UNIVERSITAT DE BARCELONA

Cardiovascular assessment in fetuses and children conceived by assisted reproductive technologies

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PhD THESIS

Departament d'Obstetrícia i Ginecologia, Pediatria, Radiologia i Anatomía

Programa Doctorat Medicina RD 1393/2007

CARDIOVASCULAR ASSESSMENT IN FETUSES AND CHILDREN CONCEIVED BY ASSISTED REPRODUCTIVE TECHNOLOGIES

Submitted by

Brenda I. Valenzuela Alcaraz

To obtain the degree of "Doctor in Medicine"

and the International Doctor Mention

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TESIS DOCTORAL

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If I have seen further it is by standing on the shoulders of giants.

- Isaac Newton

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Barcelona, September 20th, 2016.

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We declare that **Brenda I. Valenzuela Alcaraz** has performed under our supervision the studies presented in the thesis **"Cardiovascular assessment in fetuses and children conceived by Assisted Reproductive Technologies"**.

This 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 a Tribunal.

Eduard Gratacós Solsona
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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. It was approved by the *Comisión de Doctorado de la Facultad de Medicina* on the 17th of February 2012. Projects included in this thesis, led to five articles published or submitted for publication in international journals:

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Valenzuela-Alcaraz B, Crispi , 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;128:1442-50.
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Status: submitted. *Journal Impact Factor*: 3.85

Valenzuela-Alcaraz B, Crispi F, Cruz-Lemini M, Bijnens B, Garcia-Otero L, Sitges M, Balasch J, Gratacos E. "Differential effect of assisted reproductive technologies and small-for-gestational-age on fetal cardiac remodeling". Ultrasound Obstet Gynecol. 2016 doi: 10.1002/uog.16217.
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Status: draft in preparation

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1. INTRODUCTION

1. INTRODUCTION

1.1- ASSISTED REPRODUCTIVE TECHNOLOGIES AND OFFSPRING HEALTH

Assisted reproductive technologies (ART) have been introduced to overcome reproductive failures in the human being. The current prevalence of infertility (defined as a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse) is estimated to be around 9% worldwide for women aged 20-44 years(1). By definition, ART include all treatments or procedures that include the *in vitro* handling of both human oocytes and sperm, or embryos, for the purpose of establishing a pregnancy(1). Europe leads the world in ART, initiating approximately 50% of all reported treatment cycles. Globally, approximately five million children have been born as a result of IVF (*in vitro* fertilization: an ART procedure that involves extracorporeal fertilization) and the rate of application of ART is increasing at around 1 million a year. This implies the birth of 350,000 babies a year via assisted reproduction(2, 3). The most common fertilization technique is *intracytoplasmic sperm injection* (ICSI, a procedure in which a single spermatozoon is injected into the oocyte cytoplasm) which accounts for around two-thirds of all treatments worldwide, and conventional *in vitro fertilization* (IVF) around one third (3-5).

Although the majority of ART children are born healthy; there are several reports of increased rate of pregnancy complications(6-10) and worse perinatal outcomes (8, 11-13) in this population. Together with these outcomes, epigenetic changes and imprinting errors have been observed in ART children(14-16). Epigenetic alterations, resulting from these environmental exposures during early embryonic development, may contribute to long-term health consequences according to the fetal programming hypothesis(17). Due

to the fact that these techniques are relatively new (IVF offspring are at the most 38 years old and ICSI offspring 24 years old) the effect of ART on later stages of development and adult susceptibility are uncertain. However, follow up studies during childhood and adolescence have shown cardiac, vascular and metabolic differences when comparing with those spontaneously conceived children(18-22).

Based on all this evidence, we hypothesized on the existence of pregnancy complications and fetal cardiovascular changes in the ART population that would persist postnatally, which was the main motivation for developing this thesis project.

1.2.- OBSTETRIC AND PERINATAL OUTCOMES IN INFERTILE WOMEN UNDERGOING ASSISTED REPRODUCTIVE TECHNOLOGIES.

It is well documented that pregnancies resulting from ART are associated with an increased risk of pregnancy complications such as preeclampsia(10), placenta previa (7) and placental abruption, gestational diabetes and cesarean delivery(9), together with poor perinatal outcomes when compared with those after spontaneous conception(6, 11-13).

Low birthweight (LBW), small for gestational age (SGA) and preterm delivery (PTD) are well-documented outcomes mainly related to a higher incidence of multiple pregnancies in ART(23), although a worse outcomes are also present in singleton ART pregnancies. Singletons born after IVF present a higher relative risk (RR) for PTD

(gestational age at delivery <37 weeks: RR 1.84 (95% confidence interval (CI) 1.54-2-21)) and LBW (birth weight <2500 g: RR 1.60 (95% CI 1.29-1.98)) (11-13). The risk for SGA is also higher among IVF infants (birth weight < 10^{th} centile: RR 1.45 (95% CI 1.04-2.00)). In general, IVF infants had lower birth weights (-97 g (95% CI -161g to -24g)) and shorter gestations (-0.6 weeks (95% CI -0.9 weeks to -0.4 weeks)) (11-13). Recent meta-analyses demonstrated that both singletons and twins conceived after IVF are at increased risk for PTD, LBW, SGA, perinatal mortality, and other adverse perinatal health outcomes, after correction for maternal age or parity(6, 11-13, 24).

Baseline infertility characteristics, embryo manipulation, culture conditions, epigenetics changes and have been proposed as potential underlying mechanisms to explain the association of ART with perinatal complications. Studies in animal models showed that embryo culture conditions may affect perinatal outcome and offspring size (25). Human data also suggest embryo culture media to affect birthweight ((26, 27). In addition, epigenetic changes and imprinting disorders have been described in ART pregnancies. Epigenetics is defined as all heritable changes in gene expression that occur without changes in the DNA sequence.(28) A loss of methylation at a critical imprinting control region is suggested to be a molecular mechanism underlying the association between ART and imprinting defects such as Beckwith-Weidemann, and Angelman syndrome(14, 29).

Despite the potential effect of the embryo manipulation, the underlying cause of infertility (older age, known and unknown baseline diseases) could also explain the increased obstetric risk observed in ART(30-33). Evidence that suggests perinatal

outcomes among pregnancies of subfertile women who conceived with natural cycles, (pharmacological treatment of women with anovulation or oligo-ovulation with the intention of inducing normal ovulatory cycles)(1) are less favorable than those which are spontaneously conceived, although better than those achieved with an infertility treatment(30-33). The direct causes for these results are not clear yet; most of the mentioned complications could be explained by maternal and paternal underlying medical conditions associated with subfertility and infertility(34) such as sperm factors, the use of fertility hormone medications, laboratory conditions during embryo culture, culture media(26), embryo manipulation (cryopreservation and thawing, prenatal genetic diagnosis (if performed))(35), increased proportion of multiple gestations and vanishing twins (spontaneous disappearance of one or more gestational sacs or embryos in an ongoing pregnancy, documented by ultrasound)(1), or a combination of these factors(36).

Given the evidence of perinatal complications associated to ART and the controversial underlying mechanism, the first specific objective of this thesis was to examine the perinatal outcomes in our infertile population achieving pregnancy spontaneously or after ART ***(STUDY 1)***.

1.3.- ASSISTED REPRODUCTIVE TECHNOLOGIES AND OFFSPRING CARDIOVASCULAR HEALTH

Cardiovascular disease is a leading cause of morbidity and mortality worldwide (responsible for the largest number of deaths 17.5 million)(37). Most cardiovascular diseases undergo a long subclinical phase that could even start before birth. The fetal

programming hypothesis postulates that during critical periods of development (including periconception, pregnancy, and early postnatal life) organisms exhibit an enhanced plasticity that enables them to fine-tune patterns of gene expression. This so called “programming” thereby engenders an ability to adapt to novel conditions; however, these adaptive changes could conflict with postnatal environment and impair adult health (17). It could be hypothesized that ART techniques could program long-term cardiovascular changes in offspring from early stages of life.

Because IVF offspring are at most 38 years-old and ICSI offspring 24 years-old (with the first born in 1992), the effects of ART techniques on adult health and disease are uncertain. However, Ceelen(18-20) and Sakka(21) realized follow up studies investigating blood pressure levels and several indicators of insulin resistance in IVF and spontaneously conceived children. IVF children were 2.1 times more likely to be in the highest systolic blood pressure quartile (≥114.5 mm Hg) and 1.9 times more likely to be in the highest diastolic blood pressure quartile (≥65.5 mm Hg) than controls. IVF children also showed higher fasting glucose[17-19], elevated triglycerides(21), increased body fat composition[17-19] and increased incidence of subclinical primary hypothyroidism as compared to spontaneously conceived ones.

More recently, Scherrer et al(22) conducted a study in high altitude that enable them to demonstrate vascular dysfunction in ART children (30% pulmonary hypertension, 25% lower flow-mediated dilation (FMD), faster pulse wave velocity (PWV) and thicker carotid intima media thickness (cIMT)). In order to rule out confounding factors, they also studied vascular function in parents of ART children, likewise in their naturally conceived siblings.

Vascular measurements were normal in all of them, suggesting that ART *per se* induced vascular dysfunction(38). They could found no differences in arterial blood pressure, just as Belva(39) et al in ICSI adolescents.

In the above mentioned studies(18-22), cardiovascular and metabolic measurements were independent of children being born small for gestational age (SGA), a condition which is common after ART and has been shown to increase risk of adverse cardiometabolic outcomes, thus implicating a lasting effect of ART and/or probably an independent cause for cardiometabolic development of disease.

Although subtle, these changes have been detected in several studies during late childhood-adolescence. However, at that time, no studies had evaluated the presence of cardiovascular changes during the prenatal period. The second and third specific objectives of this thesis were to assess cardiovascular structure and function in singleton and twin fetuses conceived by ART. For that purpose, we designed two prospective pregnancies cohorts (in singletons STUDY 2 and twins STUDY 3) in which fetal echocardiography would be performed.

Fetal heart evaluation is challenging due to the small size of the heart, its high heart rate and restricted access to the fetus in the maternal abdomen, even though; the evaluation of cardiac function is feasible in most fetuses by experienced healthcare professionals(40). Changes in fetal cardiomyocyte maturation and the fetal circulation pattern differ from that in the adult, and these patterns may also change during

pregnancy. Despite its challenges, most fetal echocardiographic parameters have been validated and fetal echocardiography has demonstrated its utility to demonstrate cardiac remodeling and dysfunction in several fetal diseases such as fetal growth restriction(41-43), twin-to-twin transfusion syndrome(44), maternal diabetes(45) , etc. In most fetal conditions, cardiac dysfunction undergoes a long subclinical phase before reaching clinical cardiac failure (Figure 1). Fetal echocardiography enables us to assess patterns of cardiac remodeling and dysfunction (longitudinal and radial).

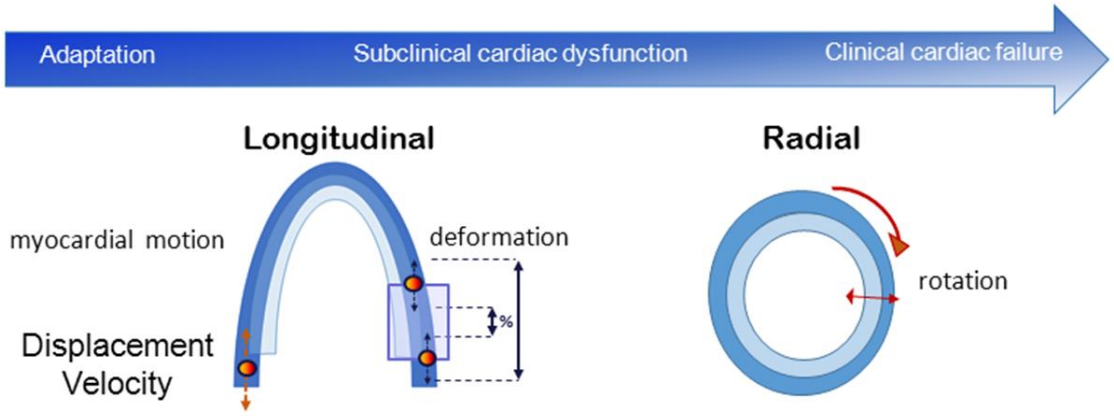
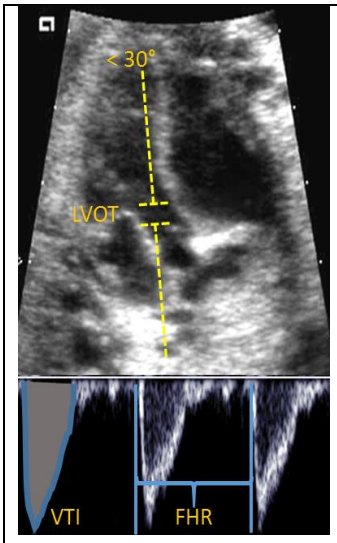


***Figure 1**. Graphic representation of the three-directional myocardial motility involving longitudinal, radial and circumferential contraction. The motion is shown as a single point motility determined by displacement and systolic (S') and early diastolic (E') annular peak velocities; and deformation by the change in length or thickness between two points represented as strain or strain rate.*

A comprehensive fetal echocardiography usually comprises cardiac morphometry and function. **Cardiac morphometry** is usually based on 2D and M-mode for quantifying cardiac chamber size, wall thickness, and also the description of ventricular shape by measurement of the basal diameter and base-to-apex length in a four chamber view,(46, 47). Cardiac function can be assessed by ultrasound using M-mode or Doppler (conventional or tissue):

- **Conventional Doppler** evaluation comprises measurements for cardiac function such as cardiac output, the early (E) and late (A) diastolic filling velocities and E/A ratio, and the myocardial performance index (MPI):

    - **Cardiac *outflow tracts (OT)*** reflect heart afterload (Figure 2). Aortic and pulmonary artery velocity-time integrals can be calculated by manual trace of the spectral Doppler area. Then, stroke volume (amount of blood ejected per heartbeat) can be estimated by multiplying velocity time integrals per outflow area. (48). Combining this information with the fetal heart rate allows estimating the left and right cardiac output (CO). In the fetus the sumatory of both is named the combined cardiac output (CCO), which should normally be expressed as the cardiac index (CI; CCO divided by estimated fetal weight)(49).



Cardiac output calculation	
Left Cardiac Output (ml/min)	π x(Ao valve diameter/2)^{2}x VTI Ao x FHR
Right Cardiac Output (ml/min)	π x(Pulm valve diameter/2)^{2}x VTI Pulm x FHR
Combined Cardiac Output	Left cardiac output + Right cardiac output
Cardiac Index	*Cardiac output / estimated fetal weight*

Figure 2. *Illustration of the left ventricular outflow tract for measuring stroke volume (SV) and cardiac output (CO). The valve diameter (D) is measured in a 2D image. Velocity time integral (VTI) of the blood flow and heart rate (HR) are evaluated in the spectral Doppler waveform.*

- ***The E/A ratio*** is known to reflect diastolic function or ventricular relaxation (Figure 3). It is performed with the spectral Doppler sample volume below the atrioventricular (AV) valves, where a biphasic wave is displayed in the normal fetus. The first wave component, the E wave (early or passive diastole) represents myocardial relaxation and negative pressure of the ventricle. The A wave (active, atrial or late diastole) represents the atrial contraction during ventricular filling. The ratio is obtained by the division of the peak velocities of the E over the A waveform and usually the value is <1(50). E/A velocities increase as pregnancy progresses(51), although the right waveforms have higher velocities than the left side(52).

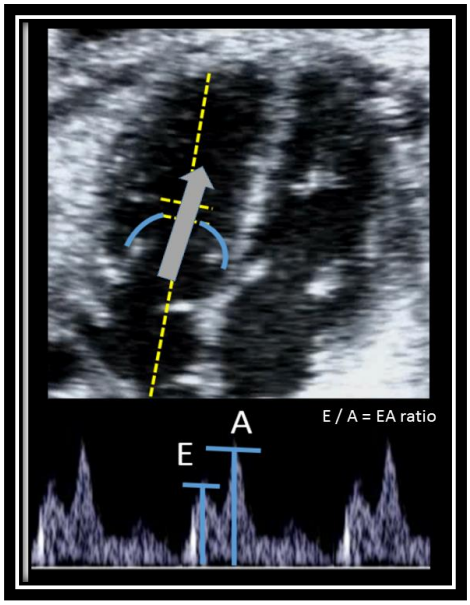


Figure 3*. Image of the E/A ratio evaluation. The sample gate is placed just below the atrio-ventricular valves in a four-chamber view in order to display biphasic inflow (including the E (early diastole) and A (atrial contraction).*

- ***The myocardial performance index*** *(MPI or Tei Index)* is considered a marker of global cardiac function and provides information on the different time periods during both systolic and diastolic phases explained above (Figure 4). These time periods are the isovolumetric contraction time (ICT), the ejection time (ET) and isovolumetric relaxation time (IRT). MPI is calculated as (ICT + IRT) / ET; normal values are reported throughout gestation(53).

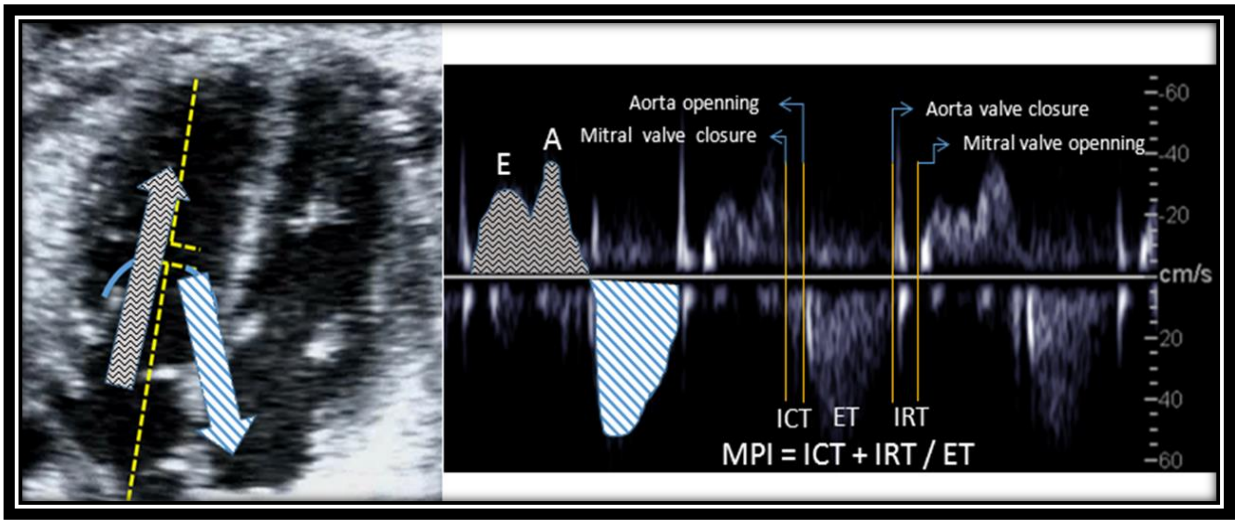


Figure 4*. Illustration of myocardial performance index (MPI) assessment by spectral Doppler. Placing the Doppler sample volume in a four-chamber view on the medial wall of the ascending aorta, the mitral biphasic inflow (grey arrow, early (E) and atrial (A) waveforms) and the aortic outflow (blue arrow and waveform (Ao)) are displayed in the same spectral image. The MPI is calculated by measuring time intervals including: 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 closure of the aorta; and isovolumic relxaxation time (IRT) from the closure of the aortic valve to the opening of the mitral valve.*

- ***M-mode*** (motion-mode) ultrasound permit the evaluation of radial or longitudinal myocardial motion: *Short-Axis* (Figure 5): Radial motion can be assessed from *a* transverse cardiac view, positioning the beam along the short axis of the heart in the four-chamber view, perpendicular to the interventricular septum(54). Using this view, M-mode can be applied to obtain measurements for the end-systolic (ESVD) and end-diastolic ventricular diameters (EDVD) and to calculate the *shortening fraction* (SF) and *ejection fraction* (EF) by applying Teicholz's formula(55, 56). Shortening fraction, that has long been considered a surrogate marker of ventricular function, is the percentage the ventricular diameters shorten during contraction and is calculated as [SF= (EDVD–ESVD)/EDVD](57). Ejection fraction is defined as the percentage of blood ejected in each heart cycle; in order to calculate it the diameters are elevated to a cube obtaining volumes instead of diameters [EF= ($EDVD^3$–$ESVD^3$)/$EDVD^3$].

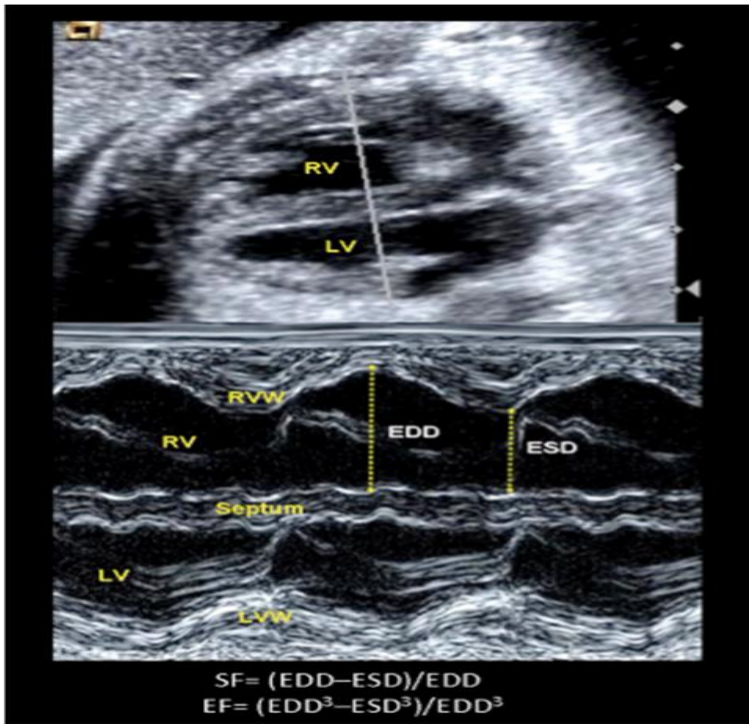


Figure 5. *Illustration of a transverse four-chamber view in order to measure shortening (SF) and ejection fractions (EF) of the right (RV) and left ventricles (LV) by M-mode. The arrows between the septal and right free walls show the measurement of end-diastolic (EDD) and end systolic (ESD) diameters required for the SF and EF calculation. RVW= right ventricle wall; LVW= left ventricle wall.*

- ***Long-Axis Cardiac Evaluation*** *(Figure 6):* M-mode can be also applied in the long axis of the heart (apical or basal 4-chamber view). Also known as long-axis displacement (LAD) or motion (LAM), this approach is most suited to right ventricle examination owing to the longitudinal nature for right ventricle muscle fibers as opposed to the mainly circumferential orientation of the left ventricle muscle fibers. The beam is positioned at 0° to measure the maximum excursion of the area of junction between the tricuspid annulus and the right ventricle free wall from end-diastole to end-systole (referred to also as TAPSE, tricuspid annular plane systolic excursion) and between the mitral annulus and the left ventricle free wall (MAPSE)(58). Normal ranges for the fetus are published(59) and recently validated for measuring longitudinal axis motion in the IUGR fetuses(60). These measurements have been proposed as sensitive markers of cardiac dysfunction as they reflect global longitudinal function(58, 59). The most important thing to take into account when using this technique is that the measurement is not reliable when done not in the correct view or angle.

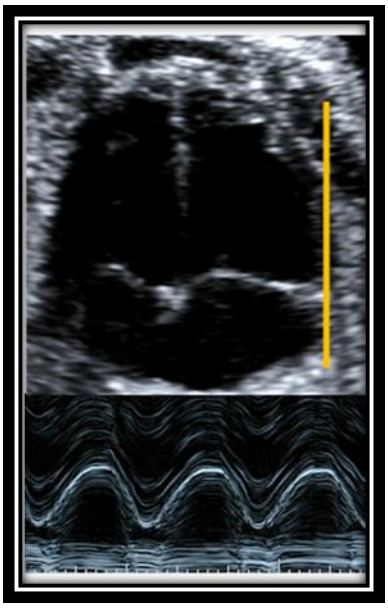


Figure 6. *Illustration of an apical four-chamber view for evaluating long-axis motion (maximum displacement) at the tricuspid annulus (TAPSE).*

- **Tissue Doppler Imaging or** Doppler myocardial imaging is a technique that evaluates myocardial velocities within the ventricular walls; particularly ventricular motion in the long-axis, from the apex to the base(61). This technique has been widely used in the adult patient to diagnose diastolic heart failure, and has also been described as applicable to fetuses(62). It is a reproducible echocardiographic technique that uses frequency shifts in ultrasound waves to calculate myocardial velocity, which is characterized by a lower velocity and higher amplitude. TDI can be applied online to evaluate annular or myocardial velocities and can be performed in spectral and color-coded modes. *Spectral TDI* (S-TDI) (Figure 7) can be performed in an apical o basal 4-chamber view, the 2D scan area is reduced, placing a sample volume (2-4mm) in the basal part of the ventricle or annulus. The insonation beam is maintained at an angle of <30°. The velocity of myocardial movement toward the Doppler cursor is displayed as a waveform moving towards the atria(61). The peak annular velocities obtained are E' or Ea (early diastolic annular peak velocity), A' or Aa (late-diastolic annular peak velocity) and S' or Sa (systolic annular peak velocity) during the ventricular systole. S-TDI also allows time periods to be calculated to obtain the myocardial performance index (MPI'): isovolumetric contraction time (ICT'), ejection time (ET'), and isovolumetric relaxation time (IRT'). MPI' is calculated as (ICT' + IRT')/ET'(63). TDI has been shown to be feasible in fetuses(62) and normal ranges have been published for velocities and MPI'(64). The peak annular velocities evaluated at mitral or tricuspid annuli reflect global systolic (S') or diastolic (E' and A') myocardial motion and have been demonstrated to early and sensitive markers of cardiac dysfunction(65).

