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# Etude de l'impact des procédés d'assistance médicale à la procréation sur la régulation des gènes soumis à l'empreinte et des séquences répétées dans le placenta et le sang de cordon chez l'homme

Cécile Samson Choux

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## THESE DE DOCTORAT

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Ecole doctorale n°554

Environnements - Santé

Doctorat de Biologie Cellulaire

Par

**M<sup>me</sup> Cécile CHOUX**

# **Etude de l'impact des procédés d'assistance médicale à la procréation sur la régulation des gènes soumis à empreinte et des séquences répétées dans le placenta et le sang de cordon chez l'Homme**

Thèse présentée et soutenue à Dijon, le 14 décembre 2018

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# ***ABSTRACT***

# RESUME

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Le nombre d'enfants nés par Assistance Médicale à la Procréation (AMP) dans le monde est estimé à plus de 5 millions, représentant jusqu'à 4% des naissances. Environ 10% des couples en âge de procréer sont actuellement infertiles, et leur apporter des techniques pour devenir parents est devenu un problème de santé publique. Cependant, l'innocuité de ces techniques n'a pas été totalement démontrée. Notamment, le risque de pathologies d'origine placentaire pourrait être augmenté. De plus, des issues périnatales défavorables, un risque majoré de malformations majeures et de pathologies liées à l'empreinte ont été rapportés chez ces enfants. Ceci soulève la question d'une éventuelle vulnérabilité épigénétique induite par l'AMP.

L'objectif de ce travail de thèse était d'étudier la régulation épigénétique des gènes soumis à empreinte (GSE) et des éléments transposables (TE) dans le placenta et le sang de cordon d'enfants conçus par AMP comparés à des enfants conçus naturellement.

En guise d'introduction, nous avons rédigé une revue détaillée des modifications phénotypiques et épigénétiques induites par l'AMP dans les embryons, le placenta et le sang de cordon chez l'Homme et sur les modèles animaux.

Au cours de cette thèse, une cohorte de presque 250 patientes a été incluse prospectivement, répartie en 4 groupes de patientes selon la technique d'AMP et 4 groupes de témoins selon la durée d'infertilité.

A partir de cette cohorte, la première question posée a été l'effet de la Fécondation *in vitro* (FIV) sur la méthylation de l'ADN et/ou la transcription des GSE et TE dans le sang de cordon et le placenta à la naissance. Pour cela, nous avons sélectionné 51 patientes enceintes après FIV avec ou sans ICSI avec transfert d'embryon frais à J2 et les avons comparées à 48 témoins enceintes dans l'année après l'arrêt de la contraception. Nous avons étudié la méthylation de l'ADN et l'expression de 3 GSE et 4 TE. Les niveaux de méthylation de l'ADN placentaire pour *H19/IGF2*, *KCNQ1OT1*, LINE-1 et ERVFRD-1 et le niveau d'expression placentaire d'ERVFRD-1 étaient plus bas dans le groupe FIV/ICSI que dans le groupe contrôle. Ces modifications épigénétiques pourraient faire partie des mécanismes de compensation développés pendant la grossesse après AMP, comme discuté dans notre revue.

Ensuite, nous avons voulu déterminer si ces changements de méthylation de l'ADN des GSE pouvaient être associés à des modifications des histones. A partir de la cohorte précédente, nous avons sélectionné 16 patientes du groupe FIV/ICSI avec des niveaux de méthylation dans le placenta inférieurs au 5<sup>ème</sup> percentile pour au moins un des GSE étudiés. Elles ont été appariées à 16 témoins sur la parité, le sexe du nouveau-né et l'âge gestationnel à l'accouchement. Des marques permissives (H3K4me2 et me3 et H3K9ac) et répressives (H3K9me2 et me3) ont été étudiées. Les résultats ont révélé une quantité significativement augmentée de H3K4me2 dans le groupe FIV/ICSI pour *H19/IGF2* et *KCNQ1OT1*. La quantité des deux marques répressives pour *H19/IGF2* et *SNURF* était significativement abaissée dans le groupe FIV/ICSI.

Ces données montrent que l'hypométhylation de l'ADN au niveau des GSE pourrait être associée à une augmentation des marques permissives et une diminution des marques répressives des histones, ce qui permettrait de favoriser un état « actif » de la chromatine au niveau de l'allèle normalement réprimé.

Nos résultats, ainsi que les données de la littérature, renforcent l'hypothèse de potentiels mécanismes mis en place dans le placenta après AMP, utiles pour compenser des anomalies précoces de la placentation, qui seraient écrits à travers des modifications épigénétiques comme la méthylation de l'ADN mais aussi les modifications des histones.

Bien que certaines questions restent en suspens, cette thèse a permis de bâtir les fondations de travaux futurs, notamment pour étudier l'impact de la congélation/décongélation des embryons et le rôle joué par l'infertilité en elle-même.

## ABSTRACT

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It is estimated that more than five million children have been born by Assisted Reproductive Technologies (ART) worldwide, representing up to 4% of all births. As around 10% of reproductive-aged couples are currently infertile, providing them with treatment options is a public health issue. However, the safety of these techniques has not been fully demonstrated. Notably, the rate of placenta-related adverse pregnancy outcomes could be increased after ART. Moreover, adverse perinatal outcomes, a higher risk of major malformations and imprinting disorders have also been reported in children born following ART. These issues combined raise the question of a potential ART-induced epigenetic vulnerability.

The aim of this thesis was to investigate the epigenetic regulation of imprinted genes (IGs) and transposable elements (TEs) in the placenta and cord blood of children conceived by ART and to compare them to children conceived naturally.

By way of introduction, we wrote a comprehensive review about phenotypic and epigenetic modifications induced by ART in embryos, placenta and cord blood either in human or animal models.

Then, an extensive cohort of almost 250 patients was prospectively included, resulting in 4 groups of ART techniques and 4 groups of controls stratified on the time to pregnancy.

From this cohort, the first question we investigated was the effect of *in vitro* fertilization (IVF) on DNA methylation and/or transcription of TEs and IGs in cord blood and placenta collected at birth. For this purpose, we selected 51 pregnant women after IVF with fresh embryo transfer at day -2 and compared them with 48 controls pregnant within 1 year of stopping contraception. We studied the DNA methylation and expression of 3 imprinted DMRs and 4 TEs. DNA methylation levels for *H19/IGF2* and *KCNQ1OT1* DMRs, LINE-1 and ERVFRD-1 in the placenta were lower in the IVF/ICSI group than in the control group. The expression level of ERVFRD-1 in the placenta was also lower in the IVF/ICSI group than in the control group. These modifications in epigenetic regulation may influence some compensation mechanisms developed throughout pregnancy after ART, as discussed in our review.

We then intended to determine if these DNA methylation changes in IGs were associated with histone modifications. From the previously mentioned cohort, we selected the 16 patients from the IVF/ICSI group who presented with below the 5<sup>th</sup> percentile of percentage placenta DNA methylation for at least one of the previously studied DMRs. These patients were compared with 16 controls matched for parity, new-born sex, and gestational age at delivery. Permissive (H3K4me2 and me3 and H3K9ac) and repressive (H3K9me2 and me3) histone marks were studied. The results revealed a significantly higher quantity of H3K4me2 in the IVF/ICSI group than in the natural conception group for *H19/IGF2* and *KCNQ1OT1* DMRs. The quantity of both repressive marks at *H19/IGF2* and *SNURF* DMRs was significantly lower in the IVF/ICSI group than in the natural conception group.

These data demonstrate that DNA hypomethylation at imprinted DMRs may be associated with an increase in permissive histone marks and a decrease in repressive histone marks. This is consistent with a more “active” chromatin conformation on the normally repressed allele.

Our findings, together with the literature data, reinforce the hypothesis that some mechanisms are established in the placenta after ART, probably to mediate placental plasticity and compensate primary disorders in trophoblastic invasion, and written through epigenetic changes such as DNA methylation but also histone modifications.

Although some questions remain unanswered, this thesis paves the way for further original studies, notably to assess the impact of frozen-thawed embryo transfer and to decipher the role of infertility *per se*.

