.001***

SC indicates spontaneously conceived pregnancies; IVF, in vitro fertilisation; PI, pulsatility index.
§ Cardiac morphometry results measured at end diastole, except for atrial areas.
Data are mean ± SD or median (interquartile range).
¥ Comparisons adjusted by linear regression analyses for nulliparity, gestational age and fetal weight at scan, and birthweight centile.
*P<0.05 as compared to fertile.
†P<0.05 as compared to subfertile.

Study 3

Stimulated versus natural cycle in vitro fertilization procedures: unravelling the origins of fetal cardiac remodeling in assisted reproductive technologies

Boutet ML, Casals G, Valenzuela-Alcaraz B, García-Otero L, Crovetto F, Carrillo P, Borrás A, Cívico MS, Manau D, Gratacós E, Crispi F.

Status: In preparation

Full title: **Stimulated versus natural cycle in vitro fertilization procedures: unravelling the origins of fetal cardiac remodeling in assisted reproductive technologies**

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ABSTRACT

Objectives

Cardiac remodeling and dysfunction have been demonstrated in fetuses conceived through *in vitro* fertilization (IVF). However, pregnancies included in previous studies were obtained following conventional stimulated IVF. Ovarian stimulation has a potential impact on oocyte quality and endometrial milieu that may affect perinatal results. Our aim was to compare cardiac morphometry and myocardial performance index (MPI) in fetuses conceived following conventional IVF with those obtained after natural cycle IVF (NC-IVF), performed without a previous ovarian stimulation.

Methods

Prospective cohort study of 102 singleton pregnancies including 34 spontaneously conceived from fertile couples, 34 following NC-IVF and 34 after conventional IVF with fresh embryo transfer, matched by maternal age, ethnicity, and gestational age at scan. Fetal echocardiography was performed at 29-33 weeks. Comparisons between groups were adjusted by gestational age and fetal weight at scan, and birthweight centile.

Results

Overall, as compared to the fertile, both IVF groups showed significant signs of fetal cardiac remodeling (cardio-thoracic ratio: conventional IVF mean 0.31 [SD 0.04] vs NC- IVF 0.31 [0.04] vs fertile 0.28 [0.04], adjusted P-value <0.001; larger right atria-to-heart ratio: conventional IVF 19.1% [3.2] vs NC- IVF 18.2% [3.3] vs fertile 17.2% [2.9], adjusted P-value <0.001; increased relative wall thickness: conventional- IVF 0.83 [0.24] vs NC- IVF 0.85 [0.3] vs fertile 0.72 [0.3], adjusted P-value<0.001) and suboptimal function (MPI: conventional IVF 0.50 [0.07] vs NC- IVF 0.48 [0.08] vs fertile 0.47 [0.08], adjusted P-value<0.001), with more pronounced changes in the conventional IVF group.

Conclusions

Fetuses conceived following IVF showed signs of cardiac remodeling as compared to the fertile group, even in NC-IVF (in absence of ovarian stimulation). These results underscore the importance of future studies for assessing the long-term cardiovascular health in fetuses conceived following IVF.

Keywords: IVF - fetal heart - cardiac remodeling - fetal echocardiography - ovarian stimulation- natural cycle IVF

METHODS

Study design and population

This is a nested case-control study within a prospective cohort of singleton pregnancies conceived by IVF and SC, who attended the Barcelona Center for Maternal Fetal and Neonatal Medicine (BCNatal, Barcelona, Spain) from 2017 to 2021.

A total of 102 singleton pregnancies, including 34 SC from fertile couples (with a time-to-pregnancy below 12 months), 34 following NC-IVF, and 34 after conventional IVF in stimulated cycles with fresh embryo transfer, was selected from a prospective cohort, matched by maternal age, ethnicity, and gestational age at scan. Oocyte donation cycles, fetal malformations and fetal chromosomal anomalies were considered exclusion criteria.

Eligible pregnancies from IVF procedures were enrolled from our Assisted Reproduction Unit (Hospital Clínic de Barcelona) at pregnancy diagnosis and eligible SC pregnancies were recruited in our Maternal-Fetal Unit (Hospital Clínic de Barcelona) at third trimester.

Natural cycle-IVF protocol

Patients undergoing NC-IVF presented regular menstrual cycles (24–36 days) and attended their first hormonal (estradiol and luteinizing hormone in plasma) and ultrasound assessment on 5^{th} or 6^{th} day of the cycle. Hormonal and ultrasound controls were repeated onwards every 24 or 48 hours, until a follicle of minimum 15 mm with a consistent rise in serum estradiol concentration was observed, after which hCG administration (Ovitrelle 250 mg s.c., Serono S.A.) was indicated. A single dose of 0.25 mg SC injection of a GnRH antagonist (cetrorelix, Cetrotide; Merck Serono, or ganirelix, Orgalutran; MSD) was indicated to prevent premature ovulation before oocyte retrieval. In our institution, gonadotropin use in NC-IVF was limited to cases with LH surge detection before reaching a minimum follicular size of 15mm. Only in these cases, 75 international units (IU) of SC FSHr (Gonal-F; Merck-Serono S.A., Madrid, Spain) or hMG (Menopur, Ferring SA, Madrid, Spain) plus GnRH antagonist were used daily until trigger indication. Thirty-six hours after hCG administration, ultrasound-guided transvaginal oocyte retrieval was performed under minimal or no sedation, and systematic follicular flushing was carried out for 3 to 6 times.

Ovarian stimulation protocols

IVF ovarian stimulation protocol and gonadotropin doses were selected considering the patient´s age and ovarian reserve markers. Either long agonist or antagonist protocols were used. Daily doses from 150 to 300 IU of FSHr (Gonal-F; Merck-Serono S.A., Madrid, Spain) alone or with the addition of 75 IU of LHr (Luveris; Merck-Serono S.A., Madrid, Spain) or hMG (Menopur, Ferring SA, Madrid, Spain) were used. In presence at least two follicles ≥18 mm of diameter, with ≥4 follicles measuring ≥14 mm in association with a consistent rise in serum estradiol concentration, hCG administration (Ovitrelle 250 mg s.c., Serono S.A.) was indicated. Thirty-six hours after hCG administration, ultrasound-guided transvaginal oocyte retrieval was performed.

Embryo culture protocol

All the laboratory procedures were identical for NC-IVF and conventional IVF. Embryo culture was performed in microdrops of Global Media (Life Global, CooperSurgical Målov, Denmark) under mineral oil at 37ºC in an atmosphere of 7% O2 and 6.5% CO2. The ASEBIR criteria (Balaban et al., 2011) was used to assess embryo quality. The Gardner and Schoolcraft criteria (Gardner et al., 2000) was used to assess the quality of blastocysts.

Embryo transfer protocol

In all IVF cycles with subsequent fresh embryo transfer, 200 mg every 8 hours of vaginal micronized natural progesterone was started the morning after the oocyte retrieval. Embryos were transferred on day 3 or day 5. Pregnancy diagnosis was performed by means of serum β-hCG test 12 days after embryo transfer. At 5-6 weeks´ gestation transvaginal ultrasonography was performed in all pregnancies.

Data collection and study protocol

The study protocol included collection of maternal and perinatal characteristics and a third trimester ultrasound scan with fetal biometry, feto-placental Doppler assessment and fetal echocardiography.

Parental demographics and clinical background were collected by personal interviews and review of medical records, including age, ethnicity, body mass index, socioeconomic status (illiterate or only primary educational level were

considered low socioeconomic status), chronic diseases (e.g. hypertension, diabetes mellitus, autoimmune disease), parity, fertility and obstetric history, mode of conception, smoking status, alcohol consumption and illicit substance abuse (heroin, cocaine, or cannabis) during pregnancy.

The IVF-related parameters collected included etiology and length of infertility, polycystic ovarian syndrome (PCOS) diagnosis according to Rotterdam criteria (Fauser, 2004), data on previous unsuccessful ART cycles, current ovarian stimulation protocol (type of aGnRH and gonadotropin, days of stimulation, total dose of gonadotropins in international units), number of oocytes retrieved, fertilization technique, number of embryos obtained, number of embryos transferred, embryonic stage at embryo transfer, vanishing twin phenomenon or selective embryonic reduction.

Pregnancy and perinatal outcomes collected included gestational age at delivery, mode of delivery, birth weight, Apgar score, umbilical artery pH, Neonatal Intensive Care Unit (NICU) admission, perinatal morbidity and mortality, and the presence of pregnancy complications such as gestational diabetes, pregnancy-induced hypertension / preeclampsia / eclampsia, HELLP syndrome, cholestasis, abruptio placentæ, placenta previa, and preterm birth (PTB, <37 weeks of gestation). Gestational age was calculated based on first trimester crown-rump length (Robinson, 1973). SGA and LGA were defined respectively as birth weight below 10^{th} centile and above the 90^{th} centile according to customized standards (Figueras et al., 2008).

Delivery prior to the 37th week of gestation was considered preterm birth. Preeclampsia was defined by new onset of hypertension of ≥ 140 mmHg systolic blood pressure and/or ≥ 90 mmHg diastolic blood pressure, on two occasions at least four hours apart, after 20 week´s gestation together with proteinuria (≥ 300 mg proteins in 24-hour urine or protein/creatinine ratio ≥ 0.3) or new onset of maternal thrombocytopenia, renal insufficiency, liver dysfunction, pulmonary edema or neurological features (ACOG Practice Bulletin, Number 222, 2020). Gestational diabetes was defined as a glucose intolerance with onset or first recognition during pregnancy and diagnosed by means of a pathologic oral glucose tolerance test (usually indicated after an altered fasting glucose determination or an altered glucose challenge test from the second trimester on), according to the National Diabetes Data Group (NDDG). The presence of at least one of the following features was used to define major neonatal morbidity: bronchopulmonary dysplasia, necrotizing enterocolitis, periventricular

leukomalacia, intraventricular hemorrhage, retinopathy, persistent ductus arteriosus, or sepsis. Minor neonatal morbidity was defined by the presence of at least one of the following, after excluding major neonatal morbidity criteria: respiratory distress, hyperbilirubinemia, or anemia. Perinatal mortality was defined as the occurrence of death between the 22nd week of pregnancy and the 28th day from birth.

Fetal ultrasound

An ultrasonographic examination at 28-34 weeks of gestation using either a Voluson 730 Expert (GE Medical Systems, Milwaukee, Wisconsin, USA) or a Voluson S10 ultrasound system (GE Healthcare, Zipf, Austria) with 2-5 MHz linear curved-array probes was performed, including fetal weight estimation, feto-placental Doppler, and fetal echocardiography. Ultrasound acquisitions were obtained by four maternal-fetal medicine specialists trained in fetal echocardiography and blinded to the mode of conception. Images were anonymized and recorded as clips, and measurements were performed off-line by a single investigator.

Fetal biometry and feto-placental Doppler

Fetal weight was estimated based on the Hadlock formula (Hadlock et al., 1985), including the following parameters: abdominal circumference, head circumference, biparietal diameter and femur length. Centile calculations were made following local reference curves (Figueras et al., 2008). Feto-placental Doppler assessment included the measurement of pulsatility index of umbilical artery, fetal middle cerebral artery and ductus venosus (Arduini and Rizzo, 1990; Bhide et al., 2013; Hecher et al., 1994) in all cases. Cerebroplacental ratio was calculated as middle cerebral artery PI divided by umbilical artery PI (Baschat and Gembruch, 2003). Z-scores of the above mentioned Doppler parameters were calculated based on previous publications (Baschat and Gembruch, 2003; Gómez et al., 2008; Kessler et al., 2006). Doppler estimations were done in the absence of fetal movements and with the mother under voluntary suspended ventilation if required.

Fetal echocardiography

A two-dimensional and Doppler echocardiographic examination was performed to assess fetal structural heart integrity, to exclude cardiac defects, and to

evaluate cardiac morphometry and function following standard protocols (Carvalho et al., 2013; Crispi et al., 2013; García-Otero et al., 2019). Cardiac, thoracic, and ventricular dimensions were measured on 2D images from an apical or basal four-chamber view at end-diastole. Ventricular sphericity indices were calculated by dividing longitudinal ventricular diameters by basal-transverse. Atrial areas were measured at maximum distention from an apical or basal four-chamber view. Atria-to-heart ratios were calculated as atrial area * 100 / heart area. Left and septal myocardial wall thicknesses were measured on 2D images from a transverse four-chamber view at end diastole. Relative wall thickness was calculated as the left plus septal wall thickness divided by left ventricular end-diastolic diameter (Foppa et al., 2005). Left myocardial performance index (MPI) was obtained from a single Doppler spectrum in a cross-sectional image of the fetal thorax, calculated as (ICT + IRT) / ET (Hernandez-Andrade et al., 2005).

Statistical analysis

Statistical analysis was performed using STATA 15.1 (StataCorp LLC, College Station, TX, USA). Fetal cardiovascular assessment parameters constituted the study outcome. The mode of conception (NC-IVF, conventional IVF or spontaneous pregnancy from fertile couples) was the independent variable of interest. Descriptive statistics and results were expressed as mean (± standard deviation), median (interquartile range), or number (percentage), as appropriate. The normal distribution of fetal cardiac parameters was verified by means of the Shapiro-Wilk test and histograms. Comparisons among the study groups were performed using ANOVA or Kruskal-Wallis with Bonferroni correction, after checking the fulfilment of each of these tests´ assumptions. Between-group differences in feto-placental data and cardiovascular parameters were assessed using multiple regression analyses to adjust for gestational age and fetal weight at scan, and birthweight centile. The level of significance was set at 0.05 for all the statistical tests. All reported p-values are 2 sided.

Ethical approval

The local ethics committee approved this clinical study protocol (HCB/2017/0714). All patients willing to participate provided their written informed consent. This study was conducted following the Declaration of

Helsinki for Medical Research involving Human Subjects (World Medical Association, 2013).

RESULTS

Table I. Baseline and infertility characteristics of the study groups.

	Fertile (n=34)	**NC-IVF** (n=34)	**Conventional IVF** (n=34)
Maternal characteristics			
Age (y)	37.4 (35.9-38.5)	37.7 (36.1-39.1)	37.8 (35.4-39.1)
Body mass index (kg/m^2)	22.5 (20.8-24.7)	22.0 (20.6-25.1)	23.2 (21.4-28.3)
Smoking habit (n(%))	6 (17.7)	8 (23.5)	6 (17.7)
Caucasian (n(%))	28 (82.4)	30 (88.2)	31 (91.2)
Nulliparity (n(%))	20 (58.8)	24 (70.6)	29 (85.3)
Polycystic ovarian syndrome (n(%))	1 (2.9)	0	4 (11.8)
Paternal characteristics			
Age (y)	37.7 (± 4.4)	40.1 (± 4.7)	38.8 (± 3.8)
Body mass index (kg/m^2)	25.9 (23.6-26.8)	24.4 (22.6-26.3)	25.2 (23.7-27.5)
Smoking habit (n(%))	5(14.7)	8 (23.5)	8 (23.5)
Caucasian (n(%))	29 (85.3)	28 (82.4)	30 (88.2)
Infertility and ART characteristics			
Infertility cause (n(%))			
Unexplained infertility		15 (44.1)	14 (41.2)
Endometriosis		6 (17.7)	5 (14.7)
Tubal factor		2 (5.9)	4 (11.8)
Male factor		10 (29.4)	13 (38.2)

IVF indicates *in vitro* fertilization; NC-IVF, natural cycle IVF.

Data are mean (± SD), median (interquartile range) or number (%), as appropriate.

Table II. Perinatal characteristics of the study groups

	Fertile (n=34)	**NC-IVF** (n=34)	**Conventional IVF** (n=34)
Pregnancy complications (n(%))			
Vanishing twin phenomenon	0	1 (2.9)	3 (8.8)
Preterm delivery	1 (2.9)	0	1 (2.9)
Preeclampsia	1 (2.9)	1 (2.9)	2 (5.9)
Gestational diabetes	1 (2.9)	4 (11.8)	5 (14.7)
Placenta previa	0	2 (5.9)	0
Abruptio placentæ	0	0	0
Prenatal corticoid exposure	0	0	0
Prenatal aspirin exposure	5 (14.7)	3 (8.8)	6 (17.7)
Delivery data			
Gestational age at birth (weeks, days)	40,3 (39,0-40,5)	39,6 (38,1-40,2)	40,0 (38,6-40,4)
Birthweight (grams)	3325 (± 328)	3146 (± 412)	3272 (± 426)
Female (n(%))	16 (47.1)	19 (55.9)	16 (47.1)
Birthweight centile	44 (24-62)	36 (17-65)	44 (19-79)
Small-for-gestational age (n(%))	3 (8.8)	6 (17.7)	6 (17.7)
Large-for-gestational age (n(%))	2 (5.9)	4 (11.8)	4 (11.8)
Induction of labor (n(%))	8 (23.5)	12 (37.5)	14 (43.8)
Cesarean section (n(%))	10 (29.4)	12 (35.3)	17 (50.0)
Neonatal outcome			
Admission to NICU (n(%))	0	4 (11.8)	1 (2.9)
Days at NICU (days)	0	17 (6-30)	3 (3-3)
Neonatal morbidity (n(%))	2 (5.9)	6 (17.7)	3 (8.8)
Minor neonatal morbidity (n(%))	2 (5.9)	6 (17.7)	3 (8.8)
Major neonatal morbidity (n(%))	0	0	0
Neonatal mortality (n(%))	0	0	0

IVF indicates *in vitro* fertilization; NC-IVF, natural cycle IVF.
Data are mean (± SD), median (interquartile range) or number (%), as appropriate.

Table III Feto-placental data at scan.

	Fertile (n=34)	**NC-IVF** (n=34)	**Conventional IVF** (n=34)	*p-value*
Gestational age (weeks,days)	31,4 (29,4-33,3)	30,5 (29,2-32,6)	31,1 (29,2-33,0)	*0.720*
Estimated fetal weight (g)	1840 (1526-2210)	1660 (1353-1991)	1780 (1404-2053)	*0.285*
Estimated fetal weight centile	69 (32-87)	45 (14-83)	63 (19-81)	*0.422*
Cerebroplacental ratio (z-score)	0.31 (± 1.04)	-0.12 (± 1.19)	0.16 (± 1.59)	*0.257*
Ductus venosus PI (z-score)	-0.48 (± 0.80)	-0.46 (± 1.01)	-0.80 (± 0.81)	*0.217*

IVF indicates *in vitro* fertilization; NC-IVF, IVF in natural cycle.

Data are mean (± SD) or median (interquartile range) as appropriate.

Table IV Fetal cardiac assessment of the study groups.

Fetal echocardiography§	**Fertile** (n=34)	**NC- IVF** (n=34)	**IVF Fresh ET** (n=34)	*Crude p-value*	*Adjusted p-value*¥	*Linear trend p-value*¥
Cardio-thoracic ratio	0.28 ± 0.04	0.31 ± 0.04*	0.31 ± 0.04*	***0.003***	***<0.001***	***<0.001***
Left atrium-to-heart area$^{\Upsilon}$	16.37 ± 3.07	17.30 ± 3.78*	16.75 ± 2.70*†	*0.512*	***<0.001***	***0.004***
Right atrium-to-heart area$^{\Upsilon}$	17.22 ± 2.92	18.23 ± 3.29	19.10 ± 3.20*†	*0.057*	***<0.001***	***<0.001***
Left ventricular sphericity index	1.94 ± 0.29	1.95 ± 0.30	1.79 ± 0.22*†	***0.025***	***<0.001***	***<0.001***
Right ventricular sphericity index	1.65 ± 0.25	1.73 ± 0.37*	1.56 ± 0.22*†	*0.065*	***<0.001***	***<0.001***
Left-free wall thickness (mm)	3.56 ± 0.95	3.95 ± 0.64*	3.87 ± 0.79*	*0.157*	***<0.001***	***<0.001***
Septal wall thickness (mm)	3.42 ± 0.82	3.70 ± 0.55*	3.63 ± 0.54*	*0.214*	***<0.001***	***<0.001***
Relative wall thickness	0.72 ± 0.32	0.85 ± 0.30*	0.83 ± 0.24*	*0.238*	***<0.001***	***<0.001***
Left myocardial performance index	0.47 ± 0.08	0.48 ± 0.08*	0.50 ± 0.07*†	*0.240*	***<0.001***	***<0.001***

IVF indicates *in vitro* fertilization; NC-IVF, IVF in natural cycle.

Data are mean ± SD.

§ Cardiac morphometry results measured at end diastole, except for atrial areas.

$^{\Upsilon}$ Atria data normalized by dividing by cardiac area and multiplied by 100.

¥ Comparisons adjusted by linear regression analyses for gestational age and fetal weight at scan, and birthweight centile:

*P<0.05 as compared with fertile.

†P<0.05 as compared to natural cycle IVF.

Study 4

Fetal neurosonography and infant neurobehavior in assisted reproductive technologies following fresh and frozen embryo transfer

Boutet ML, Eixarch E, Ahumada-Droguett P, Nakaki A, Crovetto F, Cívico MS, Borrás A, Manau D, Gratacós E, Crispi F, Casals G.
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Fetal neurosonography and infant neurobehavior in assisted reproductive technologies following fresh and frozen embryo transfer

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Short title: Neurodevelopment in offspring conceived by ART

Keywords: ART, IVF, mode of conception, prenatal imaging, fetal brain, neurosonography, cortical folding, neurodevelopment

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Contribution:

What are the novel findings of this work?

- Is the first study assessing brain cortical folding in fetuses from ART, showing differences between spontaneously conceived and ART offspring before postnatal influences on neurodevelopment occur.
- It suggests the existence of in utero brain reorganization associated to ART, which could explain their differential performance at 12 months of age.

What are the clinical implications of this work?

- Neurosonography is an appropriate tool to identify subtle brain differences among fetuses exposed and not exposed to ART.
- These findings support the relevance of a neurodevelopmental follow-up in offspring conceived by ART.

ABSTRACT

Objective: To explore and compare fetal cortical brain development and infant neurobehaviour in spontaneously conceived (SC) and assisted reproductive technologies (ART) offspring.

Methods: A prospective cohort study of 210 singleton pregnancies including 70 SC pregnancies, 70 conceived by *in vitro* fertilization (IVF) following frozen embryo transfer (ET), and 70 IVF after fresh ET. Fetal neurosonography was performed at 32±2 weeks to assess cortical development. Sulci depths were measured off-line and normalized by biparietal diameter. Additionally, *Ages & Stages Questionnaires* (ASQ) were obtained postnatally, at 12±1 months of corrected age. Comparisons were adjusted by maternal age, ethnicity, nulliparity, fetal sex, weight centile and gestational age at scan for neurosonography, and by maternal age, ethnicity, nulliparity, educational level, employment status, new-born's gestational age at birth, breastfeeding, infant's sex and age at the ASQ evaluation.

Results: In comparison to the SC, the fetuses conceived by ART showed statistically significant differences in cortical development, with reduced parieto-occipital (fresh ET mean[SD] 12.5mm[2.5] *vs.* frozen ET 13.4[2.6] *vs.* SC 13.4[2.6], p<0.001), cingulate (fresh ET 5.8[1.8] *vs.* frozen ET 6.0[2.1] *vs.* SC 6.4[1.9], p<0.001), and calcarine (fresh ET 13.3[3.9] *vs.* frozen ET 14.1[2.8] *vs.* SC 16.1[2.7], p=<0.001) sulci depth, together with lower Sylvian fissure grading score. Changes in cortical development were more pronounced in the fresh ET group as compared to the frozen ET. Additionally, ART infants showed lower ASQ scores, especially in the fresh ET group (global ASQ z-scores: fresh ET mean[SD] -0.3[0.4] *vs.* frozen ET -0.2[0.4] *vs.* SC 0[0.4], p<0.001).

Conclusions: Fetuses conceived by ART show a distinctive pattern of cortical development and suboptimal infant neurodevelopment, with more pronounced changes in fresh ET. These findings support the existence of *in utero* brain reorganization associated to ART and warrant follow-up studies to assess their long-term persistence.

INTRODUCTION

The number of pregnancies conceived by assisted reproductive technologies (ART) is currently increasing worldwide[1]. There is a growing interest in the neurodevelopment of subjects conceived by ART, which has been mostly studied in children and adolescents. Some follow-up studies have suggested a suboptimal neurodevelopment at comparing ART offspring with the general population, although there are inconsistencies in literature[2–4]. Fresh and frozen embryo transfer (ET) in *in vitro* fertilization (IVF) cycles show different perinatal risk profiles[5,6], and some registry-based studies have also revealed poorer neurologic results for the fresh ET modality as compared to the frozen ones[7,8]. The variability observed in previous studies reporting postnatal neurodevelopment in ART offspring, might be partially explained by the influence of socioeconomic and educational levels during childhood and adolescence that limit direct comparisons with spontaneously conceived (SC) offspring.

Prenatal neurosonography enables an accurate evaluation of the fetal brain cortical folding, a surrogate marker of brain maturation, avoiding the influence of postnatal factors. Interestingly, only two previous studies have explored the central nervous system in fetuses conceived by ART *versus* SC, with contradictory results, reporting variations in first-trimester brain volumes[9] and no differences during the second trimester among groups[10]. However, no previous studies have evaluated fetal brain cortical development in the third trimester of pregnancy, which would be the optimal time period to study prenatal brain maturation.

We aim to explore and compare fetal cortical brain development by neurosonography in fetuses conceived by ART -including frozen *versus* fresh ET-, and to investigate its association with postnatal neurobehaviour at 12 months of age.

MATERIALS AND METHODS

Study populations and protocol

We conducted a prospective cohort study of 210 singleton pregnancies from 2017 to 2020, including 70 SC and 140 conceived by IVF after frozen ET (n=70) and fresh ET (n=70).

All pregnancies achieved by ART were recruited from a single centre during the first trimester (Assisted Reproduction Unit, Hospital Clínic de Barcelona), ensuring homogeneity in ovarian stimulation and endometrial preparation protocols, laboratory procedures and embryo culture conditions between the study participants. In addition, a group of SC pregnancies was recruited during the third trimester from fertile couples (with a time-to-pregnancy no longer than 12 months) who attended the BCNatal Barcelona Center for Maternal Fetal and Neonatal Medicine (Hospital Clínic and Hospital Sant Joan de Déu). The enrolment of the SC population started eleven months after the beginning of the ART participants´ recruitment, and they were matched with them by maternal age (± 1 year) and gestational age at neurosonography (± 1 week). Pregnancies after oocyte-donation cycles were not eligible. Intrauterine infection, fetal malformations or chromosomal anomalies were considered exclusion criteria. **Figure 1** shows the flow diagram of the study population.

Maternal demographics, ART-related and obstetric variables were collected by direct patient´s interview and review of medical records, being the conditions associated to decreased fertility probably underdiagnosed in the SC population. Upon delivery, pregnancy and perinatal outcomes were recorded. Gestational age was calculated according to first trimester crown-rump length (CRL) measurement on first-trimester ultrasound[11]. Small for gestational age (SGA) and large for gestational age (LGA) were defined respectively as birth weight below 10th centile or above the 90th centile[12] according to local standards[13]. Preeclampsia was defined by new onset of hypertension of ≥ 140 mmHg systolic blood pressure and/or ≥ 90 diastolic blood pressure, on two occasions at least four hours apart, after 20 week´s gestation, together with proteinuria (≥ 300 mg proteins in 24 hours urine or protein/creatinine ratio ≥ 0.3), or -in the absence of proteinuria- new onset of maternal thrombocytopenia, renal insufficiency, liver dysfunction, pulmonary edema or neurological features[14]. Major neonatal morbidity was defined by the presence of at least one of the following: bronchopulmonary dysplasia, necrotizing enterocolitis, intraventricular haemorrhage, periventricular leukomalacia, retinopathy, persistent ductus arteriosus, or

sepsis. Minor neonatal morbidity was defined by the presence of at least one of the following: respiratory distress, hyperbilirubinemia, or anaemia. Perinatal mortality was defined by either intrauterine fetal death after 22 weeks of pregnancy or neonatal death within the first 28 days of life.

The study protocol included the performance of fetal neurosonography in third trimester and postnatal *Ages and Stages Questionnaires*[15] (ASQ) at 12 months of corrected age. This study was conducted according to the Declaration of Helsinki for Medical Research involving Human Subjects[16]; the study protocol was evaluated and approved by the local ethics committee (HCB/2017/0714), and all participants provided their written informed consent.

ART protocols

Ovarian stimulation protocols

The ovarian stimulation protocol for IVF and the gonadotropin doses were chosen according to age and ovarian reserve markers. Either long agonist or antagonist protocols were used. Ovarian stimulation was achieved with daily doses from 150 to 300 IU of FSHr (Gonal-F; Merck-Serono S.A., Madrid, Spain) alone or with the addition of 75 IU of LHr (Luveris; Merck-Serono S.A., Madrid, Spain) or HMG (Menopur, Ferring SA, Madrid, Spain). The hCG administration (Ovitrelle 250 mg s.c., Serono S.A.) was indicated in the presence of two or more follicles ≥18 mm of diameter, with ≥4 follicles measuring ≥14 mm in association with a consistent rise in serum estradiol concentration. US-guided transvaginal oocyte retrieval was performed 36 hours after hCG administration.

IVF laboratory procedures

Embryo culture was carried out in microdrops of Global Media (Life Global, CooperSurgical Målov, Denmark) under mineral oil at 37ºC in an atmosphere of 6.5% CO2 and 7% O2. Embryo quality was assessed according to the ASEBIR criteria[17]. The quality of blastocysts was assessed according to the criteria of Gardner and Schoolcraft[18].

Vitrification and warming protocols of both cleavage embryos and blastocysts were performed using commercially available kits (Kitazato, Tokyo, Japan) according to the method described by Kuwayama[19]. After warming, embryos were cultured in Global medium containing 10% protein substitute supplement (Life Global, CooperSurgical, Målov, Denmark) until ET. Cleavage embryos with at least 50% of their cells intact immediately after warming

and further development after a 24-hour culture period were considered as surviving embryos and transferred. Surviving blastocysts were defined as re-expanded or starting to re-expand within 2 hours after warming.

In preimplantation genetic testing for monogenic defects (PGT-M) cases, embryos underwent biopsy on day 3 and unaffected embryos were transferred or cryopreserved two days later at blastocyst stage. Trophectoderm biopsy at blastocyst stage and subsequent vitrification was performed in preimplantation genetic testing for aneuploidies (PGT-A) cases.

ET protocols

In pregnancies conceived by IVF with fresh ET vaginal natural progesterone was started the morning after the oocyte retrieval with a dose of 200 mg every 8 hours. Cleavage embryos were transferred on day 3 and blastocysts on day 5.

Frozen ET was performed either in natural cycle or under endometrial preparation. Natural cycle was chosen depending on menstrual cycle regularity and patient´s preference. For frozen ET in natural cycle ultrasound surveillance was started the day 8-9 of the cycle (depending on its duration), and once the dominant follicle reached 17-18 mm a daily follow-up was done until its disappearance, being that day consequently defined as day 0. Frozen ET under endometrial preparation was achieved using transdermal oestrogens (Evopad 50 mcg; Janssen, Toledo, Spain) with 3 patches replaced every 72 hours, or oral estradiol valerate (Progynova 2 mg every 8h; Bayer, Barcelona, Spain). Oestrogen was started the first day of the cycle and ultrasound monitoring was performed after 12-15 days of treatment. Vaginal natural progesterone (200 mg every 8 hours) was added when endometrial thickness was ≥7 mm by US (Progeffik; Effik, Alcobendas, Spain; or Utrogestan; SEID, Barcelona, Spain). The first day of progesterone treatment was considered day 0, cleavage embryos were thawed on day 3 and transferred on day 4, and blastocyst embryos were thawed and transferred on day 5. Supplementation with oestrogens and progesterone was performed until the 12th week of pregnancy.

Pregnancy was diagnosed by a positive serum β-hCG test 12 days after ET and a transvaginal ultrasound was performed in all pregnancies at 5-6 weeks of gestation.

Fetal neurosonography

Neurosonographic acquisition

A detailed two-dimensional neurosonography was performed in all fetuses during the third trimester (32 ± 2 weeks) using a GE Voluson 730 Expert (GE Medical Systems, Milwaukee, Wisconsin, USA). A complete neurosonographic examination was performed initially to confirm structural brain normality. We used a standardized protocol following the International Society of Ultrasound in Obstetrics and Gynecology (ISUOG) guidelines[20] including axial (transventricular and transthalamic) and coronal (transthalamic, transcaudate and transcerebellar) planes. Axial planes were obtained by transabdominal approach. Coronal views were obtained by transvaginal approach in cases of cephalic position and transabdominally in fetuses with breech position. Patients with poor image quality for delineation of measurements (mainly due to fetal presentation or patient´s intolerance to the transvaginal approach, but also because of the presence of severe endometriosis, previous abdominopelvic surgeries, high maternal body mass index and placenta previa) were excluded.