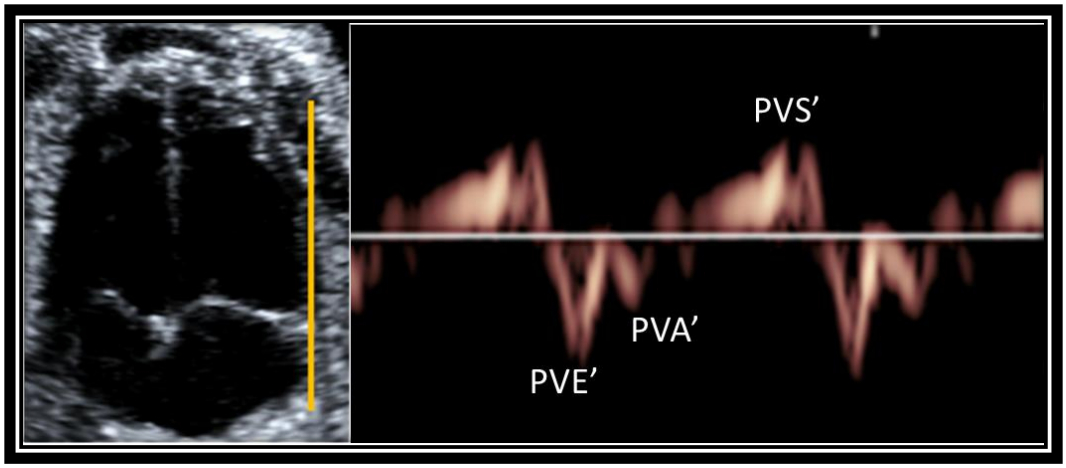


Figure 7. Example of an early (E') and late (A') diastolic and systolic (S') peak annular velocities obtained by spectral tissue Doppler at the right annulus.

In the present thesis, we planned to conduct a comprehensive fetal echocardiographic assessment including morphometric measures to assess remodeling and Doppler and M-mode techniques to also assess function.

1.4.- FETAL GROWTH IN ASSISTED REPRODUCTIVE TECHNOLOGIES

Large human studies have demonstrated a significant association of ART and SGA. Fetal growth restriction has also been associated with ART in animal models, with IVF-conceived embryos, fetuses, placenta and offspring. Findings related it directly with culture media in both, animals and humans (21, 26, 66, 67).

On the other hand, LBW associates long-term cardiovascular disease with increased risk of coronary artery disease, stroke, hypertension and diabetes in adulthood (68). It has also been described that SGA fetuses and children present less efficient and remodeled hearts (42). SGA fetuses show more globular hearts together with signs of systolic and diastolic dysfunction that persist postnatally up to preadolescence (69). Given, the frequent association of ART with SGA, it could be postulated that cardiovascular changes in ART fetuses might be mediated by fetal growth impairment. The fourth specific objective of this thesis was to compare cardiovascular structure and function in ART versus SGA fetuses ***(STUDY 4).***

1.5.- ART AND CHILD CARDIOVASCULAR HEALTH

As explained above vascular dysfunction has been associated to ART in late childhood and adolescence (18-20, 22, 39, 70, 71). However, the particular cardiac structure of children conceived by ART has not been evaluated. Therefore, the fifth and

last objective of this thesis was to evaluate cardiac structure and function of children conceived by ART ***(STUDY 5).***

Regarding pediatric cardiovascular assessment, the standard views and planes are different from fetuses; with the advantage that the evaluation can be performed directly over the heart. Left ventricle is the one routinely scanned more than the right ventricle. Cardiovascular child evaluation was performed using Vivid Q (General Electric Healthcare, Horten, Norway). Children were studied when resting quietly or asleep. A complete two-dimensional M-mode and Doppler echocardiographic examination, with a 10S-RS phased-array 4.5-11.5 MHz transducer, was performed to assess structural heart integrity and morphometry, very similar to the fetal evaluation. The imaging planes are identified by transducer location (apical, parasternal) and by the plane of examination relative to the heart (4-chamber, long-axis, and short-axis). In addition, imaging planes may be described as anatomic planes: sagittal, parasagittal, transverse, or coronal(72, 73). Cardiac morphometry was assessed by measuring atrial areas, ventricular sphericity index and wall thicknesses. Systolic function was evaluated by estimating stroke volume, heart rate, cardiac output, shortening fraction, ejection fraction, mitral and tricuspid annular plane systolic excursion (MAPSE, TAPSE) and systolic annular peak velocities (S'). Diastolic function was assessed by isovolumetric relaxation time (IRT), peak early (A) and late (A) transvalvular filling velocities, E/A ratio, E deceleration time, A wave duration time, early-diastolic (E') and atrial contraction (A') annular peak velocities, E/E' ratio, E'/A' ratio, isovolumetric relaxation time by TDI (IRT').

In addition, several vascular measurements can also be performed in postnatal life. Apart from blood pressure, ultrasound assessment of carotid arteries' intima-media thickness (cIMT) has been proposed as a risk factor for cardiovascular disease(74, 75) (76). Ultrasound assessment of carotids has become the basis for many clinical studies because it is a high-resolution, noninvasive technique. It is rapidly applicable, readily available and demonstrates the wall structure. Its measurement has been validated in infancy and childhood. Actually, Vascular changes in newborns have been assessed specifically about IMT; founding increased in those neonates experiencing compromised growth(74) and recently in those children born after ART(22); suggesting a sequence of prenatal events leading to arterial thickening.

In this thesis, we were planning to perform a comprehensive infant echocardiography (using similar parameters as in fetal life) complemented by vascular assessment using cIMT ultrasound and blood pressure measurement.

In conclusion, the general hypothesis of this thesis was that fetuses conceived by ART associate a worse perinatal outcome and cardiovascular remodeling and dysfunction. In order to explore this hypothesis, we followed five specific objectives and constructed 4 cohorts including ART and spontaneously conceived pregnancies recruited in the prenatal period and followed-up to infancy.

2. HYPOTHESIS

2. HYPOTHESIS

2.1 MAIN HYPOTHESIS:

Fetuses conceived by assisted reproductive technologies (ART) present worse perinatal outcome together with cardiovascular remodeling and dysfunction as compared to spontaneously conceived pregnancies.

2.2 SPECIFIC HYPOTHESIS:

1. Pregnancies conceived by ART present a higher prevalence of pregnancy complications (such as prematurity and SGA) as compared to spontaneously conceived pregnancies.
2. Singleton fetuses conceived by ART present cardiovascular remodeling and dysfunction.
3. Twin fetuses conceived by ART present cardiovascular remodeling and dysfunction.
4. Cardiovascular changes observed in fetuses conceived by ART are independent from fetal growth restriction.
5. Cardiovascular remodeling and dysfunction associated to ART persist postnatally.

3. OBJECTIVES

3. OBJECTIVES

3.1 MAIN OBJECTIVE:

To evaluate perinatal outcomes and cardiovascular structure/function in fetuses and children conceived by ART.

3.2 SPECIFIC OBJECTIVES:

1. To compare perinatal outcomes in spontaneously conceived pregnancies and those conceived by different types of ART.

2. To assess cardiovascular structure and function in ART and spontaneously conceived singleton fetuses and infants.

3. To assess cardiovascular structure and function in ART and spontaneously conceived twin fetuses.

4. To assess the differential effect of ART and SGA on fetal cardiac structure and function.

5. To assess cardiovascular structure and function in ART and spontaneously conceived children.

4. METHODS

4. METHODS

4.1 STUDY DESIGN AND VARIABLES

General study design

Between 2004 and 2016, 4 cohort studies were constructed including pregnant women attended at the Department of Obstetrics, Gynecology and Neonatal Medicine of Hospital Clinic, Barcelona, Spain:

Cohort 1: A retrospective cohort study comprising 1260 pregnancies including 206 fertile women with naturally conceived pregnancies sampled from a low-risk population, together with 1054 infertile women evaluated and/or treated at the Infertility and Assisted Reproduction Unit and further stratified as infertile women who spontaneously conceived before any fertility treatment was started (infertile without treatment); conception by ovarian stimulation; or conception by IVF and/or ICSI. This cohort as used to assess the first specific objective of this thesis (STUDY 1).

Cohort 2: A prospective cohort study including 100 singleton pregnancies conceived by IVF and/or ICSI in infertile patients and 100 control pregnancies conceived naturally identified in fetal life and followed up to 2 to 4 years of age. This cohort was used for objectives 2 and 5 (STUDIES 2 and 5).

Cohort 3: A prospective cohort study including 50 twin fetuses conceived by ART and 50 twins spontaneously conceived. This cohort was used for objectives 3 (STUDY 3).

Cohort 4: A prospective cohort study including singleton pregnancies born at term, subdivided into four groups: 102 appropriate for gestational age (AGA) fetuses conceived spontaneously (controls), 72 AGA fetuses conceived by ART (ART-AGA), 31 SGA fetuses conceived by ART (ART-SGA) and 28 SGA conceived naturally. This cohort was used for objectives 4 (STUDY 4).

The exclusion criteria for all the cohorts were fetal or neonatal infection, chromosomal abnormalities, structural malformations, monochorionic twins and preexisting maternal diseases such as alcohol dependence syndrome, chronic hypertension, insulin-dependent and non-insulin dependent diabetes mellitus, heart disease, type B hepatitis, positive HIV serology.

All study protocols were approved by the IRB at Hospital Clinic, and written parental consent was obtained for all study participants.

Study variables

Study variables included parental baseline and fertility characteristics, fetal ultrasound data, perinatal outcomes and infant cardiovascular data were collected

Parental baseline characteristics also were collected by parental interview and review of medical records at the time of prenatal evaluation including parental age, body mass index, smoking status, ethnicity, parity, cardiovascular history, socioeconomic status, educational level. Upon delivery, presence of pregnancy complications

(gestational diabetes, preeclampsia, placenta praevia, small for gestational, gestational age and preterm delivery), mode of delivery, birthweight, birthweight centile(77), Apgar score, umbilical artery pH and perinatal morbidity were recorded.

Fertility characteristics were recorded including infertility, type of infertility (primary or secondary), infertility cause and ART technique characteristics.

Pregnancy and perinatal characteristics were retrieved from medical records or parental interview. Gestational dating was performed by known last menstrual period (LPM), oocyte retrieval or intrauterine insemination, and gestational age was calculated based on the crown-rump length (CRL) obtained at first trimester ultrasound(78). Number of fetuses, complications (small for gestational age, preeclampsia, preterm delivery, placenta previa, gestational diabetes), date of delivery (gestational age, singleton, multiple, mode of delivery, indication, complications). Neonatal data (gender, birth weight, percentile height, Apgar score, cord venous and arterial pH, days in NICU, mechanical ventilation, neonatal morbidity) and childhood data (height, weight, BMI, blood pressure, concomitant illness, medical treatment, hospital admissions and cause).

Fetal and postnatal cardiovascular characteristics are described below.

Study variables and outcome definitions

The following definitions were established for this project:

- **Fertile**: with no difficulty to conceive before 12 months of regular unprotected sexual intercourse and/or less than 3 spontaneous abortions(1, 2)
- **Infertility**: failure to conceive after ≥12 months of regular unprotected sexual intercourse in women <35 years of age, or ≥6 months in women ≥35 years (1, 2).
    - Primary, when a woman is unable to ever bear a child, either due to the inability to become pregnant or the inability to carry a pregnancy to a live birth.
    - Secondary, inability to become pregnant following a previous pregnancy or a previous ability to carry a pregnancy to a live birth.
- **ART** (assisted reproductive technologies): all treatments or procedures that include the *in vitro* handling of both human oocytes and sperm, or embryos, for the purpose of establishing a pregnancy. ART does not include assisted insemination (artificial insemination) using sperm from either a woman's partner or a sperm donor(1, 2, 5)
- **IVF** (*in vitro* fertilization): an ART procedure that involves extracorporeal fertilization(1, 2, 5).
- **ICSI** (intracytoplasmic sperm injection): a procedure in which a single spermatozoon is injected into the oocyte cytoplasm(1, 2, 5).
- **Natural cycle**: an IVF procedure in which one or more oocytes are collected from the ovaries during a spontaneous menstrual cycle without any drug use(1, 2).

- **Ovulation Induction** (OI): pharmacological treatment of women with anovulation or oligo-ovulation with the intention of inducing normal ovulatory cycles(1, 2).
- **Embryo:** the product of the division of the zygote to the end of the embryonic stage, eight weeks after fertilization(1, 2).
- **Frozen embryo cycle**: an ART cycle in which frozen (cryopreserved) embryos are thawed and transferred to the woman(1, 2, 5).
- **Blastocyst**: an embryo, five or six days after fertilization, with an inner cell mass, outer layer of trophectoderm and a fluid-filled blastocele cavity(1, 2).
- **Vanishing twin**: spontaneous disappearance of one or more gestational sacs or embryos in an ongoing pregnancy, documented by ultrasound(1, 2).
- **Small for gestational age (SGA):** the presence of birth weight below the 10^{th} centile according to local growth curves(79)
- **Preeclampsia:** defined as resting blood pressure of above 140/90 mmHg on 2 occasions at least 4 hours apart, and the presence of proteinuria (300 mg or more in 24 hours), beyond 20 weeks of pregnancy in previously normotensive women(80)
- **Preterm delivery (PTD):** was defined by spontaneous delivery before 37 weeks of gestation.
- **Gestational diabetes (GD):** Carbohydrate intolerance of variable severity with onset or first recognition during current pregnancy(81).
- **Placenta previa:** the placenta implanted wholly or partly over the internal cervical os(82).
- **Perinatal mortality**: defined as either intrauterine death or neonatal death within the first 28 days of life.

- **Perinatal morbidity:** defined by the presence of bronchopulmonary dysplasia, hyaline membrane disease, necrotizing enterocolitis or neonatal sepsis.

4.2 ASSISTED REPRODUCTIVE TECHNOLOGIES PROTOCOL

All ART patients included in this thesis were treated accordingly to a Hospital Clinic´s routine used protocol. All patients received standard ovarian stimulation with follicle-stimulating hormone (FSH) under pituitary suppression with agonist gonadotrophin releasing hormone according to a routinely used protocol(83). In all women, pituitary desensitization was achieved by sub-cutaneous administration of triptorelin acetate (Decapeptyl 0.1 mg; Ipsen Pharma, Barcelona, Spain / 0.1 mg daily, which was reduced to 0.05 mg after ovarian arrest was confirmed) started in the mid-luteal phase of the previous cycle. Gonadotropin stimulation of the ovaries was started when serum estradiol concentrations declined to <50 pg/ml and a vaginal ultrasonographic scan showed an absence of follicles >10 mm diameter. On days 1 and 2 of ovarian stimulation, 450 IU and 300 IU/day of recombinant r-FSH (Gonal-F; Merck-Serono S.A., Madrid, Spain), respectively, were administered subcutaneously. On days 3 and 4 of ovarian stimulation, 150 IU per day of FSH were administered to each patient. From day 5 onward, FSH was administered on an individual basis according to the ovarian response as assessed by sequential transvaginal ultrasonography and serum estradiol measurements. The criteria for administration recombinant human chorionic gonadotrophin (r-hCG; 250 µg) (Ovitrelle; Merck-Serono S.A.) were the presence of ≥2 follicles ≥18 mm in diameter with ≥4 follicles measuring ≥14 mm in association with a consistent rise in serum estradiol concentration. Oocyte aspiration was performed with vaginal ultrasonography 35-36 h after r-hCG administration. Cumulus-oocyte complexes were collected in Flushing Medium (MediCult, Denmark), and cultured in IVF Medium (MediCult, Denmark). In ICSI cases, cumulus cells were removed by recombinant hyaluronidase (MediCult, Denmark) treatment after 2 hours of culture and the denuded

oocytes placed in 50 µl droplets of ISM1 Medium (MediCult, Denmark) covered with paraffin oil (Scandinavian IVF, Sweden) until insemination.

The ICSI procedure consists on the injection of a single sperm into the cytoplasm of an oocyte using a set of micromanipulation devices adapted to a microscope. The oocyte is held by a holding pipette and a microinjection pipette in the opposite side is used to collect, immobilize, aspirate and inject the sperm into the ooplasm. After the sperm is released into the oocyte, the microinjection pipette is withdrawn gently and the injected oocyte is released from the holding pipette. In conventional IVF, oocytes were inseminated with 100.000 motile spermatozoa /ml in an embryo tested four-well dish containing IVF Medium covered with paraffin oil. In both cases, conventional IVF and ICSI, insemination was performed 40 hours after hCG administration.

Fertilization was assessed by the presence of two pronuclei and two polar bodies 18-20 hours post-insemination. In conventional IVF cases, cumulus-enclosed oocytes were stripped by gentle pipetting to check fertilization. Normally fertilized ICSI / IVF oocytes were then cultured individually in 20 µl drops of ISM1/BlastAssist Medium (MediCult, Denmark) covered with oil until the day of embryo transfer. The culture of oocytes and embryos was performed at 37º C temperature in a humidified atmosphere containing 6.5% CO_2 in air to maintain the pH between 7.2 and 7.4.

Embryos were evaluated using the dynamic system of embryo scoring proposed by European Society of Human Reproduction and Embryology[84] Briefly, the criteria for assessing an embryo as morphologically optimal were as follow: (i) day 2 optimal embryos had 4 equal blastomeres, less than 20% fragmentation and no multinucleation;

(ii) day 3 optimal embryos were those with 8 equal blastomeres, less than 20% fragmentation and no multinucleation that had 4 blastomeres in day 2.

Ultrasound-guided transfer of 1 to 3 embryos per patient (depending on the age of the patient, the indication for IVF/ICSI, the quality of embryos available per replacement and couple´s decision) was performed on Day 2 / Day 3 or Day 5 after oocyte retrieval and the supernumerary good quality embryos cryopreserved between day 2 and 6.

The luteal phase was supported with vaginal micronized progesterone (600 mg/day given at 8 hours intervals) starting on the day following oocyte aspiration and continuing either up to menstruation, or if the patient became pregnant, for at least the first 3 weeks of pregnancy.

Pregnancy was diagnosed by increasing serum concentrations of β-hCG measured 12-13 and 19-20 days after embryo transfer, and the subsequent demonstration of an intrauterine gestational sac by ultrasonography carried out 12-14 days after the second β-hCG determination.

4.3 FETAL ULTRASONOGRAPHIC EVALUATION

All pregnancies from studies 2 to 4 underwent ultrasonographic examination at third trimester of gestation using a Siemens Sonoline Antares (Siemens Medical Systems, Malvern, PA, USA) with 6–4-MHz linear curved-array and 2-10 MHz phased-array probes.

4.3.1. Conventional fetoplacental evaluation

The evaluation included conventional feto-placental assessment and fetal echocardiography. Estimated fetal weight was calculated according to the method of Hadlock et al(85); both estimated fetal weight and birth weight centile were calculated using local reference curves(77). Doppler examination was performed in absence of maternal or fetal movements, and using a high-wall filter (70Hz) to avoid noise and clutter signals. The angle of insonation was maintained as close as possible to 0º. Feto-placenta Doppler evaluation included:

- ***Mean uterine artery pulsatility index (UtA-PI)*** was obtained 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; measurement was obtained 1 cm distal to the crossover point(86). The PI of the left and right arteries was measured, and the mean PI was calculated.
- ***Umbilical artery (UA)*** PI was measured from a free-floating cord loop. Normal UA was considered as a PI below the 95th percentile(87). Presence, absence (AEDV) or reversal (REDV) of the end-diastolic velocity was also recorded.

- ***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. The ***cerebroplacental ratio (CPR)*** was calculated dividing the middle cerebral artery PI by the umbilical artery PI. Both parameters were considered abnormal if below the 5th percentile, and indicative of cerebral blood flow redistribution(87, 88).
- ***Ductus venosus (DV)*** PI was measured in a mid-sagittal or a transverse section of the fetal abdomen, positioning the Doppler gate at the isthmic portion. Normal DV was considered as a PI below the 95th percentile(89). Presence (PAV), absence/reversal (RAV) of the 'a' wave was also recorded.
- ***Aortic isthmus (AoI)*** PI was measured either in a sagittal view of the fetal thorax with clear visualization of the aortic arch, placing the gate a few millimetres beyond the origin of the left subclavian artery; or in a cross-sectional view of the fetal thorax, at the three vessels and trachea view, placing the gate just before the convergence of the AoI and the arterial duct(90, 91). Normal AoI was considered as a PI below the 95th percentile(90). The ***isthmus flow index (IFI)*** was calculated dividing (systolic+diastolic)/systolic velocity-time integrals (VTI); it was considered abnormal below the 5th percentile(92).

4.3.2. Fetal echocardiography

Fetal echocardiography included a comprehensive examination to assess structural heart integrity and morphometry, and also systolic and diastolic function parameters. Cardiac dimensions were measured on 2D images from an apical four-chamber view.

- ***Cardiac morphometry*** included cardio-thoracic ratio, atrial areas, foramen ovale diameter, ventricular sphericity indices and wall thicknesses. The circumference of the heart and the circumference of the thorax were measured in end-diastole and expressed as a cardio-thoracic ratio.[93] Left and right atrial areas and foramen ovale were measured on 2D images from an apical four-chamber view at maximum point of distention.[94, 95] Left and right atrial areas were normalized by heart area and estimated fetal weight. Left and right ventricular sphericity indices were calculated as base-to-apex length / basal diameter.[96] Ventricular end-diastolic septal and free wall thicknesses were measured by M-mode from a transverse four-chamber view and normalized by the transverse cardiac diameter.[94]

- ***Ventricular systolic function*** evaluation included ejection fraction, heart rate, stroke volume, cardiac output, mitral/tricuspid displacement (MAPSE/TAPSE) and systolic annular peak velocities (S'). Left and right ventricular ejection fraction (%) was obtained from M-mode transverse four chamber view and estimated by Teicholz's rule.[97] Left and right stroke volumes were calculated as π / 4 × (aortic or pulmonary valve diameter) × (aortic or pulmonary artery systolic velocity-time

integral).[98] Then, left and right cardiac outputs were calculated as left/right stroke volume x heart rate. Finally, combined cardiac output was calculated as the sum of both.[98] Diameters of the aortic and pulmonary valves were measured in three different cardiac cycles in still frame images at systole from the leading-edge to the leading-edge and their mean value was averaged and used for further analysis. Aortic and pulmonary artery systolic velocity-time integral were obtained in a long or short axis view of the fetal heart, and were calculated by planimetering the area underneath the Doppler spectrum. MAPSE and TAPSE were assessed by M-mode from an apical or basal four chamber view by placing the cursor at a right angle to the atrioventricular junction, marked by the valve rings at the mitral, tricuspid and basal septum respectively.[99] Tissue Doppler Imaging was applied in the spectral Doppler mode to record systolic peak velocities (S') at mitral lateral and septal annulus, and tricuspid lateral annulus from an apical or basal four-chamber view, and measured in real time during echocardiographic study.[100]

- ***Diastolic function*** was evaluated by peak early and late transvalvular filling (E/A) ratio, deceleration time of E velocity, A duration, early (E') diastolic annular peak velocities, E/E' ratio and left isovolumetric relaxation time (IRT). Atrioventricular (AV) flows were obtained from a basal or apical four-chamber view, placing the pulsed Doppler sample volume at the tip of AV valve leaflets. Right and left E/A ratios were estimated by calculating the ratio between early ventricular filling (E wave) to late ventricular filling (A-wave).[101] Deceleration time of the E wave was measured from mitral and tricuspid inflow velocities from an apical four-chamber view. Tissue Doppler Imaging was applied in the spectral Doppler mode to record early diastolic (E') peak velocity at mitral lateral and septal annulus, and tricuspid

lateral annulus from an apical or basal four-chamber view.[100] Mitral lateral and septal E/E' ratios were measured as previously described.[102] Left IRT was measured from the closure of the aortic valve to the opening of the mitral valve.[103]

All fetal cardiac parameters were evaluated as crude values and also normalized by gestational age at scan into z-scores(99, 100, 103) or adjusted by estimated fetal weight or cardiac size.(98)

4.4 INFANT CARDIOVASCULAR ASSESSMENT

Postnatal evaluation in studies 2 and 5 included anthropometric measurements (infants' height, weight, body mass index and body surface area), echocardiography, blood pressure measurement and vascular ultrasound.

4.4.1. Infant's echocardiography

Echocardiography was performed following a standardized protocol(104) using a Vivid q (General Electric Healthcare, Horten, Norway) with 2-10 MHz phased-array transducer. Infants were studied when resting quietly or asleep. A complete two-dimensional M-mode and Doppler echocardiographic examination was performed initially to assess structural heart integrity and morphometry.