# ***SUMMARY***

# SUMMARY

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# ABBREVIATIONS LIST

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## A

**ABM:** Agence de la Biomédecine  
**ADN:** Acide DésoxyriboNucléique  
**AMP:** Assistance Médicale à la Procréation  
**ART:** Assisted Reproductive Technologies

## B

**BMI:** Body Mass Index

## C

**CB:** Cord Blood  
**ChIP:** Chromatin Immuno-Precipitation  
**cIVF:** Conventional IVF

## D

**DMRs:** Differentially Methylated Regions  
**DNA:** DeoxyriboNucleic Acid  
**DNMTs:** DNA MethylTransferase  
**DOHaD:** Developmental Origin of Health and Diseases

## E

**E:** Embryonic day  
**EDTA:** EthyleneDiamineTetraAcetic Acid  
**ET:** Embryo Transfer

## F

**FET:** Frozen-thawed Embryo Transfer  
**FIV:** Fécondation *in vitro*  
**FSH:** Follicle Stimulating Hormone

## G

**GA:** Gestational Age  
**GnRH:** Gonadotrophin Releasing Hormone  
**GSE:** Gènes Soumis à Empreinte

## H

**hCG:** Human Chorionic Gonadotrophin  
**HERV:** Human endogenous retrovirus

## I

**ICRs:** Imprinting Control Regions  
**ICSI:** Intra-Cytoplasmic Sperm Injection  
**IGR:** Imprinting Gene Network  
**IGs:** Imprinted Genes  
**IUGR:** Intra-Uterine Growth Retardation  
**IUI:** Intra-Uterine Insemination  
**IVC:** *In Vitro* Culture  
**IVF:** *In Vitro* Fertilization

## **L**

**L1:** LINE-1

**LGA:** Large for Gestational Age

**LINE:** Long Interspersed Nucleotide Element

**LTR:** Long Terminal Repeat

## **N**

**NC:** Naturally Conceived

## **O**

**OI:** Ovulation Induction

**ORF:** Open Reading Frame

## **P**

**PAPP-A:** Pregnancy-Associated Plasma Protein A

**PBS:** Phosphate Buffered Saline

**PCOS:** PolyCystic Ovary Syndrome

**PCR:** Polymerase Chain Reaction

**PMSF:** PhenylMethylSulfonyl Fluoride

## **R**

**RNA:** RiboNucleic Acid

## **S**

**SD:** Standard Deviation

**SDS:** Sodium Dodecyl Sulfate

**SGA:** Small for Gestational Age

**SINE:** Short Interspersed Nucleotide Element

**SO:** SuperOvulation

## **T**

**TEs:** transposable elements

## **U**

**UTR:** *UnTranslated Region*

## **W**

**WG:** Weeks of Gestation

# ***INTRODUCTION***


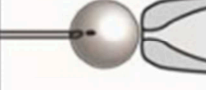





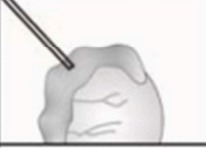
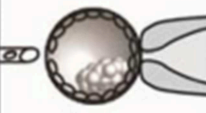
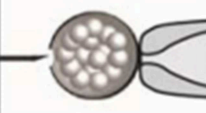
## INTRODUCTION

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The development of *in vitro* fertilization (IVF) has been a major breakthrough of the 20<sup>th</sup> century. After various attempts on animals in the 1960's, the first pregnancy obtained by IVF in humans was described in the Lancet in 1973 (De Kretzer *et al.*, 1973) but it developed only a few days and was indeed what we could now call a 'biochemical pregnancy'. The following attempts were not a great success either, as in 1976 when Steptoe and Edwards reported the successful implantation of a blastocyst after IVF, but in the fallopian tubes (Presl, 1977). Finally, the same team was able to claim victory with the birth of the world's first "test-tube baby", Louise Brown, on 25 July 1978 in UK (Clarke, 2006).

The subsequent use of clomiphene citrate and human Chorionic Gonadotrophin (hCG) to stimulate cycles and control oocyte maturation and collection has led IVF to a shift from research towards clinical treatment. Nowadays, from an oocyte collected after ovarian stimulation with Follicle Stimulating Hormone (FSH), preceded by ovarian blockage by Gonadotrophin Releasing Hormone (GnRH) or accompanied by GnRH antagonists, and prepared semen, IVF generates embryos which are cultured in media for 2 to 5-6 days. This procedure is then followed by intra-uterine transfer of one or two (maximum three) embryos.

Over the last 40 years, the basic process has been improved with new stimulation protocols, new culture media, the possibility of slow-freezing and, more recently, the intra-cytoplasmic sperm injection (ICSI), a new fertilization technique described for the first time in 1992 by Palermo *et al.* (Palermo *et al.*, 1992), and the vitrification of embryos and oocytes (**Figure 1**).

<b>In Vitro Fertilization</b>	
<b>In vitro fertilization (IVF):</b> Eggs are retrieved from the ovary and fertilization occurs in vitro.	
	<b>Conventional IVF:</b> Sperm are added to culture medium containing eggs for insemination.
	<b>Intracytoplasmic sperm injection (ICSI):</b> A single sperm head is injected into the cytoplasm of the egg. Used for: male factor infertility, if sperm have been obtained surgically, a history of unsuccessful cycles, procedures using either cryopreserved sperm or eggs, and/or if PGD/PGS will be performed. 69% of ART cycles in the United States utilized ICSI.
<b>Associated Procedures</b>	
	<b>Controlled ovarian hyperstimulation (COH):</b> Hormones are administered to stimulate the maturation of several oocytes. Protocols vary. More than 99% of ART cycles in the United States used ovarian stimulation.
	<b>Oocyte retrieval:</b> Eggs are collected by transvaginal ultrasound-guided follicle aspiration.
	<b>Embryo culture:</b> Zygotes are cultured for 3 or 5 days using commercially-available culture medium in a 37°C incubator with low oxygen.
	<b>Embryo transfer:</b> Embryos are non-surgically transferred into the uterus through the cervix. Transfers can be performed using cleavage or blastocyst stage embryos.
<b>Additional Optional Procedures</b>	
	<b>Gamete/Embryo freezing:</b> Freezing occurs by slow-cooling or a rapid cooling method known as vitrification.
	<b>Surgical sperm retrieval:</b> Sperm are aspirated from the epididymis or testis. In instances of low sperm count, larger portions of testicular tissue can be biopsied. ICSI is exclusively used for sperm obtained by sperm retrieval.
	<b>Preimplantation genetic diagnosis/screening (PGD or PGS):</b> Analyses for single genetic defects and aneuploidy are performed on a single blastomere (day 3 embryos) or trophoctoderm cell (day 5 embryos). Trophoctoderm biopsy is favored over blastomere biopsy, which has been shown to negatively affect embryo development. PGD was performed in 6% of ART cycles in the US.
	<b>Assisted hatching:</b> The outer barrier of the embryo, known as the zona pellucida, is manually or chemically penetrated, in hopes of improving implantation.

**Figure 1: ART and its associated procedures**

From (Vrooman *et al.*, 2017). This figure explains the different procedures of ART.

Basically, although IUI implies that a woman's fallopian tubes and a man's sperm are normal, IVF enables to bypass non-functioning fallopian tubes. Concerning ICSI, the main indication is the abnormality of a man's semen, particularly severe teratozoospermia, oligozoospermia and/or abnormal survival rate. Indications are also discussed according to other female or male diseases and/or age.

All in all, IVF has become the ideal solution for most fertility problems, enabling birth of more than 5 million children and totalling up to 4% of births worldwide (Messerlian *et al.*, 2017).

According to the ABM (*Agence de la BioMédecine*), in France, 46,347 intra-uterine insemination (IUI), 62,623 cycles of IVF and 32,739 frozen-thawed embryo transfers (FET) were carried out in 2016.

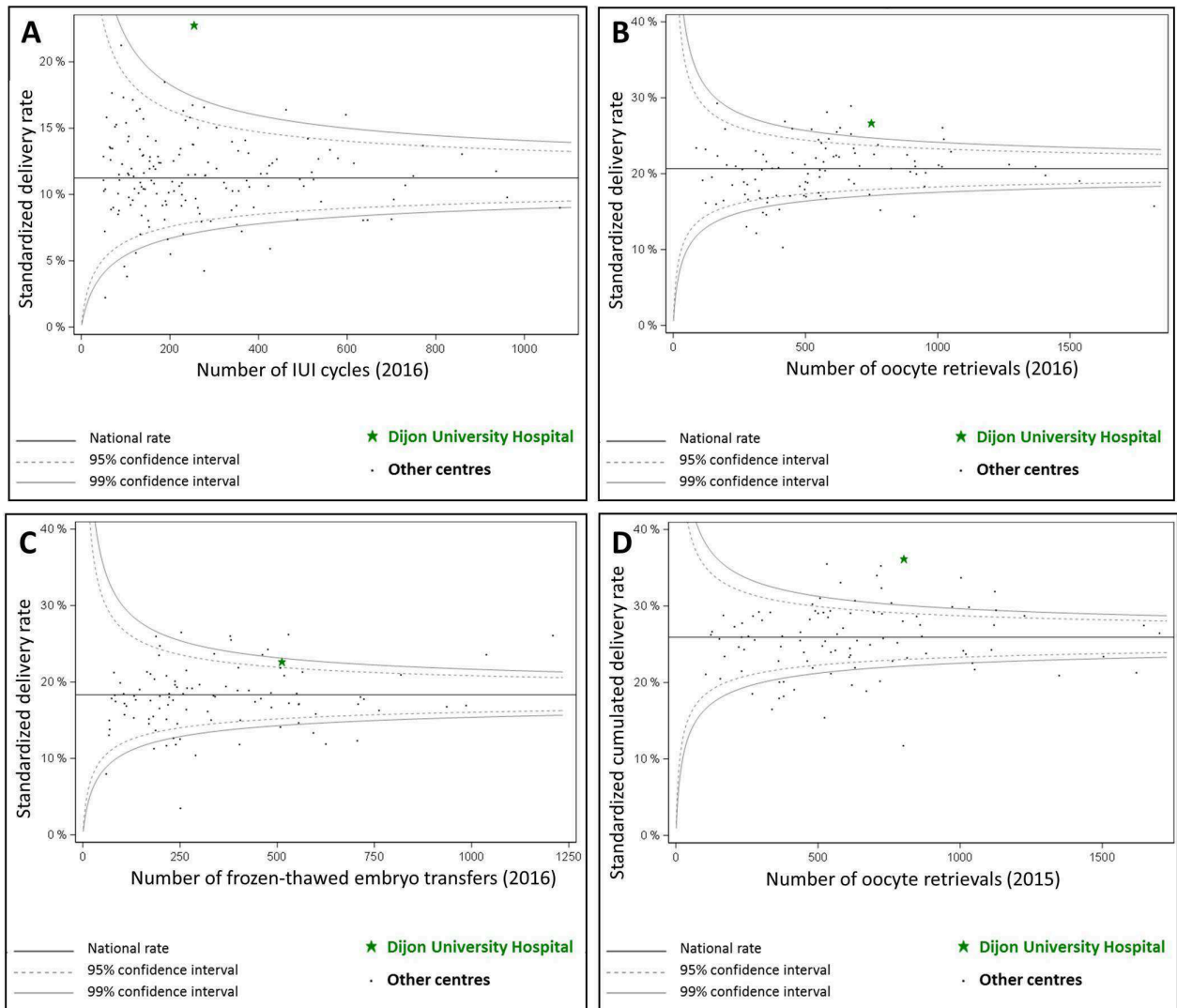
At Dijon University hospital, 255 IUI, 787 cycles of IVF with or without ICSI with oocyte retrieval and 513 frozen-thawed embryo transfers were done in 2016.