Image processing, linear measurements, and assessment of cortical folding

Measurements were performed offline using the OsiriX MD 12.0 imaging software (Pixmeo SARL, Geneva, Switzerland) by a single experienced examiner blinded to study groups. To provide rigorous perpendicular measurements to the midline, a straight line projecting the interhemispheric fissure was traced in every plane going from frontal bone to occipital bone in axial views and from cranial to caudal bone in coronal views. Brain structures were measured according to previous studies[21–24]. In brief, fissures/sulci depths were measured in millimetres (**Figure 2**), and values were corrected by BPD and multiplied by 100 to normalize by head size[21,22]. Parieto-occipital sulcus depth was evaluated in a slightly cranial plane above the transventricular plane, where the full depth or triangle shape of the sulcus was visualized, drawing a perpendicular line from midline to the apex of the sulcus excluding the cortex[22]. Cingulate sulcus depth was measured in the coronal transthalamic plane tracing a perpendicular line from the interhemispheric midline until the apex of the sulcus without including the cortex. Calcarine sulcus depth was measured in the coronal view using the transcerebellar plane, drawing a perpendicular line from midline to the apex of the sulcus excluding the cortex.

Cortical fissures/sulci were graded according to previously described methodology[25]. The degree of cortical development of Sylvian fissure, parieto-occipital, cingulate and calcarine

sulci was evaluated, and grading scores in a scale from 0 (no maturation) to 5 (maximum degree of maturation) were assigned to each case.

For these sulci depth measurements, an intraclass correlation coefficient (ICC) between 0.685 and 0.971 intraobserver, and between 0.773 and 0.917 interobserver were reported in a previous publication of our research group[23]. In the same report, the Cohen´s kappa coefficient calculated for the sulci grading scores lied between 0.894 y 0.955 for intraobserver, and between 0.765 and 0.906 for interobserver variability.

Postnatal ASQ assessment

Postnatal neurobehaviour was assessed at a mean of 12±1 months of corrected age by means of a Spanish version of the ASQ 2nd edition, a first-level comprehensive screening program widely used to determine the child´s performance compared with standards taken from typically developing children of the same age, and validated for its application both by parents or primary caregivers[15,26]. The ASQ screening system is composed of several questionnaires designed from four to sixty months, providing information on five different domains for each age: communication, personal-social, problem-solving, gross and fine-motor skills. In most cases these questionnaires accurately identify infants or young children who need further evaluation to determine whether they are eligible for early intervention services. In the current study, parents completed the questionnaires by electronic mail under researchers' supervision. In order to assess the ASQ scores results among groups, data were compared by means of Z-scores using the SC population as a reference group.

Statistical analysis

Data management and statistical analyses were performed using STATA 16 (Statacorp, College Station, Texas, US). The pre-specified study outcomes were fetal neurosonographic measurements and postnatal neurobehavioural scores per domain. The independent or exposure variable of interest was the mode of conception (spontaneous, ART with frozen ET, ART with fresh ET). Normal distribution of continuous variables was checked using the Shapiro-Wilk test and histograms. Descriptive statistics and results were expressed as mean (± standard deviation), median (interquartile range) or number (percentage), as appropriate. After checking the fulfilment of each test´s assumptions, comparisons among the study groups were assessed by ANOVA or Kruskal-Wallis tests with Bonferroni correction for

continuous variables and Pearson chi^2 test for the categorical ones. Neurosonographic differences between groups were adjusted for confounding factors such as maternal age, ethnicity, nulliparity, fetal sex, gestational age and weight centile at scan by multiple regression analyses. Normalized neurobehavioural Z-scores from each area (communication, gross-motor, fine-motor, problem-solving and personal-social) and from the global performance (summatory of the five domains) were calculated using the correspondent SC group mean and standard deviation (SD) by means of the following formula: Z-score = (Score - Mean score for SC)/ SD for SC. Neurobehavioural comparisons among groups were adjusted by linear regression for maternal age, ethnicity, nulliparity, educational level, employment status, new-born´s gestational age at birth, breastfeeding, infant´s sex and age at postnatal evaluation. Correlations between neurosonographic and ASQ findings were investigated using the Spearman correlation coefficient ρ. All reported p-values are 2 sided. The significance level was set at 0.05 for all the statistical tests.

Sample size calculation

Since there are no data about cortical brain assessment by ultrasound in ART fetuses compared to those SC, the sample size was calculated based on a previous publication that acknowledged ultrasonographic changes in cortical folding between third-trimester fetuses with ventriculomegaly *versus* controls[23]. In a proportion 1:1, for a two-sided 95% confidence interval and 80% power, samples of 36 and 63 patients per exposition group were calculated for detecting a 15% difference in calcarine and parieto-occipital sulci depths respectively. Therefore, the study design contemplated an initial recruitment of at least 70 patients per study group.

RESULTS

Baseline and perinatal characteristics of the study populations

Figure 1 shows the flow chart of the study populations. Baseline characteristics (**Table I**) and perinatal outcomes (**Table II**) were similar among the study groups, with the exception of higher rates of nulliparity and induction of labour among the ART groups as compared to the SC. Regarding the infertility causes, a higher proportion of unexplained infertility was found within the fresh ET group, with no differences for endometriosis, tubal obstruction, male factor, and preimplantation genetic diagnosis, intracytoplasmic sperm injection (ICSI) rates, nor the number of embryos transferred. There was a higher proportion of blastocysts transferred in the frozen ET group. Frozen ET was performed in programmed cycles in 78.6% of cases (n=55) and in natural cycle in 21.4% (n=15). There were no significant differences between groups regarding the gestational age at CRL assessment for pregnancy dating (SC media[SD] 12.5[0.6] weeks of gestation *vs.* ART frozen ET 12.5[0.6] *vs.* ART fresh ET 12.5[0.5], p=0.505).

Fetal neurosonographic results

As shown in **Table III**, gestational age and estimated fetal weight were similar at neurosonography assessment. Cephalic dimensions were similar between groups. Both ART populations showed less profound parieto-occipital, cingulate and calcarine sulci depth (**Table III**). In addition, lower cortical grading scores were also observed in both ART groups at the level of Sylvian fissure, and parieto-occipital and calcarine sulci (**Figure 3**). Overall, differences in cortical development were more pronounced in the fresh ET group as compared to frozen ET (**Table III** and **Figure 3**). The neurosonographic differences reported here were statistically significant after adjustment by maternal age, ethnicity, nulliparity, fetal sex, gestational age and estimated fetal weight centile at ultrasound scan.

Infant ASQ scores

Infant ASQ results among study groups are displayed in **Figure 4**. Both groups of infants conceived by ART showed significantly lower scores in the communication, personal-social and gross-motor domains as compared to those conceived naturally. In addition, the fresh ET group also showed significantly lower scores for fine-motor and problem-solving skills as

compared to the SC and frozen ET groups. Results were statistically significant after adjustment by maternal age, ethnicity, nulliparity, educational level, employment status, breastfeeding, infant´s sex, gestational age at birth and age at postnatal evaluation. The distribution of cases below 2 SD of the SC population in the whole sample is displayed by domain in **Table SI** in *Supporting information*.

Fetal neurosonographic cortical features and infant global ASQ scores showed a weak but statistically significant positive correlation in the overall population (with parieto-occipital sulcus depth: ρ=0.23, p=0.003; with cingulate sulcus depth: ρ=0.21, p=0.008; with calcarine sulcus depth: ρ=0.32, p<0.001) and within the ART population (with parieto-occipital sulcus depth: ρ=0.22, p=0.017; with cingulate sulcus depth: ρ=0.20, p=0.032; with calcarine sulcus depth: ρ=0.29, p=0.001). In addition, statistically significant positive correlations were also found between the above-mentioned parameters and each of the ASQ domains (**Table SII** in *Supporting information*).

DISCUSSION

We report third-trimester neurosonographic results in fetuses from ART, suggesting a distinctive pattern of cortical development, associated to suboptimal infant neurobehaviour at 12 months of age.

To our knowledge, this is the first study assessing prenatal brain cortical development in ART offspring. We report less profound parieto-occipital, cingulate and calcarine sulci depth together with lower cortical grading scores. Cortical folding is a complex process of prenatal brain organization in which the smooth brain surface evolves to a system of sulci and gyri associated to fast cortical expansion in functional areas in ventricular and subventricular zones[27]. Parieto-occipital, cingulate and calcarine sulci increase in depth with gestational age[22,25], and Sylvian fissure undergoes a process of operculization[28]. Cortical development changes have been previously described in fetuses with fetal growth restriction (FGR), congenital heart defects (CHD), and non-severe ventriculomegaly. Whereas FGR with or without preeclampsia has been associated to reduced Sylvian fissure depth[24,29,30], fetuses with CHD[31] or isolated ventriculomegaly[23,32] were reported to experiment a widespread suboptimal cortical folding process, with shallower sulci depths and delayed operculization, similar to those observed in the ART population.

The reported differences in fetal cortical development in ART weakly correlate with a suboptimal neurobehaviour in infancy, particularly in communication, personal-social, problem-solving, and motor areas. Complex functions at cortical levels are distributed across neural networks[33]. Calcarine sulcus is part of the primary visual cortex. Parieto-occipital sulcus participates in visuospatial working memory[34]. Cingulate sulcus is involved in cognitive[35], motor[36], emotional and social-behavioural[37,38] processing; its deficiency has been described in psychiatric disorders as schizophrenia, attention deficit hyperactivity disorder and autism spectrum disorder (ASD)[39,40]. Therefore, the changes observed in our population for abovementioned parameters may partially contribute to the neurobehavioural features found at 12 months. Previous follow-up studies on infants and children from ART show inconsistent cognitive, psychomotor, and behavioural results[41–46]. These inconsistencies may be explained by differences in the ART populations, the influence of cofactors such as infertility, multiplicity, prematurity or other perinatal complications associated to ART[8,47–51], and also by the effect of postnatal socioeconomic and educational level[49]. Couples

undergoing fertility treatments may intrinsically differ from those conceiving spontaneously in demographic characteristics such as age, educational level, and socioeconomic position[49], and also in the way they encourage learning[52,53] and acknowledge health issues on their offspring[54]. As many factors influence neurodevelopment after birth, the uniqueness of the current study is the ability to describe fetal brain changes *in utero*, occurring before postnatal influences may arise.

It has been proposed that neurodevelopment in fetuses from ART might be influenced by parental underlying subfertility, ovarian stimulation and/or IVF procedures. The reported differences could be triggered by the differential intrauterine vasoactive and hormonal milieu among the study groups[55–60], but also by changes in cardiac function: brain and heart development take place simultaneously *in utero* and often share morphogenetic programs[61]. Fetuses from ART show differences in growth, cardiac shape and function as compared to those SC, being the fresh ET modality associated to fetal smallness[6,62] and cardiac remodeling and dysfunction[63,64], and the frozen ET related to macrosomy[62], hypertensive disorders of pregnancy[5,65] and milder cardiac features[64,66].

Interestingly, our findings were more evident in the fresh ET as compared to the frozen ET group, in line with a register-based study reporting higher mental disorders´ risks for ART, particularly in this modality. Fresh ET ART treatments using ICSI due to male factor infertility showed increased risks of ASD and intellectual disability[8]. Nevertheless, no differences were acknowledged between fresh and frozen ET at comparing academic performance in adolescents, in an uncontrolled follow-up study, in which mild conditions may have been underrepresented[67]. A birth cohort study with a 2-year follow-up reported reassuring results for ART, however it was underpowered to detect differences between these two techniques[68].

Strengths and limitations

Among the strengths, this is the first study examining cortical development in fetuses from ART. We present a well-phenotyped cohort from a single centre, with all study groups included prospectively. All patients underwent a detailed neurosonography to exclude any additional abnormality of the central nervous system, preventing the inclusion of conditions that could potentially bias our results. Another strength of this study is the use of both depth measurements and grading of fissures/sulci, providing quantitative objective data along with

maturation status. To ensure high image-quality and accurate assessment of cortical folding parameters, ultrasonography was measured off-line by a single trained neurosonographer blinded to the mode of conception, following a strict and reliable protocol. Finally, relevant confounders have been considered in our models for both prenatal and postnatal outcomes. Among the limitations, we underscore that infertility factors contribution to the outcome cannot be unravelled from the ART procedure itself. Moreover, regarding embryonic stage at ET there is a different proportion of blastocysts transferred between the ART groups. To overcome this issue, we have performed a sub-analysis excluding the cases of transfer in blastocyst stage (**Tables SIII, SIV,** and **Figure S1** in Supporting information). Even though the power decreased, significant differences between the study groups remained after excluding those cases. Also, the frozen ET group presents certain heterogeneity itself, with 15% of ET in natural cycle. On the other hand, the reported neurosonographic differences are subtle, with most outcomes lying within normal ranges, and their postnatal persistence remain to be elucidated. Due to technical reasons, particularly shadowing from fetal skull, we have measured only the brain´s distal side from the transducer, which could have biased our results in case of asymmetry. The ASQ for assessing postnatal performance at 12 months is mainly a screening tool subject to reporting bias and it was obtained once rather than longitudinally. Finally, although the mass-significance effect has been attenuated by applying Bonferroni corrections to each comparison, we cannot exclude that some of them could be due to chance.

Conclusions

Our results provide new evidence about the existence of *in utero* brain reorganization associated to ART, which could partially explain a differential performance at 12 months and warrant follow-up studies to assess their long-term consequences. These findings support the importance of a neurodevelopmental follow-up in ART offspring.

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References

1. Wyns C, De Geyter C, Calhaz-Jorge C, Kupka MS, Motrenko T, Smeenk J, Bergh C, Tandler-Schneider A, Rugescu IA, Vidakovic S, Goossens V. ART in Europe, 2017: results generated from European registries by ESHRE. *Hum Reprod Open*. 2021;(3):1-17. doi:10.1093/hropen/hoab026
2. Berntsen S, Söderström-Anttila V, Wennerholm U-B, Laivuori H, Loft A, Oldereid NB, Bente Romundstad L, Bergh C, Pinborg A. The health of children conceived by ART: "the chicken or the egg?" *Hum Reprod Update*. 2019;25(2):137-158. doi:10.1093/humupd/dmz001
3. Pinborg A. Short- and long-term outcomes in children born after assisted reproductive technology. *BJOG An Int J Obstet Gynaecol*. 2019;126(2):145-148. doi:10.1111/1471-0528.15437
4. Bergh C, Wennerholm UB. Long-term health of children conceived after assisted reproductive technology. *Ups J Med Sci*. 2020;125(2):152-157. doi:10.1080/03009734.2020.1729904
5. Ernstad EG, Wennerholm U-B, Khatibi A, Petzold M, Bergh C. Neonatal and maternal outcome after frozen embryo transfer: Increased risks in programmed cycles. *Am J Obstet Gynecol*. 2019;221(2):126.e1-126.e18. doi:10.1016/j.ajog.2019.03.010
6. Maheshwari A, Pandey S, Raja EA, Shetty A, Hamilton M, Bhattacharya S. Is frozen embryo transfer better for mothers and babies? Can cumulative meta-analysis provide a definitive answer? *Hum Reprod Update*. 2018;24(1):35-58. doi:10.1093/HUMUPD/DMX031
7. Pinborg A, Loft A, Aaris Henningsen AK, Rasmussen S, Andersen AN. Infant outcome of 957 singletons born after frozen embryo replacement: The Danish National Cohort Study 1995-2006. *Fertil Steril*. 2010;94(4):1320-1327. doi:10.1016/j.fertnstert.2009.05.091
8. Sandin S, Nygren KG, Iliadou A, Hultman CM, Reichenberg A. Autism and mental retardation among offspring born after in vitro fertilization. *JAMA*. 2013;310(1):75-84. doi:10.1001/jama.2013.7222
9. Husen SC, Koning I V., Go ATJI, Groenenberg IAL, Willemsen SP, Rousian M, Steegers-Theunissen RPM. IVF with or without ICSI and the impact on human embryonic brain development: the Rotterdam Periconceptional Cohort. *Hum Reprod*. 2021;36(3):596-

604. doi:10.1093/humrep/deaa341

10. Yin L, Xu Y, Li H, Ling C, Wai Choy K, Xia F, Deng X. Effect of assisted reproductive technology on fetal brain development assessed by prenatal ultrasonography. *J Perinat Med*. 2015;43(1):103-109. doi:10.1515/jpm-2014-0020
11. Robinson HP. Sonar Measurement of Fetal Crown-Rump Length as Means of Assessing Maturity in First Trimester of Pregnancy. *Br Med J*. 1973;4:28-31. doi:DOI: 10.1136/bmj.4.5883.28
12. Salomon LJ, Alfirevic Z, Da Silva Costa F, Deter RL, Figueras F, Ghi T, Glanc P, Khalil A, Lee W, Napolitano R, Papageorghiou A, Sotiradis A, Stirnemann J, Toi A, Yeo G. ISUOG Practice Guidelines: ultrasound assessment of fetal biometry and growth. *Ultrasound Obstet Gynecol*. 2019;53(6):715-723. doi:10.1002/UOG.20272
13. Figueras F, Meler E, Iraola A, Eixarch E, Coll O, Figueras J, Francis A, Gratacos E, Gardosi J. Customized birthweight standards for a Spanish population. *Eur J Obstet Gynecol Reprod Biol*. 2008;136(1):20-24. doi:10.1016/j.ejogrb.2006.12.015
14. Gestational Hypertension and Preeclampsia: ACOG Practice Bulletin, Number 222. *Obs Gynecol*. 2020;135(6):e237-e260. doi:10.1097/NMC.0000000000000523
15. Bricker D, Squires J, Mounts L, Potter L, Nickel R, Twombly E, Farrell J. *Ages & Stages Questionnaires: A Parent-Completed Child-Monitoring System - Spanish Version*. 2nd ed. Paul H Brookes publishing Co.; 1999.
16. World Medical Association. Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects. *JAMA*. 2013;310:2191-2194. doi:10.1001/jama.2013.281053
17. Balaban B, Brison D, Calderón G, Catt J, Conaghan J, Cowan L, Ebner T, Gardner D, Hardarson T, Lundin K, Magli MC, Mortimer D, Mortimer S, Munné S, Royere D, Scott L, Smitz J, Thornhill A, Van Blerkom J, Van Den Abbeel E. Istanbul consensus workshop on embryo assessment: Proceedings of an expert meeting. *Reprod Biomed Online*. 2011;22(6):632-646. doi:10.1016/j.rbmo.2011.02.001
18. Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: Towards a single blastocyst transfer. *Fertil Steril*. 2000;73(6):1155-1158. doi:10.1016/S0015-0282(00)00518-5
19. Kuwayama M. Highly efficient vitrification for cryopreservation of human oocytes and embryos: The Cryotop method. *Theriogenology*. 2007;67(1):73-80.

doi:10.1016/j.theriogenology.2006.09.014

20. Paladini D, Malinger G, Monteagudo A, Pilu G, Timor-Tritsch I, Toi A. Sonographic examination of the fetal central nervous system: Guidelines for performing the "basic examination" and the "fetal neurosonogram." *Ultrasound Obstet Gynecol*. 2007;29(1):109-116. doi:10.1002/uog.3909
21. Egaña-Ugrinovic G, Sanz-Cortes M, Figueras F, Bargalló N, Gratacós E. Differences in cortical development assessed by fetal MRI in late-onset intrauterine growth restriction. *Am J Obstet Gynecol*. 2013;209(2):126.e1-126.e8. doi:10.1016/j.ajog.2013.04.008
22. Alonso I, Borenstein M, Grant G, Narbona I, Azumendi G. Depth of brain fissures in normal fetuses by prenatal ultrasound between 19 and 30 weeks of gestation. *Ultrasound Obstet Gynecol*. 2010;36(6):693-699. doi:10.1002/uog.7660
23. Hahner N, Puerto B, Perez-Cruz M, Policiano C, Monterde E, Crispi F, Gratacos E, Eixarch E. Altered cortical development in fetuses with isolated nonsevere ventriculomegaly assessed by neurosonography. *Prenat Diagn*. 2018;38(5):365-375. doi:10.1002/pd.5240
24. Paules C, Miranda J, Policiano C, Crovetto F, Youssef L, Hahner N, Nakaki A, Crispi F, Gratacos E, Eixarch E. Fetal neurosonography detects differences in cortical development and corpus callosum in late-onset small fetuses. *Ultrasound Obstet Gynecol*. Published online 2021. doi:10.1002/uog.23592
25. Pistorius LR, Stoutenbeek P, Groenendaal F, De Vries L, Manten G, Mulder E, Visser G. Grade and symmetry of normal fetal cortical development: A longitudinal two- and three-dimensional ultrasound study. *Ultrasound Obstet Gynecol*. 2010;36(6):700-708. doi:10.1002/uog.7705
26. Sarmiento Camposa JA, Squires J, Ponte J. Universal developmental screening: Preliminary studies in Galicia, Spain. *Early Child Dev Care*. 2011;181(4):475-485. doi:10.1080/03004430903458007
27. Lui JH, Hansen D V, Kriegstein AR. Development and evolution of the human neocortex. *Cell*. 2011;146(1):18-36. doi:10.1016/j.cell.2011.06.030
28. Quarello E, Stirnemann J, Ville Y, Guibaud L. Assessment of fetal Sylvian fissure operculization between 22 and 32 weeks: A subjective approach. *Ultrasound Obstet Gynecol*. 2008;32(1):44-49. doi:10.1002/uog.5353

29. Husen SC, Koning I V., Go ATJI, Van Graafeiland AW, Willemsen SP, Groenenberg IAL, Steegers-Theunissen RPM. Three-dimensional ultrasound imaging of fetal brain fissures in the growth restricted fetus. *PLoS One*. 2019;14(5):1-14. doi:10.1371/journal.pone.0217538

30. Basso A, Youssef L, Nakaki A, Paules C, Miranda J, Casu G, Salazar L, Gratacos E, Eixarch E, Crispi F, Crovetto F. Fetal neurosonography at 31-35 weeks reveals altered cortical development in pre-eclampsia with and without small-for-gestational-age fetus. *Ultrasound Obstet Gynecol*. Published online 2022:0-3. doi:10.1002/uog.24853

31. Lee FT, Seed M, Sun L, Marini D. Fetal brain issues in congenital heart disease. *Transl Pediatr*. 2021;10(8):2182-2196. doi:10.21037/tp-20-224

32. Hahner N, Benkarim OM, Aertsen M, Perez-Cruz M, Piella G, Sanroma G, Bargallo N, Deprest J, Gonzalez Ballester MA, Gratacos E, Eixarch E. Global and regional changes in cortical development assessed by MRI in fetuses with isolated nonsevere ventriculomegaly correlate with neonatal neurobehavior. *Am J Neuroradiol*. 2019;40(9):1567-1574. doi:10.3174/ajnr.A6165

33. Nieuwenhuys R. *The Insular Cortex. A Review.* Vol 195. 1st ed. Elsevier B.V.; 2012. doi:10.1016/B978-0-444-53860-4.00007-6

34. Pollmann S. Working memory dependence of spatial contextual cueing for visual search. *Br J Psychol*. 2019;110(2):372-380. doi:10.1111/bjop.12311

35. Leech R, Sharp DJ. The role of the posterior cingulate cortex in cognition and disease. *Brain*. 2014;137(1):12-32. doi:10.1093/brain/awt162

36. Michelet T, Badets A. The anterior midcingulate cortex might be a neuronal substrate for the ideomotor mechanism. *Exp Brain Res*. 2021;(1). doi:10.1007/s00221-021-06159-9

37. Hadland KA, Rushworth MFS, Gaffan D, Passingham RE. The effect of cingulate lesions on social behaviour and emotion. *Neuropsychologia*. 2003;41(8):919-931. doi:10.1016/S0028-3932(02)00325-1

38. Lockwood PL. The anatomy of empathy: Vicarious experience and disorders of social cognition. *Behav Brain Res*. 2016;311:255-266. doi:10.1016/j.bbr.2016.05.048

39. Chiu PH, Kayali MA, Kishida KT, Tomlin D, Klinger LG, Klinger MR, Montague PR. Self Responses along Cingulate Cortex Reveal Quantitative Neural Phenotype for High-Functioning Autism. *Neuron*. 2008;57(3):463-473. doi:10.1016/j.neuron.2007.12.020

40. Rubia K, Alegría AA, Brinson H. Brain abnormalities in attention-deficit hyperactivity disorder: a review. *Rev Neurol*. 2014;58 Suppl 1(Supl 1):S3-16.

41. Middelburg KJ, Heineman MJ, Bos AF, Hadders-Algra M. Neuromotor, cognitive, language and behavioural outcome in children born following IVF or ICSI - A systematic review. *Hum Reprod Update*. 2008;14(3):219-231. doi:10.1093/humupd/dmn005

42. Bay B, Mortensen EL, Kesmodel US. Assisted reproduction and child neurodevelopmental outcomes: A systematic review. *Fertil Steril*. 2013;100(3):844-853. doi:10.1016/j.fertnstert.2013.05.034

43. Hart R, Norman RJ. The longer-term health outcomes for children born as a result of ivf treatment. Part II-mental health and development outcomes. *Hum Reprod Update*. 2013;19(3):244-250. doi:10.1093/humupd/dmt002

44. Liu L, Gao J, He X, Cai Y, Wang L, Fan X. Association between assisted reproductive technology and the risk of autism spectrum disorders in the offspring: A meta-analysis. *Sci Rep*. 2017;7(April):1-8. doi:10.1038/srep46207

45. Rumbold AR, Moore VM, Whitrow MJ, Oswald TK, Moran LJ, Fernandez RC, Barnhart KT, Davies MJ. The impact of specific fertility treatments on cognitive development in childhood and adolescence: A systematic review. *Hum Reprod*. 2017;32(7):1489-1507. doi:10.1093/humrep/dex085

46. Rönö K, Rissanen E, Bergh C, Wennerholm U-B, Opdahl S, Romundstad LB, Henningsen A-KA, Spangmose AL, Pinborg A, Gissler M, Tiitinen A. The neurodevelopmental morbidity of children born after assisted reproductive technology: a Nordic register study from the Committee of Nordic Assisted Reproductive Technology and Safety group. *Fertil Steril*. Published online 2022. doi:10.1016/j.fertnstert.2022.01.010

47. Kissin DM, Zhang Y, Boulet SL, Fountain C, Bearman P, Schieve L, Yeargin-Allsopp M, Jamieson DJ. Association of assisted reproductive technology (ART) treatment and parental infertility diagnosis with autism in ART-conceived children. *Hum Reprod*. 2015;30(2):454-465. doi:10.1093/humrep/deu338

48. Drenth Olivares M, Kuiper DB, Haadsma ML, Heineman KR, Heineman MJ, Hadders-Algra M. IVF procedures are not, but subfertility is associated with neurological condition of 9-year-old offspring. *Early Hum Dev*. 2019;129(December 2018):38-44.

doi:10.1016/j.earlhumdev.2018.12.017

49. Carson C, Kelly Y, Kurinczuk JJ, Sacker A, Redshaw M, Quigley MA. Effect of pregnancy planning and fertility treatment on cognitive outcomes in children at ages 3 and 5: Longitudinal cohort study. *BMJ*. 2011;343(7820). doi:10.1136/bmj.d4473
50. Hvidtjørn D, Grove J, Schendel D, Værke C, Schieve LA, Uldall P, Ernst E, Jacobsson B, Thorsen P. Multiplicity and early gestational age contribute to an increased risk of cerebral palsy from assisted conception: A population-based cohort study. *Hum Reprod*. 2010;25(8):2115-2123. doi:10.1093/humrep/deq070
51. Källén AJB, Finnström OO, Lindam AP, Nilsson EME, Nygren KG, Otterblad Olausson PM. Is there an increased risk for drug treated attention deficit/hyperactivity disorder in children born after in vitro fertilization? *Eur J Paediatr Neurol*. 2011;15(3):247-253. doi:10.1016/j.ejpn.2010.12.004
52. Agarwal P, Loh SKE, Lim SB, Sriram B, Daniel ML, Yeo SH, Heng D. Two-year neurodevelopmental outcome in children conceived by intracytoplasmic sperm injection: Prospective cohort study. *BJOG An Int J Obstet Gynaecol*. 2005;112(10):1376-1383. doi:10.1111/j.1471-0528.2005.00663.x
53. Spangmose AL, Malchau SS, Schmidt L, Vassard D, Rasmussen S, Loft A, Forman J, Pinborg A. Academic performance in adolescents born after ART - A nationwide registry-based cohort study. *Hum Reprod*. 2017;32(2):447-456. doi:10.1093/humrep/dew334
54. Källén B, Finnström O, Nygren KG, Olausson PO. In vitro fertilization in Sweden: Child morbidity including cancer risk. *Fertil Steril*. 2005;84(3):605-610. doi:10.1016/j.fertnstert.2005.03.035
55. Zhou CL, Xu GF, Yang Q, Wang HH, Guo MX, Xiong YM, Guo XY, Hou M, Jin LY, Sheng JZ, He L, Jin L, Huang HF. Diminished verbal ability among children conceived through ART with exposure to high serum estradiol in utero. *J Assist Reprod Genet*. 2020;37(8):1931-1938. doi:10.1007/s10815-020-01835-1
56. Chen X, Kong L, Piltonen TT, Gissler M, Lavebratt C. Association of polycystic ovary syndrome or anovulatory infertility with offspring psychiatric and mild neurodevelopmental disorders: a Finnish population-based cohort study. *Hum Reprod*. 2020;35(10):2336-2347. doi:10.1093/humrep/deaa192
57. Mainigi M, Rosenzweig JM, Lei J, Mensah V, Thomaier L, Talbot CC, Olalere D, Ord T,

Rozzah R, Johnston M V., Burd I. Peri-Implantation Hormonal Milieu: Elucidating Mechanisms of Adverse Neurodevelopmental Outcomes. *Reprod Sci*. 2016;23(6):785-794. doi:10.1177/1933719115618280

58. Gore AC, Martien KM, Gagnidze K, Pfaff D. Implications of prenatal steroid perturbations for neurodevelopment, behavior, and autism. *Endocr Rev*. 2014;35(6):961-991. doi:10.1210/er.2013-1122
59. Lester BM, Marsit CJ. Epigenetic mechanisms in the placenta related to infant neurodevelopment. *Epigenomics*. 2018;10(3):321-333. doi:10.2217/epi-2016-0171
60. Li H, Liu L, Dang M, Zhang W, Liu J. Increased susceptibility of mice obtained from in vitro fertilization to global cerebral ischemia-reperfusion injury: possible role of hydrogen sulphide and its biosynthetic enzymes. *Int J Neurosci*. 2020;130(6):533-540. doi:10.1080/00207454.2019.1667797
61. McQuillen PS, Miller SP. Congenital heart disease and brain development. *Ann N Y Acad Sci*. 2010;1184:68-86. doi:10.1111/j.1749-6632.2009.05116.x
62. Terho AM, Pelkonen S, Opdahl S, Romundstad LB, Bergh C, Wennerholm UB, Henningsen AA, Pinborg A, Gissler M, Tiitinen A. High birth weight and large-for-gestational-age in singletons born after frozen compared to fresh embryo transfer, by gestational week: a Nordic register study from the CoNARTaS group. *Hum Reprod*. 2021;36(4):1083-1092. doi:10.1093/humrep/deaa304
63. Valenzuela-Alcaraz B, Serafini A, Sepulveda-Martínez A, Casals G, Rodríguez-López M, Garcia-Otero L, Cruz-Lemini M, Bijnens B, Sitges M, Balasch J, Gratacós E, Crispi F. Postnatal persistence of fetal cardiovascular remodelling associated with assisted reproductive technologies: a cohort study. *BJOG An Int J Obstet Gynaecol*. 2019;126(2):291-298. doi:10.1111/1471-0528.15246
64. Boutet ML, Casals G, Valenzuela-Alcaraz B, García-Otero L, Crovetto F, Cívico MS, Borrás A, Manau D, Gratacós E, Crispi F. Cardiac remodeling in fetuses conceived by ARTs: fresh versus frozen embryo transfer. *Hum Reprod*. 2021;36(10):2697-2708. doi:10.1093/humrep/deab159
65. Sites CK, Wilson D, Barsky M, Bernson D, Bernstein IM, Boulet S, Zhang Y. Embryo cryopreservation and preeclampsia risk. *Fertil Steril*. 2017;108(5):784-790. doi:10.1016/j.fertnstert.2017.08.035
66. Rizzo G, Pietrolucci ME, Mappa I, Bitsadze V, Khizroeva J, Makatsariya A, D'Antonio F.