- ***Cardiac morphometry*** included atrial areas, left and right sphericity indexes and wall thicknesses. Left and right atrial planimetric areas were measured on a 2D image from an apical four-chamber view at end-systole (greatest dimension, just before mitral or tricuspid valve opening). Ventricular base-to-apex length and transverse diameter were measured on a 2D image from an apical four-chamber view at end-diastole. Left and right ventricular sphericity indexes were calculated as base-to-apex length/mid-transverse diameter, Ventricular end-diastolic septal and lateral free wall thicknesses were measured by M-mode from a parasternal long-axis view.[104, 105] Systolic function evaluation included shortening fraction,

cardiac output, TAPSE and annular systolic peak velocities by tissue Doppler.[106] Left shortening fraction was calculated from internal ventricular diameters obtained from a parasternal long-axis view by M-mode, using the equation (end-diastolic dimension – end-systolic dimension)/ end-diastolic dimension.

- ***Left and right stroke volumes*** were calculated as π/4 x (aortic or pulmonary valve diameter)^2 x (aortic or pulmonary artery systolic flow velocity-time integral). Left and right cardiac outputs were calculated as stroke volume*heart rate. Diameters of the aortic and pulmonary valves were measured in frozen real-time images during early to mid-systole by the leading-edge-to-edge method; aortic diameter was obtained from the parasternal long-axis view, while the pulmonary artery diameter was obtained in a parasternal short-axis view.[106] Ascending aorta flow velocity integral was measured with pulsed Doppler from an apical five-chamber view, and the pulmonary artery flow velocity integral was recorded from a standard parasternal short-axis view with the sample volume placed immediately distal to the pulmonary valve. Velocity-time integrals were calculated by manual trace of the spectral Doppler area. TAPSE was measured real time in an apical four-chamber view, by placing the M-mode cursor at the atrioventricular junction, marked by the tricuspid valve rings at the right free wall. Maximum amplitude of motion was taken as the extent of displacement between end-systole and end-diastole, and measured in millimeters. Tissue Doppler was applied at mitral and tricuspid lateral annuli from an apical four-chamber view, to record S' in centimeters/second.[106]

- ***Diastolic function*** was evaluated by E/A ratios, deceleration time of E velocity, E' and left IRT. Atrioventricular flow velocities were obtained from an apical four-chamber view, placing the pulsed Doppler sample volume just below the valve leaflets. E deceleration time was measured as the time from the maximum mitral/tricuspid velocity to the baseline. Tissue Doppler was applied at mitral and tricuspid lateral annuli from an apical four-chamber view, to record E' in centimeters/second. Left IRT was obtained from the pulsed Doppler waveform of the aortic blood flow, from the end of the aortic wave to the beginning of the mitral early filling wave.

4.4.2. Blood pressure assessment

- ***Systolic and diastolic blood pressure*** was obtained from the brachial artery using a validated ambulatory automated Omron 5 Series device, at the beginning of the medical evaluation by a trained nurse, while the neonate was resting.

4.4.3. Vascular ultrasound

Carotid and aorta ultrasound assessment was performed by skilled sonographers using a Siemens Sonoline Antares (Siemens Medical Systems, Malvern, PA, USA). Longitudinal clips of the far wall of both carotid arteries were obtained approximately 1

cm proximal to the bifurcation using a 13-MHz linear-array transducer. Longitudinal clips of the far wall of the proximal abdominal aorta were obtained in the upper abdomen by a 10-MHz linear probe. Carotid and aorta IMT measurements were performed offline according to a standardized protocol based on a trace method with the assistance of a computerized program (Siemens Syngo Arterial Health Package). To obtain IMT, three end-diastolic still frames were selected across a length of 10 mm and analyzed for mean and maximum IMT, and the average reading from these three frames was calculated (107). IMT results were normalized by neonatal weight.

4.5 STATISTICAL ANALYSES

Data was analyzed for all cohorts using the IBM SPSS Statistics 19 statistical package. Comparisons between the study groups for descriptive statistics were done with Student's t test or χ^2 test where appropriate, and are presented as mean SD, median (interquartile range) or percentage (%). Specific analysis realized for each study are described below.

5. STUDIES

5. STUDIES

STUDY 1.

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ORIGINAL ARTICLE

Differential effect of mode of conception and infertility treatment on fetal growth and prematurity

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Abstract

Objectives To examine perinatal outcomes in pregnancies conceived by different methods: fertile women with spontaneous pregnancies, infertile women who achieved pregnancy without treatment, pregnancies achieved by ovulation induction (OI) and *in vitro* fertilization or intra-cytoplasmic sperm injection (IVF/ICSI).
Methods Retrospective single-center cohort study including 200 fertile and 748 infertile women stratified according to infertility treatment. The outcome measurements were preterm delivery (PTD), small-for-gestational-age (SGA), gestational diabetes, placenta previa or preeclampsia.
Results The overall rate of pregnancy complications was significantly increased in all infertility groups regardless of the infertility treatment (adjusted odds ratio (OR): infertile without treatment 2.3 versus OI 2.2 versus IVF/ICSI 3.4). While PTD was mainly associated to IVF/ICSI (adjusted OR: infertile without treatment 1.3 versus OI 1.6 versus IVF/ICSI 3.3), SGA was significantly associated to both OI and IVF/ICSI (adjusted OR: infertile without treatment 1.9 versus OI 2.7 versus IVF/ICSI 2.6). All these associations remained statistically significant after adjusting by maternal age and twin pregnancy.
Conclusions This study confirms the higher prevalence of pregnancy complications in infertile women irrespectively of receiving infertility treatment or not, and further describes a preferential association of prematurity with IVF/ICSI, and SGA with treated infertility (OI and IVF/ICSI).

Keywords

Assisted reproductive technologies, intrauterine growth restriction, low birth weight, perinatal outcome, small for gestational age

History

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Introduction

Assisted reproductive technologies (ART) are currently used worldwide, with the estimated number of children born using these techniques per year ranging between 173 000 and 230 000, and multiple pregnancy rates ranging from 5.7% (Sweden) to 38.3% (Serbia) [1]. Although most pregnancies after infertility therapies result in normal healthy outcomes, an increased risk for obstetric and perinatal complications such as preterm delivery (PTD), low birth weight (LBW), small for gestational age (SGA) [2–4], preeclampsia [5–7], gestational diabetes and placenta praevia [8] has been reported in singleton and twin ART pregnancies [6,9–11] as compared to spontaneously conceived ones. While most studies have focused on *in vitro* fertilization (IVF) or intra-cytoplasmic sperm injection (ICSI), perinatal outcomes by other infertility treatments such as ovulation induction (OI) seem also to be affected. Studies evaluating the effect of OI with gonadotropins on perinatal outcomes, controlling for maternal age and parity, have demonstrated an increased risk for PTD, LBW and SGA in singletons compared with spontaneous conception. Evidence that suggests perinatal outcomes among pregnancies conceived in cycles stimulated with clomiphene citrate (CC) are less favorable than those which are spontaneously conceived, but better than those of children born after IVF [12,13].

The mechanism responsible for adverse perinatal outcomes in ART is unknown and conflicting results have been published maintaining unresolved whether ART procedures or subfertility itself leads to these changes [3]. In order to evaluate the differential effect of infertility and mode of conception on perinatal outcomes, we designed a study

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including four different groups: a) fertile women with naturally conceived (spontaneous) pregnancies, b) infertile women achieving pregnancy without treatment, c) infertile women achieving pregnancy with OI medication and d) infertile women achieving pregnancy with IVF or ICSI. Perinatal results were retrieved and compared among the different groups.

Materials and methods

Study population

The study design was a retrospective single-center cohort study comprising 1260 pregnancies including 206 fertile women with naturally conceived pregnancies sampled from the general population, together with 1054 infertile women evaluated and/or treated at the Infertility and Assisted Reproduction Unit, Hospital Clínic (Spain) from 2004 to 2010. Only pregnancies that were treated, followed-up and delivered at our center were included. The study population was stratified according to the type of conception and infertility treatment into the following: (1) fertile women with naturally conceived pregnancies; (2) infertile women who spontaneously conceived before any fertility treatment was started (infertile without treatment); (3) conception by OI with CC or injectable gonadotrophin; and (4) conception by IVF and/or ICSI. The study protocol was approved by the Hospital's Ethical Committee and written patient consent was obtained from all patients. Fertile women were defined as pregnant patients with no history of infertility, no difficulty to conceive before 12 months and less than three spontaneous abortions. Infertility was defined as those patients who initiated care with failure to conceive for $\geq$12 months in women <35 years of age, or $\geq$6 months in women $\geq$35 years [14]. Ovarian stimulation was defined as the use of ovulation inducing drugs (gonadotropins, CC) in order to obtain an adequate follicular response. All IVF/ICSI patients received standard ovarian stimulation, with follicle-stimulating hormone (FSH), under pituitary suppression with agonist gonadotropin releasing hormone according to a routinely used protocol (Supplementary Digital Content, Supplementary Methods). Oocyte aspiration was performed through vaginal ultrasound guidance. Number and timing of embryo transfer was individualized according to clinical indicators.

Baseline and perinatal characteristics

Parental baseline characteristics were collected by parental interview and review of medical records, including maternal characteristics such as age, smoking, gravidity, parity, medical disease and infertility history. Maternal medical disease was defined as the presence of any disease diagnosed previous to infertility treatment, such as lupus, hypertension or diabetes. Fertility characteristics were recorded including infertility time period before initiating treatment, type of infertility (primary or secondary), cause of infertility and need for spousal treatment.

Gestational dating was performed by known last menstrual period (LPM), oocyte retrieval or intrauterine insemination, and gestational age was calculated based on the crown-rump length (CRL) obtained at first trimester ultrasound [15]. The presence of the following pregnancy characteristics was recorded: twin pregnancy, gestational diabetes, preeclampsia, placenta praevia, PTD, SGA and LBW. PTD was defined as delivery between 20 and 37 weeks' gestation. SGA was defined as the presence of birth weight below the 10th centile according to local growth curves [16] and LBW as birth weight below 2500 g. Adverse pregnancy outcome was defined by the presence of any of the following: gestational diabetes, placenta praevia, PTD, preeclampsia or SGA. Upon delivery, gestational age at delivery, mode of delivery, neonatal gender, birth weight, birth weight centile, Apgar score, umbilical artery pH and data on perinatal morbidity were recorded.

Statistical analysis

Data were analyzed using the IBM SPSS Statistics 19 statistical package. Comparisons between the study groups were done with Student's t test or χ^2 test where appropriate, and are presented as median (interquartile range) or percentage (%). To compare outcomes across all four study groups, analysis of variance (ANOVA) was performed with Bonferroni post-hoc contrasts conducted on all variables that were significantly different. Parameters were adjusted with linear regression for maternal age, smoking, maternal disease and twin pregnancy. p values below 0.05 were considered statistically significant.

Logistic regression analysis was used to explore the association of the different infertility treatments with pregnancy outcome (adverse pregnancy outcome, PTD, SGA), in order to obtain odds ratio (OR) and 95% confidence intervals (CI) when compared to the spontaneous conception group. Decision tree analysis was performed using the CHAID (χ^2 automatic interaction detection) method, which creates a tree based classification model where, by means of regression models, the best predictor variables for an outcome are selected and presented. The significance level was established at 0.05, where at each step or level CHAID chose the independent or predictor variable (i.e. infertility group) that had the strongest interaction with the dependent variable (perinatal outcomes).

Results

Study population characteristics

This was a retrospective cohort study including 206 fertile women with spontaneously conceived pregnancies were initially included as controls, but six ended with miscarriage leaving 200 fertile pregnancies. From the initial infertile cohort of 1054 pregnant women, 306 pregnancies ended as first trimester miscarriages and were excluded from further data analysis. From the remaining 748 pregnancies, 260 infertile women conceived spontaneously before starting any treatment, 265 conceived using medications to induce ovulation (homologous artificial insemination was not included) and 223 conceived through IVF/ICSI techniques (Figure S1, Supplementary Digital Content).

Maternal basal characteristics and infertility diagnoses of the study populations are listed in Table 1. As expected, the

infertile groups had older mothers and higher prevalence of medical disease, smoking and primiparity as compared to fertile women. Infertile women were more likely to have an ovarian or ovulatory dysfunction diagnosis (25%), most of these in the non-treatment (24%) and OI (44%) groups. In the IVF/ICSI group, the most common infertility causes were due to tubarian (33%) and/or masculine (30%) factors. Spouse treatment was offered for the 8% of the whole infertile cohort.

Perinatal characteristics

Pregnancy characteristics and perinatal results for all study groups are shown in Tables 1 and 2. The prevalence of twins was higher in the infertile groups, particularly in the IVF/ICSI group with a 22% of twins. As expected, infertile groups presented a higher prevalence of overall pregnancy complications with PTD mainly increased in the IVF/ICSI group, while SGA and LBW were more common in both

Table 1. Baseline and pregnancy characteristics of the study groups.

	Fertile n=200	Infertile without treatment n=260	OI n=265	IVF/ICSI n=223	Adjusted p values
Maternal baseline characteristics					
Age (years)	31 (28–35)	32 (29–35)	33 (30–35)*†	34 (32–36)*†‡	<0.001
Age ≥35 years (%)	27	30	30	41* †‡	<0.001
Medical disease (%)	2	21*	22*	18*	0.001
Smoking (%)	11	28*	29*	15†‡	0.816
Primiparity (%)	50	75*	87*†	93*†	<0.001
Fertility characteristics					
Infertility period ≥6months (%)	–	31‡	20†	34‡	<0.001
Infertility cause					
Unexplained (%)	–	34‡	28†	9†‡	0.005
Female (%)	–	53	56	57	0.099
Male (%)	–	12	15	30†‡	<0.001
Female + Male (%)	–	1	1	4†‡	0.953
Spouse treatment (%)	–	6‡	11†	6‡	0.554
Pregnancy characteristics of the study groups					
Twins (%)	1.6	2*‡	5*†	22*†‡	<0.001
Preterm delivery (%)	4	6	8	21*†‡	0.008
Small-gestational age (%)	6	12	18*	21*†	0.056
Low birth weight (%)	3	7	13*	22*†‡	0.008
Gestational Diabetes (%)	4	10	9	8	0.269
Preeclampsia (%)	0	4	1	5	0.630
Placenta praevia (%)	0	2	1	5	0.032
Adverse pregnancy outcome (%)	13	29*	30*	46*†‡	<0.001

Data shown as median (interquartile range) or percentage within the treatment group. p values calculated as ANOVA with Bonferroni correction or χ^2 among the 4 study groups. OI: ovulation induction; IVF: *in vitro* fertilization; ICSI: intracytoplasmic sperm injection. Preterm delivery defined by delivery <37 weeks of gestation. Small for gestational age defined by birth weight <10th centile. Low birth weight defined as birth weight <2500 g. Adverse pregnancy outcome defined by the presence of at least one of the following: gestational diabetes, preeclampsia, placenta praevia, preterm delivery or small for gestational age.

*p <0.05 as compared with controls;

†p<0.05 as compared with non treatment group;

‡p<0.05 as compared with OI group. Adjusted p values calculated by linear regression including maternal age and twin pregnancy.

Table 2. Delivery characteristics of the study groups.

	Fertile n=200	Infertile without treatment n=260	OI n=265	IVF/ICSI n=223	Adjusted p values
Induction of labor (%)	17	23*‡	24	31*‡	0.284
Cesarean section (%)	20	31*	28*	42*	0.797
Gestational age at delivery (weeks)	40 (39–41)	39 (38–40)	39 (38–40)	38 (37–40)*†‡	<0.001
Male (%)	51	54	54	60	0.180
Birth weight (g)	3325 (3040–3550)	3250 (2900–3600)	3160* (2800–3500)	2960*†‡ (2590–3340)	<0.001
Birth weight centile	54 (19–66)	55 (23–84)	52 (20–75)	43†(20–67)	0.696
5 min Apgar score	10 (9–10)	10 (9–10)	10 (9–10)	10 (9–10)	0.236
Umbilical artery pH	7.25 (7.2–7.3)	7.24 (7.2–7.27)	7.26 (7.2–7.3)	7.26 (7.23–7.29)	0.948
Perinatal death (%)	0	1	2	1.5	0.185

Data shown as percentage within the treatment group. p values calculated as χ^2 among the four study groups. Adjusted p values calculated by linear regression including maternal age, smoking and twins.

*p<0.05 as compared with fertile pregnancies;

†p<0.05 as compared with non-treatment group;

‡p<0.05 as compared with OI group.

OI: ovulation induction; IVF: *in vitro* fertilization; ICSI: intracytoplasmic sperm injection.

OI and IVF/ICSI groups as compared to spontaneously conceived pregnancies. There was also a non-significant trend to increased prevalence of gestational diabetes, preeclampsia and placenta praevia. Infertile groups presented a higher rate of induction of labor and cesarean section as compared to the fertile group. Mean gestational age at delivery was significantly lower in the IVF/ICSI group when compared to the other study groups. Birth weight was significantly lower in both OI and IVF/ICSI groups, with no differences between the non-treatment group and fertile groups. All study groups presented similar neonatal gender, Apgar score, umbilical artery pH and perinatal mortality rate.

Factors associated to pregnancy outcomes

Multivariable regression analysis was performed to assess the effect of infertility treatments on those pregnancy outcomes that showed significant differences in the univariate analysis. While preterm delivery was mainly associated to IVF/ICSI

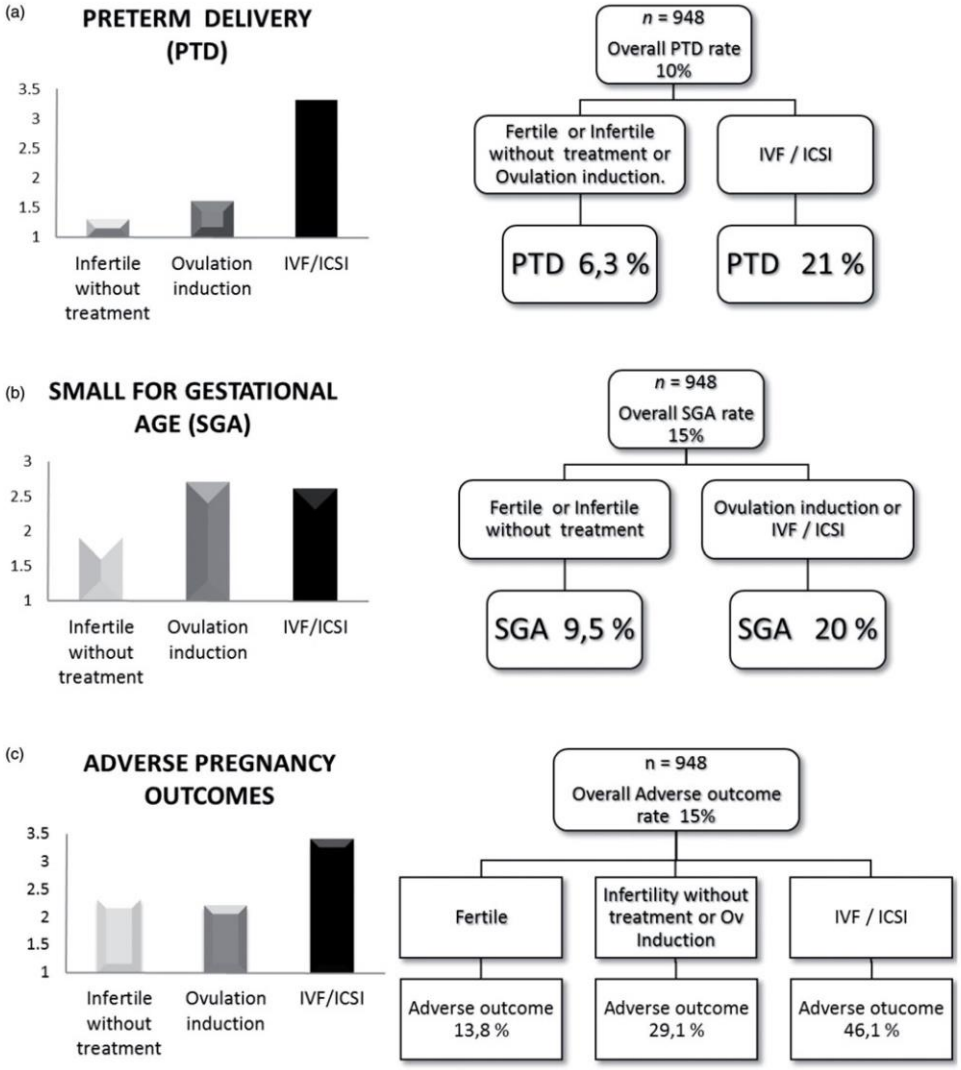

Figure 1. Decision tree analysis for risk of presenting (A) preterm delivery, (B) a small for gestational age fetus, and (B) adverse pregnancy outcomes according to the different infertility groups. Adjusted odds ratio (left) and decision-tree analysis (right) are shown, according to the different infertility groups. a). Increased risk of preterm delivery higher in IVF/ICSI (21%) compared with the rest of the groups (6.3%). b). Higher proportion of SGA (20%) in infertile with OI and IVF/ICSI groups versus fertile. c). Higher risk for adverse pregnancy outcomes in IVF/ICSI (46.1%) and OI (29.1%) groups compared to fertile.

with an 3.3 OR adjusted by maternal age and twin, 95% confidence interval (Figure 1B), SGA and LBW were significantly associated to both OI (2.7 OR and 3.1 OR) and IVF/ICSI 2.6 OR and 3.4 OR, respectively (Figure 1C). Adverse pregnancy outcome risk showed a linear trend as treatment progresses (Figure 1A), with higher risk in the IVF/ICSI group (3.4 OR). All these associations remained statistically significant even after adjusting by maternal age and twin pregnancy (Table S1, Supplementary Digital Content).

Decision tree analysis was performed in order to identify the best predictive variable for adverse outcomes (Figure 1), showing a significant difference in the proportion of PTD between IVF/ICSI and the rest of the groups (21% versus 6.3%, $p<0.001$). SGA also showed a different proportion in those groups who presented infertility treatment versus those without (20 versus 9.5%, $p < 0.001$). Finally, adverse perinatal outcome showed a significant linear behavior, with 3.3% in the spontaneous pregnancy group, 11.5% in the infertility non-treatment/induction group and 29% in the IVF/ICSI group ($p < 0.001$).

Discussion

This study confirms the higher prevalence of pregnancy complications in all infertile women (with or without treatment) and further describes a preferential association of prematurity with IVF/ICSI, and SGA with treated infertility (OI and IVF/ICSI).

In our population, the overall rate of pregnancy complications was significantly increased in all infertility groups regardless the infertility treatment. Our data goes in line with previous studies reporting worse perinatal outcomes in infertile women with no treatment [10,17], suggesting that adverse perinatal outcome may be more related to maternal factors associated with infertility, rather than the type of ART used. In addition, we are also confirming that the prevalence of adverse outcomes increases with the use of more intensive treatment [3,18]. Our results confirm previous data [19] reporting a higher rate of cesarean section in the infertility groups, but fail to demonstrate particular differences in preeclampsia, placenta previa or gestational diabetes, most probably due to the limited sample size of our study. The potential role of infertility status as the origin of these increased risks, is further supported by the study undertaken by Zhu et al. [20] which revealed that subfertile couples with a time to pregnancy of more than 12 months, who conceived without the need of any infertility treatment, gave birth to singletons with an increased prevalence of congenital malformations.

In our study, prematurity was mainly associated to IVF/ICSI, with a lower gestational age at delivery and a higher prevalence of PTD. In addition, IVF/ICSI was the only group that remained as main predictor of PTD in the decision tree analysis. Our results are in agreement with several previous studies demonstrating a significant association of prematurity with IVF/ICSI, both in singleton and twin pregnancies [5,21,22]. The etiology for PTD is complex and multifactorial; women undergoing IVF/ICSI often have more embryos transferred, which may increase the chance of having multiple pregnancies and/or a vanishing twin, both conditions associated with PTD. In our study, the IVF/ICSI group presented the highest prevalence of twin pregnancies. However, differences in PTD remained significant after adjusting by twins and maternal age. Besides twins, other factors such as older maternal age, previous maternal disease or ovarian hyperstimulation have been proposed to explain this increase in prematurity, as they may lead to poorer early embryonic and placental development, increasing the risks of developing complications that also increase the risk of PTD [2,23,24]. Furthermore, our data showed a non-significant tendency to increased PTD in the OI group. Data regarding the prevalence of PTD in OI and other infertile groups is more scarce and controversial, but recent studies have suggested that singleton pregnancies conceived using OI with or without intrauterine insemination, are at risk of moderate and very preterm birth [17,25,26].