For IUI, the pregnancy rate was 29.6%, with a clinical pregnancy rate (*ie.* pregnancy with gestational sac and cardiac activity visible with US scan) of 23.5% and a delivery rate of 20.4%. Concerning IVF, the clinical pregnancy and delivery rates per oocyte retrieval were 41.8% and 30.8%, respectively. For FET, the pregnancy and delivery rates per frozen-thawed embryo were 37.6% and 23.2%, respectively.

The standardized results of our centre (normalized according to the characteristics of the population) compared to the national average are displayed in **Figure 2** (A: IUI; B: IVF; C: FET and D: cumulated results of fresh ET and FET for the same oocyte retrieval).

All in all, in France, out of 798,948 new-borns in 2016, 24,609, or 3.1%, were the result of ART. As between 8 and 12% of reproductive-aged couples are currently infertile (Vander Borgh *et al.*, 2018), providing them with safe treatment options has become a public health issue.

However, all over the recent years, the scientific community has started to look more closely at the potential consequences of the treatments and manipulations used for IVF.



**Figure 2:** Results obtained by our centre “Dijon University Hospital” according to the last ABM (*Agence de la Biomédecine*) report.

The results are displayed as funnel plots of the Standardized national Mean Difference. These results are normalized according to the characteristics of the population in each centre. Standardized delivery rate, A: by IUI cycles; B: by oocyte retrieval and C: by FET. D: Standardized Cumulated Delivery rates, from fresh or frozen-thawed embryo transfers, per oocyte retrieval.

## **I. The potential link between ART and adverse pregnancy outcomes (Tables 1 and 2)**

In both humans and animals, ART has been associated with increased incidence of placenta-related adverse pregnancy outcomes.

Indeed, in a number of animal models, more abortions are reported after IVF, culture or superovulation than with natural conception (Van der Auwera *et al.*, 2001; Delle Piane *et al.*, 2010; Fauque *et al.*, 2010). In the same way, Intra-Uterine Growth Retardation (IUGR) as well as overgrowth have been described in animals following ART procedures (Young *et al.*, 1998; Sinclair *et al.*, 2000; Hiendleder *et al.*, 2004; Miles *et al.*, 2004; Delle Piane *et al.*, 2010; Hori *et al.*, 2010; Bloise *et al.*, 2012; Chen *et al.*, 2013; Grazul-Bilska *et al.*, 2013; Ptak *et al.*, 2013).

Similarly, in humans, after adjusting for several confounding factors, the risk of spontaneous abortion is higher in ART cohorts than in spontaneous pregnancies (**Table 1**).

However, the studies that evidenced higher levels of abortions did not distinguish between different techniques of ART. Of note, when each ART technique was studied separately, no difference was found except that the risk was higher in FET when compared with naturally conceived pregnancy (**Table 1**).



**Table 1: Spontaneous abortions in human after ART**

Groups compared	Population	Study design	Adjustment	Measure of association	Comments	References
ART vs NC	554 vs 34286	Prospective multicentre cohort	None	0.8 (0.1-5.6)	ART=cIVF, ICSI and GIFT	(Shevell et al., 2005)
	1945 vs 549 (Ford cohort)	Retrospective cohort	Age	<b>1.20 (1.03-1.46)</b>	ART=cIVF, ICSI and GIFT, including sperm donation	(Wang et al., 2004)
	1945 vs 4265 (Treloar cohort)		Age, number of previous spontaneous abortions	<b>1.34 (1.19-1.51)</b>		
OI vs NC	682 vs 40261	Prospective cohort	Maternal characteristics, obstetrics history	<b>5.37 ( 3.71-7.77)</b>		(Chaveeva et al., 2011)
	266 vs 864	Prospective multicentre cohort	Maternal and paternal age, hospital, obstetrics history, smoking habits, menstrual cycle type, infertility diagnosis, man alcohol use	1.3 (0.79-2.18)	Infertile couples, excluding oocyte and sperm donation	(Brandes et al., 2011)
	1222 vs 34286	Prospective multicentre cohort	Age, parity, maternal race, marital status, years of education, prior preterm delivery or pregnancy with anomaly, BMI, smoking habits, bleeding	1.6 (0.6-4.4)		(Shevell et al., 2005)
IUI vs NC	203 vs 864	Prospective multicentre cohort	Maternal and paternal age, hospital, obstetrics history, smoking habits, menstrual cycle type, infertility diagnosis, man alcohol use	1.3 (0.85-2.10)	Infertile couples, excluding oocyte and sperm donation	(Brandes et al., 2011)
IVF vs NC	561 vs 600	Prospective cohort	Age, maternal education, obstetric history, hypertension and diabetes prior to pregnancy, BMI, smoking habits	0.94 (0.44-2.54)	cIVF and ICSI and FET	(Farhi et al., 2013)
	634 vs 40261	Prospective cohort	Maternal characteristics, obstetrics history	1.20 ( 0.58-2.47)		(Chaveeva et al., 2011)
cIVF vs NC	223 vs 600	Prospective cohort	Age, maternal education, obstetric history, hypertension and diabetes prior to pregnancy, BMI, smoking habits	1.04 (0.43-1.63)	Including FET	(Farhi et al., 2013)
	190 vs 864	Prospective multicentre cohort	Maternal and paternal age, hospital, obstetrics history, smoking habits, menstrual cycle type, infertility diagnosis, man alcohol use	1.1 (0.67-1.75)	Infertile couples, excluding oocyte and sperm donation	(Brandes et al., 2011)
ICSI vs NC	338 vs 600	Prospective cohort	Age, maternal education, obstetric history, hypertension and diabetes prior to pregnancy, BMI, smoking habits	0.89 (0.40-1.98)	Including FET	(Farhi et al., 2013)
	202 vs 864	Prospective multicentre cohort	Maternal and paternal age, hospital, obstetrics history, smoking habits, menstrual cycle type, infertility diagnosis, man alcohol use	1.0 (0.60-1.62)	Infertile couples, excluding oocyte and sperm donation	(Brandes et al., 2011)

FET vs NC	61 vs 864	Prospective multicentre cohort	Maternal and paternal age, hospital, obstetrics history, smoking habits, menstrual cycle type, infertility diagnosis, man alcohol use	<b>2.2 (1.14-4.19)</b>	Infertile couples, excluding oocyte and sperm donation	(Brandes et al., 2011)
IVF with EPE <sub>2</sub> vs IVF without EPE <sub>2</sub>	299 vs 2696	Retrospective cohort	None	Miscarriage rate: 8.4 vs 7.1% (NS)	cIVF and ICSI, only fresh embryo transfer, excluding oocyte donation	(Imudia et al., 2014)
IVF with Progesterone level at oocyte retrieval $\geq 12$ ng/mL vs $< 12$	51 vs 135	Prospective cohort	None	Implantation rate: <b>31.6 vs 43.9 % (P=0.01)</b> Pregnancy rate: <b>20.0 vs 38.6% (P=0.02)</b> Miscarriage rate: 16.7 vs 12.1% (P=0.64)	Only antagonist protocols, cIVF and ICSI, only fresh embryo transfer	(Nayak et al., 2014)
IVF with 1 <sup>st</sup> trimester serum hCG $< 10^{\text{th}}$ percentile vs IVF with normal serum hCG	46 vs 161	Retrospective cohort	None	<b>6.5 (4.1-10.1)</b>		(Haddad et al., 1999)
Letrozole-induced cycles vs natural cycles	792 vs 3136	Retrospective cohort	Maternal age and calendar year	<b>0.37 (0.30-0.47)</b>	Only single embryo transfer, excluding twin pregnancies	(Tatsumi et al., 2017)
IVF with fresh blastocyst transfer vs IVF with cleavage embryo transfer	194 vs 194	Randomized clinical trial	None	19.72 vs 12% (ns)	Patients $< 39$ years of age with $> 3$ fertilized oocytes and $< 4$ previous ART attempts	(Levi-Setti et al., 2018)

ART: Assisted Reproductive Technologies, cIVF : conventional *In Vitro* Fertilization, EPE<sub>2</sub>: Elevated Peak serum Estradiol level ( $> 90^{\text{th}}$  percentile, 3450-4500 pg/mL), GIFT: Gamete Intra-Fallopian Transfer, ICSI: Intra-Cytoplasmic Sperm Injection, IVF: *In Vitro* Fertilization (cIVF + ICSI), NC: Naturally Conceived, NS: Not Significant.