Fetal Cardiac Remodeling Is Affected by the Type of Embryo Transfer in Pregnancies Conceived by in vitro Fertilization: A Prospective Cohort Study. *Fetal Diagn Ther*. 2020;47(10):772-778. doi:10.1159/000508987

67. Spangmose AL, Malchau SS, Henningsen AA, Forman JL, Rasmussen S, Loft A, Schmidt L, Pinborg A. Academic performance in adolescents aged 15–16 years born after frozen embryo transfer compared with fresh embryo transfer: a nationwide registry-based cohort study. *BJOG An Int J Obstet Gynaecol*. 2019;126(2):261-269. doi:10.1111/1471-0528.15484

68. Balayla J, Sheehy O, Fraser WD, Séguin JR, Trasler J, Monnier P, MacLeod AA, Simard MN, Muckle G, Bérard A. Neurodevelopmental outcomes after assisted reproductive technologies. *Obstet Gynecol*. 2017;129(2):265-272. doi:10.1097/AOG.0000000000001837

FIGURE LEGENDS

Figure 1 Flow diagram of the study populations. SC indicates spontaneously conceived; ART, assisted reproductive technologies; ET, embryo transfer.

Figure 2 Illustrative neurosonographic measurements. (a) Axial transthalamic plane showing the parieto-occipital sulcus depth measurement, (b) coronal transthalamic plane showing the cingulate sulcus measurement, and (c) coronal transcerebellar plane showing the calcarine sulcus depth measurement. The dashed line corresponds to the interhemispheric fissure.

Figure 3 Distribution of main fissures/sulci grading scores in spontaneously conceived, frozen ET and fresh ET fetuses. SC indicates spontaneously conceived; ET, embryo transfer. P-values are adjusted by maternal age, ethnicity, nulliparity, fetal sex, gestational age, and weight centile at scan. * $P<0.05$ as compared to SC.

Figure 4 Adjusted Z-scores of the different infant Ages & Stages Questionnaires domains at 12 months of age among the study groups. SC indicates spontaneously conceived; ET, embryo transfer. Z-scores values and comparisons are adjusted by maternal age, ethnicity, nulliparity, educational level, employment status, new-born´s gestational age at birth, breastfeeding, infant´s sex and age at evaluation. † $P<0.05$ as compared to SC. ‡ $P<0.05$ as compared to fresh ET.

Table I Baseline and fertility characteristics in the study populations.

	SC (n=70)	ART Frozen ET (n=70)	ART Fresh ET (n=70)
Maternal characteristics			
Age (y)	34.4 (30.1-37.3)	35.8 (33.7-37.6)	35.9 (33.9-37.7)
Body Mass Index	23.2 (20.6-25.7)	23.4 (20.3-25.9)	23.1 (20.9-27.2)
Caucasian (n(%))	48 (68.6)	58 (82.9)	**63.0 (90.0)***
Nulliparity (n(%))	40 (57.1)	**58 (82.9)***	**65 (92.9)***
Hypertension (n(%))	1 (1.4)	2 (2.9)	0
Cardiovascular disease (n(%))	1 (1.4)	2 (2.9)	3 (4.3)
Diabetes (n(%))	0	1 (1.4)	1 (1.4)
Autoinmune disease (n(%))	3 (4.3)	3 (4.3)	2 (2.9)
Thyroid disease (n(%))	2 (2.9)	2 (2.9)	5 (7.1)
Kidney disease (n(%))	1 (1.4)	1 (1.4)	0
Epilepsy (n(%))	1 (1.4)	0	1 (1.4)
Psychopharmaceuticals exposure during pregnancy (n(%))	3 (4.3)	1 (1.4)	3 (4.3)
Smoking habit during pregnancy (n(%))	13 (19.7)	9 (13.0)	11 (16.2)
Declared alcohol intake during pregnancy (n(%))	9 (13.9)	8 (11.8)	5 (7.4)
Declared drug use during pregnancy (n(%))	0	0	0
University studies (n(%))	37 (58.7)	41 (60.3)	40 (58.8)

Employment rate (n(%))	46 (78.0)	52 (77.6)	56 (81.2)
Fertility and ART characteristics			
Infertility cause (n(%))			
Unexplained	0	20 (28.6)	**35 (50.0)†**
Endometriosis	0	7 (10.0)	11 (15.7)
Tubal obstruction	0	7 (10.0)	7 (10.0)
Male factor	0	35 (50.0)	27 (38.6)
Preimplantation genetic diagnosis	0	4 (5.7)	5 (7.3)
Transferred embryos (n)	0	2.0 (1.0-2.0)	2.0 (2.0-2.0)
Transfer of ICSI embryos (n(%))	0	68 (97.1)	68 (97.1)
Transfer in blastocyst stage (n(%))	0	41 (58.6)	**14 (20.0)†**
Vanishing twin phenomenon	0	29 (41.4)	14 (20.0)

SC indicates spontaneously conceived pregnancies; ART, pregnancies achieved by assisted reproductive technologies; ET, embryo transfer; ICSI, intracytoplasmic sperm injection.

Data are median (interquartile range) or number (percentage), as appropriate.

Significant differences are in bold font.

* $P<0.05$ as compared to SC.

† $P<0.05$ as compared to ART frozen ET.

Table II Perinatal characteristics in the study populations.

	SC (n=70)	**ART Frozen ET** (n=70)	**ART Fresh ET** (n=70)
Perinatal characteristics			
Pregnancy complications (n(%))			
Preterm delivery	2 (2.9)	5 (7.1)	2 (2.9)
Preeclampsia	2 (2.9)	7 (10.0)	2 (2.9)
Gestational diabetes	4 (5.8)	5 (7.1)	6 (8.6)
Placenta previa	0	1 (1.4)	0
Abruptio placentæ	0	1 (1.4)	0
Aspirin exposure from first trimester (n(%))	9 (12.9)	10 (14.3)	4 (5.7)
Prenatal corticoid exposure (n(%))	1 (1.4)	4 (5.7)	2 (2.9)
Delivery data			
Gestational age at birth (weeks, days)	40.0 (39.0-40.4)	39.5 (38.4-41.0)	40.2 (39.1-41.0)
Birthweight (grams)	3280 (3030-3540)	3280 (3050-3550)	3235 (2876-3630)
Birthweight (centile)	41 (19-67)	40 (18-60)	36 (11-64)
Birthweight below the 10th centile (n(%))	11 (15.7)	6 (8.6)	12 (17.1)
Birthweight above the 90th centile (n(%))	4 (5.7)	5 (7.1)	5 (7.1)
Induction of labour (n(%))	24 (34.3)	**40 (59.7)***	**35 (53.0)***
Caesarean section (n(%))	21 (30.4)	29 (41.4)	19 (27.1)
Female (n(%))	37 (52.9)	28 (40.0)	39 (55.7)

Neonatal outcome (n(%))			
Admission to NICU	9 (13.2)	6 (8.7)	2 (2.9)
Minor neonatal morbidity	11 (16.4)	11 (15.9)	4 (5.7)
Major neonatal morbidity	0	0	0
Neonatal mortality	0	0	0

SC indicates spontaneously conceived pregnancies; ART, pregnancies achieved by assisted reproductive technologies; ET, embryo transfer; NICU, neonatal intensive care unit.

Data are median (interquartile range) or number (percentage), as appropriate.

Significant differences are in bold font.

* $P<0.05$ as compared to SC.

† $P<0.05$ as compared to ART frozen ET.

Table III Fetal neurosonographic results in the study populations.

Fetal neurosonography	**SC** (n=70)	**ART Frozen ET** (n=70)	**ART Fresh ET** (n=70)	*p-value*
Gestational age at scan (weeks, days)	31.6 (29.3-32.5)	31.2 (29.3-32.2)	30.5 (29.4-32.0)	*0.192*
Estimated fetal weight at scan (g)	1775 ± 435	1716 ± 340	1678 ± 346	*0.325*
Estimated fetal weight at scan (centile)	43 (20-79)	57 (33-82)	46 (21-72)	*0.303*
EFW below 10th centile (n(%))	8 (12.3)	8 (11.4)	9 (12.9)	*0.967*
EFW above 90th centile (n(%))	16 (22.9)	11 (15.7)	8 (11.4)	*0.188*
Cephalic presentation (n(%))	60 (85.7)	64 (91.4)	58 (82.9)	*0.315*
Left laterality (n(%))	42 (60.0)	45 (64.3)	39 (55.7)	*0.585*
Biparietal diameter (mm) §	78.3 ± 6.4	78.1 ± 4.5	78.4 ± 4.5	*0.910*
Occipito-frontal diameter (mm) §	102.9 ± 6.9	101.8 ± 5.3	101.1 ± 5.9	*0.171*
Cephalic circumference (mm) §	289.8 ± 19.6	288.0 ± 14.4	286.8 ± 15.1	*0.401*
Parieto-occipital sulcus depth¶§	13.4 ± 2.6	13.4 ± 2.6	**12.5 ± 2.5*†**	***<0.001***
Cingulate sulcus depth¶§	6.5 (4.8-7.8)	**5.8 (4.1-7.5)***	**5.8 (4.2-7.4)***	***0.001***
Calcarine sulcus depth¶§	16.4 (14.3-17.9)	**14.5 (12.1-15.8)***	**13.5 (10.1-16.1)*†**	***<0.001***

ET indicates embryo transfer. Data are mean ± SD, median (interquartile range), or number (percentage), as appropriate.

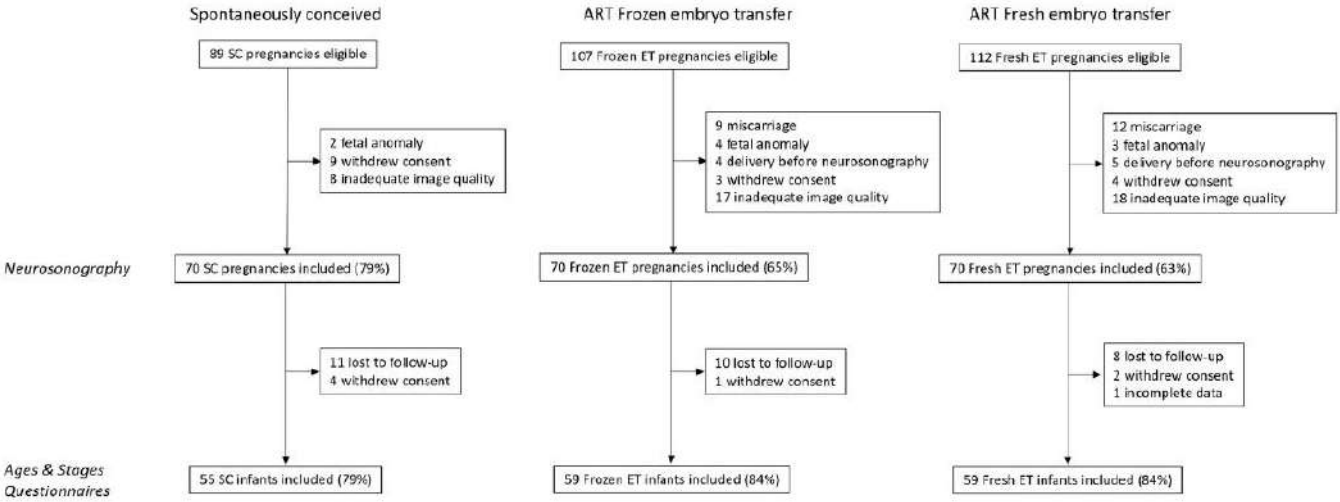
¶ Data normalized by dividing per biparietal diameter and multiplied by 100.

§ Comparisons adjusted by maternal age, ethnicity, nulliparity, fetal sex, gestational age, and fetal weight centile at scan. Significant differences are in bold font.

* $P<0.05$ as compared to SC.

† $P<0.05$ as compared to ART frozen ET.

SUPPORTING INFORMATION



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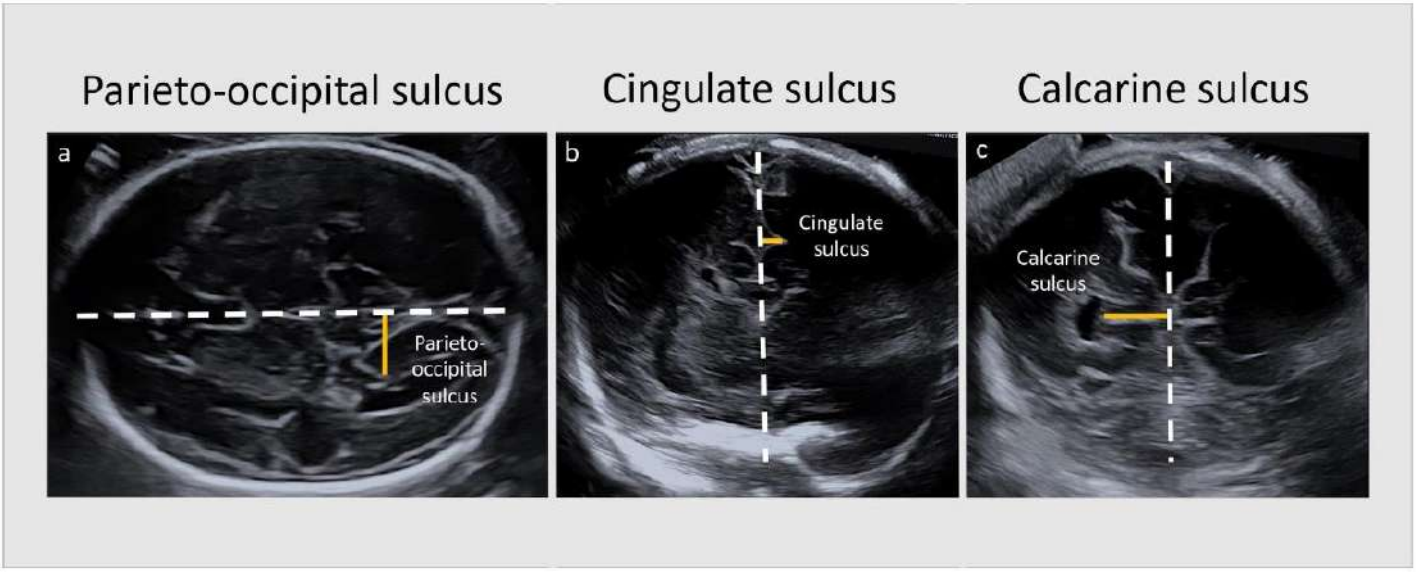

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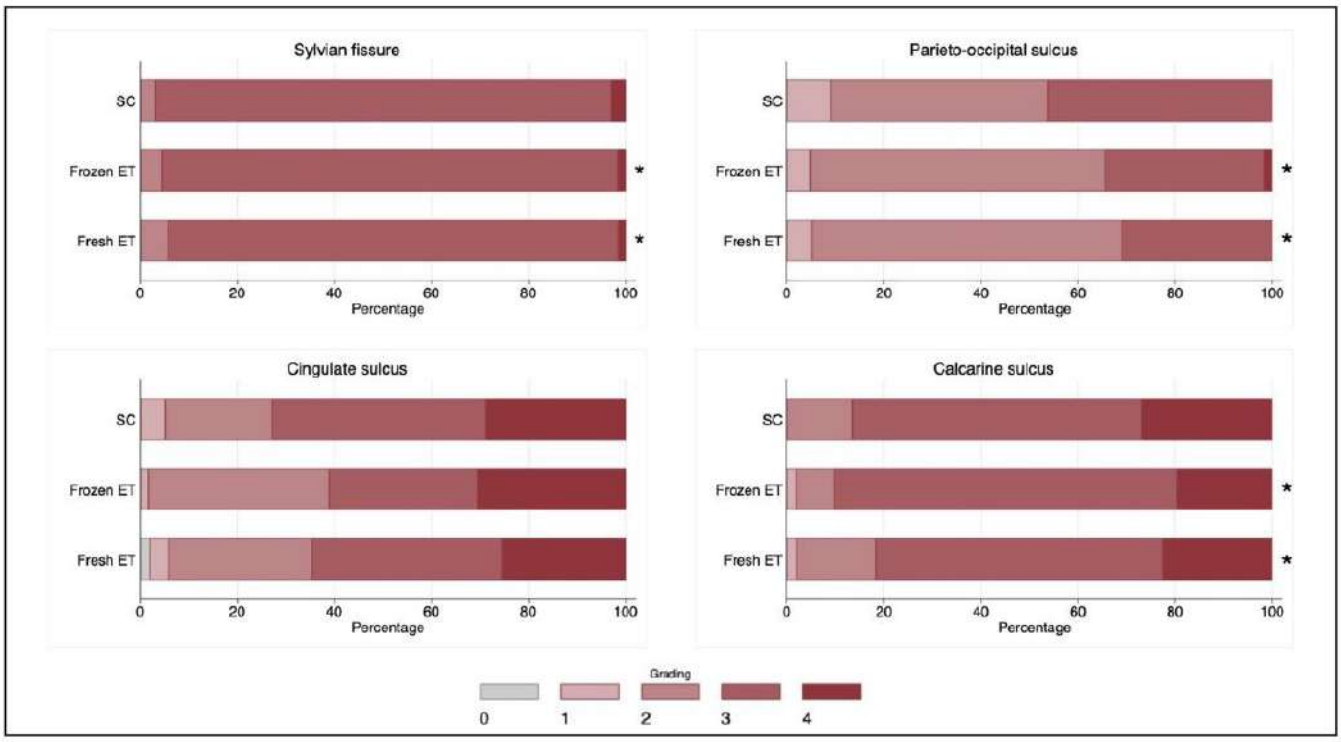

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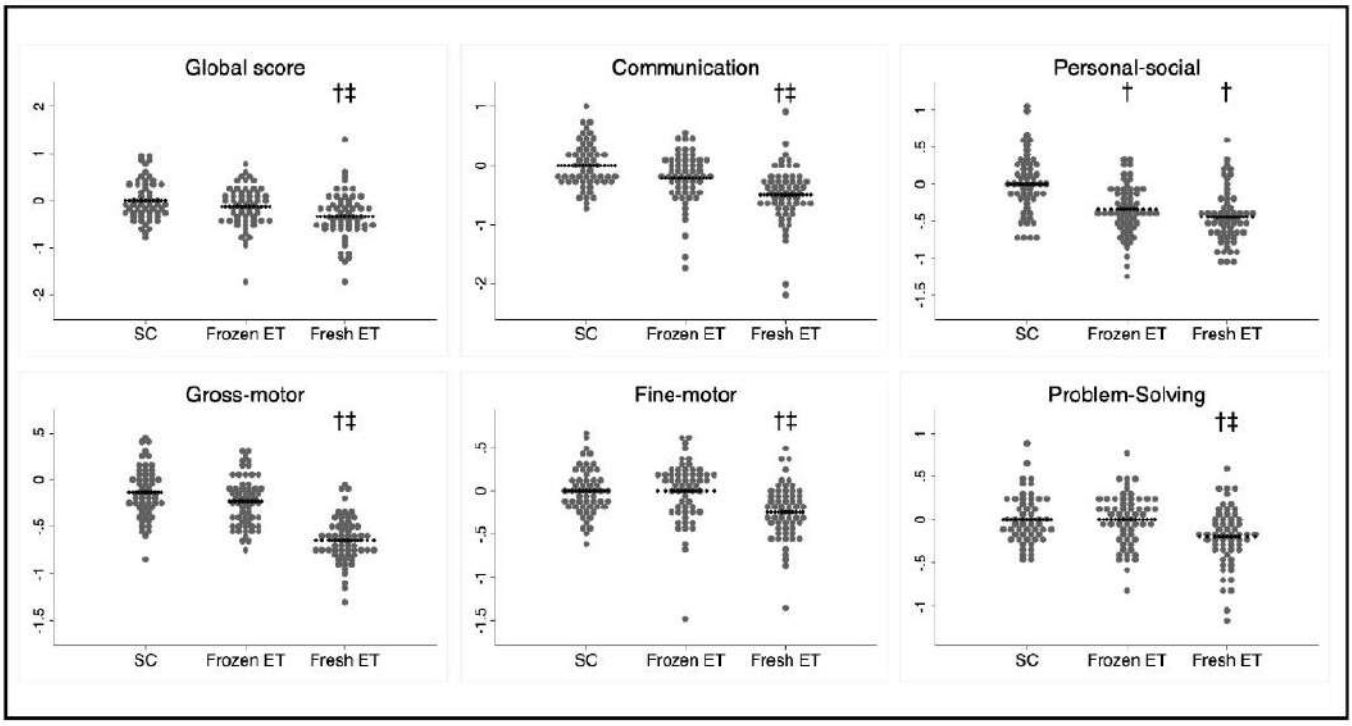

Figure 4.jpg

Table SI Distribution of crude ASQ Z-scores below -2 standard deviations from the SC population mean.

	SC (n=55)	**ART Frozen ET (n=59)**	**ART Fresh ET (n=59)**
Communication domain (n(%))	0	7 (11.9)	8 (13.6)
Gross-motor domain (n(%))	2 (3.6)	2 (3.4)	0
Fine-motor domain (n(%))	3 (5.5)	3 (5.1)	4 (6.8)
Problem-solving domain (n(%))	3 (5.5)	0	4 (6.8)
Personal-social domain (n(%))	3 (5.5)	9 (15.3)	8 (13.6)
At least one of the domains (n(%))	8 (14.5)	10 (16.9)	13 (22.0)
Global score (n(%))	1 (1.8)	6 (10.2)	5 (8.5)

SC indicates spontaneously conceived pregnancies; ART, assisted reproductive technologies pregnancies; ET, embryo transfer.

Data are number (percentage).

Table SII Pearson´s correlations between each *Ages & Stages Questionnaires´* score domain and sulci depth.

Whole sample			**ART population**		
Sulci depth	**p-value**	ρ coefficient	**Sulci depth**	**p-value**	ρ coefficient
Communication score					
Parieto-occipital	**<0.001**	0.40	Parieto-occipital	**<0.001**	0.40
Cingulate	**0.003**	0.23	Cingulate	**0.017**	0.22
Calcarine	**<0.001**	0.51	Calcarine	**<0.001**	0.49
Gross-motor score					
Parieto-occipital	**<0.001**	0.30	Parieto-occipital	**0.045**	0.19
Cingulate	**<0.001**	0.34	Cingulate	0.100	0.15
Calcarine	**<0.001**	0.43	Calcarine	**<0.001**	0.36
Fine-motor score					
Parieto-occipital	**0.045**	0.16	Parieto-occipital	**0.049**	0.18
Cingulate	**0.033**	0.17	Cingulate	0.069	0.17
Calcarine	**<0.001**	0.29	Calcarine	**<0.001**	0.32
Problem-solving score					
Parieto-occipital	**<0.001**	0.26	Parieto-occipital	**<0.001**	0.33
Cingulate	0.188	0.10	Cingulate	0.096	0.16
Calcarine	**<0.001**	0.35	Calcarine	**<0.001**	0.39
Personal-social score					
Parieto-occipital	**<0.001**	0.37	Parieto-occipital	**0.003**	0.28
Cingulate	**<0.001**	0.28	Cingulate	**0.045**	0.19
Calcarine	**<0.001**	0.47	Calcarine	**<0.001**	0.34

ART indicates pregnancies conceived by assisted reproductive technologies.

Significant correlations are in bold font.

Table SIII Baseline, fertility and perinatal characteristics of the study populations excluding the cases of transfer in blastocyst stage.

	SC (n=70)	**ART Frozen ET** (n=41)	**ART Fresh ET** (n=56)
Maternal characteristics			
Age (y)	34.4 (30.1-37.3)	35.5 (33.6-37.4)	**36.0 (34.0-37.8)***
Body Mass Index	23.2 (20.6-25.7)	23.5 (20.8-26.2)	23.4 (21.2-27.8)
Caucasian (n(%))	48 (68.6)	32 (78.0)	**49 (87.5)***
Nulliparity (n(%))	40 (57.1)	**34 (82.9)***	**52 (92.9)***
Hypertension (n(%))	1 (1.4)	1 (2.4)	0
Cardiovascular disease (n%))	1 (1.4)	2 (4.9)	2 (3.6)
Diabetes (n(%))	0	0	0
Autoimmune disease (n(%))	3 (4.3)	0	2 (3.6)
Thyroid disease (n(%))	2 (2.9)	2 (4.9)	4 (7.1)
Kidney disease (n(%))	1 (1.4)	1 (2.4)	0
Epilepsy (n(%))	1 (1.4)	0	0
Psychopharmaceuticals exposure during pregnancy (n(%))	3 (4.3)	1 (2.4)	3 (5.4)
Self-reported smoking habit during pregnancy (n(%))	13 (18.6)	7 (17.1)	10 (17.9)
Self-reported alcohol intake during pregnancy (n(%))	9 (12.9)	4 (9.8)	4 (7.1)
Self-reported drug use during pregnancy (n(%))	0	0	0
University level education (n(%))	37 (53.9)	29 (70.7)	40 (71.4)
Employee rate (n(%))	46 (65.7)	28 (68.3)	44 (78.6)

Fertility and ART characteristics			
Infertility cause (n(%))			
Unexplained	0	12 (29.3)	26 (46.4)
Endometriosis	0	4 (9.8)	8 (14.3)
Tubal obstruction	0	5 (12.2)	7 (12.5)
Male factor	0	20 (48.8)	22 (39.3)
Preimplantation genetic diagnosis	0	1 (2.4)	0
Transferred embryos (n)	0	2.0 (2.0-2.0)	2.0 (2.0-2.0)
Transfer of ICSI embryos (n(%))	0	39 (95.1)	55 (98.2)
Perinatal characteristics			
Pregnancy complications (n(%))			
Preterm delivery	2 (2.9)	2 (4.9)	2 (3.6)
Preeclampsia	2 (2.9)	4 (9.8)	1 (1.8)
Gestational diabetes	4 (5.7)	3 (7.3)	4 (7.1)
Placenta previa	0	0	0
Abruptio placentæ	0	1 (2.4)	0
Aspirin exposure from first trimester (n(%))	9 (12.9)	5 (12.2)	3 (5.4)
Prenatal corticoid exposure (n(%))	1 (1.4)	2 (4.9)	1 (1.8)
Delivery data			
Gestational age at birth (weeks, days)	40.0 (39.0-40.4)	40.0 (38.6-41.0)	40.2 (39.2-41.0)
Birthweight (grams)	3280 (3030-3540)	3240 (3030-3540)	3245 (2860-3650)
Birthweight (centile)	41 (19-67)	37 (18-58)	36 (12-68)
Birthweight below the 10th centile (n(%))	11 (15.7)	5 (12.2)	10 (17.9)

Birthweight above the 90th centile (n(%))	4 (5.7)	1 (2.4)	5 (8.9)
Induction of labour (n(%))	24 (34.3)	**25 (61.0)***	29 (51.8)
Caesarean section (n(%))	21 (30.4)	17 (41.5)	15 (26.8)
Female (n(%))	37 (52.9)	17 (41.5)	29 (51.8)
Neonatal outcome (n(%))			
Admission to NICU	9 (12.8)	4 (9.8)	2 (3.6)
Minor neonatal morbidity	11 (15.7)	6 (14.6)	4 (7.1)
Major neonatal morbidity	0	0	0
Neonatal mortality	0	0	0

SC indicates spontaneously conceived pregnancies; ART, pregnancies conceived by assisted reproductive technologies; ET, embryo transfer; ICSI, intracytoplasmic sperm injection; NICU, neonatal intensive care unit.

Data are median (interquartile range) or number (percentage), as appropriate.

Significant differences are in bold font.

* $P<0.05$ as compared to SC.

Study 5

Maternal and fetal haemopexin and α_1-microglobulin concentrations in pre-eclamptic IVF pregnancies according to presence of corpus luteum at embryo transfer

Boutet ML, Youssef L, Erlandsson L, Hansson E, Manau D, Crispi F, Casals G, Hansson SR.
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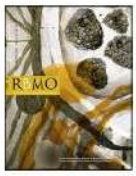

ARTICLE

Maternal and fetal haemopexin and α_1-microglobulin concentrations in pre-eclamptic IVF pregnancies according to presence of corpus luteum at embryo transfer

BIOGRAPHY

María Laura Boutet is an obstetrics and gynaecology specialist currently working as a pre-doctoral researcher at University of Barcelona and Lund University, awarded by the FetalMed PhD Erasmus Mundus Joint Doctorate scholarship from the Erasmus+Programme of the European Union. Her main research interests are safety in ART and fetal programming.

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KEY MESSAGE

The presence of corpus luteum may influence heme-induced oxidative stress in pregnancy and preeclampsia risk. Well-designed randomized controlled trials in IVF pregnancies are warranted to investigate whether applying potential strategies to develop a corpus luteum might reduce the perinatal complications associated with programmed cycles of IVF.

ABSTRACT

Research question: Do pregnancies with corpus luteum show different maternal and fetal plasma concentrations of the scavenger proteins haemopexin and α_1-microglobulin compared with pregnancies without corpus luteum in preeclampsia?

Design: Case-control study of 160 singleton pregnancies: 54 naturally conceived, 50 by IVF after fresh embryo transfer or frozen embryo transfer (FET) in natural cycle (presence of corpus luteum) and 56 after fresh oocyte donation or FET in programmed cycles (absence of corpus luteum). Pregnancies were subclassified into normotensive, preeclampsia and severe preeclampsia cases. Heme-scavenger concentrations were measured by ELISA in maternal and cord plasma collected at delivery.

Results: After adjustment, maternal haemopexin was higher in IVF with corpus luteum than in naturally conceived pregnancies in normotensive ($P = 0.038$) and preeclampsia ($P = 0.011$) populations, and lower in preeclampsia for IVF pregnancies lacking corpus luteum compared with IVF with corpus luteum ($P = 0.002$). Maternal α_1-microglobulin levels were higher in the absence of corpus luteum only in severe cases of preeclampsia compared with naturally conceived pregnancies ($P = 0.014$) and IVF with corpus luteum pregnancies ($P = 0.041$). In cord blood, haemopexin was higher in IVF with corpus luteum compared with naturally conceived pregnancies in preeclampsia ($P = 0.039$) and α_1-microglobulin was higher in the group lacking corpus luteum compared with IVF with corpus luteum in the normotensive population ($P < 0.001$).

Conclusions: The physiological differences shown for these heme-scavengers between pregnancies after embryo transfer in the presence or absence of corpus luteum support the hypothesis that corpus luteum activity could influence perinatal outcomes. Future research is needed on whether applying potential strategies to develop a corpus luteum might reduce the perinatal complications associated with programmed cycles of IVF.

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KEYWORDS

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INTRODUCTION

Preeclampsia is a multi-system hypertensive disorder of pregnancy, with a complex pathogenesis based on a multi-step aetiology. According to the current theory, the disease evolves in two stages (*Staff, 2019*; *Redman et al., 2021*). Stage one is characterized by a deficient placentation, involving incomplete transformation of the spiral arteries by failed intravascular trophoblast invasion of the myometrial junctional zone (*Brosens et al., 2002*) or a later syncytiotrophoblast dysfunction, which promotes imbalanced blood perfusion, hypoxia and the consequent oxidative stress. The second stage of the disease is characterized by maternal hypertension in combination with de-novo maternal end-organ damage, after generalized endothelial dysfunction and systemic inflammation.

Free fetal haemoglobin (HbF) has been associated with preeclampsia pathophysiology (*Centlow et al., 2008*; *Masoumi et al., 2017*; *Anderson et al., 2018*), by promoting placental tissue damage and oxidative stress, consequently leading to its leakage through the blood–placenta barrier into the maternal circulation (*Anderson et al., 2011, 2018*; *Gram et al., 2015*). Free fetal haemoglobin is, therefore, potentially a causative factor that links stage one and two of the two-stage model.

Haemopexin and α_1-microglobulin are two scavenger proteins synthesized in the liver, and are involved in the defence mechanisms against the toxicity of free HbF and its degradation metabolites heme and free iron (*Chiabrando et al., 2011*). In preeclampsia, elevated levels of heme and free HbF were associated with a decrease in haemopexin plasma concentration, suggesting consumption of this molecule, with maternal levels correlating to blood pressure (*Gram et al., 2015*; *Anderson et al., 2018*), and α_1-microglobulin has been shown to be up-regulated (*Anderson et al., 2011*). Multiple studies have reported an increased risk of adverse maternal outcomes, such as hypertensive disorders of pregnancy in women who have conceived through IVF, especially those undergoing IVF with frozen embryo transfer (FET) (*Ernstad et al., 2019*; *Waschkies et al., 2021*). The reason for this increased risk, however, is not completely understood. Recent reports have shown a higher incidence of hypertensive disorders in pregnancies after embryo transfer in programmed cycles compared with those undergoing embryo transfer in natural or stimulated cycles, suggesting a link between the absence of corpus luteum and adverse pregnancy outcomes (*Von Versen-Hoÿnck et al., 2019*; *Waschkies et al., 2021*).