We also report a significant association of SGA and LBW with both OI and IVF/ICSI. There is mixed information in the literature about the definition of SGA, intrauterine growth restriction and LBW, with SGA usually defined by birthweight less than 10th or 5th centile and LBW as less than 2500 g. Regardless of the definition, our data and previous literature [2,3,18] support the association of ART with fetal growth restriction in both term and preterm pregnancies. While this association was initially explained by the higher incidence of multiples pregnancies in ART, recent studies have also demonstrated higher rates of SGA/LBW in singleton pregnancies. Other studies have reported a higher incidence of SGA in groups with OI, particularly in twin pregnancies [13]. In contrast, in our study both OI and IVF/ICSI showed a similar increase in PTD. The mechanism underlying this association is unclear. As some studies have reported a higher incidence of SGA when utilizing fresh embryos versus vitrified ones [27], it has been attributed to culture media required for the gametes [28], but this would not explain the higher incidence of SGA in OI. It was not possible to evaluate the potential effect of this variable in our cohort, as the majority of ART procedures in our center were performed with fresh embryos. Another potential explanation could be superovulation, genetic imprinting and methylation errors that have been reported in IVF/ICSI but also in OI and that could impair oocyte quality and thus impact fetal growth and development [26,29,30].

This study has several strengths and limitations. The study design allowed to include a fertile group sampled from the general population and an infertile group who conceived spontaneously together with the OI and IVF/ICSI groups. However, we acknowledge the limited sample size of our cohort, which may have prevented to demonstrate potential associations with low prevalence complications such as preeclampsia or placenta praevia. In addition, although statistical analyzes were adjusted by maternal age and twin pregnancies, other potential confounders (such as socio-economic status, ethnicity, pregestational maternal weight or uterine anomalies) could not be included in the multivariate analysis due to the limited sample size and lack of statistical power.

Conclusion

Adverse pregnancy outcomes seem to be present in infertile women, regardless of the use of ART, supporting the concept of maternal underlying factors related to infertility rather than the ART technique. While prematurity is more related to IVF/ICSI, SGA seems to depend on fertility treatment. This study confirms previous literature and provides further evidence on the differential effect infertility management has on pregnancy complications, emphasizing the need of specific follow-up clinical protocols for managing these high-risk pregnancies.

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Declaration of interest

The authors report no declarations of interest.

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Supplementary material available online

STUDY 2.

Circulation

American Heart Association

Assisted Reproductive Technologies Are Associated With Cardiovascular Remodeling In Utero That Persists Postnatally

Brenda Valenzuela-Alcaraz, Fàtima Crispi, Bart Bijnens, Monica Cruz-Lemini, Montserrat Creus, Marta Sitges, Joaquim Bartrons, Salvadora Civico, Juan Balasch and Eduard Gratacós

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Assisted Reproductive Technologies Are Associated With Cardiovascular Remodeling In Utero That Persists Postnatally

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Background—Assisted reproductive technologies (ARTs) have been shown to be associated with general vascular dysfunction in late childhood. However, it is unknown whether cardiac remodeling is also present and if these changes already manifest in prenatal life. Our aim was to assess fetal and infant (6 months of age) cardiovascular function in ART pregnancies.

Methods and Results—This prospective cohort study included 100 fetuses conceived by ART and 100 control pregnancies. ART fetuses showed signs of cardiovascular remodeling, including a more globular heart with thicker myocardial walls, decreased longitudinal function (tricuspid ring displacement in controls: median, 6.5 mm [interquartile range, 6.1–7.1 mm]; tricuspid ring displacement in ART: 5.5 mm [interquartile range, 5.1–6.1]; $P<0.001$), impaired relaxation, and dilated atria (atrial area in controls, 1.46 cm^2 [interquartile range, 1.2–1.5 cm^2]; atrial area in ART, 1.6 cm^2 [interquartile range, 1.3–1.8 cm^2]; $P<0.001$). Additionally, ART infants showed persistence of most cardiac changes and a significant increase in blood pressure and aortic intima-media thickness (systolic blood pressure in controls, 74 mm Hg [interquartile range, 67–83 mm Hg]; systolic blood pressure in ART, 83 mm Hg [interquartile range, 75–94 mm Hg]; $P<0.001$; aortic intima-media thickness in controls, 0.52 mm [interquartile range, 0.45–0.56 mm]; aortic intima-media thickness in ART, 0.64 mm [interquartile range, 0.62–0.67]; $P<0.001$). We could not demonstrate that our findings were directly caused by ART because of their association with various confounding factors, including intrauterine growth restriction or factors related to the cause of infertility.

Conclusions—Children conceived by ART manifest cardiac and vascular remodeling that is present in fetal life and persists in postnatal life, suggesting opportunities for early detection and potential intervention. The underlying mechanisms and the effect of potential confounders such as growth restriction or prematurity remain to be elucidated. **(*Circulation*. 2013;128:1442-1450.)**

Key Words: fertilization in vitro ■ pediatrics ■ pregnancy ■ reproductive techniques, assisted ■ ventricular remodeling

Assisted reproductive technologies (ARTs), mainly standard in vitro fertilization or intracytoplasmic sperm injection, permit childbirth in many infertile couples and nowadays represent 1% to 4% of births in developed countries.[1] Although these technologies are generally considered safe, the potential association of ART with poorer pregnancy outcomes has long been investigated. There is evidence that ART is associated with increased risk for adverse perinatal outcome and congenital malformations.[2] This notwithstanding, it is not possible to separate ART-related risks from those secondary to the underlying reproductive pathology of the infertile couple.[3–5] In this scenario, preliminary evidence has recently suggested that ART could be associated with long-term cardiovascular changes. Ceelen et al[6] first suggested the presence of increased blood pressure in late childhood after ART conception. More recently, another study demonstrated the presence of signs of systemic and pulmonary vascular dysfunction in 12-year-old children conceived by ART.[7]

Editorial see p 1398
Clinical Perspective on p 1450

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Cardiovascular remodeling has previously been described to be associated with low birth weight (LBW),[8,9] and it is regarded as a manifestation of fetal programming defined as the permanent alteration of tissue structures and functions as a result of fetal environment. It is unknown whether cardiovascular changes in ART children occur already in fetal life. In addition, fetal cardiovascular programming associated with LBW has been demonstrated to be accompanied by cardiac remodeling, which constitutes an additional risk factor in later life. However, the potential association of ART with cardiac structural and functional remodeling was not investigated in previous studies. This information is relevant to advance our understanding of the long-term impact of ART on cardiovascular function and on the design of preventive strategies.

In the present study, we evaluated the hypothesis that pregnancies conceived by ART are associated with both cardiac and vascular remodeling in the offspring and that changes can be detected already during fetal life. We designed a prospective cohort study including 100 ART and 100 spontaneously conceived fetuses to comprehensively assess cardiac and vascular structure and function in the fetal and postnatal periods.

Methods

Study Populations and Study Protocol

The study design was a prospective cohort study including 100 singleton pregnancies conceived by in vitro fertilization or intracytoplasmic sperm injection in infertile patients and 100 control pregnancies conceived naturally identified in fetal life and followed up to 6 months of age. The ART group was a consecutive sample of patients with a normal first-trimester scan who accepted participation in the study. Cases were considered noneligible if any of the following were present: preimplantation genetic diagnosis, oocyte donation, multiple pregnancies, or any maternal medical disease. Likewise, later diagnosis of fetal malformations or any pregnancy complications leading to delivery before 34 weeks of gestation were considered exclusion criteria. The control group was recruited at 28 to 30 weeks' gestation among women with low-risk pregnancies attending our Maternal–Fetal Medicine Unit for normal pregnancy follow-up. Controls were matched for maternal age (±1 year) with cases. Eligibility and exclusion criteria for controls were the same as for cases, and controls underwent the same study protocol as cases. The study protocol was approved by the Institutional Review Board at Hospital Clinic, and written parental consent was obtained for all study participants. Figure 1 shows a flow diagram of the study population. Cardiovascular assessment included echocardiography in fetal life, vascular assessment in the neonatal period, and both echocardiography and vascular assessment at 6 months of age.

Parental baseline and ART characteristics were collected by parental interview and review of medical records at the time of prenatal evaluation. LBW was defined as birth weight below the 10th percentile.

Fetal Assessment

All pregnancies underwent ultrasonographic examination at 28 to 30 weeks of gestation using a Siemens Sonoline Antares (Siemens Medical Systems, Malvern, PA) with 6- to 4-MHz linear curved-array and 2- to 10-MHz phased-array probes, including the assessment of estimated fetal weight, fetoplacental Doppler, and fetal echocardiography.

Fetoplacental Doppler assessment included pulsatility index measurement of the umbilical artery and middle cerebral artery according to a previously published methodology.

Fetal echocardiography included a comprehensive examination to assess structural heart integrity and morphometry, as well as systolic and diastolic function parameters. Cardiac dimensions were measured on 2-dimensional images from an apical 4-chamber view. Left and right atrial areas were taken at maximum point of atrial distension and ventricular base-to-apex lengths and basal diameters at end diastole. Left and right ventricular sphericity indexes were calculated as base-to-apex length divided by basal diameter.[10] Ventricular end-diastolic septal and free wall thicknesses were measured by M mode from a transverse 4-chamber view. Left and right ventricular ejection fractions (in percent) were obtained from M-mode long-axis transverse 4-chamber views using the Teichholz formula. Left and right stroke volumes were calculated as follows: $\pi/4\times$(aortic or pulmonary valve diameter)$^2\times$(aortic or pulmonary artery systolic time-velocity integral).[11] Then, left and right cardiac outputs were calculated as left or right stroke volume times heart rate.[11] Mitral/tricuspid annular displacement was assessed by M mode from an apical or basal 4-chamber view. Tissue Doppler was applied to record systolic peak velocities (S′) at mitral and tricuspid lateral annuli from an apical or basal 4-chamber view and measured in real time during the echocardiographic study.[12] Right and left E/A ratios were estimated by calculating the ratio of early ventricular filling (E) to late ventricular filling (A).[13] Deceleration time of the E wave was measured from mitral and tricuspid inflow velocities from an apical 4-chamber view. Tissue Doppler was applied to record early diastolic (E′) peak velocity at mitral and tricuspid lateral annuli from an apical or basal 4-chamber view.[12] Left isovolumic relaxation time was measured from the closure of the aortic valve to the opening of the mitral valve.

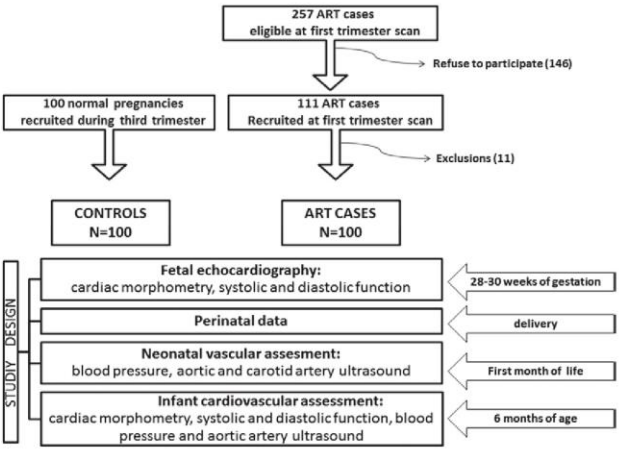

Figure 1. Flow diagram of the study populations. ART indicates pregnancies conceived by assisted reproductive technologies.

Neonatal Assessment

Neonatal vascular assessment was performed within the first month of life, including the measurements of blood pressure and vascular intima-media thickness (IMT). Blood pressure centiles were calculated according to standard normograms.[14]

Carotid and aorta ultrasound assessment was performed by skilled sonographers using a Siemens Sonoline Antares (Siemens Medical Systems). Longitudinal clips of the far walls of both carotid arteries and abdominal aorta were obtained with a linear-array transducer. Carotid and aorta IMT measurements were performed offline according to a standardized protocol based on a trace method with the assistance of a computerized program (Siemens Syngo Arterial Health Package). IMT results were normalized by neonatal weight.

Table 1. Baseline Characteristics of the Study Groups

Characteristic	Controls (n=100)	ART (n=100)	*P* Value*
Maternal characteristics			
Age, y	35 (32–37)	36 (35–38)	0.066
BMI, kg/m²†	22 (21–25)	23 (21–25)	0.161
Smoking, %	4	3	1
White, %	84	90	0.293
Primiparity, %	40	43	0.774
Early cardiovascular history, %‡	2	2	0.614
Low socioeconomic level, %	15	14	1
University education, %	63	52	0.153
Paternal characteristics			
Age, y	36 (33–39)	38 (36–40)	0.077
BMI, kg/m²†	25 (23–26)	25 (24–28)	0.442
Smoking, %	12	17	0.422
White, %	84	92	0.128
Early cardiovascular history, %‡	4	5	1
Low socioeconomic level, %	7	5	0.766
University education, %	30	35	0.546
Fertility and ART characteristics, %			
Infertility cause			
Unexplained	NA	32	NA
Female	NA	25	NA
Male	NA	34	NA
Female+male	NA	9	NA
ART technique			
Standard IVF	NA	9	NA
ICSI	NA	85	NA
IVF+ICSI	NA	7	NA
Transferred embryos, n			
1	NA	12	NA
2	NA	70	NA
3	NA	18	NA

ART indicates pregnancy conceived by assisted reproductive technologies; BMI, body mass index; ICSI, intracytoplasmic sperm injection; IVF, in vitro fertilization; and NA, not applicable. Data are median (interquartile range) when appropriate.

*P value calculated by the Student t test or Pearson χ^2 test.

†BMI was calculated as weight in kilograms divided by the square of height in meters.

‡Early cardiovascular disease was defined by the presence of congenital heart disease, coronary disease, hypertension, diabetes mellitus, or hypercholesterolemia in men <55 years of age and women <65 years of age.

Assessment at 6 Months of Age

Infants' follow-up evaluation, including anthropometric data, echocardiography, and vascular assessment, was scheduled at 6 months of age. Anthropometric data included the infants' height and weight measured at the time of the examination.

Echocardiography was performed following a standardized protocol[15] using a Vivid q (General Electric Healthcare, Norway) with a 2- to 10-MHz phased-array transducer. Infants were studied when resting quietly or asleep. A complete echocardiography was performed initially to assess structural heart integrity. Left and right atrial planimetric areas were measured on a 2-dimensional image from an apical 4-chamber view at end systole (greatest dimension, just before mitral or tricuspid valve opening). Ventricular base-to-apex length and transverse diameter were measured on a 2-dimensional image from an apical 4-chamber view at end diastole. Left and right ventricular sphericity indexes were calculated as base-to-apex length divided by midtransverse diameter. Ventricular end-diastolic wall thicknesses were measured by M mode from a parasternal long-axis view.[15,16]

Left shortening fraction was calculated from internal ventricular diameters obtained from a parasternal long-axis view by M mode using the following equation: (end-diastolic dimension−end-systolic dimension)/end-diastolic dimension. Left and right stroke volumes were calculated as follows: $\pi/4\times$(aortic or pulmonary valve

Table 2. Perinatal Characteristics of the Study Groups

Characteristic	Controls (n=100)	ART (n=100)	*P* Value*
Pregnancy complications, %			
Vanishing twin	0	8	0.012
Preeclampsia	0	4	0.123
Low birth weight	1	17	0.001
Spontaneous preterm delivery	2	5	0.442
Gestational diabetes	4	6	0.746
Placenta previa	0	3	0.245
Obstetric cholestasis	0	0	1
Prenatal corticoid exposure	2	1	1
Delivery data			
Gestational age at delivery, wk	40 (39–40)	38 (37–39)	0.032
Maternal systolic blood pressure, mm Hg	111 (110–115)	114 (111–116)	0.437
Maternal diastolic blood pressure, mm Hg	70 (68–71)	71 (65–72)	0.891
Cesarean section, %	24	31	0.342
Male, %	49	51	0.888
Birth weight, g	3355 (3020–3550)	2740 (2585–2920)	0.022
Birth weight percentile	49 (31–73)	26 (5–55)	0.033
5-min Apgar score	10 (9–10)	10 (9–10)	0.102
Umbilical artery pH	7.2 (7.1–7.2)	7.2 (7.2–7.3)	0.920
Neonatal outcome, %			
Admission to neonatal intensive care unit	1	1	0.477
Major neonatal morbidity†	0	0	1
Perinatal mortality	0	0	1

ART indicates pregnancy conceived by assisted reproductive technologies. Data are median (interquartile range) when appropriate.

*P value calculated by the Student t test or Pearson χ^2 test.

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

Table 3. Fetal Assessment in the Study Groups

Characteristic	Controls (n=100)	ART (n=100)	Crude *P* Value	Adjusted *P* Value*
Gestational age at scan, wk	29 (28–29)	28 (28–29)	0.062	0.130
Fetoplacental data				
Estimated fetal weight at scan, g	1375 (1205–1508)	1300 (1173–1446)	0.061	0.078
Estimated fetal weight percentile at scan	53 (26–80)	54 (29–69)	0.710	0.656
Umbilical artery PI	1.10 (0.98–1.25)	1.09 (0.95–1.25)	0.979	0.706
Middle cerebral artery PI	2.18 (1.8-1.3)	2.0 (1.5–2.3)	0.961	0.806
Fetal echocardiography				
Cardiac morphometry				
Left atrial area, cm^2	1.35 (1.1–1.4)	1.48 (1.2–1.7)	<0.001	<0.001
Right atrial area, cm^2	1.46 (1.2–1.5)	1.60 (1.3–1.8)	0.002	<0.001
Left sphericity index	1.77 (1.61–1.92)	1.71 (1.54–1.78)	0.002	0.003
Right sphericity index	1.58 (1.4–1.72)	1.37 (1.25–1.5)	<0.001	<0.001
Left free wall thickness, mm	2.7 (2.4 - 3)	2.9 (2.7- 3.1)	0.001	0.068
Interventricular septum thickness, mm	2.4 (2.4–2.8)	2.7 (2.4–2.9)	<0.001	<0.001
Right free wall thickness, mm	2.8 (2.6–3.2)	3.2 (2.9–3.3)	0.026	0.038
Systolic function				
Left ejection fraction, %	69 (63–73)	63 (57–68)	<0.001	<0.001
Right ejection fraction, %	68 (63–73)	67 (60–73)	0.657	0.659
Heart rate, bpm	140 (136–148)	143 (136–150)	0.836	0.749
Left cardiac output, mL/min	25.6 (20–30)	25.5 (21–30)	0.838	0.798
Right cardiac output, mL/min	31 (26–37)	33 (28–38)	0.385	0.727
Mitral ring displacement, mm	4.7 (4.2–5.3)	4.2 (3.7–4.9)	<0.001	<0.001
Tricuspid ring displacement, mm	6.5 (6.1–7.1)	5.5 (5.1–6.1)	<0.001	<0.001
Mitral S′, cm/s	6.9 (6–7.4)	6 (6–7)	0.031	0.038
Tricuspid S′, cm/s	7.9 (6.7–8.8)	7 (6–8)	0.148	0.179
Diastolic function				
Mitral E/A ratio	0.71 (0.66–0.76)	0.74 (0.67–0.78)	0.681	0.320
Tricuspid E/A ratio	0.80 (0.70–0.90)	0.80 (0.72–0.91)	0.806	0.810
Mitral E deceleration time, ms	73 (55–91)	63 (51–78)	0.003	0.002
Tricuspid E deceleration time, ms	64 (51–77)	51 (44–66)	<0.001	0.001
Mitral E′, cm/s	7.6 (6.9–8)	7 (6–8)	0.061	0.049
Tricuspid E′, cm/s	8.3 (7.9–9.1)	8 (7–9)	0.003	0.002
Left isovolumic relaxation time, ms	30 (42–52)	48 (41.5–54.5)	0.031	0.003

A indicates ventricular inflow during atrial contraction; ART, pregnancy conceived by assisted reproductive technologies; E, ventricular inflow in early diastole; E′, annular peak velocity in early diastole; PI, pulsatility index; and S′, systolic annular peak velocity. Data are median (interquartile range).

**P* value calculated by linear regression adjusted for gestational age at delivery, birth weight percentile, and preeclampsia.

diameter)^2×(aortic or pulmonary artery systolic flow velocity-time integral). Left and right cardiac outputs were calculated as stroke volume times heart rate. Tricuspid annular displacement was measured in real time in an apical 4-chamber view by placing the M-mode cursor at the atrioventricular junction, marked by the tricuspid valve rings at the right free wall. Maximum amplitude of motion was taken as the extent of displacement between end systole and end diastole and measured in millimeters. Tissue Doppler was applied at mitral and tricuspid lateral annuli from an apical 4-chamber view to record S′. E/A ratios were calculated. E deceleration time was measured as the time from the maximum mitral/tricuspid velocity to baseline. Tissue Doppler was applied at mitral and tricuspid lateral annuli from an apical 4-chamber view to record E′. Left isovolumic relaxation time was obtained from the pulsed Doppler waveform of the aortic blood flow from the end of the aortic wave to the beginning of the mitral early filling wave.

Vascular assessment included blood pressure and aortic wall thickness by ultrasound. Aortic IMT measurement involved obtaining longitudinal clips of the far wall of the proximal abdominal aorta in the upper abdomen with a 10-MHz linear probe. Aortic IMT measurements were performed offline according to a standardized protocol based on a trace method with the assistance of a computerized program (GE EchoPAC PC 108.1.x, General Electric Healthcare). IMT results were normalized by infant weight.

Statistical Analysis

SPSS Statistics 19 (IBM) was used for the statistical analysis. The study outcome was fetal and postnatal cardiovascular assessment. The independent variable of interest was the type of conception (natural or ART), and the covariates were birth weight percentile,

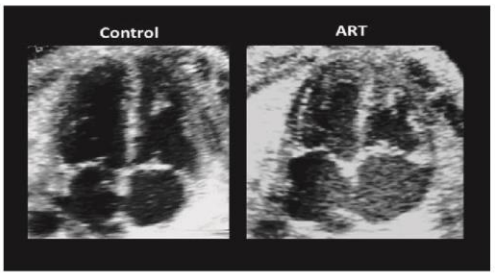

Figure 2. Echocardiographic images in fetuses conceived naturally (control) and by assisted reproductive technologies (ART). The images are 2-dimensional apical 4-chamber views at end diastole illustrating larger atria and a more globular right ventricular shape in ART compared with controls.

gestational age at delivery, and the presence of preeclampsia. Annular peak velocities by tissue Doppler were chosen to calculate sample size because of their high sensitivity for preclinical cardiac dysfunction in fetuses and children. On the basis of previous studies measuring fetal cardiac function,[15] sample size was calculated to allow observation of a difference of 25% in tricuspid E′ values in ART fetuses. For a power of 80% and an α risk of 0.05, a minimum of 91 subjects per study group were required. We decided to include 100 fetuses in each study group. Data are presented as median (interquartile range) or percentage as appropriate. Statistics for baseline and perinatal data included comparison of means by the Student *t* test or proportions by the Pearson χ^2 test. To evaluate the influence of covariates, comparisons of the cardiovascular parameters between the study and control groups were adjusted for association to preeclampsia, birth weight percentile, and gestational age at delivery by linear regression. All reported *P* values are 2 sided. All fetoplacental Doppler and cardiac parameters are shown as crude values (this document) and normalized into *z* scores by previously published reference values or adjusted by heart size, estimated fetal weight (in fetuses), or body surface area (in infants; see the online-only Data Supplement).

Results

Baseline and Perinatal Characteristics

Baseline and perinatal characteristics of the study population are shown in Tables 1 and 2. The study groups were similar in terms of maternal and paternal baseline characteristics compared with controls (Table 1). As expected, ART pregnancies had a higher occurrence of pregnancy complications, mainly a higher prevalence of LBW and a tendency to increased incidence of preeclampsia (Table 2). Delivery and perinatal characteristics were similar among the study groups except for an earlier gestational age at delivery and lower birth weight percentile in ART compared with controls. Maternal blood pressure values were similar among the study groups.

Fetal Assessment

Results are shown in Table 3. Gestational age at evaluation, estimated fetal weight, and fetoplacental Doppler were similar between groups. As illustrated in Figure 2, fetuses conceived by ART showed increased atrial size and myocardial wall thickness and lower ventricular sphericity indexes compared with controls. Although cardiac output was similar between groups, ART fetuses showed a significant decrease in left ejection fraction, ring displacement, tricuspid E′, E deceleration time, and isovolumic relaxation time compared with controls. Most cardiovascular changes in ART fetuses remained significant after adjustment for gestational age at delivery, birth weight percentile, and association with preeclampsia.

Neonatal Assessment

Results are displayed in Table 4 and Figure 3. Systolic blood pressure was similar among the study groups, whereas diastolic blood pressure percentile was significantly higher after ART pregnancy compared with controls. Aorta and carotid IMTs were significantly increased in ART children, even after normalizing

Table 4. Neonatal Vascular Outcome of the Study Groups Within the First Month of Life

Characteristic	Controls (n=75)	ART (n=60)	Crude *P* Value	Adjusted *P* Value*
Blood pressure				
Systolic blood pressure, mm Hg	82 (75–90)	84 (78–91)	0.163	0.489
Systolic blood pressure percentile	47 (28–74)	54 (30–79)	0.892	0.614
Diastolic blood pressure, mm Hg	47 (39–57)	53 (46–61)	0.548	0.256
Diastolic blood pressure percentile	55 (21–85)	71 (44–91)	0.004	0.042
Vascular wall thickness†				
Aortic mean IMT, mm	0.45 (0.36–0.51)	0.55 (0.53–0.61)	<0.001	0.035
Aortic mean IMT/weight, mm/kg	0.12 (0.10–0.14)	0.16 (0.14–0.18)	<0.001	0.011
Aortic maximum IMT, mm	0.57 (0.47–0.64)	0.65 (0.62–0.66)	<0.001	0.002
Aortic maximum IMT/weight, mm/kg	0.14 (0.13–0.17)	0.19 (0.16–0.22)	0.001	0.024
Carotid mean IMT, mm	0.24 (0.21–0.27)	0.28 (0.25–0.30)	<0.001	0.091
Carotid mean IMT/weight, mm/kg	0.06 (0.05–0.07)	0.07 (0.07–0.08)	0.001	0.035
Carotid maximum IMT, mm	0.29 (0.20–0.33)	0.32 (0.31–0.33)	<0.001	0.005
Carotid maximum IMT /weight, mm/kg	0.07 (0.05–0.08)	0.09 (0.07–0.09)	0.003	0.018

ART indicates pregnancy conceived by assisted reproductive technologies; and IMT, intima-media thickness. Data are median (interquartile range).