In addition, throughout ART pregnancy, placental-related defects can also occur (Thomopoulos *et al.*, 2013). For example, human studies found an increased risk of gestational hypertension, preeclampsia, placenta praevia and placental abruption (**Table 2**). Moreover, the risks of low birth weight (Jackson *et al.*, 2004) and prematurity (Jackson *et al.*, 2004; Pinborg *et al.*, 2013; Cavoretto *et al.*, 2018) were increased after ART (**Table 2**). Interestingly, according to the ART technique used, the potential adverse effects on pregnancy may be different. For example, after FET, the risks of large for gestational age (Sazonova *et al.*, 2012; Wennerholm *et al.*, 2013; Ishihara *et al.*, 2014; Korosec *et al.*, 2014; Litzky *et al.*, 2018), gestational hypertension (Ishihara *et al.*, 2014; Roque *et al.*, 2018) and preeclampsia (Sazonova *et al.*, 2012) are increased but the risk of small for gestational age (SGA) is decreased (Wennerholm *et al.*, 2013; Ishihara *et al.*, 2014; Korosec *et al.*, 2014; Litzky *et al.*, 2018) as compared to superovulation followed by fresh embryo transfer (**Table 2**). The risks are therefore dependent on the specific ART technique used, which emphasises the importance of studying ART techniques differentially and not pooling ART patients into a single group.

However, regarding **Table 2**, the results of different studies are quite heterogeneous and most of them are based on retrospective cohorts. Recently, some meta-analyses that compiled the existing studies on the obstetrical outcomes of ART were performed. The first one concluded that the rates of gestational hypertension and preeclampsia were increased after ART compared to natural conception (Thomopoulos *et al.*, 2017), but different methods of ART were mixed. A meta-analysis comparing IVF and natural conception reported higher rates of gestational hypertension, placenta praevia, *abruptio placentae*, SGA and preterm birth after IVF (Qin *et al.*, 2016). The increase in the rate of preterm birth after IVF was also demonstrated by another recent meta-analysis (Cavoretto *et al.*, 2018). As for the comparison between IVF with fresh or frozen-thawed embryo transfer, another recent meta-analysis found an increase in gestational hypertension and preeclampsia after FET but no difference in placenta praevia rates (Roque *et al.*, 2018).

**Table 2: Adverse and perinatal outcomes after ART potentially involving placenta in humans**

Outcome	Groups compared	Population	Study design	Adjustment	Measure of association	Comments	References
GH	ART vs NC	129 vs 2348	Meta-analysis	None	<b>2.06 (1.30-3.26)</b>	Including IUI, FET and oocyte donation	(Thomopoulos et al., 2017)
		364 vs 304	Retrospective case-control	Matched for maternal characteristics	NS		(Caserta et al., 2008)
		267 vs 4680	Retrospective cohort	BMI, parity	1.3 (0.9-1.9)		(Hernandez-Diaz et al., 2007)
		554 vs 34286	Prospective multicentre cohort	None	<b>1.6 (1.0-2.5)</b>	ART=cIVF, ICSI and GIFT	(Shevell et al., 2005)
		322 vs 322	Retrospective case-control	Age, parity, date of conception	<b>4.1 (1.1-18)</b>	ART= cIVF, ICSI and GIFT	(Ochsenkuhn et al., 2003)
	OI vs NC	4111 vs 4468	Retrospective cohort	Matched for age, parity, BMI, smoking habits, alcohol consumption, pre-existing diseases	1.06 (0.87-1.28)		(Hayashi et al., 2012)
		682 vs 40261	Prospective cohort	Maternal characteristics, obstetrics history	1.07 (0.66-1.73)		(Chaveeva et al., 2011)
		129 vs 4762	Retrospective cohort	BMI, parity, multiple gestations	<b>1.7 (1.0-2.7)</b>		(Hernandez-Diaz et al., 2007)
		1222 vs 34286	Prospective multicentre cohort	Age, parity, maternal race, marital status, years of education, prior preterm delivery or pregnancy with anomaly, BMI, smoking habits, bleeding	0.8 (0.5-1.2)		(Shevell et al., 2005)
		646 vs 1902	Retrospective case-control	Matched for age, gestational age, parity	<b>1.5 (1.04-2.02)</b>		(Maman et al., 1998)
		263 vs 5096	Retrospective case-control	None	<b>14.1 vs 9.7% (P&lt;0.02)</b>		(Olivennes et al., 1993)
	IUI vs NC	50 vs 215	Retrospective case-control	Excluding PCOS, age≥40years, family history of diabetes, risk factors for diabetes, history of stillbirth, recurrent miscarriage, history of macrosomia, parity>3, Cushing syndrome, congenital adrenal hyperplasia, hypothyroidism	14 vs 7.2% (NS)		(Ashrafi et al., 2014)
		2351 vs 5305	Retrospective cohort	Matched for age, parity, BMI, smoking habits, alcohol consumption, pre-existing diseases	0.88 (0.70-1.09)	Only IUI without ovulation stimulation, including sperm donation	(Hayashi et al., 2012)
		83 vs 4762	Retrospective cohort	BMI, parity, multiple gestations	1.4 (0.8-2.6)		(Hernandez-Diaz et al., 2007)

GH	IVF vs NC	26652 vs 68948	Meta-analysis	None	<b>1.30 (1.04-1.62)</b>	Including cIVF and ICSI	(Qin et al., 2016)
		1659 vs 5193	Retrospective cohort	Matched for maternal age and birth year	<b>1.99 (1.56-2.53)</b> <b>P&lt;0.001</b>	Excluding birth<28 WG, donor oocytes/sperm/embryos, preimplantation genetic diagnosis, chronic hypertension, diabetes or heart disease and fetal anomalies Including cIVF and ICSI	(Zhu et al., 2016)
		116 vs 664	Retrospective case-control	None	Age≥40: <b>3.02 (1.49-6.09)</b> Age 30-34: <b>6.23 (1.63-19.8)</b>	2 populations (age≥40 and age 30-34), ICSI and cIVF	(Toshimitsu et al., 2014)
		95 vs 215	Retrospective case-control	Excluding PCOS, age≥40years, family history of diabetes, risk factors for diabetes, history of stillbirth, recurrent miscarriage, history of macrosomia, parity>3, Cushing syndrome, congenital adrenal hyperplasia, hypothyroidism	<b>21 vs 7.2% (P=0.01)</b>	cIVF and ICSI	(Ashrafi et al., 2014)
		561 vs 600	Prospective cohort	Age, maternal education, obstetric history, hypertension and diabetes prior to pregnancy, BMI, smoking habits	1.49 (0.93-2.38)	FET and cIVF and ICSI,	(Farhi et al., 2013)
		4570 vs 4264	Retrospective cohort	Matched for age, parity, BMI, smoking habits, alcohol consumption, pre-existing diseases	<b>0.74 (0.62-0.89)</b>	cIVF and ICSI	(Hayashi et al., 2012)
		634 vs 40261	Prospective cohort	Maternal characteristics, obstetrics history	1.38 ( 0.92-2.06)		(Chaveeva et al., 2011)
		269 vs 15037	Retrospective case-control	Matched for year of delivery and mother's place of residence. Adjustment on age, parity, socio-economic status	<b>2.35 (1.57-3.53)</b>	Only fresh SET, cIVF and ICSI	(Poikkeus et al., 2007)
		307 vs 307	Retrospective case-control	Matched for maternal age, parity, ethnic origin, date of parturition, height, smoking habits, obstetric history	13.7 vs 11.1% (NS)	Excluding FET	(Koudstaal et al., 2000)