Most pregnancies achieved by assisted reproductive technology (ART) treatments begin in a non-physiological endocrine environment (*Conrad and Baker, 2013*). In contrast to natural singleton pregnancies occurring in the presence of one corpus luteum, IVF cycles with transfer of autologous fresh embryos result in a supraphysiologic number of corpus luteum, and in some embryo transfer protocols, the pituitary–ovarian axis is purposely suppressed by oestradiol supplementation in the context of a programmed cycle, resulting in the absence of corpus luteum. In naturally conceived pregnancies, the corpus luteum plays an important role in producing and releasing crucial hormones for implantation, placentation and pregnancy maintenance. In ART protocols, some of these hormones, e.g. progesterone and oestrogen, are provided to support gestation. The corpus luteum activity, however, is not limited to these hormones (*Conrad and Baker, 2013*). Interestingly, animal models have demonstrated that relaxin, a potent vasodilator and stimulus of decidualization, is also secreted by the corpus luteum and contributes to the cardiovascular and renal adaptations seen in pregnancy (*Debrah et al., 2006*; *Conrad, 2011*). In humans, plasma relaxin has also been reported to be undetectable in a non-corpus luteum cohort, but markedly elevated in a multiple-corpus luteum cohort throughout pregnancy (*Conrad et al., 2019*).

The absence of corpus luteum and altered maternal plasma levels of the scavenger proteins haemopexin and α_1-microglobulin have been individually associated with preeclampsia development (*Ernstad et al., 2019*; *Von Versen-Hoÿnck et al., 2019*; *Bellos et al., 2020*). The aim of the present study was to assess the scavenger proteins haemopexin and α_1-microglobulin in maternal and fetal blood at birth in naturally conceived pregnancies and in two different IVF populations (exposed and not exposed to the corpus luteum activity). These results were analysed according to the occurrence of preeclampsia in these gestations.

MATERIALS AND METHODS

Ethical approval

This clinical study was conducted according to the Declaration of Helsinki for Medical Research involving Human Subjects (*World Medical Association, 2013*), and the study protocol was approved by the local Ethics Committee (reference: HCB/2016/0253 dated 15 April 2016, reference: HCB/2017/0714 dated 26 September 2017). All patients agreeing to participate provided written informed consent.

Study design, size, duration and setting

This is a nested case-control study of 160 singleton pregnancies. The women attended the Hospital Clínic de Barcelona, Barcelona, Spain, and were recruited prospectively between 2016 and 2020. Fifty-four were naturally conceived pregnancies from fertile couples, 50 were conceived by IVF after fresh embryo transfer and FET in natural cycle (presence of corpus luteum) and 56 resulted from IVF after fresh oocyte donation or FET in programmed cycles (absence of corpus luteum). Pregnancies were subclassified according to their outcomes as normotensive, preeclampsia and severe preeclampsia.

Participants and materials

The study population is presented in FIGURE 1. This is a case-control study nested within a prospective cohort of singleton pregnancies composed of three different populations (naturally conceived, IVF corpus luteum and IVF without corpus luteum). All patients from the original cohort were enrolled prospectively. Naturally conceived pregnancies from fertile couples (with a time to pregnancy no longer than 12 months) were prospectively recruited after the first trimester of pregnancy. The IVF pregnancies were recruited in the Assisted Reproduction Unit (Hospital Clínic de Barcelona) during the participant's first trimester, warranting homogeneity in ovarian stimulation and FET endometrial preparation protocols, laboratory procedures and embryo culture conditions between the study

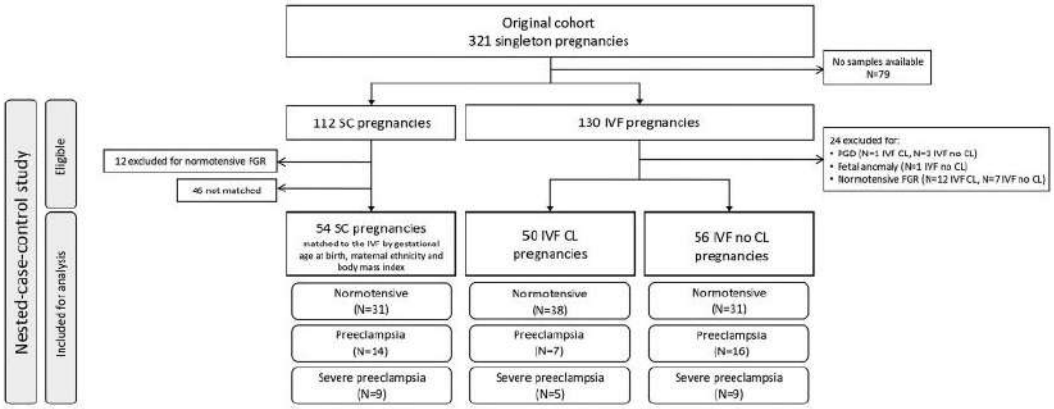

FIGURE 1 The study population. FGR, fetal growth restriction; IVF CL, IVF pregnancies after embryo transfer with the presence of corpus luteum; IVF no CL, IVF pregnancies after embryo transfer without corpus luteum; NC, naturally conceived pregnancies; PGT, preimplantation genetic testing.

participants. The IVF patients were categorized as IVF with corpus luteum and IVF without corpus luteum according to the corpus luteum presence at embryo transfer. Ovarian stimulation, embryo culture, cryopreservation and embryo transfer protocols are explained in the Supplementary methods. The assignment to the perinatal outcome group (normotensive, preeclampsia and severe preeclampsia) was carried out at least 1 month after birth, to identify puerperal preeclampsia cases.

For the nested case-control study, only the pregnancies followed and delivered in our centre (Hospital Clínic de Barcelona) with available plasma samples were considered eligible. Pregnancies that met any exclusion criteria (preimplantation genetic testing, fetal malformations, fetal chromosomal anomalies, intrauterine infection during pregnancy and normotensive fetal growth restriction pregnancies) were removed, and the naturally conceived pregnancies were matched to the IVF populations by gestational age at birth (± 1 week), maternal body mass index (± 2 kg/m^2) and ethnicity for normotensive and both preeclampsia groups.

Data collection and study protocol

Maternal epidemiological and obstetric parameters were collected by interviews and review of their medical records, including data on parental age, ethnicity, body mass index (obesity was defined as body mass index above 30 kg/m^2), educational level, working status, preeclampsia risk factors (prior preeclampsia, chronic hypertension, pregestational diabetes mellitus, antiphospholipid syndrome), other chronic diseases, parity, obstetric history, mode of conception, smoking status and illicit substance abuse (heroin, cocaine or cannabis), aspirin and corticosteroid exposures during pregnancy.

The IVF-related parameters included cause and length of infertility, diagnosis of polycystic ovary syndrome (PCOS) according to the Rotterdam 2003 criteria (*Fauser, 2004*), number of previous failed ART cycles, ovarian stimulation data, number of follicles punctured, number of oocytes retrieved, number of embryos obtained and transferred, endometrial preparation protocol, use of cryopreserved or fresh gametes, embryonic stage at the time of embryo transfer, vanishing twin detection or selective embryonic reduction.

All included pregnancies were followed and delivered in Hospital Clínic de Barcelona. Upon delivery, pregnancy and perinatal outcomes were recorded, including gestational age at delivery, mode of delivery, birth weight, Apgar score, umbilical artery pH, neonatal admission to the neonatal intensive care unit, perinatal morbidity and mortality, and the presence of the following pregnancy complications: gestational diabetes; pregnancy-induced hypertension; preeclampsia; eclampsia; syndrome of haemolysis; elevated liver enzymes; low platelet count (haemolysis, elevated liver enzymes and low platelets syndrome); cholestasis; placenta previa; abruptio placentae; and preterm birth (<37 weeks of gestation). Gestational age was calculated according to first-trimester crown–rump length (*Robinson, 1973*). Small for gestational age and large for gestational age were defined, respectively, as birth weight below 10th centile or above the 90th centile according to local standards (*Figueras et al., 2008*).

Preeclampsia was defined by new onset of hypertension of more than 140 mmHg systolic blood pressure or more than 90 diastolic blood pressure after 20 weeks' gestation together with more than 300 mg proteins in 24 h urine, evidence of maternal acute kidney injury, liver dysfunction, neurological features, haemolysis or thrombocytopaenia, or fetal growth restriction (FGR), or all (*Brown et al., 2018*). Both early (gestational age under 34 weeks at clinical onset) and late onset (34 weeks or over) preeclamptic pregnancies were included (*Valensise et al., 2008*). Preeclampsia cases were classified as severe in case of early onset, or severe hypertension that does not respond to treatment or in association with ongoing or recurring severe headaches, visual scotomata, nausea or vomiting, epigastric pain, oliguria and severe hypertension, as well as progressive deterioration in laboratory blood tests, i.e. rising creatinine or liver transaminases or falling platelet count, or failure of fetal growth or abnormal Doppler findings (*NICE guideline, 2019*).

Major neonatal morbidity was defined by the presence of bronchopulmonary dysplasia, necrotizing enterocolitis, intraventricular haemorrhage, periventricular leukomalacia, retinopathy, persistent ductus arteriosus or sepsis. Minor neonatal morbidity was defined

by the presence of respiratory distress, hyperbilirubinaemia or anaemia. Perinatal mortality was defined by either intrauterine fetal death after 22 weeks of pregnancy or neonatal death within the first 28 days of life.

Paired maternal and cord blood sampling

Maternal blood samples were drawn from peripheral veins at delivery room. Cord blood was obtained after the cord was clamped at delivery. All blood samples were collected in EDTA-treated tubes. Plasma was separated by centrifugation at 1500 g for 10 min at 4°C, and samples were immediately stored at −80°C until analysed.

Heme scavengers

Haemopexin concentrations were determined using a Human Hemopexin ELISA Kit (GWB-4B6D1A) from Genway Biotech Inc (San Diego, CA, USA) according to the manufacturer's instructions. Standards and unknown samples were diluted with 1x diluent from kit, run in duplicates, and the absorbance was read at 450 nm using a GloMax Discover microplate reader (Promega, Madison, WI, USA). For every plate, a standard curve was used to quantify haemopexin concentrations in each sample. The sensitivity of the test was 2.23 ng/ml, the interassay coefficient of variation was 9.74%, and the average intra-assay coefficient of variation was 2.57% and 2.60% for maternal and fetal samples, respectively.

An in-house developed sandwich enzyme-linked immunosorbent assay with a sensitivity of 1.95 ng/ml was used for the quantification of α_1-microglobulin. Microtitre plates were coated with an anti-human α_1-microglobulin antibody (mouse monoclonal, clone 35.14; 5 µg/ml in phosphate buffered saline [PBS]) overnight at +4°C under sealing film with 100 µl per well. After washing three times with PBS + 0.05% tween 20, 100 µl of human urinary α_1-microglobulin reference standard samples (1.56–100 ng/ml in PBS + 0.05% tween 20 + 0.5% bovine serum albumin [BSA]) or unknown plasma samples (diluted 2500x with PBS + 0.05% tween 20 + 0.05% BSA) were added to the wells in duplicates and incubated under sealing film for 1 h at room temperature, darkness and rotational shaking 250–500 revolutions per min. After washing three times with PBS 0.05% tween 20, 100 µl/well of a horseradish peroxidase-conjugated detection antibody (mouse monoclonal, clone 57.10; 5 ng/ml) was added and incubated under sealing film for 1 h at room temperature, darkness, and rotational shaking 250–500 revolutions per min. Finally, after washing three times with PBS 0.05% tween 20, 100 µl/well of a ready-to-use 3,3′,5,5′-Tetramethylbenzidine, TMB (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) substrate solution was added, sealed and incubated in darkness without shaking. The reaction was stopped after 15 min using 1 M sulphuric acid and the absorbance was read at 450 nm using a GloMax Discover microplate reader (Promega, Madison, WI, USA). Both anti-human α_1-microglobulin and horseradish peroxidase-conjugated detection antibodies were produced in-house by Agrisera AB (Vännäs, Sweden) by immunization with human urinary α_1-microglobulin prepared as previously described (*Berggård et al., 2008*). For every plate, a standard curve was used to quantify α_1-microglobulin concentrations in each sample, with an interassay coefficient of variation of 6.04%, and an average intraassay coefficient of variation of 2.07% and 1.90% for maternal and fetal samples, respectively, in congruence with previous studies (*Anderson et al., 2011*; *Youssef et al., 2020*).

Statistical analysis

Statistical software STATA 16 (StataCorp LLC, Texas, USA) was used to analyse the data. The study outcomes were haemopexin and α_1-microglobulin maternal and cord plasma concentrations. The independent or exposure variable of interest was corpus luteum status at conception (natural, IVF treatments with corpus luteum and IVF treatments without corpus luteum). On the basis of previous reports (*Gram et al., 2015*; *Youssef et al., 2020*), a sample size of 46 patients per group was calculated by expecting differences of 2.5 SD in α_1-microglobulin concentrations between the normotensive and the preeclampsia cases in a proportion 3:1, for a two-sided 95% confidence interval, a confidence interval width no larger than 4 µg/ml with a 90% probability of achieving the target confidence interval width.

Descriptive statistics and results were expressed as mean (± SD), median (interquartile range), or number (%), as appropriate. Normal distribution of continuous variables was checked using the Shapiro–Wilk test and histograms. Comparisons among the study groups were assessed by analysis of variance or Kruskal–Wallis tests with Bonferroni correction for continuous variables and Pearson chi-squared test for the categorical variables. Each group of cases (normotensive, preeclampsia and severe preeclampsia) was assessed separately by corpus luteum status at conception. Alternatively, comparisons between naturally conceived, preeclampsia and severe preeclampsia cases were carried out, including the whole study population. Differences between groups were adjusted for confounding factors such as maternal age, ethnicity, oocyte-donation, FET cycle, prematurity, gestational age and weight centile at time of sampling by linear regression. Correlations between the maternal and fetal heme-scavenger concentrations were investigated using the Spearman correlation coefficient. All reported *P*-values are two-sided. Fetal sex contribution was evaluated by comparing the biomarkers' results from women compared with men for each exposure and perinatal group using Student t-test or Mann–Whitney U test, as appropriate. The significance level was set at $P < 0.05$ for all the statistical tests.

RESULTS

Baseline and perinatal characteristics of the study population

The baseline characteristics of the study population are presented in TABLE 1. No significant differences were found between the study groups in maternal ethnicity, body mass index and clinical risk factors for preeclampsia, such as prior preeclampsia, chronic hypertension, pregestational diabetes mellitus and antiphospholipid syndrome. The maternal age was higher in the IVF group without corpus luteum compared with the naturally conceived pregnancies and the IVF with corpus luteum that developed preeclampsia (Supplementary Table 1). The IVF with corpus luteum group presented a higher rate of nulliparity compared with the naturally conceived pregnancies only in normotensive pregnancies (Supplementary Table 1). No significant differences were found for infertility causes, and number of embryos transferred between the IVF study groups. Regarding IVF techniques, the

TABLE 1 BASELINE CHARACTERISTICS OF THE STUDY GROUPS ACCORDING TO CORPUS LUTEUM STATUS

Characteristics	NC (*n* = 54)	IVF CL (*n* = 50)	IVF no CL (*n* = 56)
Maternal age, years	35.9 (32.0–38.3)	36.9 (35.1–39.5)	38.9 (36.1–40.5)[a]
White, *n* (%)	45 (83.3)	44 (88.0)	45 (80.4)
Nulliparity, *n* (%)	35 (64.8)	44 (88.0)[a]	44 (78.6)
Maternal body mass index, kg/m^2	23.1 (21.3–25.9)	23.3 (20.3–26.8)	23.9 (22.0–26.6)
Maternal obesity, *n* (%)	19 (35.2)	19 (38.0)	21 (37.5)
Prior preeclampsia, *n* (%)	4 (7.4)	0	2 (3.6)
Chronic hypertension, *n* (%)	1 (1.9)	1 (2.0)	4 (7.1)
Pregestational diabetes mellitus, *n* (%)	5 (9.3)	1 (2.0)	1 (1.8)
Antiphospholipid syndrome, *n* (%)	5 (9.3)	3 (6.0)	3 (5.4)
PCOS, *n* (%)	2 (3.7)	11 (22.0)[a]	11 (19.6)
Endometriosis, *n* (%)	NA	4 (8.0)	3 (5.4)
Tubal factor, *n* (%)	NA	4 (8.0)	6 (10.7)
Male factor, *n* (%)	NA	22 (44.0)	22 (39.3)
Unexplained infertility, *n* (%)	NA	22 (44.0)	28 (50.0)
Oocyte donation, *n* (%)	NA	1 (2.0)	21 (37.5)[b]
FET, *n* (%)	NA	10 (20.0)	44 (78.6)[b]
Embryos transferred, *n* (%)	NA	2 (1–2)	2 (1–2)

Data are median (interquartile range) or number (%), as appropriate.

[a] $P < 0.05$ compared NC: maternal age, NC versus IVF no CL: $P < 0.001$; nulliparity, NC versus IVF CL: $P = 0.015$; PCOS, NC versus IVF CL: $P = 0.026$.

[b] $P < 0.001$ compared with IVF CL.

FET, frozen embryo transfer; IVF CL, IVF with corpus luteum pregnancies; IVF no CL, IVF without corpus luteum pregnancies; NA, not applicable; NC, naturally conceived pregnancies; PCOS, polycystic ovary syndrome.

IVF without corpus luteum had a higher proportion of FET and oocyte donation cycles. A higher proportion of blastocyst transfers took place in the IVF without corpus luteum group compared with the IVF with corpus luteum group (55.4% versus 12.0%, $P < 0.001$).

Perinatal outcomes are presented in TABLE 2. The occurrence of preterm delivery and pregnancy complications. i.e. preeclampsia, severe preeclampsia, gestational diabetes, placenta previa and placental abruption, was not significantly different among the study groups. No differences were found in gestational age at birth, birth weight and birth weight centile between the exposure groups for normotensive, preeclampsia nor severe preeclampsia cases (Supplementary Table 2), although globally the IVF with corpus luteum group had a higher gestational age at birth than naturally conceived pregnancies and programmed cycles (TABLE 2). A higher proportion of caesarean sections occurred in the programmed cycle population compared with the naturally conceived pregnancies and IVF with corpus luteum groups only in the non-severe preeclampsia cases (Supplementary Table 2). No other significant differences were found in delivery and neonatal characteristics between the study populations either overall or when comparing the normotensive, preeclampsia and severe preeclampsia cases.

Heme scavenger results

The distribution of plasma haemopexin and α_1-microglobulin across the exposure groups are shown in TABLE 3, and the values adjusted for maternal age, ethnicity, oocyte-donation, frozen embryo transfer cycle, prematurity, gestational age and weight centile at sampling are shown in FIGURE 2. No significant differences for haemopexin and α_1-microglobulin were obtained when the exposure groups in the unadjusted model were compared (TABLE 3). The adjusted model showed significant differences among groups (TABLE 3 and FIGURE 2). As shown in FIGURE 2, both in normotensive and preeclampsia populations, IVF with corpus luteum pregnancies presented significantly higher maternal haemopexin levels than the naturally conceived pregnancy group ($P = 0.038$ and $P = 0.011$, respectively). In preeclamptic pregnancies, maternal haemopexin levels were significantly lower in IVF treatments without corpus luteum compared with the IVF group with one corpus luteum or several corpus lutea ($P = 0.002$). In severe preeclampsia cases, a tendency existed towards lower haemopexin levels in the IVF pregnancies lacking corpus luteum. The adjusted haemopexin profile in cord blood showed the same trend as seen in maternal blood, although differences between exposure groups were not significant except for the preeclampsia group (naturally conceived pregnancies versus IVF corpus luteum: $P = 0.039$).

Furthermore, maternal α_1-microglobulin levels were similar among the exposure groups in normotensive and preeclampsia populations, but significantly higher in the absence of corpus luteum in severe preeclamptic cases compared with naturally conceived pregnancies and IVF treatments with corpus luteum pregnancies ($P = 0.014$ and $P = 0.041$, respectively). This pattern was also observed in cord blood for severe preeclampsia cases in the adjusted model, although no significant difference was found between groups ($P = 0.744$ for multiple comparison). In umbilical cord samples from normotensive pregnancies, IVF pregnancies without corpus luteum showed significantly higher adjusted α_1-microglobulin levels compared with

TABLE 2 PERINATAL CHARACTERISTICS OF THE STUDY GROUPS ACCORDING TO CORPUS LUTEUM STATUS

Characteristics	NC ($n = 54$)	IVF CL ($n = 50$)	IVF no CL ($n = 56$)
Gestational diabetes, *n* (%)	4 (7.4)	8 (16.0)	8 (14.3)
Preeclampsia, *n* (%)	24 (44.4)	12 (24.0)	25 (44.6)
Severe preeclampsia, *n* (%)	9 (16.7)	5 (10.0)	9 (16.1)
Placenta previa, *n* (%)	0	1 (2.0)	4 (7.1)
Placental abruption, *n* (%)	1 (1.9)	0	1 (1.8)
Preterm delivery, *n* (%)	7 (13.0)	3 (6.0)	9 (16.1)
Prenatal corticoid exposure, *n* (%)	3 (5.6)	4 (8.0)	6 (10.7)
Prenatal aspirin exposure, *n* (%)	17 (31.5)	7 (14.0)	10 (17.9)
Gestational age at birth, weeks, days	39.0 (37.4–40.0)	39.6 (38.3–40.4)[a,c]	39.0 (37.4–40.1)
Birthweight, g	3208 (2640–3420)	3155 (2925–3490)	3217 (2805–3563)
Birthweight, centile	35 (12–59)	36 (15–65)	55 (17–73)
Birthweight below the 10th centile	12 (22.2)	5 (10.0)	8 (14.3)
Birthweight above the 90th centile	4 (7.4)	2 (4.0)	7 (12.5)
Induction of labour	29 (53.7)	26 (52.0)	33 (58.9)
Caesarean section	20 (37.0)	15 (30.0)	35 (62.5)[a,b]
Female	29 (53.7)	26 (52.0)	34 (60.7)
NICU admission	7 (13.0)	4 (8.0)	12 (21.4)

[a] $P < 0.05$ compared with NC: gestational age at birth, NC versus IVF CL: $P = 0.034$; caesarean section, NC versus IVF no CL: $P = 0.018$.
[b] $P < 0.05$ compared with IVF CL: caesarean section, IVF CL versus IVF no CL: $P = 0.002$.
[c] $P < 0.05$ compared with IVF no CL: gestational age at birth, IVF CL versus IVF no CL: $P = 0.036$.
IVF CL, IVF with corpus luteum pregnancies; IVF no CL, IVF without corpus luteum pregnancies; NC, naturally conceived pregnancies; NICU, neonatal intensive care unit.
Data are median (interquartile range) or number (percentage), as appropriate.

the IVF pregnancies with corpus luteum ($P < 0.001$).

Overall, in the adjusted model, the preeclampsia group showed significantly decreased concentrations of haemopexin and significantly increased concentrations of α_1-microglobulin in maternal and fetal plasma compared with normotensive pregnancies, regardless of the corpus luteum status at the time of conception ($P < 0.001$), except for fetal α_1-microglobulin ($P = 0.585$), although a significant difference was seen for fetal α_1-microglobulin between the severe preeclampsia population compared with naturally conceived pregnancies ($P < 0.001$). In general, these differences were more pronounced in severe preeclampsia cases (FIGURE 3).

Within the preeclamptic cases, 25 women received prophylactic aspirin during pregnancy and 35 women did not. Women with preeclampsia who received aspirin had no differences in haemopexin or α_1-microglobulin levels compared with women with preeclampsia who did not receive it (median haemopexin concentration 1204 µg/ml versus 1388 µg/ml, adjusted $P = 0.899$; median α_1-microglobulin concentration 21.9 µg/ml versus 23.6 µg/ml, adjusted $P = 0.372$). Similar results were found after conducting the same analysis within each exposure group for each of the biomarkers (data not shown).

Maternal and fetal haemopexin concentrations only showed a significant moderate correlation in severe preeclampsia cases ($\rho = 0.55$; $P = 0.028$). On the other hand, maternal and fetal α_1-microglobulin concentrations showed a significant weak correlation in the whole population ($\rho = 0.38$; $P < 0.001$), with this correlation being stronger within the preeclampsia group ($\rho = 0.46$; $P = 0.001$). All findings were independent of fetal sex (data not shown).

DISCUSSION

To the best of our knowledge, this is the first study assessing the profile of heme scavengers haemopexin and α_1-microglobulin in IVF compared with naturally conceived pregnancies. These preeclampsia-associated molecules were assessed considering the presence or absence of corpus luteum at conception, which varies according to the ART treatment. Our data are consistent with previous reports that have suggested an association between preeclampsia and changes in α_1-microglobulin and haemopexin (*Olsson et al., 2010*; *Gram et al., 2015*) and provide further evidence of physiological differences between pregnancies after embryo transfer in stimulated and natural versus programmed cycles, supporting the hypothesis that corpus luteum activity could influence perinatal outcomes.

In the present study, as expected, both maternal and fetal haemopexin levels were significantly lower at birth in preeclamptic pregnancies as well as in severe preeclampsia cases compared with normotensive pregnancies, and independent of the mode of conception. This supports the hypothesis that heme-induced oxidative stress contributes to the pathogenesis in preeclampsia.

Maternal constitutional characteristics can either predispose or protect against the increased oxidative stress during pregnancy. Depending on the individual, the same oxidative insult may cause diverse signs and forms of preeclampsia, as well as varying severity of the disease (*Hansson et al., 2015*; *Staff, 2019*). Interestingly, in the present study, the IVF with corpus luteum (with one or

TABLE 3 CONCENTRATIONS OF HEME SCAVENGERS IN MATERNAL AND FETAL BLOOD ACCORDING TO CORPUS LUTEUM STATUS

Perinatal groups	NC	IVF CL	IVF no CL	*P*-value	Adjusted *P*-value[a]
Maternal plasma					
Haemopexin, μg/ml					
Normotensive	1520 (1054–1746)	1554 (1315–1778)[b]	1401 (1130–1750)	0.453	0.002
Preeclampsia	1362 (1121–1667)	1372 (403–2558)[b]	1215 (971–1498)[c]	0.881	0.004
Severe preeclampsia	1388 (1204–1633)	1033 (995–1446)	1249 (1098–1275)	0.596	0.561
α_1-microglobulin, μg/ml					
Normotensive	18 (17–20)	19 (17–21)	17 (16–20)	0.761	0.145
Preeclampsia	22 (20–24)	23 (19–26)	22 (20–24)	0.164	0.988
Severe preeclampsia	23 (20–24)	24 (24–26)	26 (25–28)[b,c]	0.435	0.023
Umbilical cord plasma					
Haemopexin, μg/ml					
Normotensive	293 (164–353)	267 (197–323)	222 (161–281)	0.373	0.242
Preeclampsia	180 (96–205)	155 (48–220)[b]	167 (135–307)	0.507	0.044
Severe preeclampsia	95 (69–205)	91 (11–349)	136 (85–164)	0.730	0.084
α_1-microglobulin, μg/ml					
Normotensive	11 (10–14)	11 (9–13)	12 (9–15)[c]	0.279	0.001
Preeclampsia	11 (8–13)	12 (7–13)	11 (8–17)	0.622	0.071
Severe preeclampsia	15 (11–15)	20 (18–21)	12 (11–18)	0.063	0.744

Unadjusted data are expressed as median (interquartile range).

[a] Comparisons adjusted by multiple linear regression for maternal age, ethnicity, oocyte-donation, frozen embryo transfer cycle, prematurity, gestational age and weight centile at sampling.

[b] $P < 0.05$ compared with NC: maternal haemopexin, normotensive group, NC versus IVF CL: $P = 0.038$; maternal haemopexin, preeclampsia group, NC versus IVF CL: $P = 0.011$; maternal α_1-microglobulin, severe preeclampsia group, NC versus IVF no CL: $P = 0.014$; umbilical cord haemopexin, preeclampsia group, NC versus IVF CL: $P = 0.039$.

[c] $P < 0.05$ compared with IVF CL: maternal haemopexin, preeclampsia group, IVF CL versus IVF no CL: $P = 0.002$; maternal α_1-microglobulin, severe preeclampsia group, IVF CL versus IVF no CL: $P = 0.041$; umbilical cord α_1-microglobulin, normotensive group, IVF CL versus IVF no CL: $P < 0.001$.

IVF CL, IVF with corpus luteum pregnancies; IVF no CL, IVF without corpus luteum pregnancies; NC, naturally conceived pregnancies.

multiple corpus) group showed less altered values for haemopexin than the naturally conceived pregnancy group in preeclampsia cases, compared with normotensive pregnancies, which could suggest a protective effect towards heme-induced oxidative stress. Alternatively, maternal haemopexin levels were lower in the IVF group lacking corpus luteum both in normotensive and preeclampsia cases compared with the IVF with corpus luteum population. We did not see the same effect in the α_1-microglobulin profile as for haemopexin levels, probably owing to the lower variability of α_1-microglobulin compared with the haemopexin concentrations. Consistent with previous investigations (*Kalapotharakos et al., 2019*), α_1-microglobulin levels in women with severe preeclampsia were significantly higher compared with non-severe preeclampsia cases. Here, when assessing only severe cases, the α_1-microglobulin levels were significantly higher in the absence of corpus luteum compared with natural pregnancies and IVF treatments in the presence of corpus luteum. In the present study, a higher proportion of women with PCOS was found among non-natural pregnancy groups, although this diagnosis could have been underestimated in fertile patients. Polycystic ovary syndrome has a three-to four-fold increased risk of pregnancy-induced hypertension and preeclampsia (*Boomsma et al., 2006*; *Kjerulff et al., 2011*; *Qin et al., 2013*; *Palomba et al., 2015*) among other perinatal complications. The potential causes of these increased risks are PCOS features (hyperandrogenism, insulin resistance, chronic low-grade inflammation), comorbidities (obesity), infertility interventions (infertility *per se*, drugs, ART) and primary placental alteration, which may act directly or through an altered trophoblast invasion and placentation (*Palomba et al., 2015*; *2021*; *Palomba, 2021*). In assessing aspirin prophylaxis, in contrast to previous reports (*Kalapotharakos et al., 2019*), we found no differences in haemopexin or α_1-microglobulin concentrations between patients with preeclampsia with or without aspirin prophylactic treatment. The current findings were independent of fetal sex, in line with previous reports on comparable haemopexin and α_1-microglobulin levels among male and female fetuses (*Masoumi et al., 2017*; *2019*; *Youssef et al., 2020*).

We report significant correlations between maternal and fetal biomarkers concentrations, with higher Pearson correlation coefficients in preeclampsia cases, supporting the hypothesis of a disruption of the blood–placental barrier, consequently with the leakage of free fetal hemoglobin (HbF) into the maternal circulation. Free HbF has also been associated with FGR (*Brook et al., 2018*). In the present study, we excluded the normotensive FGR cases, which have been reported to present lower concentrations in cord blood for both biomarkers compared with preeclampsia without FGR and normotensive pregnancies, suggesting a differential

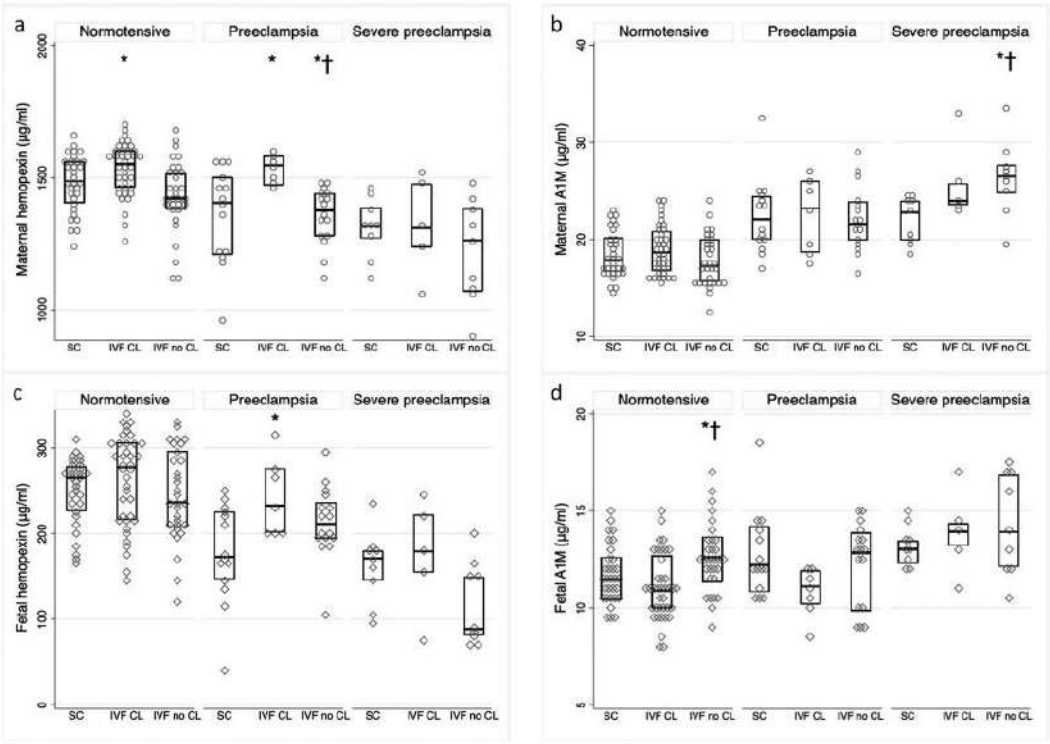

FIGURE 2 Adjusted concentrations of heme scavengers in maternal and fetal blood in the study population. Dot plots represent (A) maternal haemopexin concentrations; (B) maternal α_1-microglobulin concentrations; (C) fetal haemopexin concentrations; (D) fetal α_1-microglobulin concentrations. Boxes show median and interquartile range. Values and comparisons are adjusted by multiple linear regression for maternal age, ethnicity, oocyte donation, frozen embryo transfer cycle, prematurity, gestational age and weight centile at sampling. Exposure groups are compared for each perinatal result (normotensive, preeclampsia, severe preeclampsia). A1M, α_1-microglobulin; IVF CL, IVF pregnancies after embryo transfer with corpus luteum; IVF no CL, IVF pregnancies after embryo transfer without corpus luteum, NC, naturally conceived pregnancy.

role in the pathophysiology of placental mediated diseases in accordance with their clinical presentation (*Youssef et al., 2020*).