*P value calculated by linear regression adjusted for gestational age at delivery, birth weight percentile, and preeclampsia.

†Vascular IMT normalized by neonatal weight.

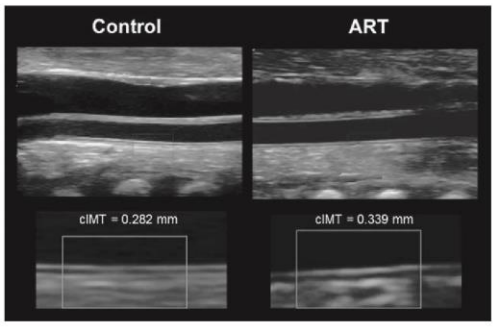

Figure 3. Ultrasound carotid images in neonates conceived naturally (control) and by assisted reproductive technologies (ART) illustrating the increase in carotid intima-media thickness (cIMT) in ART compared with controls.

for neonatal weight and adjusting for gestational age at delivery, birth weight percentile, and association with preeclampsia.

Assessment at 6 Months of Age

Follow-up characteristics and cardiovascular results are shown in Table 5. ART infants showed anthropometric results at the time of evaluation similar to those of controls. ART infants showed increased right atrial size, lower right sphericity index, and thicker right ventricular wall. Although cardiac output was similar among the study groups, ART infants showed a significantly decreased shortening fraction and increased heart rate. ART cases also showed signs of both systolic and diastolic dysfunction as measured by significant decreases in ring displacement, E deceleration time, and tissue Doppler velocities and a significant increase in isovolumic relaxation time. Most cardiac changes remained significant after adjustment for gestational age at delivery, birth weight percentile, and preeclampsia.

Blood pressure was significantly higher in the ART group compared with controls. Aortic IMT was also significantly increased, even after normalizing by infant weight and adjustment for gestational age at delivery, birth weight percentile, and preeclampsia.

Discussion

This study demonstrates the presence of cardiac and vascular remodeling in fetuses and infants of pregnancies obtained by ART. These findings are consistent with previous reports demonstrating signs of vascular dysfunction in children conceived by ART[6,7] and provide evidence for the existence of fetal cardiovascular programming in these pregnancies. We could not determine that our findings were caused by ART itself, by intrauterine growth restriction or prematurity in ART pregnancies, or by other confounders related to the indications for ART.

Fetuses from pregnancies conceived by ART showed more globular hearts together with increased myocardial wall thickness, decreased right longitudinal function, impaired relaxation, and dilated atria. The differences persisted after birth and were more prominent in the right side of the heart compared with the left side. The cardiac findings are consistent with experimental data showing an increased heart weight in an in vitro fertilization bovine model.[17] From a pathophysiological viewpoint, more globular and hypertrophic ventricles with decreased longitudinal function are the usual ventricular response to pressure overload. Therefore, fetal observations are in line with postnatal findings of elevated blood pressure and increased IMT. In addition, cardiac remodeling described in our ART population resembles other fetal conditions with known pressure overload such as twin-to-twin transfusion syndrome[18] or ductus arteriosus restriction.[19] These clinical entities and experimental models of systemic pressure loading[20] have been reported to show more pronounced changes in the right side of the heart. This might reflect the dominance of the right side of the heart during fetal life and a higher susceptibility to pressure overload of the right compared with the left ventricle.[21,22] The dilated atria and impaired relaxation (decrease in E′ and E deceleration time) could be explained by a decrease in ventricular compliance, leading to higher end-diastolic pressures and increased atrial pressures. Finally, the changes described in vascular function and structure in neonates and infants reproduce the findings of previous reports in late childhood[6,7] and support the development and presence of these differences from early life.

Fetal cardiovascular programming has previously been described in fetuses and children who suffered from LBW.[8,9] LBW is associated with globular hearts and longitudinal dysfunction in utero,[20] and these changes, accompanied by increased blood pressure and vascular wall thickness, have been described to persist into childhood in humans[9] and to adulthood in animal models.[23] Direct cardiac effects of fetal growth restriction have been proposed to provide a link to explain the long-described epidemiological association of this prenatal condition with increased cardiovascular mortality in adults.[8] Because of the high and expected prevalence of LBW in ART cases, it has been suggested that fetal growth restriction could be a potential confounder for cardiovascular remodeling in ART offspring.[24] However, we believe that the results of this study strongly support a direct effect of ART on fetal and infant cardiovascular changes. First, ART fetuses and infants presented changes that have not previously been reported in LBW such as myocardial hypertrophy and increased atrial size.[8,9] Second, most cardiovascular changes in ART remained significant even after adjustment by birth weight percentile. Finally, the differences between ART pregnancies and control pregnancies remained virtually unchanged after the LBW pregnancies were excluded from the study group (online-only Data Supplement).

The mechanisms driving fetal and postnatal cardiovascular remodeling in ART pregnancies remain to be elucidated. Parental predisposing factors, epigenetic changes secondary to the early embryo manipulation, hormonal effects, and postnatal environmental factors have been postulated as potential factors.[3–5] Changes in fetuses and infants in this study were similar to those described in late childhood. Consequently, the role of postnatal environment as a potential factor determining long-term vascular dysfunction in ART children is possibly negligible. Advanced maternal age in ART has been proposed as a major contributor of childbearing ability/difficulties.[4] In this study, cases and controls were matched by maternal age; however, we acknowledge that other parental factors related to their subfertility could still play a role.

Concerning epigenetic mechanisms, there is clinical and mainly experimental evidence that the processes involved in

Table 5. Anthropometric Data and Cardiovascular Assessment at 6 Months of Age

Characteristic	Controls (n=50)	ART (n=50)	Crude *P* Value	Adjusted *P* Value*
Age at evaluation, mo	6.1 (6.1–6.4)	6.1 (6–6.2)	0.092	0.102
Anthropometric data				
Height, cm	68 (65–69)	66 (65–68)	0.302	0.794
Weight, g	7650 (7170–8000)	7600 (6995–8200)	0.772	0.806
Infant echocardiography				
Cardiac morphometry				
Left atrial area, cm^2	2.71 (2.6–3)	2.75 (2.6–3.1)	0.782	0.574
Right atrial area, cm^2	2.50 (2.2–2.9)	2.70 (2.5 - 3.2)	0.018	0.005
Left sphericity index	1.81 (1.7–1.8)	1.83 (1.7–1.9)	0.271	0.650
Right sphericity index	1.91 (1.8–2)	1.82 (1.5–2)	0.021	0.010
Left ventricular wall thickness, mm	4.80 (4.4–5.4)	4.58 (4–5.2)	0.880	0.908
Septum thickness, mm	4.15 (3.4–4.5)	3.60 (3.3–4.1)	0.555	0.605
Right free wall thickness, mm	2.59 (2.3–3.2)	3.21 (2.9–3.5)	0.009	0.019
Systolic function				
Left shortening fraction, %	36 (32–40)	29 (26–35)	0.001	<0.001
Heart rate, bpm	132 (124–144)	141 (131- 148)	0.001	0.002
Left cardiac output, mL/min	25 (21.9–30)	25 (21–29.7)	0.744	0.204
Right cardiac output, mL/min	32 (25–38)	33 (28–38)	0.208	0.587
Mitral ring displacement, mm	10.8 (10.1–11.8)	9.4 (7.4–10.3)	<0.001	<0.001
Tricuspid ring displacement, mm	16.3 (15.1–17.2)	13.1 (11.9–14.1)	<0.001	<0.001
Mitral S′, cm/s	7.7 (7–8.9)	6.9 (5.7–7.5)	0.062	0.339
Tricuspid S′, cm/s	11.5 (10.9–13.2)	10.9 (9.7–13.3)	0.462	0.381
Diastolic function				
Mitral E/A ratio	1.3 (1.2–1.4)	1.2 (1.1–1.3)	0.374	0.133
Tricuspid E/A ratio	1 (0.8–1.1)	1.1 (1–1.3)	0.014	0.132
Mitral E deceleration time, ms	66 (52–90)	63 (49–78)	0.068	0.014
Tricuspid E deceleration time, ms	62 (51–77)	52 (44–66)	<0.001	<0.001
Mitral E′, cm/s	13.7 (12–14)	12.2 (10–13)	0.016	0.207
Tricuspid E′, cm/s	15 (14–17)	13 (11–16)	0.015	0.077
Left isovolumic relaxation time, ms	50 (41–59)	63 (55–67)	<0.001	<0.001
Vascular assessment				
Blood pressure, mm Hg				
Systolic blood pressure	74 (67–83)	83 (75–94)	<0.001	<0.001
Diastolic blood pressure	50 (49–59)	50.5 (50–62)	0.070	0.214
Aortic wall thickness†				
Aortic mean IMT, mm	0.52 (0.45–0.56)	0.64 (0.62–0.67)	<0.001	0.003
Aortic mean IMT/weight, mm/kg	1.4 (1.2–1.5)	1.8 (1.60–1.9)	<0.001	<0.001
Aortic maximum IMT, mm	0.60 (0.52–0.64)	0.72 (0.68–0.75)	<0.001	<0.001
Aortic maximum IMT/weight, mm/kg	1.6 (1.4–1.8)	2.0 (1.9–2.1)	<0.001	<0.001

A indicates ventricular inflow during atrial contraction; ART, pregnancy conceived by assisted reproductive technologies; E, ventricular inflow in early diastole; E′, annular peak velocity in early diastole; IMT, intima-media thickness; and S′, systolic annular peak velocity. Data are median (interquartile range).

*P value calculated by linear regression adjusted for gestational age at delivery, birth weight percentile, and preeclampsia.

†Aortic wall thickness normalized by infant weight.

egg manipulation might be associated with epigenetic changes, mediated mainly by changes in the DNA methylation pattern. The majority of the changes described affect imprinted genes, which have been involved mainly with fetal and placental growth.[25–27] However, it has been suggested that methylation might be relevant for other functions not yet characterized.[26] The importance of DNA methylation in the regulation of vascular endothelial function is being increasingly demonstrated, including nitric oxide expression and synthesis and endothelial angiogenesis. As indirect evidence, experimental models suggest that fetal cardiovascular programming occurring in LBW is associated with specific epigenetic signatures involving abnormal

methylation.[28] Therefore, molecular pathways involved in cardiovascular regulation deserve further research to ascertain their potential involvement in the vascular changes described in ART pregnancies. However, because of the variability in ART protocols and the rarity of imprinting disorders, it can be challenging to determine reliably the causative relationship between and increased risk for imprinting disorders and ART exposure.[29]

Concerning hormonal factors, the effect of supraphysiological estradiol levels on the outcome of in vitro fertilization–embryo transfer and subsequent pregnancies is a matter of controversy in the literature. Estradiol concentrations are not correlated with oocyte yield and quality, embryological outcome, implantation and pregnancy rates, abortion rate, congenital malformations, and birth weight.[30] However, associations with pregnancy complications related to abnormal placentation such as LBW, preeclampsia, and abnormal implantation of the placenta have been reported.[31] The relationship between estradiol levels in ART and long-term cardiovascular function is unknown. As indirect evidence, a recent study reported no association between ovarian hyperstimulation, a condition associated with a dramatic increase in estrogen levels, with neuromotor development at 3 months of age, but again, a potential independent effect of a history of subfertility was suggested.[32] Progesterone, another important hormone in human reproduction, has not been shown to have effects on fetal placental circulation or any association with the presence of LBW.

There are several limitations and considerations with regard to the present study. The changes reported here are subclinical, with most cardiovascular indexes lying within normal ranges. Although these differences are recognized as potential cardiovascular risk factors, their long-term persistence and association with adult cardiovascular function and disease remain to be proven. Therefore, longer follow-up of these ART pregnancies to ascertain whether ART pregnancy remains a risk factor in later life is crucial. We acknowledge that several potential confounders could have interfered with our results. However, cases and controls were matched by maternal age, and twin pregnancies and mothers with medical diseases were excluded. The analysis was adjusted for other potential influences, including prematurity, birth weight percentile, and preeclampsia. Additionally, other potential confounders such as sex, race, cardiovascular history, socioeconomic status, parity, and parental smoking were similar among study groups. However, we acknowledge that analysis correcting for birth weight percentile may inadequately control for the differences in causality because children conceived by ART may be more likely to have in utero growth restriction from placental failure. In addition, there is increasing evidence that current definitions of fetal growth restriction most likely do not detect all instances of true restriction.[33] Consequently, one could argue that by the same token, we cannot exclude that the whole distribution of fetal weights in our population was shifted to the left, reflecting a more general effect on fetal growth in ART fetuses. If this were the case, there would be forms of true fetal growth restriction that have been missed because of the lack of sensitivity of currently used definitions; therefore, the impact of fetal growth restriction on our results would be greater than is now apparent because hidden forms of growth restriction not detected by conventional criteria[33] might have affected the cardiac outcome of the ART pregnancies. We fully acknowledge that prematurity and fetal growth restriction may have significantly contributed to the cardiac findings rather than ART via a mechanism of altered fetal programming. This concept deserves further clarification in future studies. Finally, we acknowledge that future studies might unveil nonobvious confounders not considered in the design of this study that might have affected the present results.

Conclusions

This study provides evidence that the use of ART in infertile couples is associated with fetal and postnatal cardiovascular remodeling, suggesting prenatal exposure to pressure overload. From a clinical perspective and regardless of the need to clarify the specific mechanisms, the existence of fetal programming in these infants presents important opportunities to improve cardiovascular health in a relevant proportion of the population. Nowadays, 1% to 4% of all newborns in developed countries are conceived by ART[1]; therefore, the findings of this study involve thousands of children yearly. The importance of early identification of and the impact of interventions in pediatric risk factors for cardiovascular disease are now well recognized.[34,35] Moreover, ART in infertile patients should be regarded as a potential cardiovascular risk factor, and strategies to detect and improve cardiovascular remodeling could be explored in children conceived with ART. The underlying mechanisms and the effect of the potential confounders in the primary observation reported here remain to be elucidated in future research.

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Disclosures

None.

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CLINICAL PERSPECTIVE

This study provides evidence that the use of assisted reproductive technologies in infertile couples is associated with fetal and postnatal cardiovascular remodeling. Although the underlying mechanism remains to be elucidated, these data are of clinical relevance and have important implications for public health. From a clinical perspective, the existence of fetal programming in these infants presents important opportunities to improve cardiovascular health in a relevant proportion of the population. Nowadays, 1% to 4% of all newborns in developed countries are conceived by assisted reproductive technologies; therefore, the findings of this study involve thousands of children yearly. The importance of early identification and the impact of interventions on pediatric risk factors for cardiovascular disease are now well recognized. Moreover, assisted reproductive technologies in infertile patients should be regarded as a potential cardiovascular risk factor, and strategies to detect and improve cardiovascular remodeling could be explored in children conceived with assisted reproductive technologies.

STUDY 3.

Ultrasound in Obstetrics and Gynecology

Fetal cardiac remodeling in twin pregnancies conceived by assisted reproductive technologies

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TITLE PAGE

Fetal cardiac remodeling in twin pregnancies conceived by assisted reproductive technologies.

Short title: Cardiac remodeling in ART twins.

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ABSTRACT

Background: Recent data suggest that singleton fetuses conceived by assisted reproductive technologies (ART) present cardiovascular remodeling that may persist postnatally. However, it is unknown whether these cardiac changes are also present in twin pregnancies conceived by ART. Our aim was to assess the presence of fetal cardiac remodeling and dysfunction in twin pregnancies conceived by ART as compared to spontaneously conceived (SC) ones.

Material and Methods: A prospective cohort study including 50 dichorionic twin fetuses conceived by ART and 50 SC twins. The study protocol included structural and functional fetal echocardiography at 28-30 weeks of gestation.

Results: ART twin fetuses showed significant cardiac changes, predominantly affecting the right heart, such as dilated atria (right atrial/heart area: controls mean 15.7 (SD 3.1) vs ART 18.4 (3.2), p<0.001), more globular ventricles (right ventricular sphericity index: SC 1.57 (0.25) vs ART 1.41 (0.23), p=0.001) and thicker myocardial walls (septal wall thickness: SC 2.57 mm (0.45) vs ART 2.84 mm (0.41), p=0.034) together with reduced longitudinal motion (tricuspid annular plane systolic excursion: SC 6.36 mm (0.89) vs ART 5.18 mm (0.93), p<0.001) as compared to SC twins.

Conclusions: ART twin fetuses present signs of fetal cardiac remodeling and dysfunction. These changes are similar to those observed in ART singletons and reinforce the concept of fetal cardiac programming in ART. These results open opportunities for early detection and intervention in infants conceived by ART.

Keywords: fetal echocardiography, twin pregnancy, assisted reproductive technologies, *in vitro* fertilization.

MANUSCRIPT

INTRODUCTION

Assisted reproductive technologies (ART) are widely practiced in all regions of the world, representing almost 4% of all newborns, with an increasing rate of application of about 1 million per year[1] worldwide. All currently used methods of ART increase the presence of multiple pregnancies; the twinning rate after *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) is 20% to 40%, compared with approximately 1% after natural conception[1-3].

Both singleton and twin ART pregnancies have been associated with adverse perinatal outcomes such as increased risk of preeclampsia, placenta previa, preterm birth, low birth weight, cesarean section and perinatal mortality when compared with those spontaneously conceived[4-7]. Recent data suggest long-term cardiovascular consequences in ART offspring with systemic and pulmonary vascular dysfunction in childhood[8-10] in addition to a characteristic fetal cardiovascular remodeling [9-12]. All these studies were conducted in singleton pregnancies, and therefore it remains unknown whether offspring twins conceived by ART also present cardiovascular changes.

Accordingly, our objective was to assess fetal cardiovascular structure and function of dichorionic twin pregnancies conceived by ART as compared to those spontaneously conceived (SC).

MATERIALS AND METHODS

Study population and protocol

This was a prospective cohort study including 50 twin fetuses conceived by ART and 50 twins SC. Cases and controls were recruited from April 2014 to April 2016 at the Department of Maternal-Fetal Medicine of BCNatal in Barcelona, Spain. Conception by ART included all treatments or procedures that involve surgically removing eggs from a woman's ovaries and combining them with sperm to help with conception (standard IVF or ICSI). Patients were considered non eligible if any of the following were present: monochorionic pregnancy, more than 2 fetuses, any maternal medical disease including asthma, chronic hypertension, diabetes mellitus, heart disease, human immunodeficiency virus or hepatitis infection, lupus and thyroid disease. Likewise, later diagnoses of fetal malformations or vanishing twin were also considered exclusion criteria. The study protocol was approved by Hospital Clinic's Ethical Committee, and written consent was obtained for all study participants.

The study protocol included collection of baseline/perinatal data and fetal ultrasound (biometrics, fetoplacental Doppler and echocardiography) at 28-30 weeks of gestation. Parental and ART characteristics were collected by interview and review of medical records at the time of prenatal evaluation. Upon delivery, presence of pregnancy complications, gestational age and mode of delivery, birth weight, birth weight centile, Apgar score, umbilical artery pH, admission to the neonatal intensive care unit (NICU) and major neonatal morbidity were recorded. Preeclampsia was defined as *de novo* blood pressure of $\geq$ 140/90 mmHg on two occasions 4h apart after the 20th gestational week, with concurrent proteinuria ($\geq$300 mg in a 24-h urine

specimen). Small-for-gestational age (SGA) was defined as birth weight below the 10th centile according to local reference curves[13]. Major neonatal morbidity was defined as the presence of bronchopulmonary dysplasia, necrotizing enterocolitis, intraventricular hemorrhage, periventricular leukomalacia, retinopathy, persistent ductus arteriosus or sepsis in the first 28 days of life.

Fetal ultrasound

All pregnancies underwent ultrasonographic examination at 28-30 weeks of gestation using a Siemens Sonoline Antares (Siemens Medical Systems, Malvern, PA, USA) with 6–4-MHz linear curved-array and 2-10 MHz phased-array probes. Gestational age at scan was calculated based on the first trimester crown-rump length[14]. Fetal ultrasonographic evaluation included assessment of estimated fetal weight, feto-placental Doppler and fetal echocardiography.

Estimated fetal weight was calculated according to Hadlock et al[15] and estimated fetal weight centile was calculated using local reference curves[13]. Fetoplacental Doppler assessment included pulsatility index (PI) measurement of uterine arteries, umbilical artery, middle cerebral artery, ductus venosus and aortic isthmus, according to previously published methodology[16-19]. Cerebroplacental ratio was calculated by dividing middle cerebral artery and umbilical artery PI[20].

Fetal echocardiography included a comprehensive structural and functional evaluation. Cardiothoracic ratio was calculated as heart area/thoracic area[21]. Left and right atrial areas were measured at maximum atrial distension and atrial/heart ratios were calculated as atrial area/heart area*100. Left and right ventricular sphericity indexes were calculated as the ratios base-to-apex length/basal ventricular diameters

measured at end-diastole[22]. Ventricular end-diastolic septal and free wall thicknesses were measured by M-mode from a transverse four-chamber view[23, 24].Mitral/tricuspid annular-plane systolic excursions (MAPSE/TAPSE) were assessed by M-mode from an apical or basal four-chamber view by placing the cursor at a right angle to the atrioventricular junction, and maximum amplitude of displacement was measured in millimeters[25]. Systolic (S') and early diastolic (E') peak velocities at mitral and tricuspid lateral annuli were measured by real time tissue Doppler from an apical or basal four-chamber view[26]. Atrioventricular flows were obtained from a basal or apical four-chamber view, placing the pulsed Doppler sample volume at the tip of atrioventricular valve leaflets. Right and left E/A ratios were estimated by calculating the ratio between early ventricular filling (E wave) to late ventricular filling (A-wave)[27]. Deceleration time of the E wave was measured from mitral and tricuspid inflow velocities from an apical four-chamber view. Left isovolumic relaxation time (IRT) was obtained from a 4-chamber view, placing the Doppler sample volume between the aortic and mitral valve; valvular clicks in the Doppler wave were used as landmarks to measure from the closure of the aortic valve to the opening of the mitral valve[28, 29].

Statistical analysis

Data was analyzed using IBM SPSS Statistics 21 statistical package. Sample size calculation was based on mitral S' because of its high sensitivity for detecting preclinical cardiac dysfunction in fetuses and children[30]. An estimated sample size of 20 individuals per group was calculated to enable us to observe a difference of 25% in mitral S' between cases and controls, with 80% power and a 5% type-I risk. Data is presented as mean ± standard deviation (SD), or percentage (%) where appropriate. P-

values below 0.05 were considered statistically significant. Baseline comparisons among the study groups and controls were calculated by Student's t-test or Pearson's χ^2 test. Multilevel analyses were used to compare perinatal and ultrasonographic parameters, since individual twin data are by definition related and multilevel analysis takes this dependency into account. Comparisons of echocardiographic variables were adjusted by parental age, paternal BMI and preeclampsia. Supplementary material includes cardiovascular comparisons of SC and ART twins versus singletons.

RESULTS

Baseline and perinatal characteristics

Baseline and perinatal characteristics of the study population are shown in Table 1. Study groups were similar in terms of maternal and paternal characteristics, with the exception of older parental age and higher paternal BMI in ART as compared to SC twins. Causes of infertility among the ART cases included 52% unexplained, 6% female factor, 34% male factor and 8% both female and male factors. 92% of ART pregnancies were conceived through ICSI and 8% by a combination of FIV and ICSI. Both groups showed similar perinatal characteristics with the exception of higher rates of preeclampsia, cesarean section, admission to the NICU and major neonatal morbidity in ART twin pregnancies as compared to those SC.

Fetal ultrasonographic results

Results of fetal echocardiography in the study groups are shown in Table 2. There were no differences in gestational age at scan, estimated fetal weight, estimated fetal weight centile or feto-placental Doppler parameters. Twins conceived by ART

showed similar sized hearts with larger atria; more globular and thicker right ventricles as compared to SC twins (Figure 1). Regarding functional parameters, ART twins presented a reduced systolic and diastolic longitudinal motion (by M-mode and tissue Doppler) with preserved ejection fraction as compared to SC twins. Fetal cardiovascular changes in ART twins were more prominent in the right heart and remained significantly different after adjusting by parental age, BMI and preeclampsia.