GH		169 vs 469	Retrospective case-control	Matched for age, gestational age, parity	<b>2.1 (1.04-4.10)</b>		(Maman et al., 1998)
		260 vs 260	Retrospective case-control	Matched for age, parity, ethnic origin, location and date of delivery	11.2 vs 8.1% (NS)	14% FET, 6.2% ovum donation	(Reubinoff et al., 1997)
		62 vs 62	Retrospective case-control	Matched for age, race, order of gestation, insurance	<b>21 vs 4% (P&lt;0.05)</b>		(Tallo et al., 1995)
		162 vs 5096	Retrospective case-control	None	9.2 vs 9.7% (NS)		(Olivennes et al., 1993)
	cIVF vs NC	223 vs 600	Prospective cohort	Age, maternal education, obstetric history, hypertension and diabetes prior to pregnancy, BMI, smoking habits	1.63 (0.89-2.99)	Including FET	(Farhi et al., 2013)
	ICSI vs NC	338 vs 600	Prospective cohort	Age, maternal education, obstetric history, hypertension and diabetes prior to pregnancy, BMI, smoking habits	1.41 (0.84-2.38)	Including FET	(Farhi et al., 2013)
	IVF FET vs IVF fresh	31479 vs 17447	Meta-analysis	None	<b>1.82 (1.24-2.68), P=0.002</b>		(Roque et al., 2018)
		31249 vs 16909	Retrospective cohort	Adjusted for maternal age, type of embryo transferred (cleaved or blastocyst) and sex of the infant	<b>1.58 (1.35-1.86)</b>	Only SET	(Ishihara et al., 2014)
PE	ART vs NC	2987 vs 75958	Meta-analysis	None	<b>1.65 (1.53-1.77)</b>	Including IUI, FET, oocyte donation	(Thomopoulos et al., 2017)
		267 vs 4680	Retrospective cohort	BMI, parity	1.2 (0.6-2.4)		(Hernandez-Diaz et al., 2007)
		554 vs 34286	Prospective multicentre cohort	None	<b>2.7 (1.7-4.4)</b>	ART=cIVF, ICSI and GIFT	(Shevell et al., 2005)
	OI vs NC	296 vs 9559	Meta-analysis	None	<b>1.48 (1.12-1.96)</b>		(Thomopoulos et al., 2017)
		682 vs 40261	Prospective cohort	Maternal characteristics, obstetric history	Early: 1.29 (0.39-4.20) Late: 1.32 (0.78-2.24)		(Chaveeva et al., 2011)
		777 vs 3103	Retrospective case-control	Matched for age and parity, adjustment on smoking, delivery hospital level, initiating time of prenatal care, average neighbourhood income, foetal sex, previous caesarean delivery	0.72 (0.31-1.68)		(Sun et al., 2009)
		1222 vs 34286	Prospective cohort	Age, race, marital status, years of education, prior preterm delivery, prior foetal anomaly, BMI, smoking history, bleeding	1.1 (0.6-1.8)		(Shevell et al., 2005)

PE	IUI vs NC	471 vs 1884	Retrospective case-control	Matched for age and parity, adjustment on smoking, delivery hospital level, initiating time of prenatal care, average neighbourhood income, foetal sex, previous caesarean delivery	<b>2.28 (1.04-5.02)</b>		(Sun et al., 2009)
	IVF vs NC	1659 vs 5193	Retrospective cohort	Matched for maternal age and birth year	<b>1.71 (1.34-2.19)</b>	Excluding birth<28 WG, donor oocytes/sperm/embryos, preimplantation genetic diagnosis, chronic hypertension, diabetes or heart disease and fetal anomalies Including cIVF and ICSI	(Zhu et al., 2016)
		474 vs 474	Retrospective case-control	Matched with the closest propensity score adjusting for 27 maternal and paternal variables	2.50 (0.49-12.89)		(Watanabe et al., 2014)
		474 vs 2610		Adjusted for 27 maternal and paternal confounders	<b>2.32 (1.08-4.99)</b>		
		825 vs 111264	Retrospective multicentre case-control	Birth gestational age, parity, birth weight, maternal and paternal age, education, smoking habits, alcohol consumption	<b>1.48 (1.16-1.87)</b>	cIVF, ICSI and FET, including PE and eclampsia	(Yang et al., 2014)
		634 vs 40261	Prospective cohort	Maternal characteristics, obstetrics history	<b>Early: 3.28 (1.39-7.74)</b> Late: 1.48 (0.91-2.41)		(Chaveeva et al., 2011)
		870 vs 3433	Retrospective case-control	Matched for age and parity, adjustment on smoking, delivery hospital level, initiating time of prenatal care, average neighbourhood income, foetal sex, previous caesarean delivery	1.12 (0.56-2.22)	cIVF and ICSI	(Sun et al., 2009)
		269 vs 15037	Retrospective case-control	Matched for year of delivery and mother's place of residence Adjustment on age, parity, socio-economic status	2.2 vs 1.6% (NS)	Only fresh SET, cIVF and ICSI	(Poikkeus et al., 2007)
		554 vs 34286	Prospective cohort	Age, race, marital status, years of education, prior preterm delivery, prior foetal anomaly, BMI, smoking history, bleeding	<b>2.7 (1.7-4.4)</b>		(Shevell et al., 2005)
		13261 vs 2013633	Register study	Age, parity, smoking habits, year of pregnancy	<b>1.2 (1.1-1.3)</b>	cIVF and ICSI	(Kallen et al., 2005)
		144 vs 39112	Retrospective case-control	None	<b>5.2 (1.7-15.9)</b>		(Tabs et al., 2004)
	IVF FET vs NC	2348 vs 571914	Retrospective case-control	Age, parity, BMI, smoking habits, year of birth, years of involuntary childlessness	<b>1.25 (1.03-1.51)</b>	SET and DET, excluding oocyte donation	(Sazonova et al., 2012)

<b>PE</b>	IVF FET vs IVF fresh	211 vs 915	Retrospective cohort	None	0.9 vs 3.1% ( <i>P</i> =0.098)	Mostly blastocyst-stage transfers, cIVF and ICSI	( <i>Korosec et al., 2014</i> )
		2348 vs 8944	Retrospective case-control	Age, parity, BMI, smoking habits, year of birth, years of involuntary childlessness	<b>1.32 (1.07-1.63)</b>	SET and DET, excluding oocyte donation	( <i>Sazonova et al., 2012</i> )
	IVF with EPE <sub>2</sub> vs IVF without EPE <sub>2</sub>	27 vs 265	Retrospective cohort	Age, parity, total dose of gonadotropin	<b>4.79 (1.55-14.84)</b>	Excluding FET	( <i>Imudia et al., 2012</i> )
	IVF with hCG<50 IU/L vs hCG≥150 IU/L at day 12	220 vs 673	Prospective cohort	Age, parity, BMI, smoking habits, type of IVF (cIVF, IVF-ICSI or FET), number of transferred embryos, year of transfer, offspring sex	Any PE: <b>2.2 (1.0-4.9)</b> Severe PE: <b>3.7 (1.2-11.6)</b> Mild PE: 1.3 (0.4-4.0)	Including FET, cIVF and ICSI, excluding oocyte donation	( <i>Asvold et al., 2014</i> )
	IVF with 1 <sup>st</sup> trimester serum hCG<10 <sup>th</sup> percentile vs IVF with normal serum hCG	46 vs 161	Retrospective cohort	None	9 vs 1.4% (NS)		( <i>Haddad et al., 1999</i> )
<b>GH or PE</b>	ICSI vs NC	2055 vs 7861	Prospective controlled cohort	None	<b>1.3 (1.11-1.52)</b>	Excluding FET	( <i>Katalinic et al., 2004</i> )
<b>Placenta praevia</b>	ART vs NC	1408 vs 53939	Retrospective cohort	None	<b>4.7 vs 1.5% (P=0.0001)</b>	ART=cIVF, ICSI, GIFT	( <i>Fujii et al., 2010</i> )
		554 vs 34286	Prospective multicentre cohort	None	<b>6.0 (3.4-10.7)</b>	ART=cIVF, ICSI, GIFT	( <i>Shevell et al., 2005</i> )
	OI vs NC	4111 vs 4468	Retrospective cohort	Matched for age, parity, BMI, smoking habits, alcohol consumption, pre-existing diseases	<b>1.77 (1.24-2.54)</b>		( <i>Hayashi et al., 2012</i> )
		1222 vs 34286	Prospective cohort	Age, race, marital status, years of education, prior preterm delivery, prior foetal anomaly, BMI, smoking history, bleeding	0.9 (0.3-2.3)		( <i>Shevell et al., 2005</i> )
	IUI vs NC	2351 vs 5305	Retrospective cohort	Matched for age, parity, BMI, smoking habits, alcohol consumption, pre-existing diseases	<b>1.46 (1.03-2.08)</b>	Only IUI without ovulation stimulation, including sperm donation	( <i>Hayashi et al., 2012</i> )
	IVF vs NC	22920 vs 961703	Meta-analysis	None	<b>3.71 (2.67-5.16)</b>	Including cIVF and ICSI	( <i>Qin et al., 2016</i> )