Recently, it has been reported that high-risk pregnancies without preeclampsia also presented an altered profile of heme scavengers compared with low-risk gestations (*Kalapotharakos et al., 2019*). Moreover, both lower haemopexin and higher α_1-microglobulin levels have been described already at early pregnancy in patients who later developed the disorder, just before the luteal-placental shift at the end of the first trimester (*Anderson et al., 2011, 2016; Gram et al., 2015*). In women undergoing IVF in the absence of corpus luteum, the cardiovascular and endothelial function are impaired and preeclampsia risk elevated from the first trimester, suggesting that relaxin or another corpus luteum vasoactive factor contributes to the transformation of the maternal circulation before the corpus luteal-placental shift, after which placental hormones may intervene (*Conrad et al., 2019*). Interestingly, from 32 weeks' gestation, even normotensive pregnancies progressively develop some grade of syncytiotrophoblast stress expressed by alterations on sFlt-1 and PlGF (*Redman et al., 2021*), and the amount of stress has been shown to be higher in IVF pregnancies with aberrant corpus luteum numbers (*Conrad et al., 2019*). The corpus luteum activity has been demonstrated to persist after the first trimester. In a longitudinal follow-up study, circulating relaxin concentrations were upheld at one-half of the peak levels observed in the first trimester until at least 32–35 weeks of gestational age, indicating that the corpus luteum continues functioning after the luteal-placental shift (*Conrad et al., 2019*). Furthermore, it has been reported that relaxin concentrations correlated with abnormal vascular reactivity (*Von Versen-Hoÿnck et al., 2019*) and prorenin, an inactive precursor of renin, also secreted by the corpus luteum (*Wiegel et al., 2020*). Together with any direct effect on maternal vascular adaptations by the early corpus luteum activity, there may also be an effect on placentation contributing to the later development of preeclampsia. Overall, the absence of corpus luteum seems to be related to maternal vascular compromise and abnormal placentation since early pregnancy, conditions known to promote hypoxia with the potential release of free HbF into maternal circulation.

Strengths and limitations

The present study has some strengths and limitations that merit comment. As strengths, cases and controls are part of a well-phenotyped prospective cohort followed to the end of the neonatal period, including extensive fetal and perinatal data. All pregnancies were followed up and delivered in the Hospital Clínic de Barcelona, and all IVF pregnancies were achieved in the Assisted Reproduction unit from the Hospital Clínic de Barcelona, warranting homogeneity in healthcare protocols.

As limitations, we first acknowledge that research into fetal outcomes in IVF pregnancies faces difficulties in unravelling

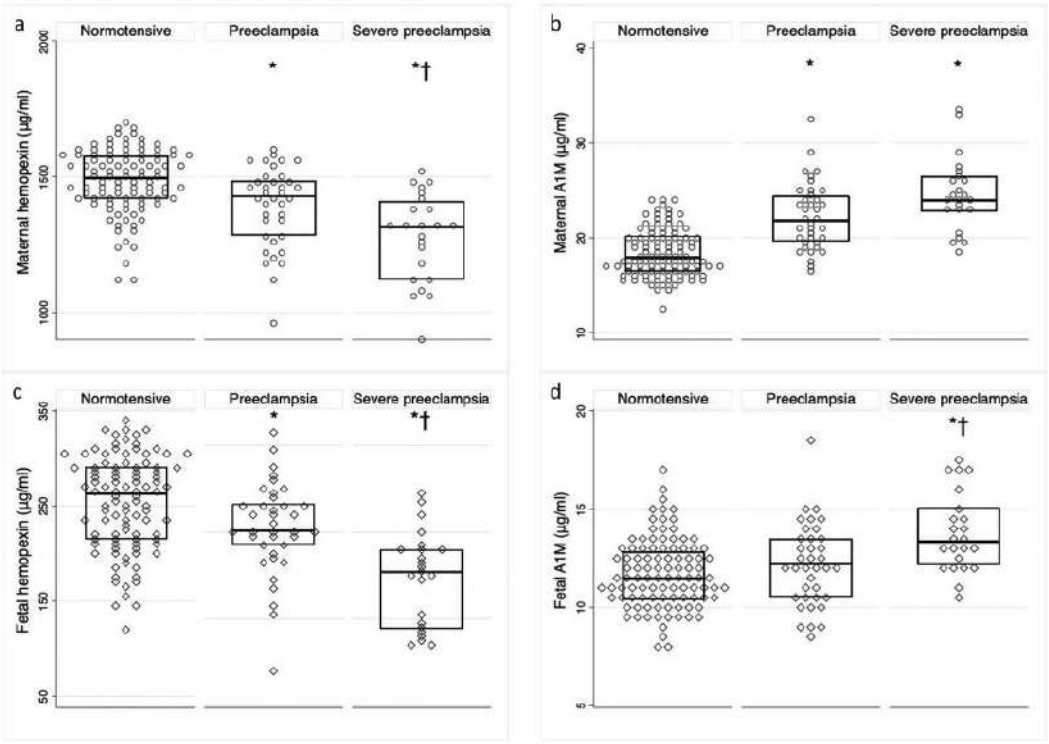

FIGURE 3 Adjusted concentrations of heme scavengers in maternal and fetal blood in the study population by the occurrence of preeclampsia. Dot plots represent (A) maternal haemopexin concentrations; (B) maternal α_1-microglobulin concentrations; (C) fetal haemopexin concentrations; (D) fetal α_1-microglobulin concentrations. Boxes show median and interquartile range. A1M indicates α_1-microglobulin. Values and comparisons are adjusted by linear regression for maternal age, ethnicity, oocyte donation, FET cycle, prematurity, gestational age and weight centile at sampling.

any contribution of the infertility factors from the IVF treatment itself. In the present study, although the main infertility causes were equally distributed in both IVF groups, we compared IVF populations with naturally conceived pregnancies from fertile couples. If infertility factors influence pregnancy outcomes, then pregnancies achieved naturally by subfertile parents (with a time-to-pregnancy of more than 1 year) may constitute a more appropriate naturally conceived pregnancy group. Nevertheless, at our centre, subfertility is most often not included in clinical records and consequently these patients are more difficult to identify for recruitment. Second, in this case-control study, naturally conceived pregnancies were matched to the IVF by gestational age at birth, maternal ethnicity, body mass index, preeclampsia occurrence and severity to limit heterogeneity; nevertheless, a selection bias cannot be fully discarded. Third, based on previous studies and to reduce the heterogeneity of the cases, we designed the study categorizing the preeclampsia cases according to severity. Probably because of the limited sample size, differences in plasma levels of the scavengers between the exposure groups decrease when all the preeclampsia cases are merged, especially in the case of maternal α_1-microglobulin (Supplementary Figure). Fourth, regarding the ART techniques, in the present study, the IVF groups presented some differences, with a higher proportion of oocyte-donation cycles, which constitutes a risk factor for preeclampsia *per se*, and FET in the embryo transfer in programmed cycles group. Therefore, adjustments for oocyte donation and FET cycles were carried out at data analysis. Fifth, the proportion of PCOS could have been underestimated in the naturally conceived pregnancy group because they were fertile patients. This, together with the limited sample size, prevented adjustment of the analysis for this condition, in which a higher risk of hypertensive disorders of pregnancy has been reported. Finally, we acknowledge that the presence or absence of corpus luteum was only determined for the IVF population at embryo transfer.

Clinical relevance and conclusions

Congruent with previous studies, our findings provide additional evidence for heme-induced oxidative stress contributing to pathogenesis in preeclampsia, additionally showing physiological differences between pregnancies achieved with or without corpus luteum. We report more altered heme scavenger concentrations in the group lacking corpus luteum at the time of conception, supporting the hypothesis that corpus luteum activity could influence the perinatal outcome through lower levels of oxidative stress.

Future research, including well-designed randomized controlled trials in IVF pregnancies, are warranted to investigate whether applying potential strategies to develop a corpus luteum might reduce the perinatal complications associated with programmed cycles of IVF and to increase knowledge on corpus luteum involvement to the development of hypertensive disorders of pregnancy.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2022.01.005.

REFERENCES

Anderson, U.D., Gram, M., Ranstam, J., Thilaganathan, B., Åkerström, B., Hansson, S.R. **Fetal hemoglobin, α_1-microglobulin and haemopexin are potential predictive first trimester biomarkers for preeclampsia.** Pregnancy Hypertens 2016; 6: 103–109. doi:10.1016/j.preghy.2016.02.003

Anderson, U.D., Jälmby, M., Faas, M.M., Hansson, S.R. **The hemoglobin degradation pathway in patients with preeclampsia – Fetal hemoglobin, heme, heme oxygenase-1 and haemopexin – Potential diagnostic biomarkers?** Pregnancy Hypertens 2018; 14: 273–278. doi:10.1016/j.preghy.2018.02.005

Anderson, U.D., Olsson, M.G., Rutardttir, S., Centlow, M., Kristensen, K.H., Isberg, P.E., Thilaganathan, B., Kerstrm, B., Hansson, S.R. **Fetal hemoglobin and α_1-microglobulin as first- and early second-trimester predictive biomarkers for preeclampsia.** Am. J. Obstet. Gynecol. 2011; 204: 520.e1–520.e5. doi:10.1016/j.ajog.2011.01.058

Bellos, I., Pergialiotis, V., Loutradis, D., Papapanagiotou, A., Daskalakis, G. **The role of hemoglobin degradation pathway in preeclampsia: A systematic review and meta-analysis.** Placenta 2020; 92: 9–16. doi:10.1016/j.placenta.2020.01.014

Berggård, T., Lindqvist, A., Cedervall, T., Åkerström, B., Thøgersen, I.B., Enghild, J.J., Cohen, A., Persson, P., Jönsson, J.-Å., Silow, M. **α_1-Microglobulin chromophores are located to three lysine residues semiburied in the lipocalin pocket and associated with a novel lipophilic compound.** Protein Sci. 2008; 8: 2611–2620. doi:10.1110/ps.8.12.2611

Boomsma, C.M., Eijkemans, M.J.C., Hughes, E.G., Visser, G.H.A., Fauser, B.C.J.M., Macklon, N.S. **A meta-analysis of pregnancy outcomes in women with polycystic ovary syndrome.** Hum. Reprod. Update 2006; 12: 673–683. doi:10.1093/humupd/dml036

Brook, A., Hoaksey, A., Gurung, R., Yoong, E.E.C., Snoyd, R., Baynes, G.C., Bischof, H., Jones, S., Higgins, L.E., Jones, C., Greenwood, S.L., Jones, R.L., Gram, M., Lang, I., Desoye, G., Myers, J., Schneider, H., Hansson, S.R., Crocker, I.P., Brownbill, P. **Cell free hemoglobin in the fetoplacental circulation: a novel cause of fetal growth restriction?** FaseB J. 2018; 32: 5436–5446. doi:10.1096/fj.201800264R

Brosens, J.J., Pijnenborg, R., Brosens, I.A. **The myometrial junctional zone spiral arteries in normal and abnormal pregnancies.** Am. J. Obstet. Gynecol. 2002; 187: 1416–1423. doi:10.1067/mob.2002.127305

Brown, M.A., Magee, L.A., Kenny, L.C., Karumanchi, S.A., McCarthy, F.P., Saito, S., Hall, D.R., Warren, C.E., Adoyi, G., Ishaku, S. **Hypertensive disorders of pregnancy: ISSHP classification, diagnosis, and management recommendations for international practice.** Hypertension 2018; 72: 24–43. doi:10.1161/HYPERTENSIONAHA.117.10803

Centlow, M., Carninci, P., Nemeth, K., Mezey, E., Brownstein, M., Hansson, S.R. **Placental expression profiling in preeclampsia: local overproduction of hemoglobin may drive pathological changes.** Fertil. Steril. 2008; 90: 1834–1843. doi:10.1016/j.fertnstert.2007.09.030

Chiabrando, D., Vinchi, F., Fiorito, V., Tolosano, E. **Haptoglobin and Haemopexin in Heme Detoxification and Iron Recycling.** Veas Prof. Francisco Acute Phase Proteins - Regulation and Functions of Acute Phase Proteins. InTech 2011. doi:10.5772/18241

Conrad, K.P. **Emerging Role of Relaxin in the Maternal Adaptations to Normal Pregnancy: Implications for Preeclampsia.** Semin. Nephrol. 2011; 31: 15–32. doi:10.1016/j.semnephrol.2010.10.003

Conrad, K.P., Baker, V.L. **Corpus luteal contribution to maternal pregnancy physiology and outcomes in assisted reproductive technologies.** Am. J. Physiol. Regul. Integr. Comp. Physiol. 2013; 304: 69–72. doi:10.1152/ajpregu.00239.2012

Conrad, K.P., Graham, G.M., Chi, Y.-Y., Zhai, X., Li, M., Stan Williams, R., Rhoton-Vlasak, A., Segal, M.S., Wood, C.E., Keller-Wood, M. **Potential influence of the corpus luteum on circulating reproductive and volume regulatory hormones, angiogenic and immunoregulatory factors in pregnant women.** Am. J. Physiol. Endocrinol. Metab. 2019; 317: e677–e685. doi:10.1152/ajpendo.00225

Debrah, D.O., Novak, J., Matthews, J.E., Ramirez, R.J., Shroff, S.G., Conrad, K.P. **Relaxin is essential for systemic vasodilation and increased global arterial compliance during early pregnancy in conscious rats.** Endocrinology 2006; 147: 5126–5131. doi:10.1210/en.2006-0567

Ernstad, E.G., Wennerholm, U.-B., Khatibi, A., Petzold, M., Bergh, C. **Neonatal and maternal outcome after frozen embryo transfer: Increased risks in programmed cycles.** Am. J. Obstet. Gynecol. 2019; 221: 126.e1–126.e18. doi:10.1016/j.ajog.2019.03.010

Fauser, B.C.J.M. **Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome.** Fertil. Steril. 2004; 81: 19–25. doi:10.1016/j.fertnstert.2003.10.004

Figueras, F., Meler, E., Iraola, A., Eixarch, E., Coll, O., Figueras, J., Francis, A., Gratacos, E., Gardosi, J. **Customized birthweight standards for a Spanish population.** Eur. J. Obstet. Gynecol. Reprod. Biol. 2008; 136: 20–24. doi:10.1016/j.ejogrb.2006.12.015

Gram, M., Dolberg Anderson, U., Johansson, M.E., Edström-Hägerwall, A., Larsson, I., Jälmby, M., Hansson, S.R., Åkerström, B. **The Human Endogenous Protection System against Cell-Free Hemoglobin and Heme Is Overwhelmed in Preeclampsia and Provides Potential Biomarkers and Clinical Indicators.** PLoS One 2015; 10e0138111. doi:10.1371/journal.pone.0138111

Hansson, S.R., Nääv, Å., Erlandsson, L. **Oxidative stress in preeclampsia and the role of free fetal hemoglobin.** Front Physiol. 2015; 5: 516. doi:10.3389/fphys.2014.00516

Hypertension in pregnancy: diagnosis and management (NICE guideline NG133), 2019.

Kalapotharakos, G., Murtoniemi, K., Åkerström, B., Hämäläinen, E., Kajantie, E., Räikkönen, K., Villa, P., Laivuori, H., Hansson, S.R. **Plasma heme scavengers α_1-microglobulin and haemopexin as biomarkers in high-risk pregnancies.** Front. Physiol. 2019; 10: 300. doi:10.3389/fphys.2019.00300

Kjerulff, L.E., Sanchez-Ramos, L., Duffy, D. **Pregnancy outcomes in women with polycystic ovary syndrome: A metaanalysis.** Am. J. Obstet. Gynecol. 2011; 204: 558.e1–558.e6. doi:10.1016/j.ajog.2011.03.021

Masoumi, Z., Familari, M., Källén, K., Ranstam, J., Olofsson, P., Hansson, S.R. **Fetal hemoglobin in umbilical cord blood in preeclamptic and normotensive pregnancies: A cross-sectional comparative study.** PLoS One 2017; 12: 1–10. doi:10.1371/journal.pone.0176697

Masoumi, Z., Maes, G.E., Herten, K., Cortés-Calabuig, Á., Alattar, A.G., Hanson, E., Erlandsson, L., Mezey, E., Magnusson, M., Vermeesch, J.R., Familari, M., Hansson, S.R. **Preeclampsia is associated with sex-specific transcriptional and proteomic changes in fetal erythroid cells.** Int. J. Mol. Sci. 2019; 20: 2038. doi:10.3390/ijms20082038

Olsson, M.G., Centlow, M., Rutardóttir, S., Stenfors, I., Larsson, J., Hosseini-Maaf, B., Olsson, M.L., Hansson, S.R., Åkerstrom, B. **Increased levels of cell-free hemoglobin, oxidation markers, and the antioxidative heme scavenger α_1-microglobulin in preeclampsia. Free Radic.** Biol. Med. 2010; 48: 284–291. doi:10.1016/j.freeradbiomed.2009.10.052

Palomba, S. **Is fertility reduced in ovulatory women with polycystic ovary syndrome? An opinion paper.** Hum. Reprod. 2021; 36: 2421–2428. doi:10.1093/humrep/deab181

Palomba, S., De Wilde, M.A., Falbo, A., Koster, M.P.H., La Sala, G.B., Fauser, B.C.J.M. **Pregnancy complications in women with polycystic ovary syndrome.** Hum. Reprod. Update 2015; 21: 575–592. doi:10.1093/humupd/dmv029

Palomba, S., Piltonen, T.T., Giudice, L.C. **Endometrial function in women with polycystic ovary syndrome: a comprehensive review.** Hum. Reprod. Update 2021; 27: 584–618. doi:10.1093/humupd/dmaa051

Qin, J.Z., Pang, L.H., Li, M.J., Fan, X.J., Huang, R.D., Chen, H.Y. **Obstetric complications in women with polycystic ovary syndrome: A systematic review and meta-analysis.** Reprod. Biol. Endocrinol. 2013; 11. doi:10.1186/1477-7827-11-56

Redman, C.W.G., Staff, A.C., Roberts, J.M. **Syncytiotrophoblast stress in preeclampsia: the convergence point for multiple pathways.** Am. J. Obstet. Gynecol. 2021: 1–21. doi:10.1016/j.ajog.2020.09.047

Robinson, H.P. **Sonar Measurement of Fetal Crown-Rump Length as Means of Assessing Maturity in First Trimester of Pregnancy.** Br. Med. J. 1973; 4: 28–31. doi:10.1136/bmj.4.5883.28

Staff, A.C. **The two-stage placental model of preeclampsia: An update.** J. Reprod. Immunol. 2019; 134–135: 1–10. doi:10.1016/j.jri.2019.07.004

Valensise, H., Vasapollo, B., Gagliardi, G., Novelli, G.P. **Early and Late preeclampsia: Two different maternal hemodynamic states in the latent phase of the disease.** Hypertension 2008; 52: 873–880. doi.10.1161/HYPERTENSIONAHA.108.117358

Von Versen-Hoÿnck, F., Narasimhan, P., Selamet Tierney, E.S., Martinez, N., Conrad, K.P., Baker, V.L., Winn, V.D. **Absent or excessive corpus luteum number is associated with altered maternal vascular health in early pregnancy.** Hypertension 2019; 73: 680–690. doi:10.1161/HYPERTENSIONAHA.118.12046

Waschkies, F., Kroning, L., Schill, T., Chandra, A., Schippert, C., Töpfer, D., Ziert, Y., von Versen-Höynck, F. **Pregnancy Outcomes After Frozen-Thawed Embryo Transfer in the Absence of a Corpus Luteum.** Front Med. 2021; 8: 1–11. doi:10.3389/fmed.2021.727753

Wiegel, R.E., Danser, A.H.J., Steegers-Theunissen, R.P.M., Laven, J.S.E., Willemsen, S.P., Baker, V.L., Steegers, E.A.P., Von Versen-Höynck, F. **Determinants of Maternal Renin-Angiotensin-Aldosterone-System Activation in Early Pregnancy: Insights From 2 Cohorts.** J. Clin. Endocrinol. Metab. 2020; 105: 3505–3517. doi:10.1210/clinem/dgaa582

World Medical Association. **Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects.** JAMA 2013; 310: 2191–2194. doi:10.1001/jama.2013.281053

Youssef, L., Erlandsson, L., Åkerström, B., Miranda, J., Paules, C., Crovetto, F., Crispi, F., Gratacos, E., Hansson, S.R. **Haemopexin and α_1-microglobulin heme scavengers with differential involvement in preeclampsia and fetal growth restriction.** PLoS One 2020; 15e0239030. doi:10.1371/journal.pone.0239030

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Differential concentrations of maternal and fetal hemopexin and α_1-microglobulin in preeclampsia from IVF pregnancies depending on the presence of corpus luteum at embryo transfer

Appendix A

Methods

Ovarian stimulation protocols
The ovarian stimulation protocol for IVF and the gonadotropin doses were chosen according to age and ovarian reserve markers. Either long agonist or antagonist protocols were used. Ovarian stimulation was achieved with daily doses from 150 to 300 IU of recombinant follicle stimulating hormone (FSHr, Gonal-F; Merck-Serono S.A., Madrid, Spain) alone or with the addition of 75 IU of recombinant luteinizing hormone (LHr, Luveris; Merck-Serono S.A., Madrid, Spain) or human menopausal gonadotropin (hMG, Menopur, Ferring SA, Madrid, Spain). The human chorionic gonadotropin (hCG) administration (Ovitrelle 250 mg s.c., Serono S.A.) was indicated in the presence of two or more follicles ≥18 mm of diameter, with ≥4 follicles measuring ≥14 mm in association with a consistent rise in serum estradiol concentration. Ultrasound (US)-guided transvaginal oocyte retrieval was performed 36 hours after hCG administration.

Embryo culture and cryopreservation protocols
Embryo culture was carried out in microdrops of Global Media (Life Global, CooperSurgical Målov, Denmark) under mineral oil at 37ºC in an atmosphere of 6.5% CO2 and 7% O2. Embryo quality was assessed according to the ASEBIR criteria (Balaban et al., 2011). The quality of blastocysts was assessed according to the criteria of Gardner and Schoolcraft (Gardner et al., 2000).
Vitrification and warming protocols of both cleavage embryos and blastocysts were performed using commercially available kits (Kitazato, Tokyo, Japan) according to the method described by Kuwayama (Kuwayama, 2007). After warming, embryos were cultured in Global medium containing 10% protein substitute supplement (Life Global, CooperSurgical, Målov, Denmark) until embryo transfer. Cleavage embryos with at least 50% of their cells intact immediately after warming and further development after a 24 hours culture period were considered as surviving embryos and transferred. Surviving blastocysts were defined as re-expanded or starting to re-expand within 2 h after warming.

ET protocols
FET was performed in all cases under endometrial preparation with transdermal estrogens (Evopad 50 mcg; Janssen, Toledo, Spain) with 3 patches replaced every 72 hours, or oral estradiol valerate (Progynova 2 mg every 8h; Bayer, Barcelona, Spain). Estrogen was started the first day of the cycle and US monitoring was performed after 12-15 days of treatment. Vaginal natural progesterone (200 mg every 8 hours) was added when endometrial thickness was ≥7 mm by US (Progeffik; Effik, Alcobendas, Spain; or Utrogestan; SEID, Barcelona, Spain). The first day of progesterone treatment was considered day 0, cleavage embryos were thawed on day 3 and transferred on day 4, and blastocyst embryos were thawed and transferred on day 5. Supplementation with estrogens and progesterone was performed until the 12th week of pregnancy. FET in natural cycles were not included in the present investigation to achieve more homogeneity in this group. Artificial cycles were chosen depending on patient's preference, or in the absence of regular menstrual cycles.
In fresh ET cases, vaginal natural progesterone was started the morning after the oocyte retrieval with a dose of 200 mg every 8 hours. Cleavage embryos were transferred on day 3 and blastocysts on day 5. Pregnancy was diagnosed by a positive serum β-hCG test 12 days after ET and a transvaginal ultrasonography was performed in all pregnancies at 5-6 weeks of gestation.

Appendix B

Table B.1. Baseline characteristics of the study groups.

	Study groups according to perinatal results								
	Normotensive (n=100)			**Preeclampsia (n=37)**			**Severe preeclampsia (n=23)**		
	SC (n=31)	**IVF CL (n=38)**	**IVF no CL (n=31)**	**SC (n=14)**	**IVF CL (n=7)**	**IVF no CL (n=16)**	**SC (n=9)**	**IVF CL (n=5)**	**IVF no CL (n=9)**
Maternal age (y)	37.6 (35.3-40.4)	37.9 (35.6-39.5)	38.3 (35.6-40.1)	31.7 (29.2-36.0)	37.2 (34.7-40.2)	39.3 (35.6-40.9)*	34.2 (30.3-35.8)	35.1 (34.6-36.1)	39.3 (38.8-42.4)*†
Caucasian (N(%))	24 (77.4)	33 (86.8)	25 (80.7)	13 (92.9)	7 (100.0)	15 (93.8)	8 (88.9)	4 (80.0)	5 (55.6)
Nulliparity (N(%))	17 (54.8)	33 (86.8)*	23 (74.2)	11 (78.6)	7 (100.0)	14 (87.5)	7 (77.8)	4 (80.0)	7 (77.8)
Maternal body mass index (kg/m^2)	24.1 (21.5-26.0)	23.2 (20.2-26.8)	23.3 (21.2-26.3)	22.8 (21.3-26.4)	24.1 (21.9-27.7)	24.9 (22.9-32.4)	21.6 (19.5-22.8)	22.5 (20.3-25.2)	25.1 (24.1-25.7)
Maternal obesity (N(%))	12 (38.7)	15 (39.5)	8 (25.8)	6 (42.9)	3 (42.9)	8 (50.0)	1 (11.1)	1 (20.0)	5 (55.6)
Prior preeclampsia (N(%))	0	0	0	2 (14.3)	0	0	2 (22.2)	0	2 (22.2)
Chronic hypertension (N(%))	0	0	0	0	0	1 (6.3)	1 (11.1)	1 (20.0)	3 (33.3)
Pregestational diabetes mellitus (N(%))	2 (6.5)	0	1 (3.2)	2 (14.3)	0	0	1 (11.1)	1 (20.0)	0
Antiphospholipid syndrome (N(%))	2 (6.5)	0	0	1 (7.1)	3 (42.9)	2 (12.5)	2 (22.2)	0	1 (11.1)
PCOS (N(%))	2 (6.5)	6 (15.8)	7 (22.6)	0	2 (28.6)	4 (25.0)	0	3 (60.0)*	0†
Endometriosis (N(%))	NA	4 (10.5)	2 (6.5)	NA	0	1 (6.3)	NA	0	0
Tubal factor (N(%))	NA	4 (10.5)	2 (6.5)	NA	0	3 (18.8)	NA	0	1 (11.1)
Male factor (N(%))	NA	17 (44.7)	12 (38.7)	NA	3 (42.9)	7 (43.8)	NA	2 (40.0)	3 (33.3)
Unexplained infertility (N(%))	NA	15 (39.5)	16 (51.6)	NA	4 (57.1)	7 (43.8)	NA	3 (60.0)	5 (55.6)
Oocyte donation (N(%))	NA	1 (2.6)	7. (22.6)†	NA	0	9 (56.3)†	NA	0	5 (55.6)†
FET (N(%))	NA	9 (23.7)	30 (96.8)†	NA	0	8 (50.0)†	NA	1 (20.0)	6 (66.7)
Number of embryos transferred	NA	2 (2-2)	2 (1-2)	NA	2 (1-2)	1 (1-2)	NA	1 (0-1)	1 (0-1)
Blastocyst stage (N(%))	NA	2 (5.3)	18 (62.1)†	NA	1 (14.3)	8 (57.1)	NA	3 (100.0)	5 (62.5)
Vanishing twin phenomenon	0	1 (2.6)	3 (9.7)	0	0	2 (12.5)	0	0	0

SC indicates spontaneously conceived pregnancies; IVF CL, *in vitro* fertilisation with corpus luteum pregnancies; IVF no CL, *in vitro* fertilisation without corpus luteum pregnancies; PCOS, polycystic ovarian syndrome; FET, frozen embryo transfer. Data are median (interquartile range) or number (percentage), as appropriate.

* $P<0.05$ as compared to SC.

† $P<0.05$ as compared to IVF CL.

Table B.2. Perinatal characteristics of the study groups.