DISCUSSION

The present study provides evidence of fetal cardiac remodeling and dysfunction in twins conceived by ART. These findings are in line with previous reports demonstrating signs of cardiovascular dysfunction in singleton fetuses and children conceived by ART.[10-12, 31]

Twins conceived by ART showed larger atria and a pattern of right ventricular concentric remodeling (more globular and thicker right ventricles), together with signs of systolic and diastolic dysfunction. To our knowledge, this is the first study evaluating cardiovascular function in ART twins. The hereby described cardiac changes are similar to those reported in singleton ART pregnancies (see supplementary material) [11] and children [9, 10, 12] and are consistent with the hypothesis of specific fetal cardiovascular programming associated to ART. From a pathophysiological point of view, more globular and hypertrophic ventricles together with dilated atria are the usual cardiac response to pressure overload, being consistent with the reported high blood pressure and vascular wall thickness described in infants[11] and children[10, 12] conceived by ART. Cardiac changes were more prominent in the right as compared to the left heart, which might reflect the dominance of right heart during fetal life together with a higher

susceptibility to pressure overload of the right as compared with the left ventricle[32, 33]. The underlying cause of these cardiovascular changes remains to be elucidated. Parental predisposing factors, hormonal effects, perinatal environmental factors or epigenetic changes secondary to embryo manipulation have been postulated as potential factors. In our study, mothers with obvious chronic disease were excluded and statistical analyses were adjusted by parental age and BMI; however, we acknowledge that advance maternal age in ART pregnancies has been proposed as a major contributor of childbearing and that subfertility *per se* could still play a role[34]. Regarding perinatal characteristics, prenatal corticoid exposure, gestational diabetes, birthweight and gestational age at delivery were similar among groups, and analyses were adjusted by the rate of preeclampsia. In addition, the cardiac changes previously reported in SGA (concentric hypertrophy) are different from the ones here described in ART (larger atria and ventricular concentric remodeling)[35]. Consequently, the role of perinatal environment is possibly minor. Finally, epigenetic changes have been associated with ART[36]. Recent evidence shows that ART twins have lower DNA methylation at differentially methylated regions (PEG1, H19/IGF2)[37] related mainly with growth and development. During heart development, DNA methylation mechanisms undergo dynamic changes; and therefore, we could hypothesize that epigenetic dysregulation associated to ART, could possibly affect expression levels of genes involved in cardiovascular regulation[9, 38]. Overall, future studies are warranted to elucidate the underlying mechanisms of the increased cardiovascular risk associated to ART.

It is important to mention the significantly high prevalence of perinatal complications in the ART twin group[5]. Twin pregnancies conceived by ART presented a higher rate of preeclampsia, cesarean section, admission to NICU and neonatal

morbidity as compared to SC twins. These results, that were originally believed to be a consequence of the associated risks of a multiple gestation, have also been reported in ART singletons [10-12].

This study has some strengths and limitations that merit comment. Among the strengths of this study is the prospective performance of comprehensive fetal echocardiograms in a well-defined population recruited for this purpose. Extreme care was taken to exclude multiple pregnancies with obvious maternal disease, use of ovulation induction medication or other confounding factors that could influence our results. Multilevel analysis was performed to take into account the twin dependency. The analysis was adjusted by potential confounding factors identified such as parental age and preeclampsia, by linear regression in order to dissect the independent effect for mode of conception. However, we acknowledge that non-obvious confounders not considered in the design of the study might have affected the results. We also accept that the number of patients included in this study is small, which could allow for the lack of statistical significance of other parameters evaluated. Finally, the cardiovascular changes here reported are subclinical and, while they are recognized as potential cardiovascular risk factors, their persistence and long-term association with cardiovascular disease remains to be proven, which warrants further follow-up of this cohort.

ART is redefining biology and society; we believe that it is a great medical achievement but, from an ethical and clinical point of view, it is important to understand the potential impact of these techniques on perinatal and long lasting health. The aim of this study was to provide further evidence with regards to the effects of ART in twin pregnancies. Overall, our results showed the presence of cardiac remodeling and

dysfunction in twin fetuses conceived by ART. The hereby observed cardiac changes , albeit subtle, could be responsible for an increased cardiovascular risk later in life, in keeping with the fetal programming theory[39]. From a clinical point of view, these findings would affect approximately 5% of newborns worldwide, identifying them as a high-risk population for cardiovascular disease. Mild cardiovascular changes present in fetal life may remain subclinical during childhood, but may worsen and turn into significant health issues with certain stressors, postnatal conditions such as obesity and other lifestyle behaviors. Thus, early interventions in this group, such as promoting breastfeeding[14], lifestyle modifications, lack of exposure to other risk factors, and blood pressure surveillance could aid in reducing these risks[35, 40]. In conclusion, this study strengthens evidence that the use of ART is associated with fetal cardiovascular remodeling, opening opportunities for early identification and potential interventions in this population.[41, 42]. The underlying mechanisms and long-term cardiovascular risks related to ART remain to be elucidated in future research.

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TABLES

Table 1. Baseline and perinatal characteristics of the study groups.

	SC twins	ART twins	*p-value*
N	*50*	*50*	
Maternal characteristics			
Age (years)	32 ± 4	35 ±3	<0.001
BMI (kg/m^2)	23 ± 3.4	23± 4.2	0.643
Smoking	6	10	0.187
Primiparity	40	58	0.142
Paternal characteristics			
Age (years)	33 ± 3	38 ± 4	<0.001
BMI (kg/m^2)	25 ± 4.0	27 ± 5.6	0.023
Smoking	39	34	0.539
Pregnancy complications			
Preeclampsia	5	20	0.018
Prenatal corticoid exposure	20	30	0.117
Gestational diabetes	4	7	0.803
N	*50*	*50*	
Delivery data			
Gestational age at delivery (weeks)	37 ± 2.3	37 ± 2.5	0.298
Cesarean section	60	70	0.041
Male	60	52	0.919
Birthweight (g)	2529 ± 483	2493 ± 603	0.967
Birthweight centile	60 ± 28	57±32	0.269
Small for gestational age	6	7	0.516
Umbilical artery pH	7.27 ± 1.15	7.27 ± 0.5	0.440
Neonatal outcome			
Admission to neonatal intensive care unit	3	15	0.043
Major neonatal morbidity	2	10	0.028

Data shown as mean ± SD or percentage.
SC, spontaneously conceived; ART, assisted reproductive technologies; BMI, body mass index.

Table 2. Fetal ultrasonographic results of the study populations.

	SC twins	**ART twins**	*Adjusted p-value**
N	*50*	*50*	
Gestational age at scan (weeks)	29 ± 0.85	29 ± 0.69	0.072
Standard feto-placental data			
Estimated fetal weight (g)	1302 ± 171	1246 ± 126	0.356
Estimated fetal weight (centile)	57 ± 26	56 ± 25	0.097
Uterine arteries mean PI	0.68 ± 0.18	0.69 ± 0.17	0.241
Umbilical artery PI	1.09 ± 0.23	1.13 ± 0.26	0.443
Middle cerebral artery PI	1.93 ± 0.43	2.03 ± 0.38	0.091
Cerebroplacental ratio	1.61 ± 0.71	1.78 ± 0.69	0.070
Ductus venosus PI	0.55 ± 0.13	0.58 ± 0.16	0.421
Aortic isthmus PI	2.35 ± 0.28	2.47 ± 0.34	0.720
Cardiac morphometric data			
Cardiothoracic ratio	0.25 ± 0.06	0.24 ± 0.03	0.438
Left atrial/heart ratio	12.5 ± 0.3	13.2 ± 0.3	0.040
Right atrial/heart ratio	15.7 ± 3.1	18.4 ± 3.2	<0.001
Left ventricular sphericity index	1.77 ± 0.30	1.67 ± 0.20	0.466
Right ventricular sphericity index	1.57 ± 0.25	1.41 ± 0.23	0.001
Left ventricular free wall thickness (mm)	2.70 ± 0.54	2.99 ± 0.42	0.285
Septal wall thickness (mm)	2.57 ± 0.45	2.84 ± 0.41	0.034
Right ventricular free wall thickness (mm)	2.80 ± 0.48	3.10 ± 0.38	0.146
Systolic Function			
Left ejection fraction (%)	68.5 ± 9.0	69.5 ± 9.1	0.690
Right ejection fraction (%)	66.8 ± 9.4	69.8 ± 9.3	0.740
MAPSE (mm)	4.87 ± 0.69	4.70 ± 0.89	0.725
TAPSE (mm)	6.36 ± 0.89	5.18 ± 0.93	<0.001
Mitral S' (cm/s)	6.9 ± 0.9	6.8 ± 1.0	0.341
Tricuspid S' (cm/s)	9.1 ± 10.7	7.2 ± 1.0	0.376
Diastolic Function			
Mitral E/A ratio	0.74 ± 0.10	0.74 ± 0.09	0.866
Tricuspid E/A ratio	0.79 ± 0.37	0.77 ± 0.13	0.793
Mitral E deceleration time (ms)	74 ± 28	66 ± 21	0.119
Tricuspid E deceleration time (ms)	69 ± 28	60 ± 15	0.243
Mitral E' (cm/s)	7.5 ± 1.3	6.9 ± 1.1	0.009

Tricuspid E' (cm/s)	8.4 ± 1.5	7.8 ± 1.0	0.012
Left isovolumic relaxation time (ms)	50 ± 11	49 ± 9	0.613

Data shown as mean ± SD or percentage.

*adjusted by parental age, paternal BMI and preeclampsia.

SC, spontaneously conceived; ART, assisted reproductive technologies; PI, pulsatility index; MAPSE, mitral annular-plane systolic excursion; TAPSE, tricuspid annular-plane systolic excursion; S', systolic peak velocity; E, early ventricular inflow; A, ventricular inflow during atrial contraction; E', early diastolic peak velocity.

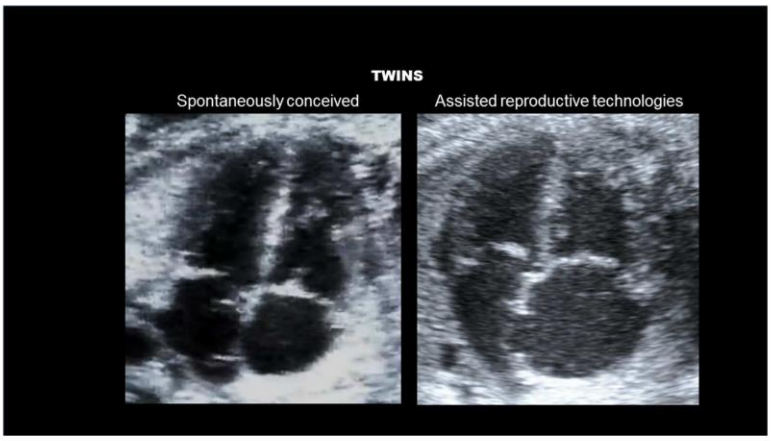

Figure 1. Illustrative four-chamber views of the study groups. Twin fetuses conceived spontaneously (control), and conceived by assisted reproductive technologies (ART), illustrating the dilated atria and shorter ventricles as compared to control.

338x190mm (96 x 96 DPI)

Differential effect of assisted reproductive technologies and small-for-gestational-age on fetal cardiac remodeling

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ABSTRACT

Objective

Assisted reproductive technologies (ART) and small-for-gestational-age (SGA) fetuses show cardiovascular remodeling *in utero*; however, these conditions are often associated. We evaluated the differential effect of ART and SGA on fetal cardiac remodeling.

Methods

We performed a prospective cohort study including term singleton pregnancies, divided into four groups: 102 appropriate for gestational age (AGA) fetuses conceived spontaneously (controls), 72 AGA fetuses conceived by ART (ART-AGA), 31 SGA fetuses conceived by ART (ART-SGA) and 28 SGA fetuses conceived naturally (SGA). SGA was defined as birthweight below 10th centile. Fetal echocardiography was performed at 29 ± 0.9 weeks of gestation, assessing cardiac dimensions, geometry and function.

Results

ART fetuses presented dilated atria (left atrium to heart ratio: controls mean 15(SD 2.7)% vs ART 18(4.1)% vs SGA 14(3.7)%) and more globular ventricles (left ventricular sphericity index: controls 1.77(0.2) vs ART 1.68(0.2) vs SGA 1.72(0.2)) with normally sized hearts. In contrast, SGA fetuses had enlarged hearts (cardiothoracic ratio: controls 24(3)% vs ART 24(4)% vs SGA 29(6)%), preserved atrial size, more globular and concentric hypertrophic ventricles (left relative wall thickness: controls 0.48(0.17) vs ART 0.54(0.13) vs SGA 0.63(0.23)). Both ART and SGA had decreased longitudinal motion (tricuspid annular ring displacement: controls 6.5(0.8)mm vs ART 5.5(0.7)mm vs SGA 5.9(0.6)mm) and impaired relaxation (left isovolumetric relaxation time: controls 47(7)ms vs ART 50(8)ms vs SGA 50(9)ms). ART-SGA fetuses presented a combination of features from both groups.

Conclusions

SGA and ART were associated with distinct patterns of fetal cardiac remodeling, which supports that they are independent causes of cardiac programming.

Keywords: cardiac remodeling, pregnancy, assisted reproductive technologies, small for gestational age.

INTRODUCTION

The concept that perinatal environment may influence fetal cardiovascular structure and function, has been extensively described[1]. This notion was first demonstrated in small for gestational age (SGA) fetuses, which complicates 7-10% of all pregnancies, and constitutes a major cause of perinatal morbidity and mortality[2]. SGA fetuses have remodeled and less efficient hearts, more globular, with decreased longitudinal motion and impaired relaxation. These changes are already present *in utero* and persist after birth[3, 4]. Fetal cardiovascular remodeling is thought to partially explain the association demonstrated by numerous epidemiological studies, between low birth weight and subsequent cardiovascular disease and mortality in adulthood[5, 6].

Recent data also suggests that the use of assisted reproductive technologies (ART) can lead to fetal cardiovascular remodeling[7-9]. ART are currently used worldwide and represent 1% to 4% of births in developed countries[10]. Fetuses conceived by ART have been shown to present globular hearts, dilated atria and decreased longitudinal function *in utero*[9] together with changes in vasculature that persist until adolescence[11-13]. However, given the higher incidence of SGA among pregnancies conceived by ART, it is unclear to what extent SGA may contribute to the degree and nature of cardiac remodeling observed. On the other hand, most studies reporting cardiovascular changes in SGA fetuses did not account for the potential effect of mode of conception.

We aimed to evaluate the differential effects of SGA and ART on fetal cardiovascular development. For that purpose, fetal echocardiography was performed in a large cohort of fetuses which included four clinical groups: normally grown spontaneously conceived fetuses, normally grown ART fetuses, SGA fetuses conceived by ART, and spontaneously conceived SGA fetuses.

METHODS

Study populations and protocol

This was a prospective cohort study including singleton pregnancies born at term, subdivided into four groups: 102 appropriate for gestational age (AGA) fetuses conceived spontaneously (controls), 72 AGA fetuses conceived by ART (ART-AGA), 31 SGA fetuses conceived by ART (ART-SGA) and 28 SGA conceived naturally. The source population comprised all pregnancies between 28-32 weeks' gestation from April 2011 to September 2013

from the Department of Maternal-Fetal Medicine at BCNatal in Barcelona, Spain. Pregnancies with structural/chromosomal anomalies, evidence of infection or any maternal medical disease including asthma, chronic hypertension, diabetes mellitus, heart disease, human immunodeficiency virus, hepatitis infection, lupus or thyroid disease were excluded. For the purpose of this study, only fetuses who delivered >37 weeks of gestation were finally included, in order to avoid the potential interaction of prematurity. Gestational age was calculated according to crown-rump length at first trimester scan. Conception by ART was defined as those fertility treatments which involve surgically removing eggs from a woman's ovaries, combining them with sperm in a laboratory, and returning them to the woman's body in order to become pregnant, i.e. standard in vitro fertilization (IVF) or intracitoplasmic sperm injection (ICSI)[14]. SGA was defined as birth weight below the 10th centile according to local reference curves[15]. A reference cohort of fetuses spontaneously conceived with normal estimated weight and birth weight (>10th centile) were randomly sampled from pregnancies at our institution and frequency paired with ART and SGA cases by gestational age at fetal scan (±1 week). One hundred sixty-two fetuses (68%) included in this study were part of previously published data[9, 16].

Our study protocol included standard feto-placental Doppler ultrasound and echocardiography at 28-32 weeks of gestation performed by 3 experienced operators in fetal echocardiography. Parental baseline and ART characteristics were collected by interview and review of medical records at the time of the prenatal evaluation, including parental age, body mass index, smoking status, ethnicity, nulliparity, socioeconomic status and ART characteristics. Familiar cardiovascular history was defined as the presence of congenital heart disease, coronary disease, hypertension, diabetes or hypercholesterolemia in men <55 years and women <65 years. Low socioeconomic status was defined as routine occupation, long-term unemployment or never worked, for both the pregnant woman and her partner.

Upon delivery, presence of pregnancy complications, gestational age at delivery, mode of delivery, birth weight, birth weight centile, Apgar score, umbilical artery pH and perinatal morbidity were recorded. Major neonatal morbidity was defined as the presence of bronchopulmonary dysplasia, necrotizing enterocolitis, intraventricular hemorrhage, periventricular leukomalacia, retinopathy, persistent ductus arteriosus or sepsis. Perinatal mortality was defined as either intrauterine death or neonatal death within the first 28 days of

life. The study protocol was approved by Hospital Clinic's Ethics Committee, and written parental consent was obtained for all study participants.

Fetal ultrasound

All pregnancies underwent ultrasonographic examination at 28-32 weeks of gestation, using a Siemens Sonoline Antares (Siemens Medical Systems, Malvern, PA, USA) with 6–4-MHz linear curved-array and 2-10 MHz phased-array probes, including the evaluation of feto-placental Doppler and echocardiography. Feto-placental Doppler evaluation included pulsatility index (PI) measurement of uterine arteries, umbilical artery, middle cerebral artery, ductus venosus and aortic isthmus according to a previously published methodology[17-20]. The cerebroplacental ratio was calculated by dividing middle cerebral artery and umbilical artery PI[21].

Fetal echocardiography included a comprehensive morphometric and functional assessment by two-dimensional, M-mode, conventional and tissue Doppler.

Fetal cardiac morphometry included cardiothoracic ratio, atrial areas, ventricular sphericity indices and relative wall thicknesses. Cardiothoracic ratio was measured from a four-chamber view and calculated as heart area/thoracic area[22]. Left and right atrial areas were traced on 2D images at the maximum point of atrial distension[23]. Ventricular base-to-apex lengths and basal diameters were measured on 2D images from an apical four-chamber view at end-diastole. Left and right ventricular sphericity indexes were calculated as base-to-apex length/basal diameters. Septal and free wall thicknesses, and ventricular dimensions were measured by M-mode from a transverse four-chamber view at end-diastole[24, 25]. Calculation of relative wall thickness was performed for left and right ventricular walls, with the formula (2*wall thickness) / (ventricular inner diameter at end-diastole)[26].

Fetal cardiac systolic function evaluation included ejection fraction, mitral/tricuspid annular-plane systolic excursion (MAPSE/TAPSE) and systolic annular peak velocity (S′). Left and right ejection fraction were obtained from a transverse four-chamber view by M-mode using the Teicholz formula[27]. MAPSE and TAPSE were assessed by M-mode from an apical or basal four-chamber view[28]. Spectral tissue Doppler imaging was used to record systolic peak velocities (S′) at mitral and tricuspid lateral annuli from an apical or basal four-chamber view, and measured in real time during the echocardiographic study[29].

Fetal cardiac diastolic function was evaluated by peak early and late transvalvular filling (E/A) ratio, deceleration time of E velocity, early (E′) diastolic annular peak velocities and left isovolumic relaxation time (IRT). Atrioventricular flows were obtained from a basal or apical four-chamber view, placing the pulsed Doppler sample volume at the tip of atrioventricular leaflets. Left and right E/A ratios were calculated as the ratio between early ventricular filling velocity (E) and late ventricular filling velocity (A)[30]. Deceleration time of the E wave was measured from mitral and tricuspid inflow velocities from an apical four-chamber view[20]. Spectral tissue Doppler imaging was used to record early diastolic (E′) peak velocity at mitral and tricuspid lateral annuli from an apical or basal four-chamber view[29]. Left IRT was measured in a cross-section of the fetal thorax at the four-chamber view, placing the Doppler sample volume on the medial wall of the ascending aorta, including the aortic and mitral valve; valvular clicks in the Doppler wave were used as landmarks and IRT was measured from the closure of the aortic valve to the opening of the mitral valve[31].

Statistical analysis

Data was analysed using IBM SPSS Statistics 21 statistical package. Data is presented as mean ± standard deviation (SD) or percentage(n) where appropriate. *p*-values below 0.05 were considered statistically significant. Comparisons among the study groups and controls were performed with analysis of variance (ANOVA), with Bonferroni correction for multiple comparisons. Categorical variables were analyzed using Fisher's exact test, and $p < 0.05$ was considered statistically significant. Linear regression was also performed in order to compare echocardiographic parameters among groups adjusted by maternal age, gestational age at scan and prenatal corticoid exposure. Finally, a multivariate analysis of covariance (MANCOVA) was performed in order to obtain mean differences between groups adjusted by maternal age, gestational age at scan and prenatal corticoid exposure.

RESULTS

Characteristics of the study populations

Table 1 shows the baseline and perinatal characteristics of the study populations. Parental baseline characteristics were similar among the study groups, with the exception of higher maternal age in both ART groups and higher paternal age in ART and SGA groups, compared to

controls. ART and SGA groups showed an earlier gestational age at delivery, compared to controls. As expected, SGA pregnancies presented lower birth weight and higher prevalence of preeclampsia, prenatal corticoid exposure, admission to the neonatal intensive care unit and major neonatal morbidity.

Fetal ultrasound in the study groups

Fetal ultrasound data is depicted in Table 2. As expected, SGA cases presented worse feto-placental Doppler indices, with increased uterine artery mean PI and lower cerebroplacental ratio. Ductus venosus and aortic isthmus values were similar among the study groups. Naturally conceived SGA cases had larger hearts compared to the other groups. ART cases showed larger atria with preserved heart size. Both SGA and ART cases showed more globular hearts, compared to controls, and increased relative wall thickness that was more marked in the naturally conceived SGA fetuses. While ejection fraction was preserved in all cases, decreased longitudinal motion and increased IRT could be observed in both ART and SGA groups, compared to controls. Finally, mitral and tricuspid E deceleration time was prolonged in SGA and reduced in ART compared to controls. Most cardiovascular changes in ART and SGA fetuses remained significant after adjusting by maternal age, gestational at scan and corticoid exposure.

MANCOVA was performed to assess the mean differences between the study groups and controls adjusted by maternal age, gestational age at scan and corticoid exposure (Figures 1, 2 and Supplementary Material). This analysis identified the following variables as more representative of each phenotype: increased cardiothoracic ratio, relative wall thicknesses and mitral E deceleration time in SGA; dilated right atria with reduced E deceleration time in ART fetuses. All study groups showed decreased longitudinal motion (TAPSE) with regards to controls.

DISCUSSION

This study provides evidence that both ART and SGA have a direct and independent effect on the fetal cardiovascular system that translates into different fetal cardiac phenotypes.

Our findings confirm previous studies demonstrating fetal cardiovascular changes in SGA and ART and further provide evidence of the independent effect of each factor. We are describing larger, more globular and hypertrophic hearts in naturally conceived SGA fetuses

with reduced longitudinal motion (decreased MAPSE, TAPSE and mitral S') and impaired relaxation (decreased E' and prolonged IRT and E deceleration time). This data is in agreement with previous studies demonstrating significant changes in cardiac structure and function in fetuses and children born SGA[3, 4, 16, 32, 33]. Cardiac changes observed in SGA fetuses can be explained as an adaptive response to sustained undernutrition and hypoxia, together with increased placental vascular resistance that results in pressure/volume overload. It is probable the heart reacts to increased placental resistance and pressure overload by shifting into a more spherical shape (with a lower radius of curvature that would better tolerate pressure overload by reducing wall stress), and hypertrophying myocardial walls (in order to increase the force-generating units). Finally, volume overload would explain cardiomegaly. While this concentric hypertrophy maintains a normal ejection fraction, signs of reduced longitudinal motion and impaired ventricular filling can usually be observed in SGA.

We also describe dilated atria and more spherical ventricles without cardiomegaly in fetuses conceived by ART. Signs of decreased longitudinal motion (reduced TAPSE, MAPSE and S') and impaired relaxation (decreased E deceleration time) were also observed. These changes are in line with our previous report in ART fetuses[9, 34] and postnatal studies demonstrating increased vascular pressure in children and adolescents conceived by ART[11-13]. This is also consistent with the shortened E deceleration time (usually secondary to increased ventricular end-diastolic pressures) observed in ART but not in SGA. The fetal heart responds to pressure overload with concentric remodeling, by reducing the ventricular radius of curvature (more spherical ventricles) together with a mild increase in myocardial wall thickness, maintaining a normal heart size. Observed dilated atria are probably consequence of a ventricular early filling problem that needs to be compensated by larger atrial contribution; increased pressure also results in dilated atria. These changes were more prominent in the right heart, probably due to its predominance in fetal life and its higher susceptibility to pressure overload compared to the left ventricle. It is also consistent with previous reports suggesting increased pulmonary pressures in children conceived by ART[13].

Parental predisposing factors, epigenetic changes secondary to early embryo manipulation, hormonal effects and association to perinatal complications have been postulated as factors potentially mediating increased vascular pressures described in ART offspring.