Placenta praevia		1659 vs 5193	Retrospective cohort	Matched for maternal age and birth year	<b>2.25 (1.75-2.89)</b> <b>P&lt;0.001</b>	Excluding birth<28 WG, donor oocytes/sperm/embryos, preimplantation genetic diagnosis, chronic hypertension, diabetes or heart disease and fetal anomalies Including cIVF and ICSI	(Zhu et al., 2016)	
		825 vs 111264	Retrospective multicentre case-control	Birth gestational age, parity, birth weight, maternal and paternal age, education, smoking habits, alcohol consumption	<b>2.78 (1.97-13.94)</b>	cIVF and ICSI and FET	(Yang et al., 2014)	
		4570 vs 4264	Retrospective cohort	Matched for age, parity, BMI, smoking habits, alcohol consumption, pre-existing diseases	<b>2.2 (1.68-2.87)</b>	cIVF and ICSI	(Hayashi et al., 2012)	
		6730 vs 24619	Retrospective cohort	Matched on age, year of birth of the baby Adjustment on age, year of pregnancy, marital status, parity, miscarriage, hospital status	<b>2.34 (1.87-2.92)</b>	cIVF, ICSI and FET, excluding oocyte donation	(Healy et al., 2010)	
		269 vs 15037	Retrospective case-control	Matched for year of delivery and mother's place of residence Adjustment on age, parity, socio-economic status	<b>4.75 (2.57-8.78)</b>	Only fresh SET, cIVF and ICSI	(Poikkeus et al., 2007)	
		5581 vs 826909	Retrospective cohort	Age, parity, interval between deliveries, year of delivery, history of caesarean section, offspring sex, marital status	<b>5.6 (4.4-7.0)</b>	cIVF, ICSI and FET	(Romundstad et al., 2006)	
		1349 vs 1349 consecutive birth for the same mother			<b>2.9 (1.4-6.1)</b>			
		13261 vs 2013633	Register study	Age, parity, smoking habits, year of pregnancy	<b>3.8 (3.3-4.5)</b>	cIVF and ICSI	(Kallen et al., 2005)	
		260 vs 260	Retrospective case-control	Matched for age, parity, ethnic origin, location and date of delivery	0.4 vs 0.8% (NS)	14% FET, 6.2% oocyte donation	(Reubinoff et al., 1997)	
		ICSI vs NC	2055 vs 7861	Prospective controlled cohort	None	<b>6.42 (4.03-10.22)</b>	Only fresh embryo transfer	(Katalinic et al., 2004)
		IVF FET vs NC	2348 vs 571914	Retrospective case-control	Age, parity, BMI, smoking habits, year of birth, years of involuntary childlessness	1.22 (0.73-2.04)	SET and DET, excluding oocyte donation	(Sazonova et al., 2012)
		IVF FET vs IVF fresh	36455 vs 33031	Meta-analysis	None	0.70 (0.46-1.08), P=0.11		(Roque et al., 2018)
			31249 vs 16909	Retrospective cohort	Adjusted for maternal age, type of embryo transferred (cleaved or blastocyst) and sex of the infant	0.90 (0.69-1.19)	Only SET	(Ishihara et al., 2014)

<b>Placenta praevia</b>		211 vs 915	Retrospective cohort	None	<b>0 vs 3.5% (P=0.002)</b>	Mostly blastocyst-stage transfers, cIVF and ICSI	(Korosec et al., 2014)
		2348 vs 8944	Retrospective case-control	Age, parity, BMI, smoking habits, year of birth, years of involuntary childlessness	<b>0.32 (0.19-0.55)</b>	SET and DET, excluding oocyte donation	(Sazonova et al., 2012)
		2045 vs 4058	Retrospective cohort	Matched on age, year of birth of the baby Adjustment on age, year of pregnancy, marital status, country of birth, parity, miscarriage, hospital status	0.73 (0.51-1.04)	cIVF and ICSI, FET=only in natural cycles, excluding oocyte donation	(Healy et al., 2010)
	IVF vs NC in infertile population	6730 vs 2167	Retrospective cohort	Matched on age, year of birth of the baby	<b>2.6 vs 1.2% (P&lt;0.001)</b>	cIVF and ICSI and FET, excluding oocyte donation	(Healy et al., 2010)
<b>Abruptio placentae</b>	ART vs NC	554 vs 34286	Prospective multicentre cohort	None	<b>2.4 (1.1-5.2)</b>	ART=cIVF, ICSI and GIFT	(Shevell et al., 2005)
	OI vs NC	4111 vs 4468	Retrospective cohort	Matched for age, parity, BMI, smoking habits, alcohol consumption, pre-existing diseases	1.35 (0.88-2.08)		(Hayashi et al., 2012)
		777 vs 3103	Retrospective case-control	Matched for age and parity, adjustment on smoking, delivery hospital level, initiating time of prenatal care, average neighbourhood income, foetal sex, previous caesarean delivery	1.63 (0.60-4.42)		(Sun et al., 2009)
		1222 vs 34286	Prospective cohort	Age, race, marital status, years of education, prior preterm delivery, prior foetal anomaly, BMI, smoking history, bleeding	<b>2.4 (1.3-4.2)</b>		(Shevell et al., 2005)
	IUI vs NC	2351 vs 5305	Retrospective cohort	Matched for age, parity, BMI, smoking habits, alcohol consumption, pre-existing diseases	0.98 (0.59-1.62)		Only IUI without ovulation stimulation, including sperm donation (Hayashi et al., 2012)
		471 vs 1884	Retrospective case-control	Matched for age and parity, adjustment on smoking, delivery hospital level, initiating time of prenatal care, average neighbourhood income, foetal sex, previous caesarean delivery	1.57 (0.41-6.03)		(Sun et al., 2009)
	IVF vs NC	15578 vs 80396	Meta-analysis	None	<b>1.87 (1.45-2.40)</b>	Including cIVF and ICSI	(Qin et al., 2016)

<b>Abruptio placentae</b>		1659 vs 5193	Retrospective cohort	Matched for maternal age and birth year	<b>4.43 (2.28-8.61) P&lt;0.001</b>	Excluding birth<28 WG, donor oocytes/sperm/embryos, preimplantation genetic diagnosis, chronic hypertension, diabetes or heart disease and fetal anomalies Including cIVF and ICSI	(Zhu et al., 2016)
		4570 vs 4264	Retrospective cohort	Matched for age, parity, BMI, smoking habits, alcohol consumption, pre-existing diseases	1.21 (0.79-1.87)	cIVF and ICSI	(Hayashi et al., 2012)
		6730 vs 24619	Retrospective cohort	Matched on age, year of birth of the baby Adjustment on age, year of pregnancy, marital status, parity, miscarriage, hospital status	<b>2.07 (1.44-2.98)</b>	cIVF, ICSI and FET, excluding oocyte donation	(Healy et al., 2010)
		870 vs 3433	Retrospective case-control	Matched for age and parity, adjustment on smoking, delivery hospital level, initiating time of prenatal care, average neighbourhood income, foetal sex, previous caesarean delivery	1.26 (0.54-2.92)	cIVF and ICSI	(Sun et al., 2009)
		269 vs 15037	Retrospective case-control	Matched for year of delivery and mother's place of residence Adjustment on age, parity, socio-economic status	0 vs 0.4% (NS)	Only fresh SET, cIVF and ICSI	(Poikkeus et al., 2007)
		13261 vs 2013633	Register study	Age, parity, smoking habits, year of pregnancy	<b>1.9 (1.4-2.5)</b>	cIVF and ICSI	(Kallen et al., 2005)
	ICSI vs NC	2055 vs 7861	Prospective controlled cohort	None	<b>1.81 (1.26-2.60)</b>	excluding FET	(Katalinic et al., 2004)
	IVF FET vs NC	2348 vs 571914	Retrospective case-control	Age, parity, BMI, smoking habits, year of birth, years of involuntary childlessness	1.21 (0.69-2.11)	SET and DET, excluding oocyte donation	(Sazonova et al., 2012)
	IVF FET vs IVF fresh	31249 vs 16909	Retrospective cohort	Adjusted for maternal age, type of embryo transferred (cleaved or blastocyst) and sex of the infant	0.65 (0.41-1.04)	Only SET	(Ishihara et al., 2014)
		2348 vs 8944	Retrospective case-control	Age, parity, BMI, smoking habits, year of birth, years of involuntary childlessness	0.66 (0.36-1.20)	SET and DET, excluding oocyte donation	(Sazonova et al., 2012)
		2045 vs 4058	Retrospective cohort	Matched on age, year of birth of the baby Adjustment on age, year of pregnancy, marital status, country of birth, parity, miscarriage, hospital status	<b>0.39 (0.19-0.80)</b>	cIVF and ICSI, FET=only in natural cycles, excluding oocyte donation	(Healy et al., 2010)