	Study groups according to perinatal results								
	Normotensive (n=100)			**Preeclampsia** (n=37)			**Severe preeclampsia** (n=23)		
	SC (n=31)	**IVF CL** (n=38)	**IVF no CL** (n=31)	**SC** (n=14)	**IVF CL** (n=7)	**IVF no CL** (n=16)	**SC** (n=9)	**IVF CL** (n=5)	**IVF no CL** (n=9)
Gestational diabetes (N(%))	1 (3.2)	5 (13.2)	3 (9.4)	1 (7.1)	1 (16.7)	4 (25.0)	2 (22.2)	2 (40.0)	1 (11.1)
Placenta previa (N(%))	0	1 (2.6)	4 (12.9)	0	0	0	0	0	0
Placental abruption (N(%))	0	0	0	0	0	0	1 (12.5)	0	0
Preterm delivery (N(%))	0	2 (5.3)	3 (9.7)	2 (14.3)	0	1 (6.3)	5 (55.6)	1 (20.0)	5 (55.6)
Prenatal corticoid exposure (N(%))	0	2 (5.3)	0	1 (7.1)	0	1 (6.3)	2 (22.2)	2 (40.0)	5 (55.6)
Prenatal aspirin exposure (N(%))	5 (16.1)	2 (5.3)	1 (3.2)	7 (50.0)	4 (57.1)	4 (25.0)	5 (55.6)	1 (20.0)	5 (55.6)
Gestational age at birth (weeks,days)	40,0 (38,4-40,3)	40,2 (39,1-40,6)	40,0 (38,4-40,3)	37,4 (37,1-39,0)	38,5 (37,3-40,1)	38,6 (37,3-39,4)	36,6 (36,3-37,4)	37,5 (37,4-38,0)	34,4 (34,1-37,3)
Birthweight (grams)	3305 (3200-3520)	3240 (3102-3640)	3510 (3184-3630)	2705 (2125-3070)	2606 (2448-3182)	3138 (2817-3398)	2240 (2040-2660)	2870 (2576-2925)	1886 (1830-2424)
Birthweight (centile)	47 (31-73)	40 (21-65)	58 (28-87)	14 (2-39)	9 (8-34)	52 (21-77)	3 (1-15)	27 (10-66)	7 (1-18)
Birthweight below the 10th centile (N(%))	0	0	0	6 (42.9)	4 (57.1)	3 (18.8)	6 (66.7)	1 (25.0)	5 (55.6)
Birthweight above the 90th centile (N(%))	3 (9.7)	2 (5.6)	5 (16.7)	1 (7.7)	0	2 (13.3)	0	0	0
Induction of labour (N(%))	13 (41.9)	18 (47.4)	18 (58.1)	11 (78.6)	4 (57.1)	10 (62.5)	5 (55.6)	4 (80.0)	5 (55.6)
Caesarean section (N(%))	10 (32.3)	10 (26.3)	16 (51.6)	5 (35.7)	2 (28.6)	13 (81.3)*†	5 (55.6)	3 (60.0)	6 (66.7)
Female (N(%))	16 (51.6)	21 (55.3)	20 (64.5)	8 (57.1)	3 (42.9)	9 (56.3)	5 (55.6)	2 (40.0)	5 (55.6)
NICU admission (N(%))	1 (3.2)	1 (2.6)	5 (16.1)	2 (14.3)	1 (14.3)	1 (6.3)	4 (44.4)	2 (40.0)	6 (66.7)

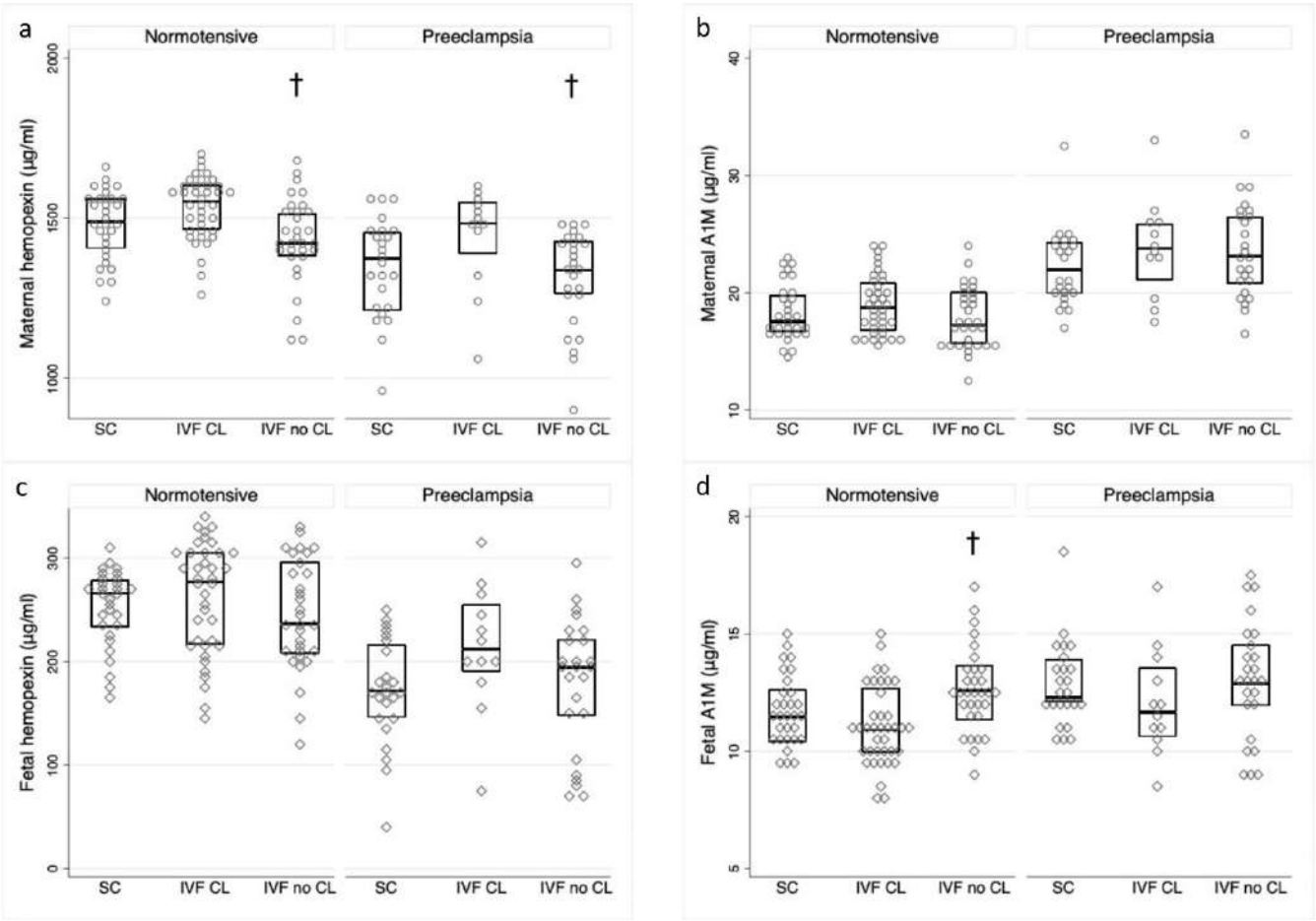
SC indicates spontaneously conceived pregnancies; IVF CL, *in vitro* fertilisation with corpus luteum pregnancies; IVF no CL, *in vitro* fertilisation without corpus luteum pregnancies; FET, frozen embryo transfer. Data are median (interquartile range) or number (percentage), as appropriate.

* $P<0.05$ as compared to SC.

† $P<0.05$ as compared to IVF CL.

Appendix C

Figure.C1. Concentrations of heme scavengers in maternal and fetal blood in the study population. Dot plots represent a) maternal hemopexin concentrations, b) maternal α_1-microglobulin concentrations, c) fetal hemopexin concentrations, d) fetal α_1-microglobulin concentrations. Boxes show median and interquartile range. SC indicates spontaneously conceived pregnancies; IVF CL, IVF pregnancies after ET with corpus luteum; IVF no CL, IVF pregnancies following ET without corpus luteum, A1M, α_1-microglobulin. Values and comparisons are adjusted by multiple linear regression for maternal age, ethnicity, oocyte-donation, frozen embryo transfer cycle, prematurity, gestational age and weight centile at sampling. Exposition groups are compared for each perinatal result (normotensive, preeclampsia). † P-value<0.05 as compared to IVF CL.



6. Discussion

6. Discussion

This thesis adds evidence on the impact of ART on fetal cardiac and brain development, as well as the importance of corpus luteum during conception.
This research is based on a comprehensive characterization of a prospectively constructed cohort of singleton pregnancies achieved by different ART techniques and also SC using fetal US assessment and a set of biomarkers measured in biological specimens collected at delivery. It provides evidence supporting that pregnancy and fetal development are differentially affected by diverse ART strategies. The current research project assesses the influences of infertility *per se*, ovarian stimulation, IVF laboratory procedures and embryo transfer protocols on specific pregnancy and offspring´s outcomes, improving our knowledge on different ART procedures´ safety in terms of the offspring's health. The main findings of each sub-study will be discussed in the following paragraphs.

6.1 Fetal cardiac remodeling and dysfunction in ART

In the first three studies (**Study 1, 2 and 3**) of this PhD, we could demonstrate that fetuses from both fresh and frozen embryo transfer present signs of fetal cardiac remodeling and suboptimal function, with more pronounced changes after fresh embryo transfer, that SC fetuses from subfertile couples show similar cardiac structure and function to those of SC fetuses from fertile couples, and that fetuses from NC-IVF cycles also show signs of cardiac remodeling and suboptimal function.

Our findings on fetal cardiac remodeling and dysfunction in the ART population are in line with those previously described in intrauterine (Bi et al., 2022; Rizzo et al., 2020; Valenzuela-Alcaraz et al., 2013) and postnatal life (Ceelen et al., 2008; Forton et al., 2019; Meister et al., 2018; Scherrer et al., 2012; Valenzuela-Alcaraz et al., 2019; von Arx et al., 2015). Fetuses from ART show a cardiac pattern of concentric ventricular remodeling together with dilated atria, reduced longitudinal motion and impaired relaxation, consistent with studies reporting increased pulmonary pressures in ART children under stressful conditions such as high altitude. These findings are subclinical, being most parameters within normal ranges.

Within pregnancies from conventional (stimulated) IVF, fresh and frozen embryo transfer cycles display distinctive perinatal risk profiles, being the fresh embryo transfer procedure associated to higher rates of FGR and PTB, and the frozen embryo transfer to increased preeclampsia rates. These ART modalities imply important differences in the early manipulation of embryos due to their eventual exposure to high concentrations of cryoprotectants, thawing procedures (Berntsen et al., 2019), and the replacement of embryos in an endometrial cavity with structural and functional differences determined by the OS exposure (Maheshwari et al., 2018). Alternatively, the adverse pregnancy outcomes mentioned above represent conditions that -although in a qualitatively different way- have been individually linked to fetal cardiac remodeling (Crispi et al., 2020b). In **Study 1**, we compared cardiac morphometry and function in fetuses from fresh and frozen embryo transfer finding the ART-associated features in both groups, with a different effect size. The frozen embryo transfer population showed milder cardiac features as compared to the fresh embryo transfer group, even after adjusting analyzes for adverse pregnancy outcomes.

All published studies reporting fetal cardiac performance in ART include SC subjects from fertile couples. However, considering that infertility *per se* and subjacent parental factors could potentially influence their cardiovascular development, singletons from subfertile couples (with a TTP over 12 months) may constitute a more suitable group for comparison (Berntsen et al., 2019). For **Study 2**, we were able to recruit a cohort of ART and SC fetuses from subfertile couples with an equivalent infertility background in terms of duration and etiology, revealing the presence of cardiac remodeling and dysfunction only in the ART population as compared to SC fetuses from subfertile and fertile couples. Moreover, our data in **Study 2** reflect that fetuses from both SC groups show consistent similar results for each parameter assessed, failing to demonstrate that the cardiac features in ART were promoted by the underlying infertility factors.

All previous reports on fetal cardiac outcomes in ART have been conducted on conventional or stimulated cycles. Because of the potential impact of the controlled OS on the oocyte and the endometrium, OS has also been proposed as a contributing factor to suboptimal cardiac performance in ART fetuses (Valenzuela-Alcaraz et al., 2019). We conducted a nested case-control study including fetuses from IVF performed in natural cycle (NC-IVF),

fetuses from IVF with controlled OS (conventional IVF), and SC fetuses from fertile couples as negative controls, finding no significant differences between both IVF groups for each of the cardiac parameters assessed (**Study 3**). Thus, our data do not support the hypothesis that avoiding ovarian stimulation would prevent the occurrence of fetal cardiac remodeling in ART subjects.

In the current research project, the fetal cardiac features reported for the ART population seem to be associated to the pregnancies achieved by IVF procedure, not to the underlying infertility nor the OS. Moreover, more pronounced features were demonstrated in stimulated cycles with fresh embryo transfer as compared to those with frozen embryo transfer.

6.2 Fetal brain cortical development in ART

The **Study 4** is the first publication exploring fetal brain cortical development in ART offspring. Due to the difference on size effect in our fetal cardiac results between fresh and frozen embryo transfer modalities, we aimed to study the neurodevelopmental outcome in each of these groups and compare it with the neurodevelopment of an SC group. Our results show that fetuses from IVF present a distinctive pattern of brain cortical folding associated to suboptimal neurodevelopment at 12 months of corrected age, particularly in communication, personal-social, problem-solving, and motor areas. These features are consistently more pronounced in the fresh embryo transfer population as compared to the frozen embryo transfer group. The novelty of this study lies in the fact that it explores the fetal brain cortex before relevant postnatal factors begin to influence neurodevelopment.

So far the neurodevelopment of IVF subjects has mostly been studied in children and adolescents with inconsistent results (Bergh and Wennerholm, 2020; Berntsen and Pinborg, 2018; Pinborg, 2019). These inconsistencies range from higher rates of neurodevelopmental deficits (Bowen et al., 1998; Goldsmith et al., 2018; Källén et al., 2005; Levin et al., 2019; Liu et al., 2017; Rumbold et al., 2017; Strömberg et al., 2002) to reassuring results (Agarwal et al., 2005; Bay et al., 2013b; Hart and Norman, 2013; Middelburg et al., 2008; Norrman et al., 2018), with no significantly different outcomes between ART and SC offspring. Overall, studies on long-term cognitive, psychomotor, and behavioral development have extensive methodological limitations. First, only few studies include infertility-related variables (such as cause and

length) to acknowledge its impact on the study outcome (Bay et al., 2014, 2013a; Carson et al., 2011a; Drenth Olivares et al., 2019; Källén et al., 2011, 2005; Kissin et al., 2015; Zhu et al., 2009). Secondly, many of the identified risk associations were only observed in subgroups or disappeared after adjustment for relevant potential confounders, such as multiplicity and gestational age at birth (Agarwal et al., 2005; Bowen et al., 1998; D'Souza et al., 1997; Ericson et al., 2002; Fountain et al., 2015; Hvidtjørn et al., 2011, 2010; Kissin et al., 2015; Klemetti et al., 2006; Knoester et al., 2008; Pinborg et al., 2004; Sandin et al., 2013; Strömberg et al., 2002). Furthermore, the factors that might affect neuropsychological development are not restricted to the perinatal period. Couples undergoing fertility treatments may differ from those conceiving spontaneously in demographic characteristics as age, highest educational level achieved, and socioeconomic status (Carson et al., 2011b), but also in the way they support learning (Spangmose et al., 2019) and acknowledge health issues on their offspring (Källén et al., 2005). In addition, some neurodevelopmental outcomes are not properly assessed: although there is enough evidence for the heritability of intelligence, most studies on child intelligence quotients have not accounted for parental intelligence level (Plomin and Von Stumm, 2018). Thirdly, most studies restrict outcomes to the risk of mental disorders that required hospitalization or early intervention services (Bay et al., 2013a; Ericson et al., 2002; Hvidtjørn et al., 2011, 2010; Källén et al., 2011, 2005; Klemetti et al., 2006; Lidegaard et al., 2005; Maimburg and Væth, 2007; Pinborg et al., 2003, 2010; Sandin et al., 2013; Strömberg et al., 2002; Sun et al., 2007). Finally, although register-based studies are able to generate reliable results on mental health disorders diagnosed with a low selection bias, this type of data may not be sensitive enough to detect more subtle neurodevelopmental deficits, and mild conditions could be underrepresented. They have also the inherent limitations associated to the recording procedures, and the retrospective nature of the studies that in several cases prevent an adequate modeling considering relevant confounding variables.

A reassuring study on long-term neurological outcome in ART subjects involving a large multinational cohort from Denmark, Finland, Sweden, and Norway has recently been published. It has merged the data from historical registries used in previous publications and has the longest follow-up period on ART neurologic outcome so far. In it, whereas the rate of some learning,

motor functioning and conduct disorders (like ASD and ADHD) is reported to be increased among ART children, the absolute risk was moderate (Rönö et al., 2022).

Our findings were more evident in the fresh as compared to the frozen embryo transfer group, in line with a registry study reporting higher mental disorders´ risk (ASD and intellectual disability) in IVF, particularly in the fresh embryo transfer population using ICSI due to male factor infertility (Sandin et al., 2013). Nevertheless, in the mentioned study, a higher risk of mental retardation was observed in ICSI with frozen embryo transfer when restricting the analysis to singleton (Sandin et al., 2013). However, a recent uncontrolled follow-up study found no differences at comparing academic performance between fresh and frozen embryo transfer in adolescents (Spangmose et al., 2019).

6.3 Maternal and fetal heme scavengers´ profile in ART according to the presence of corpus luteum

In **Study 5** we expose the different behavior of the heme scavengers hemopexin and α_1-microglobulin in normotensive and preeclampsia populations classified by mode of conception and the presence of corpus luteum at embryo transfer. In line with previous studies, we report indirect signs of hemoglobin-induced oxidative stress in preeclampsia cases, being these findings more pronounced in IVF pregnancies achieved in absense of corpus luteum. As compared to SC pregnancies, maternal hemopexin was significantly higher in IVF preeclampsia pregnancies with one or several corpora lutea and significantly lower in IVF pregnancies lacking corpus luteum. Additionally, in severe cases of preeclampsia, we found significantly higher α_1-microglobulin levels in maternal plasma in IVF pregnancies conceived in absence of corpus luteum as compared to SC and IVF treatments in which corpora lutea were preserved.

Most pregnancies obtained through ART treatments begin in a non-physiological endocrine environment (Conrad and Baker, 2013). Unlike SC singleton pregnancies occurring in the presence of a single corpus luteum, IVF cycles with replacement of autologous fresh embryos result in a supraphysiologic number of corpora lutea, and in some embryo transfer protocols, the pituitary-ovarian axis is purposely suppressed by means of

estradiol supplementation in the context of an artificial cycle, preventing the development of the corpus luteum. Therefore, in SC pregnancies the corpus luteum seems to play an important role in the production and release of crucial hormones for implantation, placentation, and pregnancy support, whereas usually in ART protocols, exogenous progesterone and estrogens are provided in order to support the gestation. However, the corpus luteum activity is not limited to these hormones (Conrad and Baker, 2013). Interestingly, animal models have demonstrated that relaxin, a potent vasodilator and stimulus of decidualization is also secreted by the corpus luteum and contributes to the cardiovascular and renal adaptations seen in pregnancy (Conrad, 2011; Debrah et al., 2006). In humans, plasma relaxin has also been reported to be untraceable in a cohort lacking corpus luteum, but significantly elevated in a multiple corpora lutea cohort throughout pregnancy (Conrad et al., 2019). In women undergoing IVF in the absence of corpus luteum, the cardiovascular and endothelial function are impaired and preeclampsia risk elevated from the first trimester, suggesting that relaxin or other vasoactive factors synthesized by this structure contributes to the transformation of the maternal circulation before the corpus luteal-placental shift, after which placental hormones may intervene (Conrad et al., 2019).

In physiological conditions, human pregnancy presents distinctive changes in the cardiovascular system, involving a reduction in mean arterial pressure together with an increase in cardiac output and a decrease in systemic vascular resistance even before the establishment of the maternal-fetal-placental unit (Chapman et al., 1998; Clapp and Capeless, 1997). Furthermore, clinical presentation of preeclampsia is preceded by maternal vascular dysfunction (Staff, 2019). Recent clinical studies have reported impaired cardiovascular function in early pregnancy in women conceiving by IVF treatments in the absence of corpus luteum (Conrad et al., 2020, 2019; Von Versen-Hoÿnck et al., 2019). The natural decline of mean arterial pressure was absent and vascular endothelial function resulted impaired in pregnancies achieved with 0 or multiple corpora lutea, albeit in different ways (Conrad et al., 2020; Von Versen-Hoÿnck et al., 2019). Altered maternal circulating levels of syncytiotrophoblast stress biomarkers such as soluble fms-like tyrosine kinase-1 (sFlt-1) and placental growth factor (PlGF) have also been reported in gestations with 0 or multiple corpora lutea as compared to SC, with higher sFlt-1/PlGF ratios thorough pregnancy (Conrad et al.,

2019). The relaxin concentrations correlated with abnormal vascular reactivity (Von Versen-Hoÿnck et al., 2019) and prorenin, renin's inactive precursor, also secreted by the corpus luteum (Wiegel et al., 2020). Furthermore, together with any direct impact on maternal vascular adaptations by the early corpus luteum activity, its hormone secretion has been demonstrated to persist after the first trimester (Conrad et al., 2019), and also an indirect impact on placentation could explain the later development of preeclampsia.

Whether the presence of corpus luteum prevents the onset of oxidative stress through the contribution of vasoactive factors prior to the establishment of the uteroplacental circulation should be further studied. Nevertheless, this approach to perinatal outcomes in ART supports the strategy of developing a corpus luteum if possible, to prevent preeclampsia occurrence.

6.4 Strengths and limitations

This research project has some strengths and limitations that should be acknowledged.

Among the strengths, it is based on a comprehensively characterized prospective cohort, including complete data from conception up to birth. All IVF pregnancies were recruited from a single center, ensuring homogeneity in IVF ovarian stimulation and endometrial preparation protocols, laboratory procedures, and embryo culture conditions. It includes different IVF treatment strategies and two unexposed groups from both fertile and subfertile couples, enabling us to increase our knowledge on fetal programming and pregnancy development related to subfertility and ART procedures. SC were matched with ART pregnancies by maternal age at last menstrual period. We have used a well-defined and strict methodology for assessing fetal growth, heart and brain by US, and maternal and fetal heme scavengers in plasma samples. At data analysis, results were carefully normalized and or corrected by relevant confounding factors to allow consistent comparisons between groups.

Among the limitations, we first acknowledge that matching ART unexposed and exposed populations by parity, a relevant variable regarding pregnancy outcomes, has not been possible due to the lower nulliparity rate in the SC pregnancies from fertile couples as compared to subfertile couples undergoing IVF procedures or not. To overcome this issue, adjustments for

nulliparity were performed at data analyses in each of the studies. Secondly, research into ART pregnancies faces difficulties in unraveling the infertility background from the IVF procedure itself. We have been able to recruit a subfertile cohort comparable with the IVF with fresh embryo transfer regarding infertility length and etiology for **Study 3**. Future studies on neurodevelopment including this subfertile cohort are warranted. Thirdly, although the data collection process through personal interviews and review of clinical records was exhaustive, due to the unique preconcepcional and early clinical management of ART pregnancies, conditions associated to decreased fertility and the presence of vanishing twin phenomenon have probably been underdiagnosed in the SC group. As opposed to most of the SC population, couples undertaking ART had a complete fertility evaluation before treatment, and they had also undergone two US examinations prior to their 11-14 weeks´ scan. We have excluded the vanishing twin cases in our case-control studies (**Studies 3 and 5**) and repeated the analysis after excluding the vanishing twin cases in each of our cohort studies (**Studies 1, 2 and 4**) obtaining similar results. Fourthly, in order to first approach this research avoiding the influence of a major confounder, we have not included ongoing multiple pregnancies in our cohort. Fifthly, in **Study 4** there was a higher proportion of embryo transfer at blastocyst stage in the frozen embryo transfer as compared to the fresh embryo transfer group, which has been object of sub-analysis as well, obtaining similar results after the exclusion of cases of transfer in this embryonic stage. Sixthly, in this perinatal cohort approximately 18% of the eligible patients declined participation when invited. Although not included and included populations were similar in terms of baseline characteristics, a potential selection bias cannot be fully discarded. Seventhly, especially in the ultrasound studies (**Studies 1 to 4**), we acknowledge multiple comparisons as a potential limitation. Despite the relatively high number of comparisons, fetal echocardiographic findings (**Studies 1 to 3**) were concordant within the populations and consistent with previously published data in fetal and children's studies. The neurosonographic features for the ART fetuses were also consistent between them and overall showed significant positive correlations with the infant outcomes (**Study 4**). In addition, we have applied Bonferroni corrections to each comparison in order to attenuate the mass-significance effect. Finally, although significantly different from the SC population, the fetal cardiac and

neurodevelopmental features presented for the ART offspring in this thesis are mostly subclinical and not necessarily pathologic. Furthermore, their long-term implications remain uncertain, as the evidence in later life stages remain controversial and this is still a relatively young population. Extrapolation from young populations with similar risk profiles and known prevalence of morbidity and mortality later in life suggest that they may be important. However, the association between fetal outcome and adult disease should be assessed in further follow-up studies.

6.5 Relevance, clinical and future research implications

This thesis contributes to the body of knowledge on safety in ART, providing additional evidence for the existence of fetal cardiac programming in pregnancies achieved by ART as well as differences in fetal brain cortical development associated to ART. We also report a differential behavior of preeclampsia-related scavengers in maternal and fetal plasma at delivery according to the presence of corpus luteum at first trimester of pregnancy, supporting the strategy of embryo transfer in the presence of corpus luteum if possible, to avoid preeclampsia and thus improve maternal and fetal health. The association between fetal outcomes and factors as infertility, OS, and embryo transfer has been studied, increasing the knowledge about safety in ART, and therefore contributing to assess the risk-benefit ratio of the different therapeutic strategies in assisted reproduction.

Our findings warrant further studies to assess the etiopathogenic mechanisms associated to cardiac remodeling, differential neurodevelopment, and occurrence of perinatal complications such preeclampsia in the ART population. Likewise, optimization of ART protocols and preventive interventional measures to be applied since intrauterine life for improving the ART offspring´s perinatal, cardiovascular, and neurodevelopmental outcomes should be further explored.

7. Conclusions

7. Conclusions

1. Fetuses from both fresh and frozen embryo transfer cycles present signs of cardiac remodeling and suboptimal function, with more pronounced changes after fresh as compared to frozen embryo transfer.

2. Fetuses conceived spontaneously by subfertile couples do not show signs of cardiac remodeling and dysfunction as those observed in IVF fetuses, and their cardiac structural and functional features are similar to those of SC fetuses from fertile couples.

3. Fetuses from ART show signs of cardiac remodeling as compared to SC fetuses from fertile couples, even in NC- IVF cycles.

4. Fetuses conceived by ART show a distinctive pattern of brain cortical development and suboptimal infant neurodevelopment, with more pronounced changes in fresh embryo transfer offspring.

5. Pregnancies lacking corpus luteum at the time of conception associate more altered heme scavenger´s concentrations in preeclampsia, suggesting that the corpus luteum activity could influence perinatal outcomes through lower levels of oxidative stress.

6. These results underscore the importance of future studies assessing adverse perinatal outcomes and long-term cardiovascular and neurological health in offspring conceived by ART.

References

REFERENCES

Agarwal, A., Rosas, I.M., Anagnostopoulou, C., Cannarella, R., Boitrelle, F., Munoz, L.V., Finelli, R., Durairajanayagam, D., Henkel, R., Saleh, R., 2022. Oxidative Stress and Assisted Reproduction: A Comprehensive Review of Its Pathophysiological Role and Strategies for Optimizing Embryo Culture Environment. Antioxidants 11. https://doi.org/10.3390/antiox11030477

Agarwal, P., Loh, S.K.E., Lim, S.B., Sriram, B., Daniel, M.L., Yeo, S.H., Heng, D., 2005. Two-year neurodevelopmental outcome in children conceived by intracytoplasmic sperm injection: Prospective cohort study. BJOG An Int. J. Obstet. Gynaecol. 112, 1376–1383. https://doi.org/10.1111/j.1471-0528.2005.00663.x

Alonso, I., Borenstein, M., Grant, G., Narbona, I., Azumendi, G., 2010. Depth of brain fissures in normal fetuses by prenatal ultrasound between 19 and 30 weeks of gestation. Ultrasound Obstet. Gynecol. 36, 693–699. https://doi.org/10.1002/uog.7660

Anderson, U.D., Gram, M., Ranstam, J., Thilaganathan, B., Åkerström, B., Hansson, S.R., 2016. Fetal hemoglobin, α1-microglobulin and hemopexin are potential predictive first trimester biomarkers for preeclampsia. Pregnancy Hypertens. 6, 103–109. https://doi.org/10.1016/j.preghy.2016.02.003

Anderson, U.D., Jälmby, M., Faas, M.M., Hansson, S.R., 2018. The hemoglobin degradation pathway in patients with preeclampsia – Fetal hemoglobin, heme, heme oxygenase-1 and hemopexin – Potential diagnostic biomarkers? Pregnancy Hypertens. 14, 273–278. https://doi.org/10.1016/j.preghy.2018.02.005

Anderson, U.D., Olsson, M.G., Rutardttir, S., Centlow, M., Kristensen, K.H., Isberg, P.E., Thilaganathan, B., Kerstrm, B., Hansson, S.R., 2011. Fetal hemoglobin and α1-microglobulin as first- and early second-trimester predictive biomarkers for preeclampsia. Am. J. Obstet. Gynecol. 204, 520.e1-520.e5. https://doi.org/10.1016/j.ajog.2011.01.058

Arduini, D., Rizzo, G., 1990. Normal values of pulsatility index from fetal vessels: A cross-sectional study on 1556 healthy fetuses. J. Perinat. Med. 18, 165–172. https://doi.org/10.1515/jpme.1990.18.3.165

Bakker, W.W., Borghuis, T., Harmsen, M.C., Van Den Berg, A., Kema, I.P., Niezen, K.E., Kapojos, J.J., 2005. Protease activity of plasma hemopexin. Kidney Int. 68, 603–610. https://doi.org/10.1111/j.1523-1755.2005.00438.x

Bakker, W.W., Donker, R.B., Timmer, A., Van Pampus, M.G., Van Son, W.J., Aarnoudse, J.G., Van Goor, H., Niezen-Koning, K.E., Navis, G., Borghuis, T., Jongman, R.M., Faas, M.M., 2007. Plasma hemopexin activity in pregnancy and preeclampsia. Hypertens. Pregnancy 26, 227–239. https://doi.org/10.1080/10641950701274896

Bakker, W.W., Henning, R.H., Van Son, W.J., Van Pampus, M.G., Aarnoudse, J.G., Niezen-Koning, K.E., Borghuis, T., Jongman, R.M., Van Goor, H., Poelstra, K., Navis, G., Faas, M.M., 2009. Vascular contraction and preeclampsia downregulation of the angiotensin receptor 1 by hemopexin in vitro. Hypertension 53, 959–964. https://doi.org/10.1161/HYPERTENSIONAHA.108.127951

Balaban, B., Brison, D., Calderón, G., Catt, J., Conaghan, J., Cowan, L., Ebner, T.,

Gardner, D., Hardarson, T., Lundin, K., Magli, M.C., Mortimer, D., Mortimer, S., Munné, S., Royere, D., Scott, L., Smitz, J., Thornhill, A., Van Blerkom, J., Van Den Abbeel, E., 2011. Istanbul consensus workshop on embryo assessment: Proceedings of an expert meeting. Reprod. Biomed. Online 22, 632–646. https://doi.org/10.1016/j.rbmo.2011.02.001

Barberet, J., Binquet, C., Guilleman, M., Romain, G., Bruno, C., Martinaud, A., Ginod, P., Cavalieri, M., Amblot, C., Choux, C., Fauque, P., 2022. Does underlying infertility in natural conception modify the epigenetic control of imprinted genes and transposable elements in newborns? Reprod. Biomed. Online 44, 706–715. https://doi.org/10.1016/j.rbmo.2022.01.004

Bardhi, E., Blockeel, C., Cools, W., Santos-Ribeiro, S., Racca, A., Mackens, S., De Vos, M., Polyzos, N.P., Popovic-Todorovic, B., De Brucker, M., Muzii, L., Panici, P.B., Tournaye, H., Drakopoulos, P., 2020. Is ovarian response associated with adverse perinatal outcomes in GnRH antagonist IVF/ICSI cycles? Reprod. Biomed. Online 41, 263–270. https://doi.org/10.1016/j.rbmo.2020.03.010

Baschat, A.A., Gembruch, U., 2003. The cerebroplacental Doppler ratio revisited. Ultrasound Obstet. Gynecol. 21, 124–127. https://doi.org/10.1002/uog.20

Bay, B., Mortensen, E.L., Hvidtjørn, D., Kesmodel, U.S., 2013a. Fertility treatment and risk of childhood and adolescent mental disorders: Register based cohort study. BMJ 347, 1–11. https://doi.org/10.1136/bmj.f3978

Bay, B., Mortensen, E.L., Kesmodel, U.S., 2014. Fertility treatment and child intelligence, attention, and executive functions in 5-year-old singletons: A cohort study. BJOG An Int. J. Obstet. Gynaecol. 121, 1642–1651. https://doi.org/10.1111/1471-0528.12907

Bay, B., Mortensen, E.L., Kesmodel, U.S., 2013b. Assisted reproduction and child neurodevelopmental outcomes: A systematic review. Fertil. Steril. 100, 844–853. https://doi.org/10.1016/j.fertnstert.2013.05.034

Bergh, C., Wennerholm, U.B., 2020. Long-term health of children conceived after assisted reproductive technology. Ups. J. Med. Sci. 125, 152–157. https://doi.org/10.1080/03009734.2020.1729904

Berntsen, S., Pinborg, A., 2018. Large for gestational age and macrosomia in singletons born after frozen/thawed embryo transfer (FET) in assisted reproductive technology (ART). Birth Defects Res. 110, 630–643. https://doi.org/10.1002/bdr2.1219

Berntsen, S., Söderström-Anttila, V., Wennerholm, U.-B., Laivuori, H., Loft, A., Oldereid, N.B., Bente Romundstad, L., Bergh, C., Pinborg, A., 2019. The health of children conceived by ART: "the chicken or the egg?" Hum. Reprod. Update 25, 137–158. https://doi.org/10.1093/humupd/dmz001

Beta, J., Khan, N., Fiolna, M., Khalil, A., Ramadan, G., Akolekar, R., 2019. Maternal and neonatal complications of fetal macrosomia: cohort study. Ultrasound Obstet. Gynecol. 54, 319–325. https://doi.org/10.1002/uog.20278

Bhide, A., Acharya, G., Bilardo, C.M., Brezinka, C., Cafici, D., Hernandez-Andrade, E., Kalache, K., Kingdom, J., Kiserud, T., Lee, W., Lees, C., Leung, K.Y., Malinger, G., Mari, G., Prefumo, F., Sepulveda, W., Trudinger, B., 2013. ISUOG practice guidelines: Use of Doppler ultrasonography in obstetrics. Ultrasound Obstet. Gynecol. 41, 233–239. https://doi.org/10.1002/uog.12371

Bi, W., Xiao, Y., Wang, X., Cui, L., Song, G., Yang, Z., Zhang, Y., Ren, W., 2022. The

association between assisted reproductive technology and cardiac remodeling in fetuses and early infants: a prospective cohort study. BMC Med. 20, 104. https://doi.org/10.1186/S12916-022-02303-6

Boutet, M.L., Casals, G., Valenzuela-Alcaraz, B., García-Otero, L., Crovetto, F., Cívico, M.S., Borrás, A., Manau, D., Gratacós, E., Crispi, F., 2021. Cardiac remodeling in fetuses conceived by ARTs: fresh versus frozen embryo transfer. Hum. Reprod. 36, 2697–2708. https://doi.org/10.1093/humrep/deab159

Bowen, J.R., Gibson, F.L., Leslie, G.I., Saunders, D.M., 1998. Medical and developmental outcome at 1 year for children conceived by intracytoplasmic sperm injection. Lancet 351, 1529–1534. https://doi.org/10.1016/S0140-6736(98)10168-X

Bricker, D., Squires, J., Mounts, L., Potter, L., Nickel, R., Twombly, E., Farrell, J., 1999. Ages & Stages Questionnaires: A parent-completed child-monitoring system - Spanish version, 2nd ed. Paul H Brookes publishing Co., Baltimore, Maryland.