However, the exact mechanisms driving fetal cardiovascular remodeling in ART pregnancies remain to be elucidated. While pressure overload seems to be a common feature in ART and SGA, the absence of fetal hypoxia/undernutrition and volume overload in ART could explain the different phenotypic response of the fetal heart in comparison to SGA. Finally, fetuses conceived by ART who developed growth restriction showed mixed characteristics from both groups, maintaining dilated atria observed in ART, but also left myocardial dysfunction present in SGA fetuses; the ART-SGA group is more similar to the normally grown ART, suggesting that changes mediated in ART are more strongly manifested in these cases and could partially counter the changes from SGA.

Epidemiological studies have provided evidence of an association of SGA with cardiovascular risk later in life, demonstrating a higher incidence of hypertension and cardiovascular mortality in this population.[6, 35] Likewise, ART has been associated with postnatal hypertension, premature subclinical atherosclerosis in the systemic circulation and pulmonary vascular dysfunction[11-13]. The findings of the present study, suggesting distinct patterns of fetal cardiac remodeling, support the hypothesis of ART and SGA being independent causes of cardiac programming. Fetal echocardiography has already demonstrated its utility in the prediction of postnatal cardiovascular health in SGA[16]. Therefore, the identification of different cardiac phenotypes in SGA and ART pregnancies may help in the diagnosis and long-term cardiovascular prognosis of these fetuses and children. The importance of early identification and the impact of interventions in pediatric populations for preventing future cardiovascular disease are now well recognized[35]. Given the high prevalence of both SGA (7-10%) and ART (2-20%), early interventions in these high-risk populations could have a tremendous effect on public health, by potentially improve the future cardiovascular health of these children.

This study has various strengths and limitations. To our knowledge, this is the first study illustrating fetal cardiovascular changes in separate populations of ART and naturally conceived SGA. Comprehensive fetal echocardiography was prospectively performed in a large proportion of well-characterized fetuses. Cases were frequency matched by gestational age with controls, and we further adjusted for other potential confounders such as maternal age and prenatal corticoid exposure. However, we acknowledge there may be other non-obvious confounders like preclampsia and birthweight, so despite the *n* per group, we decided to adjusted by these. Due to the lack of standardization and normality values for most fetal cardiac morphometric parameters,

we are displaying crude values and also results normalized by cardiac size in the supplementary material. Most geometrical differences remained statistically significant after normalization, while some functional parameters lost their significance. We acknowledge that the optimal scenario would be to establish a consensus on how to normalize these fetal echocardiographic parameters. Therefore, the differential effect of growth restriction and assisted reproductive technologies on fetal cardiac functional parameters and some of the geometrical changes could be over interpreted. The difficulty of prospectively assessing fetal echocardiography in ART pregnancies that developed growth restriction limited the sample size of our study and may have prevented some statistical differences among groups. Our findings warrant postnatal long-term follow-up to describe the prognosis and clinical significance of the described changes.

CONCLUSIONS

In summary, this study provides evidence that there is a different and independent cardiac effect in SGA and ART fetuses. Each group showed a particular cardiac phenotype, with ART-SGA cases showing a mixture of characteristics from both. Although the underlying mechanisms for these changes remain to be elucidated, the existence of different cardiac remodeling in these fetuses suggests the need for long-term cardiovascular follow-up of both SGA and ART, and opens a window of opportunity to improve cardiovascular health in these children.

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TABLES

Table 1. Baseline and perinatal characteristics of the study populations.

	Controls (n=102)	ART-AGA (n=72)	ART-SGA (n=31)	SGA (n=28)	*p*-value
Maternal characteristics					
Age (years)	33 ± 5	36 ± 3*	36 ± 3*	34 ± 4†	<0.001
BMI (kg/m^2)	23.3 ± 4.0	24.8 ± 5.6	23.5 ± 3.1	22.5 ± 3.5	0.065
Smoking (%)	9.8(10)	5.6(4)	6.5(2)	14.3(4)	0.489
Caucasian (%)	81.4(83)	88.9(64)	93.5(29)	92.9(26)	0.224
Nulliparity (%)	56.9(58)	56.3(40)	41.9(13)	50(14)	0.485
Familiar history of early cardiovascular disease (%)	1.9(2)	2.8(2)	0	7.2(2)	0.453
Low socioeconomic status (%)	15.7(16)	23.6(17)	19.4(6)	50(14)	0.007
Paternal characteristics					
Age (years)	35 ± 6	38 ± 4*	38 ± 5*	36 ± 5	0.001
BMI (kg/m^2)	25.6 ± 3.1	26.2 ± 4.2	25.0 ± 2.5	25.8 ± 4.3	0.478
Smoking (%)	31.7(32)	33.3(24)	30(9)	50(12)	0.378
Caucasian (%)	97.1(99)	95.8(69)	93.5(29)	96.3(26)	0.761
Perinatal data					
Prenatal corticoid exposure (%)	2(2)	4.2(3)	12.9(4)	32.1(9)*†‡	<0.001
Preeclampsia (%)	0	0	3.2(1)*†	3.6(1)*†	0.063
Gestational age at delivery (weeks)	40 ± 1	39 ± 1	39 ± 1	38 ± 1	0.766
Cesarean section (%)	24.5(25)	27.8(20)	45.2(14)	50(14)	0.180
Male (%)	49(50)	43.1(31)	58.1(18)	57.1(16)	0.560
Birthweight (g)	3300 ± 445	3305 ± 433	2480 ± 613*†	2105 ± 793*†‡	<0.001
Birthweight centile	50 ± 23	53 ± 27	4 ± 2*†	0 ± 3*†	<0.001
5 minutes Apgar score	9.8 ± 0.9	9.8 ± 0.1	9.8 ± 0.1	9.9 ± 0.2	0.767
Umbilical artery pH	7.2 ± 0.1	7.2 ± 0.1	7.2 ± 0.1	7.2 ± 0.1	0.584
Neonatal outcome					
Admission to neonatal intensive care unit (%)	2(2)	2.8(2)	6.5(2)	39.3(11)*†‡	<0.001
Major neonatal morbidity (%)	1(1)	1.4(1)	6.5(2)	32.1(9)*†‡	<0.001
Perinatal mortality (%)	0	0	0	3.6(1)	0.119

Data shown as mean ± standard deviation or percentage(n). Comparisons performed among groups by ANOVA, with Bonferroni corrections or Fisher's exact test where appropriate. *p<0.05 compared with controls; †p<0.05 compared with ART-AGA; ‡p<0.05 compared with ART-SGA.

ART-AGA, pregnancies conceived by assisted reproductive technologies and appropriate-for-gestational-age; ART-SGA, pregnancies conceived by assisted reproductive technologies and small-for-gestational-age; SGA, small-for-gestational-age; BMI, body mass index.

Table 2. Feto-placental Doppler and echocardiographic results in the study groups.

	Controls (n=102)	ART-AGA (n=72)	ART-SGA (n=31)	SGA (n=30)	*Adjusted p-value¶*
Gestational age at scan (weeks)	29.0 ± 0.9	29.1 ± 0.7	29.0 ± 0.7	29.3 ± 0.7	0.880
Standard feto-placental Doppler					
Uterine artery mean PI	0.64 ± 16	0.72 ± 18	0.77 ± 25	0.98 ± 31*†‡	*0.005*
Cerebro-placental ratio	1.95 ± 0.4	2.00 ± 0.4	1.60 ± 0.4†	1.62 ± 0.4*†	*0.305*
Ductus venosus PI	0.52 ± 0.1	0.55 ± 0.1	0.54 ± 0.1	0.60 ± 0.1	*0.894*
Aortic isthmus flow index	1.30 ± 0.1	1.29 ± 0.1	1.32 ± 0.1	1.25 ± 0.1	*0.646*
Cardiac morphometry					
Cardiothoracic ratio (%)	24 ± 3	24 ± 4	23 ± 3	29 ± 6*†‡	*0.921*
Left atrium/heart area ratio (%)	15 ± 2.7	18 ± 4.1*	17 ± 1.7*	14 ± 3.7†‡	*0.025*
Right atrium/heart area ratio (%)	16 ± 2.9	19 ± 3.8*	18 ± 2.3*	16 ± 3.8†‡	*0.002*
LV sphericity index	1.77 ± 0.2	1.68 ± 0.2*	1.72 ± 0.1*	1.72 ± 0.2*†	*0.085*
LV relative wall thickness	0.48 ± 0.17	0.54 ± 0.13*	0.55 ± 0.12*	0.63 ± 0.23*†	*0.002*
RV sphericity index	1.60 ± 0.2	1.40 ± 0.1*	1.52 ± 0.1*	1.54 ± 0.2*†	*0.005*
RV relative wall thickness	0.46 ± 0.10	0.52 ± 0.09*	0.51 ± 0.10*	0.62 ± 0.14*†‡	*<0.001*
Systolic function					
Heart rate (bpm)	141 ±8.5	141 ±8.4	144 ±10.8	139 ±13.7	0.922
LV ejection fraction (%)	67 ± 7.1	66 ± 8.3	64 ± 9.6*	71 ± 6.1	*0.893*
RV ejection fraction (%)	68 ± 6.7	67 ± 8.5	66 ± 7.0	65 ± 7.3	*0.266*
Mitral ring displacement (mm)	4.8 ± 0.7	4.3 ± 0.8*	4.1 ± 0.8*	4.1 ± 0.6*†‡	*0.004*
Tricuspid ring displacement (mm)	6.5 ± 0.8	5.5 ± 0.7*	5.7 ± 0.7*	5.9 ± 0.6*	*0.001*
Mitral S' (cm/s)	6.9 ± 1.0	6.4 ± 1.2*	5.9 ± 0.9*†	5.6 ± 0.6*†	*0.001*
Tricuspid S' (cm/s)	7.3 ± 1.1	7.0 ± 1.1	7.1 ± 0.9	7.1 ± 1.1	*0.162*
Diastolic function					
Mitral E/A ratio	0.72 ± 0.1	0.76 ± 0.1	0.73 ± 0.8	0.78 ± 0.9	*0.163*
Tricuspid E/A ratio	0.72 ± 0.1	0.73 ± 0.1	0.73 ± 0.1	0.76 ± 0.1	*0.087*
Mitral E deceleration time (ms)	73 ± 26.8	62 ± 19.5*	76 ± 19.1	86 ± 27.7*†‡	*0.278*
Tricuspid E deceleration time (ms)	60.5 ± 20	52.0 ± 18.7*	56.5 ± 14.4	71.0 ± 30.1*†‡	*0.960*
Mitral E' (cm/s)	7.6 ± 1.0	6.8 ± 1.2*	7.0 ± 1.0*	6.0 ± 1.1*†‡	*0.087*
Tricuspid E' (cm/s)	8.4 ± 1.12	7.7 ± 1.20*	8.0 ± 1.3*	7.1 ± 1.0*	*0.002*
Mitral A' (cm/s)	10.0±2.30	9.81±2.61*	9.66±1.71*	7.38±1.73*	*0.025*
Tricuspid A' (cm/s)	11.4±1.61	10.8±1.80	10.5±1.61*	9.61±2.00*†	*0.003*
Left isovolumic relaxation time (ms)	47.0 ± 7.3	50.0 ± 7.9*	49.0 ± 6.5*	49.5 ± 9.3*	*0.054*

Data shown as mean ± standard deviation. *p<0.05 as compared with controls; †p<0.05 as compared with ART-AGA; ‡p<0.05 as compared with ART-SGA. Adjusted p values calculated by linear regression, adjusting for maternal age, glucocorticoid exposure, preeclampsia, gestational age at delivery and birthweight. ART-AGA, pregnancies conceived by assisted reproductive technologies and appropriate-for-gestational-age; ART-SGA, pregnancies conceived by assisted reproductive technologies and small-for-gestational-age; SGA, small-for-gestational-age; PI, pulsatility index; LV, left ventricle; RV, right ventricle; S', systolic annular peak velocity; E, early diastolic inflow peak velocity; E', early diastolic annular peak velocity.

FIGURES AND LEGENDS

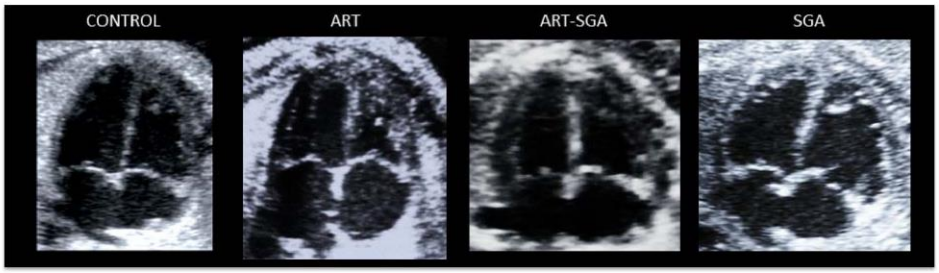

Figure 1. Illustrative four-chamber views of the study groups.

Normally-grown fetus conceived spontaneously (control), normally-grown fetus conceived by assisted reproductive technologies (ART), fetus conceived by ART born small-for-gestational age (SGA) and a spontaneously conceived SGA fetus, illustrating the enlarged concentric hypertrophic hearts in SGA as compared to the dilated atria and shorter ventricles in ART.

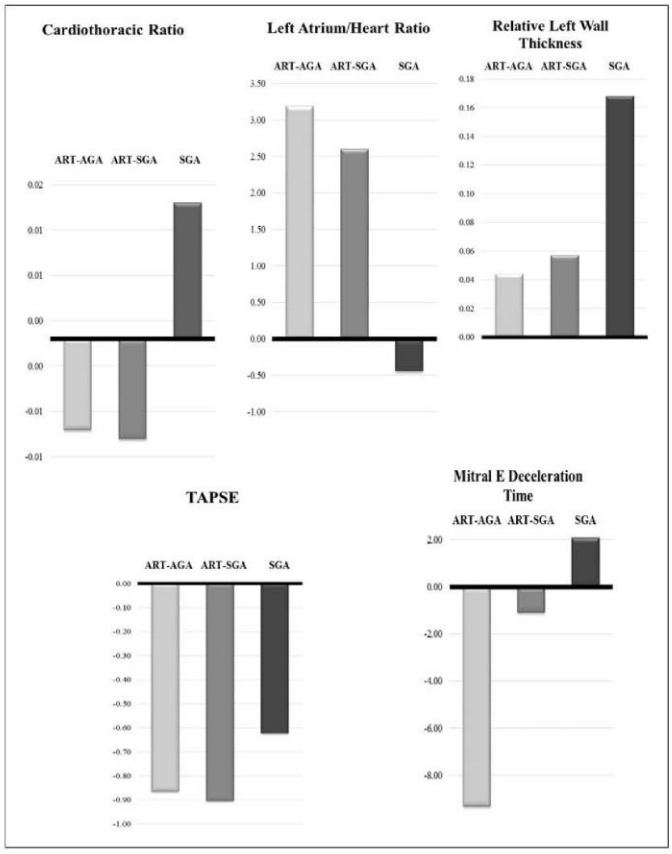

Figure 2. Adjusted mean difference bar graphs for fetal echocardiographic parameters in the study groups.

Baseline represents control group at 0 (normally-grown fetuses conceived spontaneously), with each bar quantifying the mean difference for each group with regards to controls adjusted by maternal age, gestational age at scan and prenatal corticoid exposure. This analysis illustrates the different fetal cardiac phenotypes of the study groups with

enlarged and hypertrophic hearts in SGA versus dilated atria in ART. While longitudinal motion was similar in ART and SGA, E deceleration time was prolonged in SGA and reduced in ART.

STUDY 5.

Assisted reproductive technologies and cardiovascular characteristics in 3-year-old children: a follow-up study

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Objective

Children born after assisted reproductive techniques (ART) have increased cardiovascular dysfunction. We aimed at evaluating whether signs of postnatal cardiovascular dysfunction persist at 3 years of age after being born by ART.

Methods

A cohort of 80 ART fetuses was followed into childhood and compared with 65 normally grown fetuses (controls) matched for gender and age at scan. Cardiovascular evaluation was performed at 3 years of corrected age, including blood pressure, carotid intima-media thickness (cIMT) and complete functional echocardiographic assessment. All echocardiographic parameters were adjusted by body surface area.

Results

As compared to controls, children conceived by ART showed more globular hearts (right ventricular sphericity index: control mean 1.8 (SD 0.5) vs. ART 1.6 (0.2), p<0.001) and larger atria (right atrial area: control 4.9 cm^2 (0.9) vs. ART 5.5 cm^2 (0.9), p<0.001) with signs of systolic (tricuspid annular plane systolic excursion: control 18 mm (2) vs. ART 16 mm (3), p<0.001) and diastolic dysfunction (isovolumic relaxation time: control 68 ms (12) vs. ART 79 ms (12), p<0.001). ART children also presented increased systolic blood pressure (control 90 mmHg (6) vs. ART 94 mmHg (5), p<0.003) and cIMT (control 0.52 µm (0.14) vs. ART 0.60 µm (0.16), p<0.001).

Conclusions

Primary cardiac and vascular changes previously reported in ART fetuses and 6 month-old infants persist at 3 years of age. This data support the ability to demonstrate changes early in life, which could be used to monitor early interventions to improve health in these children.

Results

For the follow-up cohort in total we recruited 80 ART infants and 65 controls.

Baseline and perinatal characteristics

Baseline and perinatal characteristics of the study are shown in Table 5.1. Parental baseline characteristics were similar among the study groups, with the exception of higher parental age in ART groups compared to controls. ART group showed an earlier gestational age at delivery, together with lower birthweight and birthweight centile as compared to controls. As expected, ART pregnancies presented higher prevalence of preeclampsia, gestational diabetes, prenatal corticoid exposure and admission to the neonatal intensive care unit, differences that were non-significant statistically. The mode of delivery by cesarean section was higher in the ART group than controls and fetal gender was similar between groups.

Infant assessment

Follow-up characteristics and cardiovascular results are shown in Table 5.2. Both groups showed similar age at scan together with anthropometric characteristics. ART infants showed larger right atrium area together with lower right sphericity index. Although cardiac output shows no differences between groups, ART infants showed a significantly decreased shortening fraction. Regarding signs of systolic and diastolic dysfunction, the ART group showed a significantly decreased ring displacement and significant increased IRT. Even after adjustment for confounding factors (parental age, gestational age at delivery and birthweight centile) cardiac changes remained significant.

Vascular assessment

Systolic blood pressure and mean blood pressure were significantly higher in the ART group than in controls; diastolic pressure showed a trend to higher values in the ART group when compared to controls. Carotid intima-media was significantly thicker in ART than in controls.

Discussion

This study demonstrated the persistence of cardiovascular changes in ART children that have been followed up from fetal life, supporting the notion that primary cardiovascular remodeling starts in fetal life and is a main determinant of postnatal cardiac and vascular changes. Cardiovascular changes observed in ART children, are consistent with previous reports demonstrating cardiac dysfunction and vascular remodeling(22, 71, 108) and also showed that cardiovascular remodeling can be evidenced by functional echocardiography *in utero* and early infancy. Although causality of these findings is not determined, the relationship between ART *per se* and cardiovascular changes is more tangible.

Children conceived by ART showed morphological cardiac changes such as larger right atrium and shorter right ventricle; cardiac dysfunction as decreased longitudinal function and impaired relaxation as a longer isovolumic relaxation time; changes that were more prominent in the right heart. All these changes go in line with those observed during fetal life(108). These findings are also well correlated with those mentioned in previous studies, like higher systolic and mean blood pressures together with a significant thicker carotid intima media thickness (cIMT)(20, 22).

A physiological explanation could be based on the hypothesis of increased vascular stiffness that leads to increased end-diastolic pressures in the ventricle (promoting myocardial hypertrophy and dilated atria)(108). In fetal life we can find similar cardiac changes due to pressure overload, like those seen in ductus arteriosus constriction(109). Regarding vascular changes, Celeen and Sakka found higher blood pressure in ART infants among another metabolic changes related as risk markers for cardiovascular diseases. Posteriorly, Scherrer et al. found significantly thicker cIMT together with a smaller flow mediated dilation and faster pulsed wave velocities; although they did not find differences in blood pressure when compared to controls. The mechanisms driving these changes in ART infants remain to be elucidated; confounding factors like advanced maternal age are well-known contributors for adverse pregnancy outcomes(110) like low birthweight, condition that has a high prevalence in ART population and is directly related with fetal cardiovascular programming(41, 42). These circumstances were present in this study and were taken into account when analyzing our data. Recently, ART and SGA have been found as different and independent conditions related to fetal cardiovascular remodeling, showing different cardiac phenotype changes and suggesting probable different etiologic pathways(111).

There are several limitations and strengths with regards to the present study. We acknowledge that in the ART population there will be always perinatal underlying confounding factors almost impossible to rule out (parental infertility per se, type of technique, embryo manipulation, culture media, epigenetic changes, etc.) and also those postnatal factors related to lifestyle (socio-economic status, food intake, exercise, etc.) In our study, although matched by infant age and gender, we found differences in parental age and parity, together with differences in birthweight and gestational age at birth. The

analysis was adjusted by these factors but even though, this correction may inadequately correct cardiac changes between groups.

In conclusion this is the first cardiovascular study in ART children that have been evaluated and followed-up from fetal life, with changes that persist postnatally and can be related to ART. To acknowledge that ART is determinant for fetal cardiovascular programming is relevant to continue with identification, and possible interventions, in pediatric risk factors for cardiovascular disease.

Table 1. Basal características

	Controls (n=65)	**ART (n=80)**	***p*-value**
Maternal características			
Maternal Age (years)	34 ± 4.4	36 ± 2.9	0.025
BMI (kg/m^2)	23.1 ± 4.5	25.0 ± 6.9	0.079
Smoking (%)	11	6	0.246
Caucasian (%)	88	93	0.173
Nulliparity (%)	55	70	0.047
Low socioeconomic status (%)	15	14	0.933
Familiar history of early cardiovascular disease (%)	4	8	0.160
Fertility and ART characteristics			
Infertility cause			
Unexplained (%)	¶	33	¶
Female (%)	¶	21	¶
Male (%)	¶	36	¶
Female + Male (%)	¶	10	¶
ART technique			
Standard IVF (%)	¶	10	¶
ICSI (%)	¶	82	¶
IVF+ICSI (%)	¶	8	¶
Number of transferred embryos			
1 (%)	¶	8	¶
2 (%)	¶	76	¶
3 (%)	¶	16	¶
Paternal características			
Paternal Age (years)	35 ± 6.0	38 ± 4.5	0.003
BMI (kg/m^2)	25.4 ±3.1	26.0 ± 4.3	0.437
Smoking (%)	31	25	0.061
Caucasian (%)	90	95	0.222
Pregnancy complications			
Prenatal corticoid exposure (%)	2	4	0.321
Preeclampsia (%)	0	13	0.119
Gestational diabetes (%)	6	14	0.205
Delivery data			
Gestational age at delivery (weeks)	40 ± 4.0	39 ± 2.2	0.010

Cesarean section (%)	23	31	0.304
Male (%)	45	52	0.263
Birthweight (g)	3403± 403	3020± 600	0.002
Birthweight centile	52 ± 28	39 ± 31	0.017
5 minutes Apgar score	10 ± 0.62	10 ± 0.12	0.091
Neonatal outcome			
Non reassuring fetal status (%)	3	5	0.280
Admission to neonatal intensive care unit (%)	1	3	0.377

Data are mean (SD) or percentage.

ART = pregnancies conceived by assisted reproductive technologies. BMI = body mass index. IVF = in vitro fertilization. ICSI = intracytoplasmic sperm injection.

* P-value calculated by Student's t-test and Pearson Chi-Square test.

† BMI calculated as weight in kilograms divided by the square of the height in meters.

††Early cardiovascular disease defined by the presence of congenital heart disease, coronary disease, hypertension, diabetes or hypercholesterolemia in male < 55 years and female < 65 years.

††† Non-reassuring fetal status

¶ Not applicable.

Table 2 . Anthropometric data and cardiovascular assessment at 3 years of age.