	IVF vs NC in infertile population	6730 vs 2167	Retrospective cohort	Matched on age, year of birth of the baby	<b>0.9 vs 0.6% (NS)</b>	cIVF and ICSI and FET, excluding oocyte donation	(Healy et al., 2010)
<b>SGA</b>	ART vs NC	1408 vs 53939	Retrospective cohort	Age, gestational age, placenta praevia, maternal pretreatment characteristics	<10 <sup>th</sup> perc 1.12 (0.95-1.31)	ART=cIVF, ICSI and GIFT	(Fujii et al., 2010)
		554 vs 34286	Prospective multicentre cohort	None	<b>&lt;10<sup>th</sup> perc 1.3 (1.0-1.8)</b>	ART=cIVF, ICSI and GIFT	(Shevell et al., 2005)
	OI vs NC	4111 vs 4468	Retrospective cohort	Matched for age, parity, BMI, smoking habits, alcohol consumption, pre-existing diseases	<b>&lt;10<sup>th</sup> perc 1.45 (1.21-1.73)</b>		(Hayashi et al., 2012)
		682 vs 40261	Prospective cohort	Maternal characteristics, obstetrics history	<5 <sup>th</sup> perc 1.76 (1.30-2.38)		(Chaveeva et al., 2011)
		777 vs 3103	Retrospective case-control	Matched for age and parity, adjustment on smoking, delivery hospital level, initiating time of prenatal care, average neighbourhood income, foetal sex, previous caesarean delivery	<b>&lt;10<sup>th</sup> perc 1.40 (1.06-1.86)</b>		(Sun et al., 2009)
		1222 vs 34286	Prospective cohort	Age, race, marital status, years of education, prior preterm delivery, prior foetal anomaly, BMI, smoking history, bleeding	<10 <sup>th</sup> perc 1.5 (0.8-2.8)		(Shevell et al., 2005)
		263 vs 5096	Retrospective case-control	None	<b>&lt;10<sup>th</sup> perc 10.6 vs 5.9% (P&lt;0.05)</b>		(Olivennes et al., 1993)
	IUI vs NC	4208 vs 229749	Retrospective cohort	Age, parity, year of pregnancy, child gender, BMI smoking habits, elective caesarean section, induction of labour	<b>&lt;10<sup>th</sup> perc 1.39 (1.18-1.65)</b>	Natural or stimulated cycle	(Malchau et al., 2014)
		2351 vs 5305	Retrospective cohort	Matched for age, parity, BMI, smoking habits, alcohol consumption, pre-existing diseases	<b>&lt;10<sup>th</sup> perc 1.27 (1.04-1.55)</b>	Only IUI without ovulation stimulation, including sperm donation	(Hayashi et al., 2012)
		471 vs 1884	Retrospective case-control	Matched for age and parity, adjustment on smoking, delivery hospital level, initiating time of prenatal care, average neighbourhood income, foetal sex, previous caesarean delivery	<10 <sup>th</sup> perc 1.07 (0.76-1.52)		(Sun et al., 2009)
	IVF vs NC	81090 vs 753771	Meta-analysis	None	<b>&lt;10<sup>th</sup> perc 1.35 (1.20-1.52)</b>	Including cIVF and ICSI	(Qin et al., 2016)

SGA		561 vs 600	Prospective cohort	Age, maternal education, obstetric history, hypertension and diabetes prior to pregnancy, BMI, smoking habits	<10 <sup>th</sup> perc 1.34 (0.97-1.83)	cIVF and ICSI and FET	(Farhi et al., 2013)
		4570 vs 4264	Retrospective cohort	Matched for age, parity, BMI, smoking habits, alcohol consumption, pre-existing diseases	<10 <sup>th</sup> perc 1.12 (0.94-1.33)	cIVF and ICSI	(Hayashi et al., 2012)
		634 vs 40261	Prospective cohort	Maternal characteristics, obstetrics history	<5 <sup>th</sup> perc 1.29 (0.92-1.81)		(Chaveeva et al., 2011)
		870 vs 3433	Retrospective case-control	Matched for age and parity, adjustment on smoking, delivery hospital level, initiating time of prenatal care, average neighbourhood income, foetal sex, previous caesarean delivery	<10 <sup>th</sup> perc 0.83 (0.63-1.10)	cIVF and ICSI	(Sun et al., 2009)
		269 vs 15037	Retrospective case-control	Matched for year of delivery and mother's place of residence Adjustment on age, parity, socio-economic status	<10 <sup>th</sup> perc 1.42 (0.74-2.71)	Only fresh SET, cIVF and ICSI	(Poikkeus et al., 2007)
		307 vs 307	Retrospective case-control	Matched for maternal age, parity, ethnic origin, date of parturition, height, smoking habits, obstetric history	<b>&lt;10<sup>th</sup> perc 16.2 vs 7.9% (P&lt;0.001)</b>	Only fresh embryo transfer	(Koudstaal et al., 2000)
		260 vs 260	Retrospective case-control	Matched for age, parity, ethnic origin, location and date of delivery	<10 <sup>th</sup> perc 12.5 vs 12.9% (NS)	14% FET, 6.2% ovum donation	(Reubinoff et al., 1997)
		62 vs 62	Retrospective case-control	Matched for age, race, order of gestation, insurance	<10 <sup>th</sup> perc 1.6 vs 1.6% (NS)		(Tallo et al., 1995)
		162 vs 5096	Retrospective case-control	None	<b>&lt;10<sup>th</sup> perc 11.2 vs 5.9% (P&lt;0.05)</b>		(Olivennes et al., 1993)
	cIVF vs NC	223 vs 600	Prospective cohort	Age, maternal education, obstetric history, hypertension and diabetes prior to pregnancy, BMI, smoking habits	<10 <sup>th</sup> perc 1.51 (0.99-2.28)	Including FET	(Farhi et al., 2013)
	ICSI vs NC	338 vs 600	Prospective cohort	Age, maternal education, obstetric history, hypertension and diabetes prior to pregnancy, BMI, smoking habits	<10 <sup>th</sup> perc 1.24 (0.87-1.77)	Including FET	(Farhi et al., 2013)
	IVF FET vs NC	6647 vs 288542	Retrospective cohort	Age, parity, year of pregnancy, child gender, country	<b>&lt;-2SD 1.18 (1.03-1.35)</b>	cIVF and ICSI	(Wennerholm et al., 2013)
		2348 vs 571914	Retrospective case-control	Age, parity, BMI, smoking habits, year of birth, years of involuntary childlessness	<10 <sup>th</sup> perc 0.80 (0.60-1.05)	SET and DET, excluding oocyte donation	(Sazonova et al., 2012)

SGA	IVF FET vs IVF fresh	55898 vs 124286	Cohort study	Infertility diagnosis, ethnicity, infant and obstetric characteristics	<b>1500-2500g</b> <b>0.52 (0.48-0.56)</b> <b>P&lt;0.001</b>	Excluding donor cycles, woman age>45, transfer>4 embryos, BW>5500 or <1500g	(Litzky et al., 2018)
		31249 vs 16909	Retrospective cohort	Adjusted for maternal age, type of embryo transferred (cleaved or blastocyst) and sex of the infant	<b>&lt;-2SD</b> <b>0.67 (0.60-0.75)</b>	Only SET	(Ishihara et al., 2014)
		211 vs 915	Retrospective cohort	None	<b>&lt;10<sup>th</sup> perc 0.9 vs 3.7% (P=0.048)</b>	Mostly blastocyst-stage transfers, cIVF and ICSI	(Korosec et al., 2014)
		6647 vs 42242	Retrospective cohort	Age, parity, year of pregnancy, child gender, country	<b>&lt;-2SD</b> <b>0.72 (0.62-0.83)</b>	cIVF and ICSI	(Wennerholm et al., 2013)
		2348 vs 8944	Retrospective case-control	Age, parity, BMI, smoking habits, year of birth, years of involuntary childlessness	<10 <sup>th</sup> perc 0.78 (0.58-1.04)	SET and DET, excluding oocyte donation	(Sazonova et al., 2012)
	IVF with EPE <sub>2</sub> vs IVF without EPE <sub>2</sub>	27 vs 265	Retrospective cohort	Age, parity, BMI, day of embryo transfer	<b>&lt;10<sup>th</sup> perc</b> <b>9.4 (3.22-27.46)</b>	Only fresh embryo transfer	(Imudia et al., 2012)
	IVF with 1 <sup>st</sup> trimester serum hCG<10 <sup>th</sup> percentile vs IVF with normal serum hCG	46 vs 161	Retrospective cohort	None	<b>&lt;10<sup>th</sup> perc</b> <b>6.5 (2.7-15.6)</b>		(Haddad et al., 1999)
LGA	OI vs NC	682 vs 40261	Prospective cohort	Maternal characteristics, obstetrics history	>95 <sup>th</sup> perc 1.05 (0.72-1.53)		(Chaveeva et al., 2011)
	IUI vs NC	4208 vs 229749	Retrospective cohort	Age, parity, year of pregnancy, child gender, BMI smoking habits, elective caesarean section, induction of labour	<b>&gt;90<sup>th</sup> perc</b> <b>0.97 (0.79-1.18)</b>	Natural or stimulated cycle	(Malchau et al., 2014)
	IVF vs NC	634 vs 40261	Prospective cohort	Maternal characteristics, obstetrics history	>95 <sup>th</sup> perc 1.11 (0.74-1.57)		(Chaveeva et al., 2011)
	IVF FET vs NC	6647 vs 288542	Retrospective cohort	Age, parity, year of pregnancy, child gender, country	<b>1.29 (1.15-1.45)</b> <b>&gt;2SD</b> <b>1.29 (1.15-1.45)</b>	cIVF and ICSI	(Wennerholm et al., 2013)
		2348 vs 571914	Retrospective case-control	Age, parity, BMI, smoking habits, year of birth, years of involuntary childlessness	<b>&gt;2SD</b> <b>1.48 (1.22-1.81)</b>	SET and DET, excluding oocyte donation	(Sazonova et al., 2012)
	IVF FET vs IVF fresh	55898 vs 124286	Cohort study	Infertility diagnosis, ethnicity, infant and obstetric characteristics	<b>&gt;4000g</b> <b>1.70 (1.64-1.76)</b> <b>P&lt;0.001</b>	Excluding donor cycles, woman age>45, transfer>4 embryos,	(Litzky et al., 2018)
		31249 vs 16909	Retrospective cohort	Adjusted for maternal age, type of embryo transferred (cleaved or blastocyst) and sex of the infant	<b>&gt;2SD</b> <b>1.48 (1.38-1.58)</b>	Only SET	(Ishihara et al., 2014)