Brook, A., Hoaksey, A., Gurung, R., Yoong, E.E.C., Sneyd, R., Baynes, G.C., Bischof, H., Jones, S., Higgins, L.E., Jones, C., Greenwood, S.L., Jones, R.L., Gram, M., Lang, I., Desoye, G., Myers, J., Schneider, H., Hansson, S.R., Crocker, I.P., Brownbill, P., 2018. Cell free hemoglobin in the fetoplacental circulation: a novel cause of fetal growth restriction? FaseB J. 32, 5436–5446. https://doi.org/10.1096/fj.201800264R

Carson, C., Kelly, Y., Kurinczuk, J.J., Sacker, A., Redshaw, M., Quigley, M.A., 2011a. Effect of pregnancy planning and fertility treatment on cognitive outcomes in children at ages 3 and 5: Longitudinal cohort study. BMJ 343. https://doi.org/10.1136/bmj.d4473

Carson, C., Kelly, Y., Kurinczuk, J.J., Sacker, A., Redshaw, M., Quigley, M.A., 2011b. Effect of pregnancy planning and fertility treatment on cognitive outcomes in children at ages 3 and 5: Longitudinal cohort study. BMJ 343, 1–16. https://doi.org/10.1136/bmj.d4473

Carvalho, J., Allan, L., Chaoui, R., Copel, J., DeVore, G., Hecher, K., Lee, W., Munoz, H., Paladini, D., Tutschek, B., Yagel, S., 2013. ISUOG Practice Guidelines (updated): sonographic screening examination of the fetal heart. Ultrasound Obstet. Gynecol. 41, 348–359. https://doi.org/10.1002/uog.12403

Cavoretto, P., Candiani, M., Giorgione, V., Inversetti, A., Abu-Saba, M.M., Tiberio, F., Sigismondi, C., Farina, A., 2018. Risk of spontaneous preterm birth in singleton pregnancies conceived after IVF/ICSI treatment: meta-analysis of cohort studies. Ultrasound Obstet. Gynecol. 51, 43–53. https://doi.org/10.1002/uog.18930

Ceelen, M., Van Weissenbruch, M.M., Vermeiden, J.P.W., Van Leeuwen, F.E., Delemarre-Van De Waal, H.A., 2008. Cardiometabolic Differences in Children Born After in Vitro Fertilization: Follow-Up Study. J Clin Endocrinol Metab 93, 1682–1688. https://doi.org/10.1210/jc.2007-2432

Centlow, M., Carninci, P., Nemeth, K., Mezey, E., Brownstein, M., Hansson, S.R., 2008. Placental expression profiling in preeclampsia: local overproduction of hemoglobin may drive pathological changes. Fertil Steril 90, 1834–43. https://doi.org/10.1016/j.fertnstert.2007.09.030

Chapman, A.B., Abraham, W.T., Zamudio, S., Coffin, C., Merouani, A., Young, D., Johnson, A., Osorio, F., Goldberg, C., Moore, L.G., Dahms, T., Schrier, R.W., 1998. Temporal relationships between hormonal and hemodynamic changes in early human pregnancy. Kidney Int. 54, 2056–2063. https://doi.org/10.1046/j.1523-

1755.1998.00217.x

Chiabrando, D., Vinchi, F., Fiorito, V., Tolosano, E., 2011. Haptoglobin and Hemopexin in Heme Detoxification and Iron Recycling, in: Prof. Veas, F. (Ed.), Acute Phase Proteins - Regulation and Functions of Acute Phase Proteins, Prof. Francisco Veas (Ed.). InTech. https://doi.org/10.5772/18241

Choux, C., Binquet, C., Carmignac, V., Bruno, C., Chapusot, C., Barberet, J., Lamotte, M., Sagot, P., Bourc'his, D., Fauque, P., 2018. The epigenetic control of transposable elements and imprinted genes in newborns is affected by the mode of conception: ART versus spontaneous conception without underlying infertility. Hum. Reprod. 33, 331–340. https://doi.org/10.1093/humrep/dex366

Clapp, J.F., Capeless, E., 1997. Cardiovascular function before, during, and after the first and subsequent pregnancies. Am. J. Cardiol. 80, 1469–1473. https://doi.org/10.1016/S0002-9149(97)00738-8

Conrad, K.P., 2020. Evidence for Corpus Luteal and Endometrial Origins of Adverse Pregnancy Outcomes in Women Conceiving with or Without Assisted Reproduction. Obstet. Gynecol. Clin. North Am. 47, 163–181. https://doi.org/10.1016/j.ogc.2019.10.011

Conrad, K.P., 2011. Emerging Role of Relaxin in the Maternal Adaptations to Normal Pregnancy: Implications for Preeclampsia. Semin. Nephrol. 31, 15–32. https://doi.org/10.1016/j.semnephrol.2010.10.003

Conrad, K.P., Baker, V.L., 2013. Corpus luteal contribution to maternal pregnancy physiology and outcomes in assisted reproductive technologies. Am J Physiol Regul Integr Comp Physiol 304, 69–72. https://doi.org/10.1152/ajpregu.00239.2012

Conrad, K.P., Graham, G.M., Chi, Y.-Y., Zhai, X., Li, M., Stan Williams, R., Rhoton-Vlasak, A., Segal, M.S., Wood, C.E., Keller-Wood, M., 2019. Potential influence of the corpus luteum on circulating reproductive and volume regulatory hormones, angiogenic and immunoregulatory factors in pregnant women. Am J Physiol Endocrinol Metab 317, e677–e685. https://doi.org/10.1152/ajpendo.00225

Conrad, K.P., Lingis, M., Sautina, L., Li, S., Chi, Y.Y., Qiu, Y., Li, M., Williams, R.S., Rhoton-Vlasak, A., Sega, M.S., 2020. Maternal endothelial function, circulating endothelial cells, and endothelial progenitor cells in pregnancies conceived with or without in vitro fertilization. Am. J. Physiol. - Regul. Integr. Comp. Physiol. 318, R1091–R1102. https://doi.org/10.1152/AJPREGU.00015.2020

Conrad, K.P., von Versen-Höynck, F., Baker, V.L., 2021. Potential role of the corpus luteum in maternal cardiovascular adaptation to pregnancy and preeclampsia risk. Am. J. Obstet. Gynecol. 226, 683–699. https://doi.org/10.1016/j.ajog.2021.08.018

Crispi, F., Bijnens, B., Figueras, F., Bartrons, J., Eixarch, E., Le Noble, F., Ahmed, A., Gratacós, E., 2010. Fetal Growth Restriction Results in Remodeled and Less Efficient Hearts in Children. Circulation 121, 2427–2436. https://doi.org/10.1161/CIRCULATIONAHA.110.937995

Crispi, F., Crovetto, F., Gratacos, E., 2018. Intrauterine growth restriction and later cardiovascular function. Early Hum. Dev. 126, 23–27. https://doi.org/10.1016/j.earlhumdev.2018.08.013

Crispi, Fatima, Crovetto, F., Rodriguez-López, M., Sepúlveda-Martinez, Á., Miranda, J., Gratacós, E., 2021. Postnatal persistence of cardiac remodeling and dysfunction in late fetal growth restriction. Minerva Obstet. Gynecol. 73, 471–481.

https://doi.org/10.23736/S2724-606X.21.04823-5

Crispi, Fàtima, Rodríguez-López, M., Bernardino, G., Sepúlveda-Martínez, Á., Prat-González, S., Pajuelo, C., Perea, R.J., Caralt, M.T., Casu, G., Vellvé, K., Crovetto, F., Burgos, F., De Craene, M., Butakoff, C., González Ballester, M., Blanco, I., Sitges, M., Bijnens, B., Gratacós, E., 2021. Exercise Capacity in Young Adults Born Small for Gestational Age. JAMA Cardiol. 6, 1308–1316. https://doi.org/10.1001/jamacardio.2021.2537

Crispi, F., Sepúlveda-Martínez, Á., Crovetto, F., Gómez, O., Bijnens, B., Gratacós, E., 2020a. Main Patterns of Fetal Cardiac Remodeling. Fetal Diagn. Ther. 47, 337–344. https://doi.org/10.1159/000506047

Crispi, F., Sepúlveda-Martínez, Á., Crovetto, F., Gómez, O., Bijnens, B., Gratacós, E., 2020b. Main Patterns of Fetal Cardiac Remodeling. Fetal Diagn. Ther. 47, 337–344. https://doi.org/10.1159/000506047

Crispi, F., Valenzuela-Alcaraz, B., Cruz-Lemini, M., Gratacós, E., 2013. Ultrasound assessment of fetal cardiac function. Australas. J. Ultrasound Med. 16, 158–167. https://doi.org/10.1002/j.2205-0140.2013.tb00242.x

Cruz-Martínez, R., Figueras, F., Bennasar, M., Garca-Posadas, R., Crispi, F., Hernández-Andrade, E., Gratacós, E., 2012. Normal reference ranges from 11 to 41 weeks' gestation of fetal left modified myocardial performance index by conventional doppler with the use of stringent criteria for delimitation of the time periods. Fetal Diagn. Ther. 32, 79–86. https://doi.org/10.1159/000330798

D'Souza, S.W., Rivlin, E., Cadman, J., Richards, B., Buck, P., Lieberman, B.A., 1997. Children conceived by in vitro fertilisation after fresh embryo transfer. Arch. Dis. Child. Fetal Neonatal Ed. 76, 70–75. https://doi.org/10.1136/fn.76.2.F70

de Geyter, C., Wyns, C., Calhaz-Jorge, C., de Mouzon, J., Ferraretti, A.P., Kupka, M., Nyboe Andersen, A., Nygren, K.G., Goossens, V., 2020. 20 years of the European IVF-monitoring Consortium registry: What have we learned? A comparison with registries from two other regions. Hum. Reprod. 35, 2832–2849. https://doi.org/10.1093/humrep/deaa250

Debrah, D.O., Novak, J., Matthews, J.E., Ramirez, R.J., Shroff, S.G., Conrad, K.P., 2006. Relaxin is essential for systemic vasodilation and increased global arterial compliance during early pregnancy in conscious rats. Endocrinology 147, 5126–5131. https://doi.org/10.1210/en.2006-0567

Demicheva, E., Crispi, F., 2014. Long-term follow-up of intrauterine growth restriction: Cardiovascular disorders. Fetal Diagn. Ther. 36, 143–153. https://doi.org/10.1159/000353633

Drenth Olivares, M., Kuiper, D.B., Haadsma, M.L., Heineman, K.R., Heineman, M.J., Hadders-Algra, M., 2019. IVF procedures are not, but subfertility is associated with neurological condition of 9-year-old offspring. Early Hum. Dev. 129, 38–44. https://doi.org/10.1016/j.earlhumdev.2018.12.017

Egaña-Ugrinovic, G., Sanz-Cortes, M., Figueras, F., Bargalló, N., Gratacós, E., 2013. Differences in cortical development assessed by fetal MRI in late-onset intrauterine growth restriction. Am. J. Obstet. Gynecol. 209, 126.e1-126.e8. https://doi.org/10.1016/j.ajog.2013.04.008

El Hajj, N., Haertle, L., Dittrich, M., Denk, S., Lehnen, H., Hahn, T., Schorsch, M., Haaf, T., 2017. DNA methylation signatures in cord blood of ICSI children. Hum. Reprod. 32, 1761–1769. https://doi.org/10.1093/humrep/dex209

Ericson, A., Nygren, K.G., Olausson, P.O., Källén, B., 2002. Hospital care utilization of infants born after IVF, Human Reproduction.

Erlandsson, L., Nääv, Å., Hennessy, A., Vaiman, D., Gram, M., Åkerström, B., Hansson, S.R., 2016. Inventory of Novel Animal Models Addressing Etiology of Preeclampsia in the Development of New Therapeutic/Intervention Opportunities. Am. J. Reprod. Immunol. 75, 402–410. https://doi.org/10.1111/aji.12460

Ernstad, E.G., Wennerholm, U.-B., Khatibi, A., Petzold, M., Bergh, C., 2019. Neonatal and maternal outcome after frozen embryo transfer: Increased risks in programmed cycles. Am. J. Obstet. Gynecol. 221, 126.e1-126.e18. https://doi.org/10.1016/j.ajog.2019.03.010

Fauser, B.C.J.M., 2004. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. Fertil. Steril. 81, 19–25. https://doi.org/10.1016/j.fertnstert.2003.10.004

Figueras, F., Meler, E., Iraola, A., Eixarch, E., Coll, O., Figueras, J., Francis, A., Gratacos, E., Gardosi, J., 2008. Customized birthweight standards for a Spanish population. Eur. J. Obstet. Gynecol. Reprod. Biol. 136, 20–4. https://doi.org/10.1016/j.ejogrb.2006.12.015

Foppa, M., Duncan, B.B., Rohde, L.E.P., 2005. Echocardiography-based left ventricular mass estimation. How should we define hypertrophy? Cardiovasc. Ultrasound. https://doi.org/10.1186/1476-7120-3-17

Forton, K., Motoji, Y., Pezzuto, B., Caravita, S., Delbaere, A., Naeije, R., Faoro, V., 2019. Decreased pulmonary vascular distensibility in adolescents conceived by in vitro fertilization. Hum. Reprod. 34, 1799–1808. https://doi.org/10.1093/humrep/dez113

Fountain, C., Zhang, Y., Kissin, D.M., Schieve, L.A., Jamieson, D.J., Rice, C., Bearman, P., 2015. Association between assisted reproductive technology conception and autism in California, 1997-2007. Am. J. Public Health 105, 963–971. https://doi.org/10.2105/AJPH.2014.302383

García-Otero, L., Gómez, O., Rodriguez-López, M., Torres, X., Soveral, I., Sepúlveda-Martínez, Á., Guirado, L., Valenzuela-Alcaraz, B., López, M., Martínez, J.M., Gratacós, E., Crispi, F., 2019. Nomograms of Fetal Cardiac Dimensions at 18–41 Weeks of Gestation. Fetal Diagn. Ther. 1–12. https://doi.org/10.1159/000494838

García-Otero, L., López, M., Gómez, O., Goncé, A., Bennasar, M., Martńez, J.M., Valenzuela-Alcaraz, B., Rodriguez-López, M., Sitges, M., Loncà, M., Bijnens, B., Crispi, F., Gratacós, E., 2016. Zidovudine treatment in HIV-infected pregnant women is associated with fetal cardiac remodelling. Aids 30, 1393–1401. https://doi.org/10.1097/QAD.0000000000001066

García-Otero, L., López, M., Goncé, A., Fortuny, C., Salazar, L., Valenzuela-Alcaraz, B., Guirado, L., César, S., Gratacós, E., Crispi, F., 2021. Cardiac Remodeling and Hypertension in HIV-Uninfected Infants Exposed in utero to Antiretroviral Therapy. Clin. Infect. Dis. 73, 586–593. https://doi.org/10.1093/CID/CIAB030

Gardiner, H.M., Pasquini, L., Wolfenden, J., Barlow, A., Li, W., Kulinskaya, E., Henein, M., 2006. Myocardial tissue Doppler and long axis function in the fetal heart. Int. J. Cardiol. 113, 39–47. https://doi.org/10.1016/j.ijcard.2005.10.029

Gardner, D.K., Lane, M., Stevens, J., Schlenker, T., Schoolcraft, W.B., 2000. Blastocyst score affects implantation and pregnancy outcome: Towards a single blastocyst transfer. Fertil. Steril. 73, 1155–1158. https://doi.org/10.1016/S0015-0282(00)00518-5

Germanakis, I., Pepes, S., Sifakis, S., Gardiner, H., 2012. Fetal longitudinal myocardial function assessment by anatomic m-mode. Fetal Diagn. Ther. 32, 65–71. https://doi.org/10.1159/000330790

Gestational Hypertension and Preeclampsia: ACOG Practice Bulletin, Number 222., 2020. . Obs. Gynecol. https://doi.org/10.1097/NMC.0000000000000523

Goldsmith, S., Mcintyre, S., Badawi, N., Hansen, M., 2018. Cerebral palsy after assisted reproductive technology: a cohort study. Dev. Med. Child Neurol. 60, 73–80. https://doi.org/10.1111/dmcn.13577

Gómez, O., Figueras, F., Fernández, S., Bennasar, M., Martínez, J.M., Puerto, B., Gratacós, E., 2008. Reference ranges for uterine artery mean pulsatility index at 11-41 weeks of gestation. Ultrasound Obstet. Gynecol. 32, 128–132. https://doi.org/10.1002/uog.5315

Gram, M., Dolberg Anderson, U., Johansson, M.E., Edström-Hägerwall, A., Larsson, I., Jälmby, M., Hansson, S.R., Åkerström, B., 2015. The Human Endogenous Protection System against Cell-Free Hemoglobin and Heme Is Overwhelmed in Preeclampsia and Provides Potential Biomarkers and Clinical Indicators. PLoS One 10, e0138111. https://doi.org/10.1371/journal.pone.0138111

Gunnarsson, R., Åkerström, B., Hansson, S.R., Gram, M., 2017. Recombinant alpha-1-microglobulin: a potential treatment for preeclampsia. Drug Discov. Today 22, 736–743. https://doi.org/10.1016/j.drudis.2016.12.005

Hadlock, F.P., Harrist, R.B., Sharman, R.S., Deter, R.L., Park, S.K., 1985. Estimation of fetal weight with the use of head, body, and femur measurements-A prospective study. Am. J. Obstet. Gynecol. 151, 333–337. https://doi.org/10.1016/0002-9378(85)90298-4

Hahner, N., Puerto, B., Perez-Cruz, M., Policiano, C., Monterde, E., Crispi, F., Gratacos, E., Eixarch, E., 2018. Altered cortical development in fetuses with isolated nonsevere ventriculomegaly assessed by neurosonography. Prenat. Diagn. 38, 365–375. https://doi.org/10.1002/pd.5240

Hart, R., Norman, R.J., 2013. The longer-term health outcomes for children born as a result of IVF treatment. Part II-mental health and development outcomes. Hum. Reprod. Update 19, 244–250. https://doi.org/10.1093/humupd/dmt002

Hecher, K., Campbell, S., Snijders, R., Nicolaides, K., 1994. Reference ranges for fetal venous and atrioventricular blood flow parameters. Ultrasound Obstet. Gynecol. 4, 381–390. https://doi.org/10.1046/j.1469-0705.1994.04050381.x

Henningsen, A.A., Gissler, M., Skjaerven, R., Bergh, C., Tiitinen, A., Romundstad, L.B., Wennerholm, U.B., Lidegaard, O., Nyboe Andersen, A., Forman, J.L., Pinborg, A., 2015. Trends in perinatal health after assisted reproduction: A Nordic study from the CoNARTaS group. Hum. Reprod. 30, 710–716. https://doi.org/10.1093/humrep/deu345

Henningsen, A.K.A., Pinborg, A., Lidegaard, Ø., Vestergaard, C., Forman, J.L., Andersen, A.N., 2011. Perinatal outcome of singleton siblings born after assisted reproductive technology and spontaneous conception: Danish national sibling-cohort study. Fertil. Steril. 95, 959–963. https://doi.org/10.1016/j.fertnstert.2010.07.1075

Hernandez-Andrade, E., López-Tenorio, J., Figueroa-Diesel, H., Sanin-Blair, J., Carreras, E., Cabero, L., Gratacos, E., 2005. A modified myocardial performance (Tei) index based on the use of valve clicks improves reproducibility of fetal left

cardiac function assessment. Ultrasound Obstet. Gynecol. 26, 227–232. https://doi.org/10.1002/uog.1959

Husen, S.C., Koning, I. V., Go, A.T.J.I., Groenenberg, I.A.L., Willemsen, S.P., Rousian, M., Steegers-Theunissen, R.P.M., 2021. IVF with or without ICSI and the impact on human embryonic brain development: the Rotterdam Periconceptional Cohort. Hum. Reprod. 36, 596–604. https://doi.org/10.1093/humrep/deaa341

Hvidtjørn, D., Grove, J., Schendel, D., Schieve, L.A., Svaerke, C., Ernst, E., Thorsen, P., 2011. Risk of autism spectrum disorders in children born after assisted conception: a population-based follow-up study. J Epidemiol Community Heal. 2011;65497e502. 65, 497–502. https://doi.org/10.1136/jech.2009.093823

Hvidtjørn, D., Grove, J., Schendel, D., Sværke, C., Schieve, L.A., Uldall, P., Ernst, E., Jacobsson, B., Thorsen, P., 2010. Multiplicity and early gestational age contribute to an increased risk of cerebral palsy from assisted conception: A population-based cohort study. Hum. Reprod. 25, 2115–2123. https://doi.org/10.1093/humrep/deq070

Källén, A.J.B., Finnström, O.O., Lindam, A.P., Nilsson, E.M.E., Nygren, K.G., Otterblad Olausson, P.M., 2011. Is there an increased risk for drug treated attention deficit/hyperactivity disorder in children born after in vitro fertilization? Eur. J. Paediatr. Neurol. 15, 247–253. https://doi.org/10.1016/j.ejpn.2010.12.004

Källén, B., Finnström, O., Nygren, K.G., Olausson, P.O., 2005. In vitro fertilization in Sweden: Child morbidity including cancer risk. Fertil. Steril. 84, 605–610. https://doi.org/10.1016/j.fertnstert.2005.03.035

Kessler, J., Rasmussen, S., Hanson, M., Kiserud, T., 2006. Longitudinal reference ranges for ductus venosus flow velocities and waveform indices. Ultrasound Obstet. Gynecol. 28, 890–898. https://doi.org/10.1002/uog.3857

Kiserud, T., Benachi, A., Hecher, K., Perez, R.G., Carvalho, J., Piaggio, G., Platt, L.D., 2018. The World Health Organization fetal growth charts: concept, findings, interpretation, and application. Am. J. Obstet. Gynecol. 218, S619–S629. https://doi.org/10.1016/j.ajog.2017.12.010

Kissin, D.M., Zhang, Y., Boulet, S.L., Fountain, C., Bearman, P., Schieve, L., Yeargin-Allsopp, M., Jamieson, D.J., 2015. Association of assisted reproductive technology (ART) treatment and parental infertility diagnosis with autism in ART-conceived children. Hum. Reprod. 30, 454–465. https://doi.org/10.1093/humrep/deu338

Klemetti, R., Gissler, M., Hemminki, E., 2006. Health of Children Born as a Result of In Vitro Fertilization. Pediatrics 118, 1819–1827. https://doi.org/10.1542/peds.2006-0735

Knoester, M., Helmerhorst, F.M., Vandenbroucke, J.P., van der Westerlaken, L.A.J., Walther, F.J., Veen, S., 2008. Cognitive development of singletons born after intracytoplasmic sperm injection compared with in vitro fertilization and natural conception. Fertil. Steril. 90, 289–296. https://doi.org/10.1016/j.fertnstert.2007.06.090

Kristensen, S.G., Pors, S.E., Andersen, C.Y., 2017. Improving oocyte quality by transfer of autologous mitochondria from fully grown oocytes. Hum. Reprod. 32, 725–732. https://doi.org/10.1093/humrep/dex043

Kuwayama, M., 2007. Highly efficient vitrification for cryopreservation of human oocytes and embryos: The Cryotop method. Theriogenology 67, 73–80. https://doi.org/10.1016/j.theriogenology.2006.09.014

Lang, R.M., Badano, L.P., Victor, M.A., Afilalo, J., Armstrong, A., Ernande, L., Flachskampf, F.A., Foster, E., Goldstein, S.A., Kuznetsova, T., Lancellotti, P., Muraru, D., Picard, M.H., Retzschel, E.R., Rudski, L., Spencer, K.T., Tsang, W., Voigt, J.U., 2015. Recommendations for cardiac chamber quantification by echocardiography in adults: An update from the American Society of Echocardiography and the European Association of Cardiovascular Imaging. J. Am. Soc. Echocardiogr. 28, 1-39.e14. https://doi.org/10.1016/j.echo.2014.10.003

Levin, S., Sheiner, E., Wainstock, T., Walfisch, A., Segal, I., Landau, D., Sergienko, R., Levitas, E., Harlev, A., 2019. Infertility Treatments and Long-Term Neurologic Morbidity of the Offspring. Am. J. Perinatol. 36, 949–954. https://doi.org/10.1055/s-0038-1675159

Liao, Z., Liu, C., Cai, L., Shen, L., Sui, C., Zhang, H., Qian, K., 2022. The Effect of Endometrial Thickness on Pregnancy, Maternal, and Perinatal Outcomes of Women in Fresh Cycles After IVF/ICSI: A Systematic Review and Meta-Analysis. Front. Endocrinol. (Lausanne). 12, 1–14. https://doi.org/10.3389/fendo.2021.814648

Lidegaard, Ø., Pinborg, A., Andersen, A.N., 2005. Imprinting diseases and IVF: Danish National IVF cohort study. Hum. Reprod. 20, 950–954. https://doi.org/10.1093/humrep/deh714

Litzky, J.F., Marsit, C.J., 2019. Epigenetically regulated imprinted gene expression associated with IVF and infertility: possible influence of prenatal stress and depression. J. Assist. Reprod. Genet. 36, 1299–1313. https://doi.org/10.1007/s10815-019-01483-0

Liu, H., Zhang, Y., Gu, H.T., Feng, Q.L., Liu, J.Y., Zhou, J., Yan, F., 2015. Association between assisted reproductive technology and cardiac alteration at age 5 years. JAMA Pediatr. 169, 603–605. https://doi.org/10.1001/jamapediatrics.2015.0214

Liu, L., Gao, J., He, X., Cai, Y., Wang, L., Fan, X., 2017. Association between assisted reproductive technology and the risk of autism spectrum disorders in the offspring: A meta-analysis. Sci. Rep. 7, 1–8. https://doi.org/10.1038/srep46207

Lopez, L., Colan, S.D., Frommelt, P.C., Ensing, G.J., Kendall, K., Younoszai, A.K., Lai, W.W., Geva, T., 2010. Recommendations for Quantification Methods During the Performance of a Pediatric Echocardiogram: A Report From the Pediatric Measurements Writing Group of the American Society of Echocardiography Pediatric and Congenital Heart Disease Council. J. Am. Soc. Echocardiogr. 23, 465–495. https://doi.org/10.1016/j.echo.2010.03.019

Magnusson, Å., Wennerholm, U.B., Källén, K., Petzold, M., Thurin-Kjellberg, A., Bergh, C., 2018. The association between the number of oocytes retrieved for IVF, perinatal outcome and obstetric complications. Hum. Reprod. 33, 1939–1947. https://doi.org/10.1093/humrep/dey266

Maheshwari, A., Pandey, S., Raja, E.A., Shetty, A., Hamilton, M., Bhattacharya, S., 2018. Is frozen embryo transfer better for mothers and babies? Can cumulative meta-analysis provide a definitive answer? Hum. Reprod. Update 24, 35–58. https://doi.org/10.1093/HUMUPD/DMX031

Maimburg, R.D., Væth, M., 2007. Do children born after assisted conception have less risk of developing infantile autism? Hum. Reprod. 22, 1841–1843. https://doi.org/10.1093/humrep/dem082

Mak, W., Kondapalli, L.A., Celia, G., Gordon, J., Dimattina, M., Payson, M., 2016. Natural cycle IVF reduces the risk of low birthweight infants compared with

conventional stimulated IVF. Hum. Reprod. 31, 789–794. https://doi.org/10.1093/humrep/dew024

Marjonen, H., Auvinen, P., Kahila, H., Tšuiko, O., Kõks, S., Tiirats, A., Viltrop, T., Tuuri, T., Söderström-Anttila, V., Suikkari, A.M., Salumets, A., Tiitinen, A., Kaminen-Ahola, N., 2018. rs10732516 polymorphism at the IGF2/H19 locus associates with genotype-specific effects on placental DNA methylation and birth weight of newborns conceived by assisted reproductive technology. Clin. Epigenetics 10, 80. https://doi.org/10.1186/s13148-018-0511-2

Mascarenhas, M.N., Flaxman, S.R., Boerma, T., Vanderpoel, S., Stevens, G.A., 2012. National, Regional, and Global Trends in Infertility Prevalence Since 1990: A Systematic Analysis of 277 Health Surveys. PLoS Med. 9, 1–12. https://doi.org/10.1371/journal.pmed.1001356

May, K., Rosenlöf, L., Olsson, M.G., Centlow, M., Mörgelin, M., Larsson, I., Cederlund, M., Rutardottir, S., Siegmund, W., Schneider, H., Åkerström, B., Hansson, S.R., 2011. Perfusion of human placenta with hemoglobin introduces preeclampsia-like injuries that are prevented by α1-microglobulin. Placenta 32, 323–332. https://doi.org/10.1016/j.placenta.2011.01.017

Mayet, J., Hughes, A., 2003. Pathophysiology in hypertension. Heart 89, 1104–1109. https://doi.org/10.1136/heart.89.9.1104

Mehta, N.U., Reddy, S.T., 2015. Role of hemoglobin/heme scavenger protein hemopexin in atherosclerosis and inflammatory diseases. Curr. Opin. Lipidol. 26, 384–387. https://doi.org/10.1097/MOL.0000000000000208

Meister, T.A., Rimoldi, S.F., Soria, R., von Arx, R., Messerli, F.H., Sartori, C., Scherrer, U., Rexhaj, E., 2018. Association of Assisted Reproductive Technologies With Arterial Hypertension During Adolescence. J. Am. Coll. Cardiol. 72, 1267–1274. https://doi.org/10.1016/j.jacc.2018.06.060

Middelburg, K.J., Heineman, M.J., Bos, A.F., Hadders-Algra, M., 2008. Neuromotor, cognitive, language and behavioural outcome in children born following IVF or ICSI - A systematic review. Hum. Reprod. Update 14, 219–231. https://doi.org/10.1093/humupd/dmn005

Mizrak, I., Asserhøj, L.L., Lund, M.A. V, Kielstrup, L.R., Greisen, G., Clausen, T.D., Main, K.M., Jensen, R.B., Vejlstrup, N.G., Madsen, P.L., Pinborg, A., 2022. Cardiovascular function in 8- to 9-year-old singletons born after ART with frozen and fresh embryo transfer. Hum. Reprod. 1–12. https://doi.org/10.1093/humrep/deab284

National Diabetes Data Group, 1997. Guide to diagnosis and classification of diabetes mellitus and other categories of glucose intolerance. Diabetes Care 20, 1039–1057.