Characteristic	**Controls** (N=65)	**ART** (N=80)	*Crude P-value*	*Adjusted P-Value**
Age at evaluation (years)	3.0 ± 0.50	2.9 ± 0.30	0.149	0.075
Anthropometric data				
Height (cm)	98 ± 6.2	96 ± 5.0	0.088	0.101
Weight (Kg)	15.8 ± 2.7	15.1 ± 2.0	0.062	0.195
BMI	16.4 ± 1.6	15.9 ± 1.6	0.601	0.900
BSA (m^2)	0.34 ± 0.03	0.33 ± 0.06	0.935	0.700
Infant echocardiography				
Cardiac morphometry				
Left atrium area (cm^2)	5.29 ± 1.04	5.10 ± 1.01	0.307	0.468
Right atrium area (cm^2)	5.10 ± 0.87	5.54 ± 0.92	0.017	0.014
Left sphericity index	1.70 ± 0.20	1.62 ± 0.27	0.060	0.013
Right sphericity index	1.84 ± 0.29	1.70 ± 0.23	0.006	<0.001
Left ventricular wall thickness(mm)	6.11 ± 1.16	6.15 ± 1.31	0.830	0.654
Septum thickness (mm)	7.44 ± 1.39	7.61± 1.49	0.835	0.409
Systolic function				
Left shortening fraction (%)	38 ± 6.13	35 ± 4.61	<0.001	0.002
Heart rate (bpm)	106 ± 14	105 ± 15	0.968	0.837
Left cardiac output (mL/min)	43 ± 11.2	44 ± 12.0	0.460	0.382
Right cardiac output (mL/min)	33 ± 25.0	28 ± 10.8	0.163	0.132
Mitral ring displacement (mm)	11.19 ± 2.74	10.23 ± 2.01	0.026	0.048
Tricuspid ring displacement (mm)	18.28 ± 2.40	16.29 ± 2.74	<0.001	<0.001
Mitral lateral S' peak velocity (cm/s)	6.89 ± 1.90	6.64 ± 1.49	0.517	0.492
Tricuspid S' peak velocity (cm/s)	11.18 ± 2.22	11.37 ± 2.16	0.560	0.414
Diastolic function				
Mitral E/A ratio	1.68 ± 0.43	1.72 ± 0.50	0.729	0.399
Tricuspid E/A ratio	1.57 ± 0.44	1.68 ± 0.44	0.182	0.185
Mitral E deceleration time (ms)	138 ± 29.5	137 ± 37.3	0.971	0.547
Tricuspid E deceleration time (ms)	173 ± 52.3	174 ± 49.2	0.902	0.678

Mitral E' (cm/s)	15.9 ± 3.09	15.5 ± 2.68	0.442	0.421
Tricuspid E' (cm/s)	17.1 ± 3.10	16.3 ± 2.74	0.132	0.089
Left isovolumic relaxation time (ms)	67.06 ± 12.4	80.25 ± 13.4	<0.001	<0.001
Vascular assessment				
Blood pressure				
Systolic blood pressure (mmHg)	90 ± 7.2	95 ± 9.7	0.019	0.011
Diastolic blood pressure (mmHg)	62 ± 9.1	66 ±9.8	0.288	0.035
Mean blood pressure (mmHg)	76 ± 7.5	80 ± 9.2	0.012	0.007
Carotid mean IMT (mm)	0.45 ± 0.09	0.52 ± 0.03	<0.001	<0.001
Carotid maximum IMT (mm)	0.49 ± 0.10	0.60 ± 0.05	<0.001	<0.001

Data are mean (SD).

ART = pregnancies conceived by assisted reproductive technologies. S' = systolic annular peak velocity. E = ventricular inflow in early diastole. A = ventricular inflow during atrial contraction. E' = annular peak velocity in early diastole. IMT = intima-media thickness

* P-value calculated by linear regression adjusted by parental age, gestational age at delivery, and birthweight centile.

6. DISCUSSION

6.- DISCUSSION

This thesis provides evidence to support the hypothesis that ART has influence on fetal cardiac remodeling that persists postnatally. This work not only confirms previous studies regarding presence of adverse perinatal outcomes, but also demonstrates the presence of cardiac remodeling and dysfunction from fetal life in single and twin fetuses. Although, the exact mechanisms that produce these changes are unknown; ART fetal cardiac changes seem to be an independent cause of the presence of SGA. Lastly, infant and children born after ART showed changes in cardiac morphometry, subclinical cardiac dysfunction and vascular remodeling that persist into childhood, supporting the fetal programing theory for cardiovascular diseases on adulthood.

Our **first study** confirms the higher prevalence of pregnancy complications in all infertile women (with or without treatment) and further describes a preferential association of prematurity with IVF/ICSI, and SGA with treated infertility (OI and IVF/ICSI). In our population, the overall rate of pregnancy complications was significantly increased in all infertility groups regardless the infertility treatment. Our data goes in line with previous studies reporting worse perinatal outcomes in infertile women with no treatment(34, 112), suggesting that adverse perinatal outcome may be more related to maternal factors associated with infertility, rather than the type of ART used. In addition, we are also confirming that the prevalence of adverse outcomes increases with the use of more intensive treatment(30, 31). Our results confirm previous data(35) reporting a higher rate of cesarean section in the infertility groups, but fail to demonstrate particular differences in preeclampsia, placenta previa or gestational diabetes, most probably due to the limited sample size of our study. In our study, prematurity was mainly associated to IVF/ICSI,

with a lower gestational age at delivery and a higher prevalence of PTD. In addition, IVF/ICSI was the only group that remained as main predictor of PTD in the decision tree analysis. Our results are in agreement with several previous studies demonstrating a significant association of prematurity with IVF/ICSI, both in singleton and twin pregnancies(113-115). The etiology for PTD is complex and multifactorial; women undergoing IVF/ICSI often have more embryos transferred, which may increase the chance of having multiple pregnancies and/or a vanishing twin, both conditions associated with PTD. In our study, the IVF/ICSI group presented the highest prevalence of twin pregnancies. However, differences in PTD remained significant after adjusting by twins and maternal age. Besides twins, other factors such as older maternal age, previous maternal disease or ovarian hyperstimulation have been proposed to explain this increase in prematurity, as they may lead to poorer early embryonic and placental development, increasing the risks of developing complications that also increase the risk of PTD(13, 116, 117). Furthermore, our data showed a non-significant tendency to increased PTD in the OI group. Data regarding the prevalence of PTD in OI and other infertile groups is more scarce and controversial, but recent studies have suggested that singleton pregnancies conceived using OI with or without intrauterine insemination, are at risk of moderate and very preterm birth(36, 112, 118).

We also report a significant association of SGA and LBW with both OI and IVF/ICSI. There is mixed information in the literature about the definition of SGA, intrauterine growth restriction and LBW, with SGA usually defined by birthweight less than 10^{th} or 5^{th} centile and LBW as less than 2500g. Regardless of the definition, our data and previous literature(30, 31, 116) support the association of ART with fetal growth restriction in both term and preterm pregnancies. While this association was initially explained by the higher

incidence of multiples pregnancies in ART, recent studies have also demonstrated higher rates of SGA/LBW in singleton pregnancies. Other studies have reported a higher incidence of SGA in groups with OI, particularly in twin pregnancies(32). In contrast, in our study both OI and IVF/ICSI showed a similar increase in PTD. The mechanism underlying this association is unclear. As some studies have reported a higher incidence of SGA when utilizing fresh embryos versus vitrified ones(119), it has been attributed to culture media required for the gametes(26), but this would not explain the higher incidence of SGA in OI. It was not possible to evaluate the potential effect of this variable in our cohort, as the majority of ART procedures in our center were performed with fresh embryos.

Regarding the strengths and limitations; the study design allowed to include a fertile group sampled from a low-risk population and an infertile group who conceived spontaneously together with the OI and IVF/ICSI groups. However, we acknowledge the limited sample size of our cohort, which may have prevented to demonstrate potential associations with low prevalence complications such as preeclampsia or placenta praevia. In addition, although statistical analyses were adjusted by maternal age and twin pregnancies, other potential confounders could not be included in the multivariate analysis due to the limited sample size and lack of statistical power.

Once we corroborated the high presence of adverse perinatal outcomes in our infertile population, our next step was conducting **STUDY 2**. This study demonstrates the presence of cardiac and vascular remodeling in fetuses and infants of pregnancies obtained by ART. These findings are consistent with previous reports demonstrating signs of vascular dysfunction in children conceived by ART[20, 120] and provide evidence for the

existence of fetal cardiovascular programming in these pregnancies. We could not determine causality of our findings by ART itself, by intrauterine growth restriction or prematurity in ART pregnancies, or by other confounders related to the indications for ART.

Fetuses from pregnancies conceived by ART showed more globular hearts together with increased myocardial wall thickness, decreased right longitudinal function, impaired relaxation and dilated atria. The differences persisted after birth and were more prominent in the right heart as compared to the left. The cardiac findings are consistent with experimental data showing an increased heart weight in an IVF bovine model.(121) From a pathophysiological viewpoint, more globular and hypertrophic ventricles with decreased longitudinal function are the usual ventricular response to pressure overload. Therefore, fetal observations are in line with postnatal findings of elevated blood pressure and increased IMT. In addition, cardiac remodeling described in our ART population resembles other fetal conditions with known pressure overload such as twin-to-twin transfusion syndrome(44) or ductus arteriosus restriction.(109) These clinical entities and experimental models of systemic pressure loading(43) have been reported to show more pronounced changes in the right heart. This might reflect the dominance of right heart during fetal life together with a higher susceptibility to pressure overload of the right as compared with the left ventricle.(122, 123) The dilated atria and impaired relaxation (decrease in E' and E deceleration time) could be explained by a decrease in ventricular compliance leading to higher end-diastolic pressures and increased atrial pressures. Finally, the changes described in vascular function and structure in neonates and infants reproduce the findings of previous reports in late childhood(20, 120) and support the development and presence of these differences from early life.

Fetal cardiovascular programming has previously been described in fetuses and children who suffered LBW.(124, 125) LBW is associated with globular hearts and longitudinal dysfunction *in utero*,(43) and these changes, accompanied by increased blood pressure and vascular wall thickness, have been described to persist into childhood in humans(125) and to adulthood in animal models.(126) Direct cardiac effects of fetal growth restriction have been proposed to provide a link to explain the long described epidemiological association of this prenatal condition with increased cardiovascular mortality in adults.(124) Due to the high and expected prevalence of LBW in ART cases, it has been suggested that fetal growth restriction could be a potential confounder for cardiovascular remodeling in ART offspring.(127) However, we believe that the results of this study strongly support a direct effect of ART on fetal and infant cardiovascular changes. Firstly, ART fetuses and infants presented changes that have not previously been reported in LBW, such as myocardial hypertrophy and increased atrial size.(124, 125) Secondly, most cardiovascular changes in ART remain significant even after adjustment by birthweight centile. Finally, the differences between ART pregnancies and controls remained virtually unchanged after excluding LBW pregnancies from the study group.

The mechanisms driving fetal and postnatal cardiovascular remodeling in ART pregnancies remain to be elucidated. Parental predisposing factors, epigenetic changes secondary to the early embryo manipulation, hormonal effects and postnatal environmental factors have been postulated as potential factors.(110, 128, 129) Changes in fetuses and infants in this study were similar to those described in late childhood. Consequently, the role of postnatal environment as a potential factor determining long term vascular dysfunction in ART children is possibly negligible. Advanced maternal age

in ART has been proposed as a major contributor of childbearing.(110) In this study, cases and controls were matched by maternal age, however we acknowledge that other parental factors related to their subfertility could still play a role.

Concerning epigenetic mechanisms, there is clinical and mainly experimental evidence that the processes involved in egg manipulation might be associated with epigenetic changes, mainly mediated by changes in the DNA methylation pattern. The majority of the changes described affect imprinted genes, which have mainly been involved with fetal and placental growth.(130-132) However, it has been suggested that methylation might be relevant for other functions as yet not characterized.(131) The importance of DNA methylation in the regulation of vascular endothelial function is being increasingly demonstrated, including nitric oxide expression and synthesis, and endothelial angiogenesis. As indirect evidence, experimental models suggest that fetal cardiovascular programming occurring in LBW is associated with specific epigenetic signatures involving abnormal methylation.(133) Therefore, molecular pathways involved in cardiovascular regulation deserve further research to ascertain their potential involvement in the vascular changes described in ART pregnancies. However, due to the variability in ART protocols and the rarity of imprinting disorders, it can be challenging to determine reliably the causative relationship between and increased risk for imprinting disorders and ART exposure.(15)

Concerning hormonal factors, the effect on supra-physiological estradiol levels on the outcome of IVF-embryo transfer and subsequent pregnancies is a matter of controversy in the literature. Estradiol concentrations are not correlated with oocyte yield

and quality, embryological outcome, implantation and pregnancy rates, abortion rate, congenital malformations and birth weight.(134) However, associations with pregnancy complications related to abnormal placentation such as LBW, preeclampsia and abnormal implantation of the placenta have been reported.(135) The relationship between estradiol levels in ART and long-term cardiovascular function is unknown. As indirect evidence, a recent study reported no association between ovarian hyperstimulation, a condition associated with a dramatic increase in estrogen levels, with neuromotor development at 3 months of age, but, again, a potential independent effect of a history of subfertility was suggested.(136) Progesterone, another important hormone in human reproduction, has not been shown to have effects on fetal placental circulation or any association with the presence of LBW.

There are several limitations and considerations with regard to the present study. The changes here reported are subclinical with most cardiovascular indices lying within normal ranges. While these differences are recognized as potential cardiovascular risk factors, their long-term persistence and association with adult cardiovascular function and disease remained to be proven. Therefore, longer follow up of these ART pregnancies to ascertain whether ART pregnancy remains a risk factor in later life was crucial. We acknowledge that several potential confounders could have interfered in our results. However, cases and controls were matched by maternal age, and twin pregnancies and mothers with medical diseases were excluded. The analysis was adjusted for other potential influences including prematurity, birthweight centile and preeclampsia. Additionally, other potential confounders, such as gender, ethnicity, cardiovascular history, socioeconomic status, parity and parental smoking were similar among study groups. However, we acknowledge that analysis correcting for birth weight percentile may

inadequately control for the differences in etiology as children conceived by ART may be more likely to have in-utero growth restriction from placental failure. In addition, there is increasing evidence that current definitions of fetal growth restriction most likely do not detect all instances of true restriction. (137) Consequently, one could argue that by the same token we cannot exclude that the whole distribution of fetal weights in our population was shifted to the left, reflecting a more general effect on fetal growth in ART fetuses. If this was the case, there would be forms of true fetal growth restriction that have been missed because of the lack of sensitivity of currently used definitions, and therefore the impact of fetal growth restriction on our result would be greater than it is now apparent, because hidden forms of growth restriction not detected by conventional criteria(137) might have affected the cardiac outcome of the ART pregnancies. In conclusion, we fully acknowledged that prematurity and fetal growth restriction may have significantly contributed to the cardiac findings, rather than ART via a mechanism of altered fetal programming and that this concept deserved further clarification.

In our **third study**, the previously described cardiac changes reported in singleton ART were similar in twin pregnancies. These findings are in line with previous reports demonstrating signs of cardiovascular dysfunction in singleton fetuses and children conceived by ART.(22, 71, 138, 139) Twins conceived by ART showed larger atria and a pattern of right ventricular concentric remodeling (more globular and thicker right ventricles), together with signs of systolic and diastolic dysfunction. To our knowledge, this is the first study evaluating cardiovascular function in ART twins. Regarding perinatal characteristics, prenatal corticoid exposure, gestational diabetes, birthweight and gestational age at delivery were similar among groups. In addition, the cardiac changes previously reported in SGA (concentric hypertrophy) are different from the ones here

described in ART (larger atria and ventricular concentric remodeling)(140). Consequently, the role of perinatal environment is possibly minor. It is important to mention the significantly high prevalence of perinatal complications in the ART twin group(11). Twin pregnancies conceived by ART presented a higher rate of preeclampsia, cesarean section, admission to NICU and neonatal morbidity as compared to SC twins. These results, that were originally believed to be a consequence of the associated risks of a multiple gestation, have also been reported in ART singletons (22, 71, 138).

ART is redefining biology and society; we believe that it is a great medical achievement but, from an ethical and clinical point of view, it is important to understand the potential impact of these techniques on perinatal and long lasting health. The aim of this study was to provide further evidence with regards to the effects of ART in twin pregnancies. Overall, our results showed the presence of cardiac remodeling and dysfunction in twin fetuses conceived by ART. The hereby observed cardiac changes, albeit subtle, could be responsible for an increased cardiovascular risk later in life, in keeping with the fetal programming theory(124). Mild cardiovascular changes present in fetal life may remain subclinical during childhood, but may worsen and turn into significant health issues with certain stressors, postnatal conditions such as obesity and other lifestyle behaviors. Thus, early interventions in this group, such as promoting breastfeeding(141), lifestyle modifications, lack of exposure to other risk factors, and blood pressure surveillance could aid in reducing these risks(111, 142)

Among the strengths of this study is the prospective performance of comprehensive fetal echocardiograms in a well-defined population recruited for this purpose. Extreme care was taken to exclude multiple pregnancies with obvious maternal disease, use of ovulation induction medication or other confounding factors that could influence our results. Multilevel analysis was performed to take into account the twin

dependency. The analysis was adjusted by potential confounding factors identified such as parental age and preeclampsia, by linear regression in order to dissect the independent effect for mode of conception. However, we acknowledge that non-obvious confounders not considered in the design of the study might have affected the results. We also accept that the number of patients included in this study is small, which could allow for the lack of statistical significance of other parameters evaluated. Finally, the cardiovascular changes here reported are subclinical and, while they are recognized as potential cardiovascular risk factors, their persistence and long-term association with cardiovascular disease remain to be proven, which warrants further follow-up of this twin cohort.

Until now, we had demonstrated that single and twin fetuses conceived by ART present cardiovascular changes together with a higher presence of adverse perinatal outcomes. Among these outcomes, SGA has an important role regarding cardiac changes; given the higher incidence of SGA in ART population STUDY 4 was conducted. This study provides evidence that both ART and SGA have a direct and independent effect on the fetal cardiovascular system that translates into different fetal cardiac phenotypes.

Our findings confirm previous studies demonstrating fetal cardiovascular changes in SGA and ART and further provide evidence of the independent effect of each factor. We are describing larger, more globular and hypertrophic hearts in naturally conceived SGA fetuses with reduced longitudinal motion (decreased MAPSE, TAPSE and mitral S') and impaired relaxation (decreased E′ and prolonged IRT and E deceleration time). This data is in agreement with previous studies demonstrating significant changes in cardiac

structure and function in fetuses and children born SGA(143-147). Cardiac changes observed in SGA fetuses can be explained as an adaptive response to sustained undernutrition and hypoxia, together with increased placental vascular resistance that results in pressure/volume overload. It is probable the heart reacts to increased placental resistance and pressure overload by shifting into a more spherical shape (with a lower radius of curvature that would better tolerate pressure overload by reducing wall stress), and hypertrophying myocardial walls (in order to increase the force-generating units). Finally, volume overload would explain cardiomegaly. While this concentric hypertrophy maintains a normal ejection fraction, signs of reduced longitudinal motion and impaired ventricular filling can usually be observed in SGA.

We also describe dilated atria and more spherical ventricles without cardiomegaly in fetuses conceived by ART. Signs of decreased longitudinal motion (reduced TAPSE, MAPSE and S') and impaired relaxation (decreased E deceleration time) were also observed. These changes are in line with our previous report in ART fetuses(71, 138) and postnatal studies demonstrating increased vascular pressure in children and adolescents conceived by ART(22, 148, 149). This is also consistent with the shortened E deceleration time (usually secondary to increased ventricular end-diastolic pressures) observed in ART but not in SGA. The fetal heart responds to pressure overload with concentric remodeling, by reducing the ventricular radius of curvature (more spherical ventricles) together with a mild increase in myocardial wall thickness, maintaining a normal heart size. Observed dilated atria are probably consequence of a ventricular early filling problem that needs to be compensated by larger atrial contribution; increased pressure also results in dilated atria. These changes were more prominent in the right heart, probably due to its predominance in fetal life and its higher susceptibility to pressure

overload compared to the left ventricle. It is also consistent with previous reports suggesting increased pulmonary pressures in children conceived by ART13.

Parental predisposing factors, epigenetic changes secondary to early embryo manipulation, hormonal effects and association to perinatal complications have been postulated as factors potentially mediating increased vascular pressures described in ART offspring. However, the exact mechanisms driving fetal cardiovascular remodeling in ART pregnancies remain to be elucidated. While pressure overload seems to be a common feature in ART and SGA, the absence of fetal hypoxia/undernutrition and volume overload in ART could explain the different phenotypic response of the fetal heart in comparison to SGA. Finally, fetuses conceived by ART who developed growth restriction showed mixed characteristics from both groups, maintaining dilated atria observed in ART, but also left myocardial dysfunction present in SGA fetuses; the ART-SGA group is more similar to the normally grown ART, suggesting that changes mediated in ART are more strongly manifested in these cases and could partially counter the changes from SGA.

Epidemiological studies have provided evidence of an association of SGA with cardiovascular risk later in life, demonstrating a higher incidence of hypertension and cardiovascular mortality in this population.(150, 151) Likewise, ART has been associated with postnatal hypertension, premature subclinical atherosclerosis in the systemic circulation and pulmonary vascular dysfunction(22, 148, 149). The findings of the present study, suggesting distinct patterns of fetal cardiac remodeling, support the hypothesis of ART and SGA being independent causes of cardiac programming. Fetal

echocardiography has already demonstrated its utility in the prediction of postnatal cardiovascular health in SGA(146). Therefore, the identification of different cardiac phenotypes in SGA and ART pregnancies may help in the diagnosis and long-term cardiovascular prognosis of these fetuses and children. The importance of early identification and the impact of interventions in pediatric populations for preventing future cardiovascular disease are now well recognized(150). Given the high prevalence of both SGA (7-10%) and ART (2-20%), early interventions in these high-risk populations could have a tremendous effect on public health, by potentially improve the future cardiovascular health of these children.

To our knowledge, this is the first study illustrating fetal cardiovascular changes in separate populations of ART and naturally conceived SGA. Comprehensive fetal echocardiography was prospectively performed in a large proportion of well-characterized fetuses. Cases were frequency matched by gestational age with controls, and we further adjusted for other potential confounders such as maternal age and prenatal corticoid exposure. However, we acknowledge there may be other non-obvious confounders like preclampsia and birthweight, so despite the *n* per group, we decided to adjusted by these. Due to the lack of standardization and normality values for most fetal cardiac morphometric parameters, we are displaying crude values and also results normalized by cardiac size in the supplementary material. Most geometrical differences remained statistically significant after normalization, while some functional parameters lost their significance. We acknowledge that the optimal scenario would be to establish a consensus on how to normalize these fetal echocardiographic parameters. Therefore, the differential effect of growth restriction and assisted reproductive technologies on fetal cardiac functional parameters and some of the geometrical changes could be over

interpreted. The difficulty of prospectively assessing fetal echocardiography in ART pregnancies that developed growth restriction limited the sample size of our study and may have prevented some statistical differences among groups. Our findings also warrant postnatal long-term follow-up to describe the prognosis and clinical significance of the described changes. Reason that motivated us to continue with **STUDY 5.**

This study demonstrated the persistence of cardiovascular changes in ART children that have been followed up from fetal life, supporting the notion that primary cardiovascular remodeling starts in fetal life and is a main determinant of postnatal cardiac and vascular changes. Cardiovascular changes observed in ART children, are consistent with previous reports demonstrating cardiac dysfunction and vascular remodeling(22, 71, 108) and also showed that cardiovascular remodeling can be evidenced by functional echocardiography *in utero* and early infancy. Although causality of these findings is not determined, the relationship between ART *per se* and cardiovascular changes is more tangible. Children conceived by ART showed morphological cardiac changes such as larger right atrium and shorter right ventricle; cardiac dysfunction as decreased longitudinal function and impaired relaxation as a longer isovolumic relaxation time; changes that were more prominent in the right heart. All these changes go in line with those observed during fetal life(108). These findings are also well correlated with those mentioned in previous studies, like higher systolic and mean blood pressures together with a significant thicker carotid intima media thickness (cIMT)(20, 22).

It is possible that IVF offspring will become healthy in adult life, or on the contrary, they could become more predisposed to cardiovascular diseases. The fact that we can recognize ART as one of the many primary causes of cardiac remodeling from fetal life,

may open opportunities for monitoring or early interventions in these children. This thesis not only showed cardiovascular changes in single and twin ART fetuses, but followed them up through different time periods as newborns, infants (6 months age) and children (3 years age) finding well-recognized pediatric cardiovascular risk factors (higher blood pressure, thicker intima media). These changes, although subclinical, could allow us to target them as a population at risk and hallmark the importance of continuing a longer follow-up. Clinical guidelines contemplate screening for hypertension in children over 3 years of age, in order to provide strategies for promoting cardiovascular health, that can be integrated into comprehensive pediatric care recommending lack of exposure to other risk factors (secondary smoking, obesity), surveillance of catch-up growth or administration of hypotensor and specially, promoting exercise and physical activity(152).

The relatively new use of ART (since 1978), the almost achieved “global protocol standardization” for the use of ART, and other unknown factors is the reason for several studies showing controversial or contradicting results. The improvements made to these techniques through the time, in order to achieve pregnancy transferring the “best” embryo, without genetic alterations, using physiologically based culture media with the best biological conditions, could be the possible cause of that the differences found in initial studies have not been reproducible or vary widely. We need to keep moving with Assisted Reproductive Technologies and adjust for all these changes.

7. CONCLUSIONS

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1. Adverse pregnancy outcomes seem to be present in infertile women, regardless of the use of ART, supporting the concept of maternal underlying factors related to infertility rather than the ART technique. While prematurity is more related to IVF/ICSI, SGA seems to depend on fertility treatment.

2. The use of ART in infertile couples is associated with fetal and postnatal cardiovascular remodeling in singleton pregnancies; suggesting prenatal exposure to pressure overload.

3. Twin fetuses conceived by ART, present cardiovascular changes like to those observed in ART single fetuses.

4. There is a different and independent cardiac effect in SGA and ART fetuses; showing a particular cardiac phenotype.

5. Infants born after ART show changes in cardiac morphometry, subclinical cardiac dysfunction and vascular remodeling; supporting the fetal programing theory for cardiovascular diseases on adulthood.

8. REFERENCES

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