Norrman, E., Petzold, M., Bergh, C., Wennerholm, U.B., 2018. School performance in singletons born after assisted reproductive technology. Hum. Reprod. 33, 1948–1959. https://doi.org/10.1093/humrep/dey273

Novakovic, B., Lewis, S., Halliday, J., Kennedy, J., Burgner, D.P., Czajko, A., Kim, B., Sexton-Oates, A., Juonala, M., Hammarberg, K., Amor, D.J., Doyle, L.W., Ranganathan, S., Welsh, L., Cheung, M., Mcbain, J., Mclachlan, R., Saffery, R., n.d. Assisted reproductive technologies are associated with limited epigenetic variation at birth that largely resolves by adulthood. https://doi.org/10.1038/s41467-019-11929-9

Olsson, M.G., Centlow, M., Rutardóttir, S., Stenfors, I., Larsson, J., Hosseini-Maaf, B.,

Olsson, M.L., Hansson, S.R., Åkerström, B., 2010. Increased levels of cell-free hemoglobin, oxidation markers, and the antioxidative heme scavenger α1-microglobulin in preeclampsia. Free Radic. Biol. Med. 48, 284–291. https://doi.org/10.1016/j.freeradbiomed.2009.10.052

Opdahl, S., Henningsen, A.A., Tiitinen, A., Bergh, C., Pinborg, A., Romundstad, P.R., Wennerholm, U.B., Gissler, M., Skjaerven, R., Romundstad, L.B., 2015. Risk of hypertensive disorders in pregnancies following assisted reproductive technology: a cohort study from the CoNARTaS group. Hum. Reprod. 30, 1724–1731. https://doi.org/10.1093/humrep/dev090

Ortigosa, N., Rodriguez-Lopez, M., Bailón, R., Sarvari, S.I., Sitges, M., Gratacos, E., Bijnens, B., Crispi, F., Laguna, P., 2016. Heart morphology differences induced by intrauterine growth restriction and preterm birth measured on the ECG at preadolescent age. J. Electrocardiol. 49, 401–409. https://doi.org/10.1016/j.jelectrocard.2016.03.011

Paladini, D., Chita, S.K., Allan, L.D., 1990. Prenatal measurement of cardiothoracic ratio in evaluation of heart disease. Arch. Dis. Child. 65, 20–23. https://doi.org/10.1136/adc.65.1_Spec_No.20

Paladini, D., Malinger, G., Monteagudo, A., Pilu, G., Timor-Tritsch, I., Toi, A., 2007. Sonographic examination of the fetal central nervous system: Guidelines for performing the "basic examination" and the "fetal neurosonogram." Ultrasound Obstet. Gynecol. https://doi.org/10.1002/uog.3909

Pandey, S., Shetty, A., Hamilton, M., Bhattacharya, S., Maheshwari, A., 2012. Obstetric and perinatal outcomes in singleton pregnancies resulting from IVF/ICSI: a systematic review and meta-analysis. Hum. Reprod. Update 18, 485–503. https://doi.org/10.1093/humupd/dms018

Paules, C., Miranda, J., Policiano, C., Crovetto, F., Youssef, L., Hahner, N., Nakaki, A., Crispi, F., Gratacós, E., Eixarch, E., 2021. Fetal neurosonography detects differences in cortical development and corpus callosum in late-onset small fetuses. Ultrasound Obstet. Gynecol. 58, 42–47. https://doi.org/10.1002/uog.23592

Pelinck, M.J., Hadders-Algra, M., Haadsma, M.L., Nijhuis, W.L., Kiewiet, S.M., Hoek, A., Heineman, M.J., Middelburg, K.J., 2010. Is the birthweight of singletons born after IVF reduced by ovarian stimulation or by IVF laboratory procedures? Reprod. Biomed. Online 21, 245–251. https://doi.org/10.1016/j.rbmo.2010.04.024

Pereira, N., Reichman, D.E., Goldschlag, D.E., Lekovich, J.P., Rosenwaks, Z., 2015. Impact of elevated peak serum estradiol levels during controlled ovarian hyperstimulation on the birth weight of term singletons from fresh IVF-ET cycles. J. Assist. Reprod. Genet. 32, 527–532. https://doi.org/10.1007/s10815-015-0434-1

Pinborg, A., 2019. Short- and long-term outcomes in children born after assisted reproductive technology. BJOG An Int. J. Obstet. Gynaecol. 126, 145–148. https://doi.org/10.1111/1471-0528.15437

Pinborg, A., Lidegaard, Ø., la Cour Freiesleben, N., Andersen, A.N., 2005. Consequences of vanishing twins in IVF/ICSI pregnancies. Hum. Reprod. 20, 2821–2829. https://doi.org/10.1093/humrep/dei142

Pinborg, A., Lidegaard, Ø., La Cour Freiesleben, N., Andersen, A.N., 2007. Vanishing twins: A predictor of small-for-gestational age in IVF singletons. Hum. Reprod. 22, 2707–2714. https://doi.org/10.1093/humrep/dem225

Pinborg, A., Loft, A., Aaris Henningsen, A.K., Rasmussen, S., Andersen, A.N., 2010.

Infant outcome of 957 singletons born after frozen embryo replacement: The Danish National Cohort Study 1995-2006. Fertil. Steril. 94, 1320–1327. https://doi.org/10.1016/j.fertnstert.2009.05.091

Pinborg, A., Loft, A., Schmidt, L., Andersen, A.N., 2003. Morbidity in a Danish National cohort of 472 IVF/ICSI twins, 1132 non-IVF/ICSI twins and 634 IVF/ICSI singletons: Health-related and social implications for the children and their families. Hum. Reprod. 18, 1234–1243. https://doi.org/10.1093/humrep/deg257

Pinborg, A., Loft, A., Schmidt, L., Greisen, G., Rasmussen, S., Andersen, A.N., 2004. Neurological sequelae in twins born after assisted conception: Controlled national cohort study. Br. Med. J. 329, 311–314. https://doi.org/10.1136/bmj.38156.715694.3a

Pinborg, A., Wennerholm, U.B., Romundstad, L.B., Loft, A., Aittomaki, K., Sö derström-Anttila, V., Nygren, K.G., Hazekamp, J., Bergh, C., 2013. Why do singletons conceived after assisted reproduction technology have adverse perinatal outcome? Systematic review and meta-analysis. Hum. Reprod. Update 19, 87–104. https://doi.org/10.1093/humupd/dms044

Pistorius, L.R., Stoutenbeek, P., Groenendaal, F., De Vries, L., Manten, G., Mulder, E., Visser, G., 2010. Grade and symmetry of normal fetal cortical development: A longitudinal two- and three-dimensional ultrasound study. Ultrasound Obstet. Gynecol. 36, 700–708. https://doi.org/10.1002/uog.7705

Plomin, R., Von Stumm, S., 2018. The new genetics of intelligence. Nat. Rev. Genet. https://doi.org/10.1038/nrg.2017.104

Pontesilli, M., Painter, R.C., Grooten, I.J., Van Der Post, J.A., Mol, B.W., Vrijkotte, T.G.M., Repping, S., Roseboom, T.J., 2015. Subfertility and assisted reproduction techniques are associated with poorer cardiometabolic profiles in childhood. Reprod. Biomed. Online 30, 258–267. https://doi.org/10.1016/j.rbmo.2014.11.006

Qin, J.B., Sheng, X.Q., Wu, D., Gao, S.Y., You, Y.P., Yang, T.B., Wang, H., 2017. Worldwide prevalence of adverse pregnancy outcomes among singleton pregnancies after in vitro fertilization/intracytoplasmic sperm injection: a systematic review and meta-analysis. Arch. Gynecol. Obstet. 295, 285–301. https://doi.org/10.1007/s00404-016-4250-3

Qin, J.B., Wang, H., Sheng, X., Xie, Q., Gao, S., 2016. Assisted reproductive technology and risk of adverse obstetric outcomes in dichorionic twin pregnancies: A systematic review and meta-analysis. Fertil. Steril. 105, 1180–1192. https://doi.org/10.1016/j.fertnstert.2015.12.131

Rizzo, G., Pietrolucci, M.E., Mappa, I., Bitsadze, V., Khizroeva, J., Makatsariya, A., D'Antonio, F., 2020. Fetal Cardiac Remodeling Is Affected by the Type of Embryo Transfer in Pregnancies Conceived by in vitro Fertilization: A Prospective Cohort Study. Fetal Diagn. Ther. 47, 772–778. https://doi.org/10.1159/000508987

Robinson, H.P., 1973. Sonar Measurement of Fetal Crown-Rump Length as Means of Assessing Maturity in First Trimester of Pregnancy. Br. Med. J. 4, 28–31. https://doi.org/DOI: 10.1136/bmj.4.5883.28

Rodriguez-Lopez, M., Osorio, L., Acosta-Rojas, R., Figueras, J., Cruz-Lemini, M., Figueras, F., Bijnens, B., Gratacós, E., Crispi, F., 2016. Influence of breastfeeding and postnatal nutrition on cardiovascular remodeling induced by fetal growth restriction. Pediatr. Res. 79, 100–106. https://doi.org/10.1038/pr.2015.182

Rönö, K., Rissanen, E., Bergh, C., Wennerholm, U.B., Opdahl, S., Romundstad, L.B., Henningsen, A.K.A., Spangmose, A.L., Pinborg, A., Gissler, M., Tiitinen, A., 2022.

The neurodevelopmental morbidity of children born after assisted reproductive technology: a Nordic register study from the Committee of Nordic Assisted Reproductive Technology and Safety group. Fertil. Steril. 117, 1026–1037. https://doi.org/10.1016/j.fertnstert.2022.01.010

Royster, G.D., Krishnamoorthy, K., Csokmay, J.M., Yauger, B.J., Chason, R.J., DeCherney, A.H., Wolff, E.F., Hill, M.J., 2016. Are intracytoplasmic sperm injection and high serum estradiol compounding risk factors for adverse obstetric outcomes in assisted reproductive technology? Fertil. Steril. 106, 363-370.e3. https://doi.org/10.1016/j.fertnstert.2016.04.023

Rumbold, A.R., Moore, V.M., Whitrow, M.J., Oswald, T.K., Moran, L.J., Fernandez, R.C., Barnhart, K.T., Davies, M.J., 2017. The impact of specific fertility treatments on cognitive development in childhood and adolescence: A systematic review. Hum. Reprod. 32, 1489–1507. https://doi.org/10.1093/humrep/dex085

Saito, K., Kuwahara, A., Ishikawa, T., Morisaki, N., Miyado, M., Miyado, K., Fukami, M., Miyasaka, N., Ishihara, O., Irahara, M., Saito, H., 2019. Endometrial preparation methods for frozen-thawed embryo transfer are associated with altered risks of hypertensive disorders of pregnancy, placenta accreta, and gestational diabetes mellitus. Hum. Reprod. 34, 1567–1575. https://doi.org/10.1093/humrep/dez079

Sandin, S., Nygren, K.G., Iliadou, A., Hultman, C.M., Reichenberg, A., 2013. Autism and mental retardation among offspring born after in vitro fertilization. JAMA 310, 75–84. https://doi.org/10.1001/jama.2013.7222

Sarmiento Camposa, J.A., Squires, J., Ponte, J., 2011. Universal developmental screening: Preliminary studies in Galicia, Spain. Early Child Dev. Care 181, 475–485. https://doi.org/10.1080/03004430903458007

Sarvari, S.I., Rodriguez-Lopez, M., Nuñez-Garcia, M., Sitges, M., Sepulveda-Martinez, A., Camara, O., Butakoff, C., Gratacos, E., Bijnens, B., Crispi, F., 2017. Persistence of Cardiac Remodeling in Preadolescents with Fetal Growth Restriction. Circ. Cardiovasc. Imaging 10, 1–9. https://doi.org/10.1161/CIRCIMAGING.116.005270

Sazonova, A., Källen, K., Thurin-Kjellberg, A., Wennerholm, U.-B., Bergh, C., 2012. Obstetric outcome in singletons after in vitro fertilization with cryopreserved/thawed embryos. Hum. Reprod. 27, 1343–1350. https://doi.org/10.1093/humrep/des036

Sazonova, A., Källen, K., Thurin-Kjellberg, A., Wennerholm, U.B., Bergh, C., 2011. Factors affecting obstetric outcome of singletons born after IVF. Hum. Reprod. 26, 2878–2886. https://doi.org/10.1093/humrep/der241

Scherrer, U., Rexhaj, E., Allemann, Y., Sartori, C., Rimoldi, S.F., 2015. Cardiovascular dysfunction in children conceived by assisted reproductive technologies. Eur. Hear. J. 36, 1583–1589. https://doi.org/10.1093/eurheartj/ehv145

Scherrer, U., Rimoldi, S.F., Rexhaj, E., Stuber, T., Duplain, H., Garcin, S., De Marchi, S.F., Nicod, P., Germond, M., Allemann, Y., Sartori, C., 2012. Systemic and pulmonary vascular dysfunction in children conceived by assisted reproductive technologies. Circulation 125, 1890–1896. https://doi.org/10.1161/CIRCULATIONAHA.111.071183

Schneider, C., McCrindle, B.W., Carvalho, J.S., Hornberger, L.K., McCarthy, K.P., Daubeney, P.E.F., 2005. Development of Z-scores for fetal cardiac dimensions from echocardiography. Ultrasound Obstet. Gynecol. 26, 599–605.

https://doi.org/10.1002/uog.2597

Sha, T., Yin, X., Cheng, W., Massey, I.Y., 2018. Pregnancy-related complications and perinatal outcomes resulting from transfer of cryopreserved versus fresh embryos in vitro fertilization: a meta-analysis. Fertil. Steril. 109, 330-342.e9. https://doi.org/10.1016/j.fertnstert.2017.10.019

Shapiro, B.S., Daneshmand, S.T., Garner, F.C., Aguirre, M., Hudson, C., Thomas, S., 2011. Evidence of impaired endometrial receptivity after ovarian stimulation for in vitro fertilization: A prospective randomized trial comparing fresh and frozen-thawed embryo transfer in normal responders. Fertil. Steril. 96, 344–348. https://doi.org/10.1016/j.fertnstert.2011.05.050

Sharma, R., Agarwal, A., Rohra, V.K., Assidi, M., Abu-Elmagd, M., Turki, R.F., 2015. Effects of increased paternal age on sperm quality, reproductive outcome and associated epigenetic risks to offspring. Reprod. Biol. Endocrinol. 13, 1–20. https://doi.org/10.1186/s12958-015-0028-x

Song, S., Ghosh, J., Mainigi, M., Turan, N., Weinerman, R., Truongcao, M., Coutifaris, C., Sapienza, C., 2015. DNA methylation differences between in vitro-and in vivo-conceived children are associated with ART procedures rather than infertility. Clin. Epigenetics 7, 1–10. https://doi.org/10.1186/s13148-015-0071-7

Spangmose, A.L., Ginström Ernstad, E., Malchau, S., Forman, J., Tiitinen, A., Gissler, M., Opdahl, S., Romundstad, L.B., Bergh, C., Wennerholm, U.B., Henningsen, A.A., Pinborg, A., 2020. Obstetric and perinatal risks in 4601 singletons and 884 twins conceived after fresh blastocyst transfers: A nordic study from the CoNARTaS group. Hum. Reprod. 35, 805–815. https://doi.org/10.1093/HUMREP/DEAA032

Spangmose, A.L., Malchau, S.S., Henningsen, A.A., Forman, J.L., Rasmussen, S., Loft, A., Schmidt, L., Pinborg, A., 2019. Academic performance in adolescents aged 15–16 years born after frozen embryo transfer compared with fresh embryo transfer: a nationwide registry-based cohort study. BJOG An Int. J. Obstet. Gynaecol. 126, 261–269. https://doi.org/10.1111/1471-0528.15484

Staff, A.C., 2019. The two-stage placental model of preeclampsia: An update. J. Reprod. Immunol. 134–135, 1–10. https://doi.org/10.1016/j.jri.2019.07.004

Strömberg, B., Dahlquist, G., Ericson, A., Finnström, O., Köster, M., Stjernqvist, K., 2002. Neurological sequelae in children born after in-vitro fertilisation: A population-based study. Lancet 359, 461–465. https://doi.org/10.1016/S0140-6736(02)07674-2

Sun, Y., Vestergaard, M., Christensen, J., Zhu, J.L., Bech, B.H., Olsen, J., 2007. Epilepsy and febrile seizures in children of treated and untreated subfertile couples. Hum. Reprod. 22, 215–220. https://doi.org/10.1093/humrep/del333

Sunkara, S.K., Lamarca, A., Polyzos, N.P., Seed, P.T., Khalaf, Y., 2016. Live birth and perinatal outcomes following stimulated and unstimulated IVF: Analysis of over two decades of a nationwide data. Hum. Reprod. 31, 2261–2267. https://doi.org/10.1093/humrep/dew184

Svahn, M.F., Hargreave, M., Nielsen, T.S.S., Plessen, K.J., Jensen, S.M., Kjaer, S.K., Jensen, A., 2015. Mental disorders in childhood and young adulthood among children born to women with fertility problems. Hum. Reprod. 30, 2129–2137. https://doi.org/10.1093/humrep/dev172

Teichholz, L., Kreulen, T., Herman, M., R, G., 1976. Problems in echocardiographic-

angiographic correlations in the presence or absence of asynergy. Am J Cardiol 37, 7–11.

Terho, A.M., Pelkonen, S., Opdahl, S., Romundstad, L.B., Bergh, C., Wennerholm, U.B., Henningsen, A.A., Pinborg, A., Gissler, M., Tiitinen, A., 2021. High birth weight and large-for-gestational-age in singletons born after frozen compared to fresh embryo transfer, by gestational week: a Nordic register study from the CoNARTaS group. Hum. Reprod. 36, 1083–1092. https://doi.org/10.1093/humrep/deaa304

Tolosano, E., Fagoonee, S., Morello, N., Vinchi, F., Fiorito, V., 2010. Heme Scavenging and the Other Facets of Hemopexin. Antioxid. Redox Signal. 12, 305–320. https://doi.org/10.1089/ars.2009.2787

Valenzuela-Alcaraz, B., Crispi, F., Bijnens, B., Cruz-Lemini, M., Creus, M., Sitges, M., Bartrons, J., Civico, S., Balasch, J., Gratacós, E., 2013. Assisted reproductive technologies are associated with cardiovascular remodeling in utero that persists postnatally. Circulation 128, 1442–1450. https://doi.org/10.1161/CIRCULATIONAHA.113.002428

Valenzuela-Alcaraz, B., Crispi, F., Cruz-Lemini, M., Bijnens, B., García-Otero, L., Sitges, M., Balasch, J., Gratacós, E., 2017. Differential effect of assisted reproductive technology and small-for-gestational age on fetal cardiac remodeling. Ultrasound Obstet. Gynecol. 50, 63–70. https://doi.org/10.1002/uog.16217

Valenzuela-Alcaraz, B., Crispi, F., Manau, D., Cruz-Lemini, M., Borras, A., Balasch, J., Gratacós, E., Nica Cruz-Lemini, M., Gratacó, E., 2016. Differential effect of mode of conception and infertility treatment on fetal growth and prematurity. J. Matern. Neonatal Med. 29, 3879–3884. https://doi.org/10.3109/14767058.2016.1151868

Valenzuela-Alcaraz, B., Cruz-Lemini, M., Rodríguez-López, M., Goncé, A., García-Otero, L., Ayuso, H., Sitges, M., Bijnens, B., Balasch, J., Gratacós, E., Crispi, F., 2018. Fetal cardiac remodeling in twin pregnancy conceived by assisted reproductive technology. Ultrasound Obstet. Gynecol. 51, 94–100. https://doi.org/10.1002/uog.17527

Valenzuela-Alcaraz, B., Serafini, A., Sepulveda-Martínez, A., Casals, G., Rodríguez-López, M., Garcia-Otero, L., Cruz-Lemini, M., Bijnens, B., Sitges, M., Balasch, J., Gratacós, E., Crispi, F., 2019. Postnatal persistence of fetal cardiovascular remodelling associated with assisted reproductive technologies: a cohort study. BJOG An Int. J. Obstet. Gynaecol. 126, 291–298. https://doi.org/10.1111/1471-0528.15246

Vannuccini, S., Clifton, V.L., Fraser, I.S., Taylor, H.S., Critchley, H., Giudice, L.C., Petraglia, F., 2016. Infertility and reproductive disorders: Impact of hormonal and inflammatory mechanisms on pregnancy outcome. Hum. Reprod. Update 22, 104–115. https://doi.org/10.1093/humupd/dmv044

Vinchi, F., Gastaldi, S., Silengo, L., Altruda, F., Tolosano, E., 2008. Hemopexin Prevents Endothelial Damage and Liver Congestion in a Mouse Model of Heme Overload. Am J Pathol 173, 289–299. https://doi.org/10.2353/ajpath.2008.071130

von Arx, R., Allemann, Y., Sartori, C., Rexhaj, E., Cerny, D., de Marchi, S.F., Soria, R., Germond, M., Scherrer, U., Rimoldi, S.F., Arx, von R., Rimoldi, S., 2015. Right ventricular dysfunction in children and adolescents conceived by assisted reproductive technologies. J Appl Physiol 118, 1200–1206. https://doi.org/10.1152/japplphysiol.00533.2014.-Assisted

Von Versen-Hoÿnck, F., Narasimhan, P., Selamet Tierney, E.S., Martinez, N., Conrad, K.P., Baker, V.L., Winn, V.D., 2019. Absent or excessive corpus luteum number is associated with altered maternal vascular health in early pregnancy. Hypertension 73, 680–690. https://doi.org/10.1161/HYPERTENSIONAHA.118.12046

Wang, Z., Liu, H., Song, H., Li, X., Jiang, J., Sheng, Y., Shi, Y., 2020. Increased Risk of Pre-eclampsia After Frozen-Thawed Embryo Transfer in Programming Cycles. Front. Med. 7, 1–5. https://doi.org/10.3389/fmed.2020.00104

Waschkies, F., Kroning, L., Schill, T., Chandra, A., Schippert, C., Töpfer, D., Ziert, Y., von Versen-Höynck, F., 2021. Pregnancy Outcomes After Frozen-Thawed Embryo Transfer in the Absence of a Corpus Luteum. Front. Med. 8, 1–11. https://doi.org/10.3389/fmed.2021.727753

Wester-Rosenlöf, L., Casslén, V., Axelsson, J., Edström-Hägerwall, A., Gram, M., Holmqvist, M., Johansson, M.E., Larsson, I., Ley, D., Marsal, K., Mörgelin, M., Rippe, B., Rutardottir, S., Shohani, B., Akerström, B., Hansson, S.R., 2014. A1M/α1-microglobulin protects from heme-induced placental and renal damage in a pregnant sheep model of preeclampsia. PLoS One 9. https://doi.org/10.1371/journal.pone.0086353

Whitelaw, N., Bhattacharya, S., Hoad, G., Horgan, G.W., Hamilton, M., Haggarty, P., 2014. Epigenetic status in the offspring of spontaneous and assisted conception. Hum. Reprod. 29, 1452–1458. https://doi.org/10.1093/humrep/deu094

Wiegel, R.E., Danser, A.H.J., Steegers-Theunissen, R.P.M., Laven, J.S.E., Willemsen, S.P., Baker, V.L., Steegers, E.A.P., Von Versen-Höynck, F., 2020. Determinants of Maternal Renin-Angiotensin-Aldosterone-System Activation in Early Pregnancy: Insights From 2 Cohorts. J. Clin. Endocrinol. Metab. 105, 3505–3517. https://doi.org/10.1210/clinem/dgaa582

World Medical Association, 2013. Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects. JAMA 310, 2191–2194. https://doi.org/10.1001/jama.2013.281053

Wyns, C., De Geyter, C., Calhaz-Jorge, C., Kupka, M.S., Motrenko, T., Smeenk, J., Bergh, C., Tandler-Schneider, A., Rugescu, I.A., Vidakovic, S., Goossens, V., 2021. ART in Europe, 2017: results generated from European registries by ESHRE. Hum. Reprod. Open 1–17. https://doi.org/10.1093/hropen/hoab026

Yin, L., Xu, Y., Li, H., Ling, C., Wai Choy, K., Xia, F., Deng, X., 2015. Effect of assisted reproductive technology on fetal brain development assessed by prenatal ultrasonography. J. Perinat. Med 43, 103–109. https://doi.org/10.1515/jpm-2014-0020

Youssef, L., Erlandsson, L., Åkerström, B., Miranda, J., Paules, C., Crovetto, F., Crispi, F., Gratacos, E., Hansson, S.R., 2020a. Hemopexin and α1-microglobulin heme scavengers with differential involvement in preeclampsia and fetal growth restriction. PLoS One 15, e0239030. https://doi.org/10.1371/journal.pone.0239030

Youssef, L., Miranda, J., Paules, C., Garcia-Otero, L., Vellvé, K., Kalapotharakos, G., Sepulveda-Martinez, A., Crovetto, F., Gomez, O., Gratacós, E., Crispi, F., 2020b. Fetal cardiac remodeling and dysfunction is associated with both preeclampsia and fetal growth restriction. Am. J. Obstet. Gynecol. 222, 79.e1-79.e9. https://doi.org/10.1016/j.ajog.2019.07.025

Yzydorczyk, C., Armengaud, J.B., Peyter, A.C., Chehade, H., Cachat, F., Juvet, C.,

Siddeek, B., Simoncini, S., Sabatier, F., Dignat-George, F., Mitanchez, D., Simeoni, U., 2017. Endothelial dysfunction in individuals born after fetal growth restriction: Cardiovascular and renal consequences and preventive approaches. J. Dev. Orig. Health Dis. 8, 448–464. https://doi.org/10.1017/S2040174417000265

Zhou, J., Liu, H., Gu, H.T., Cui, Y.G., Zhao, N.N., Chen, J., Gao, L., Zhang, Y., Liu, J.Y., 2014. Association of cardiac development with assisted reproductive technology in childhood: A prospective single-blind pilot study. Cell. Physiol. Biochem. 34, 988–1000. https://doi.org/10.1159/000366315

Zhu, J.L., Basso, O., Obel, C., Hvidtjørn, D., Olsen, J., 2009. Infertility, infertility treatment and psychomotor development: The Danish National Birth Cohort. Paediatr. Perinat. Epidemiol. 23, 98–106. https://doi.org/10.1111/j.1365-3016.2008.00989.x

Zong, L., Liu, P., Zhou, L., Wei, D., Ding, L., Qin, Y., 2020. Increased risk of maternal and neonatal complications in hormone replacement therapy cycles in frozen embryo transfer. Reprod. Biol. Endocrinol. 18, 1–9. https://doi.org/10.1186/s12958-020-00601-3

Annexes

ANNEXES

CLÍNIC
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CIF – G-08431173

DICTAMEN DEL COMITÉ ÉTICO DE INVESTIGACIÓN CLÍNICA

Neus Riba Garcia, Secretario del **Comité Ético de Investigación Clínica del Hospital Clínic de Barcelona**

Certifica:

Que este Comité ha evaluado la propuesta del promotor, para que se realice el estudio:

CÓDIGO:
DOCUMENTOS CON VERSIONES:

Tipo	Subtipo	Versión
Protocolo		V 1.0 30 MARZO 2017
Hoja Información de Paciente		Versión 1. Fecha 15 de Febrero del 2017

TÍTULO: Fetal programming and perinatal outcomes in assisted reproductive technologies
PROMOTOR:
INVESTIGADOR PRINCIPAL: EDUARD GRATACÓS SOLSONA

y considera que, teniendo en cuenta la respuesta a las aclaraciones solicitadas (si las hubiera), y que:

- Se cumplen los requisitos necesarios de idoneidad del protocolo en relación con los objetivos del estudio y están justificados los riesgos y molestias previsibles.
- La capacidad del investigador y los medios disponibles son apropiados para llevar a cabo el estudio.
- Que se han evaluado la compensaciones económicas previstas (cuando las haya) y su posible interferencia con el respeto a los postulados éticos y se consideran adecuadas.
- Que dicho estudio se ajusta a las normas éticas esenciales y criterios deontológicos que rigen en este centro.
- Que dicho estudio se incluye en una de las líneas de investigación biomédica acreditadas en este centro, cumpliendo los requisitos necesarios, y que es viable en todos sus términos.

Este CEIC acepta que dicho estudio sea realizado, debiendo ser debiendo ser comunicado a dicho Comité Ético todo cambio en el protocolo o acontecimiento adverso grave.

Reg. HCB/2017/0714

Mod_04 (V1 de 28/11/13)

Página 1 / 3

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y hace constar que:
1º En la reunión celebrada el día 21/09/2017, acta 16/2017, se decidió emitir el informe correspondiente al estudio de referencia.
2º El CEIC del Hospital Clínic i Provincial, tanto en su composición como en sus PNTs, cumple con las normas de BPC (CPMP/ICH/135/95)
3º Listado de miembros:

CIF – G-08431173

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- FRANCISCO JAVIER CARNE CLADELLAS (Médico Farmacólogo Clínico, HCB)

Vicepresidente:
- BEGOÑA GOMEZ PEREZ (Farmacéutica Hospitalaria, HCB)

Secretario:
- NEUS RIBA GARCIA (Médico Farmacólogo Clínico, HCB)

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- MONTSERRAT GONZALEZ CREUS (Trabajadora Social, Servicio de Atención al Usuario, HCB)
- MONTSERRAT NUÑEZ JUÁREZ (Enfermera, HCB)
- JOSE RIOS GUILLERMO (Estadístico. Plataforma de Estadística Médica. IDIBAPS)
- OCTAVI SANCHEZ LOPEZ (Representante de los pacientes)
- JOAQUIM FORÉS I VIÑETA (Médico Traumatólogo, HCB)
- MARIA JESÚS BERTRAN LUENGO (Médico Epidemiólogo, HCB)
- PAULA MARTIN FARGAS (Abogada, HCB)
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- JOSE LUIS BLANCO ARÉVALO (Médico Medicina Interna, HCB)

En el caso de que se evalúe algún proyecto del que un miembro sea investigador/colaborador, este se ausentará de la reunión durante la discusión del proyecto.

Para que conste donde proceda, y a petición del promotor,

Reg. HCB/2017/0714

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P.O. Xavier Carmo

Barcelona, a 26 de septiembre de 2017

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DICTAMEN DEL COMITÉ ÉTICO DE INVESTIGACIÓN CLÍNICA

NEUS RIBA GARCIA, Secretario del **Comité Ético de Investigación Clínica del Hospital Clínic de Barcelona.**

Certifica:
Que este Comité ha evaluado la propuesta del promotor, para que se realice:

Nuevas versiones en protocolo y CI

Protocolo Versión 2.0 4th April 2018 CI Versión 2. Fecha 04 de Abril de 2018.

del estudio:

CÓDIGO: NÚMERO EUDRACT:

TÍTULO: Fetal programming and perinatal outcomes in assisted reproductive technologies

PROMOTOR:

y emite

DICTAMEN FAVORABLE

Y hace constar que:

1º En la reunión celebrada el día 12/04/2018, acta 7/2018 se decidió emitir el informe correspondiente a la enmienda de referencia.

2º El CEIC del Hospital Clínic de Barcelona, tanto en su composición como en sus PNTs, cumple con las normas de BPC (CPMP/ICH/135/95).

3º Listado de miembros:

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Página 1/3

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Secretario:

- NEUS RIBA GARCIA (Médico Farmacólogo Clínico, HCB)

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- MIRIAM MÉNDEZ GARCÍA (Abogada, HCB)
- MERCÈ VIDAL FLOR (Enfermera, HCB)

Que en el caso de que se evalúe algún proyecto del que un miembro sea investigador/colaborador, éste se ausentará de la reunión durante la discusión del proyecto.

RIBA GARCIA NEUS - 46540984R

Signat digitalment per RIBA GARCIA NEUS - 46540984R
DN: c=ES, serialNumber=IDCES-46540984R, givenName=NEUS, sn=RIBA GARCIA, cn=RIBA GARCIA NEUS - 46540984R
Data: 2018.10.22 15:30:14 +02'00'

Barcelona, a 22 de octubre de 2018

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DICTAMEN DEL COMITÉ ÉTICO DE INVESTIGACIÓN CLÍNICA

ANA LUCIA ARELLANO ANDRINO, Secretario del **Comité Ético de Investigación Clínica del Hospital Clínic de Barcelona.**

Certifica:
Que este Comité ha evaluado la propuesta del promotor, para que se realice:

Canvis en el Protocol i FIP

Protocol nova versió Version 3.0 30th October 2019 - FIP nova versió Versión 3. Fecha 30 de Octubre de 2019.

del estudio:

CÓDIGO: NÚMERO EUDRACT:

TÍTULO: Fetal programming and perinatal outcomes in assisted reproductive technologies

PROMOTOR:

y emite

DICTAMEN FAVORABLE

Y hace constar que:

1º En la reunión celebrada el día 12/12/2019, acta 21/2019 se decidió emitir el informe correspondiente a la enmienda de referencia.

2º El CEIC del Hospital Clínic de Barcelona, tanto en su composición como en sus PNTs, cumple con las normas de BPC (CPMP/ICH/135/95).

3º Listado de miembros:

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- ANDREA SCALISE (Médico Farmacólogo Clínico, HCB)

Secretario:

- ANA LUCIA ARELLANO ANDRINO (Médico Farmacólogo Clínico, HCB)

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- MARINA ROVIRA ILLAMOLA (Farmacéutico Atención Primaria, CAP Eixample)
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- ELENA CALVO CIDONCHA (Farmacéutica Hospitalaria, HCB)
- CECILIA CUZCO CABELLOS (Enfermera, HCB)

Que en el caso de que se evalúe algún proyecto del que un miembro sea investigador/colaborador, éste se ausentará de la reunión durante la discusión del proyecto.

ANA LUCIA ARELLANO ANDRINO

Digitally signed by ANA LUCIA ARELLANO ANDRINO
Date: 2019.12.18 09:55:42 +01'00'

Barcelona, a 16 de diciembre de 2019

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