		211 vs 915	Retrospective cohort	None	<b>&gt;95<sup>th</sup> perc 10.5 vs 5% (P=0.003)</b>	Mostly blastocyst-stage transfers, cIVF and ICSI	(Korosec et al., 2014)
		6647 vs 42242	Retrospective cohort	Age, parity, year of pregnancy, child gender, country	<b>&gt;2SD 1.45 (1.27-1.64)</b>	cIVF and ICSI	(Wennerholm et al., 2013)
		2348 vs 8944	Retrospective case-control	Age, parity, BMI, smoking habits, year of birth, years of involuntary childlessness	<b>&gt;2SD 1.59 (1.26-1.99)</b>	SET and DET, excluding oocyte donation	(Sazonova et al., 2012)
<b>Preterm birth &lt;37 WG</b>	ART vs NC	554 vs 34286	Prospective multicentre cohort	None	<b>1.5 (1.0-2.2)</b>	ART=cIVF, ICSI and GIFT	(Shevell et al., 2005)
		322 vs 322	Retrospective case-control	Age, parity, date of conception	NS	ART= cIVF, ICSI and GIFT	(Ochsenkuhn et al., 2003)
	OI vs NC	4111 vs 4468	Retrospective cohort	Matched for age, parity, BMI, smoking habits, alcohol consumption, pre-existing diseases	<b>1.29 (1.15-1.45)</b>		(Hayashi et al., 2012)
		1222 vs 34286	Prospective cohort	Age, race, marital status, years of education, prior preterm delivery, prior foetal anomaly, BMI, smoking history, bleeding	1.1 (0.8-1.5)		(Shevell et al., 2005)
		263 vs 5096	Retrospective case-control	None	<b>6.1 vs 4.4% (P&lt;0.05)</b>		(Olivennes et al., 1993)
	IUI vs NC	4208 vs 229749	Retrospective cohort	Age, parity, year of pregnancy, child gender, BMI smoking habits, elective caesarean section, induction of labour	<b>1.26 (1.07-1.49)</b>	Natural or stimulated cycle	(Malchau et al., 2014)
		2351 vs 5305	Retrospective cohort	Matched for age, parity, BMI, smoking habits, alcohol consumption, pre-existing diseases	<b>1.16 (1.01-1.33)</b>	Only IUI without ovulation stimulation, including sperm donation	(Hayashi et al., 2012)
	IVF vs NC	8044 vs 53633	Meta-analysis	Matched for maternal age and parity	<b>1.63 (1.30-2.05)</b>		(Cavoretto et al., 2018)
		133338 vs 1289549	Meta-analysis	None	<b>1.71 (1.59-1.83)</b>	Including cIVF and ICSI	(Qin et al., 2016)
		116 vs 664	Retrospective case-control	None	Age≥40: 12.5 vs 7% (P=0.128) Age 30-34: 3.6 vs 8.1% (P=0.344)	2 populations (age≥40 and age 30-34), cIVF and ICSI	(Toshimitsu et al., 2014)

<b>Preterm birth &lt;37 WG</b>	VF vs NC	825 vs 111264	Retrospective multicentre case-control	Birth gestational age, parity, birth weight, maternal and paternal age, education, smoking habits, alcohol consumption	<b>2.21 (1.81-2.70)</b>	cIVF and ICSI and FET	(Yang et al., 2014)
		561 vs 600	Prospective cohort	Age, maternal education, obstetric history, hypertension and diabetes prior to pregnancy, BMI, smoking habits	<b>1.72 (1.04-2.87)</b>	cIVF and ICSI and FET	(Farhi et al., 2013)
		4570 vs 4264	Retrospective cohort	Matched for age, parity, BMI, smoking habits, alcohol consumption, pre-existing diseases	<b>1.29 (1.16-1.45)</b>	cIVF and ICSI	(Hayashi et al., 2012)
		875 vs 20648	Retrospective cohort compared with the general population	None	<b>2.0 (1.79-2.97)</b>	cIVF and ICSI	(Allen et al., 2008)
		269 vs 15037	Retrospective case-control	Matched for year of delivery and mother's place of residence Adjustment on age, parity, socio-economic status	<b>2.85 (1.96-4.16)</b>	Only fresh SET, cIVF and ICSI	(Poikkeus et al., 2007)
		307 vs 307	Retrospective case-control	Matched for maternal age, parity, ethnic origin, date of parturition, height, smoking habits, obstetric history	<b>15 vs 5.9% (P&lt;0.001)</b>	Only fresh embryo transfer	(Koudstaal et al., 2000)
		260 vs 260	Retrospective case-control	Matched for age, parity, ethnic origin, location and date of delivery	<b>8.8 vs 3.9% (P=0.024)</b>	14% FET, 6.2% ovum donation	(Reubinoff et al., 1997)
		62 vs 62	Retrospective case-control	Matched for age, race, order of gestation, insurance	<b>10 vs 2% (P=0.04)</b>		(Tallo et al., 1995)
		162 vs 5096	Retrospective case-control	None	<b>11.1 vs 4.4% (P&lt;0.05)</b>		(Olivennes et al., 1993)
	cIVF vs NC	223 vs 600	Prospective cohort	Age, maternal education, obstetric history, hypertension and diabetes prior to pregnancy, BMI, smoking habits	<b>2.36 (1.28-4.37)</b>	Including FET	(Farhi et al., 2013)
	ICSI vs NC	338 vs 600	Prospective cohort	Age, maternal education, obstetric history, hypertension and diabetes prior to pregnancy, BMI, smoking habits	1.40 (0.79-2.50)	Including FET	(Farhi et al., 2013)
		2055 vs 7861	Prospective controlled cohort	None	<b>1.80 (1.56-2.08)</b>	Only fresh embryo transfer	(Katalinic et al., 2004)
	IVF FET vs NC	6647 vs 288542	Retrospective cohort	Age, parity, year of pregnancy, child gender, country	<b>1.49 (1.35-1.63) &lt;32WG 2.68 (2.24-3.22)</b>	cIVF and ICSI	(Wennerholm et al., 2013)
		2348 vs 571914	Retrospective case-control	Age, parity, BMI, smoking habits, year of birth, years of involuntary childlessness	1.05 (0.88-1.25)	SET and DET, excluding oocyte donation	(Sazonova et al., 2012)
		957 vs 4800	Retrospective cohort	Matched by date of delivery Adjustment on age, parity, child year of birth and gender	1.12 (0.96-1.32)	cIVF and ICSI	(Pinborg et al., 2010)



Preterm birth <37 WG	IVF FET vs IVF fresh	211 vs 915	Retrospective cohort	None	9 vs 12.1% (NS)	Mostly blastocyst-stage transfers	(Korosec et al., 2014)
		6647 vs 42242	Retrospective cohort	Age, parity, year of pregnancy, child gender, country	<b>0.84 (0.76-0.92)</b> <b>&lt;32WG 0.79 (0.66-0.95)</b>	cIVF and ICSI	(Wennerholm et al., 2013)
		2348 vs 8944	Retrospective case-control	Age, parity, BMI, smoking habits, year of birth, years of involuntary childlessness	0.93 (0.77-1.11)	SET and DET, excluding oocyte donation	(Sazonova et al., 2012)
		957 vs 10329	Retrospective cohort	Matched by date of delivery Adjustment on age, parity, child year of birth and gender	<b>0.70 (0.53-0.92)</b>	cIVF and ICSI	(Pinborg et al., 2010)
	IVF with EPE <sub>2</sub> vs IVF without EPE <sub>2</sub>	27 vs 265	Retrospective cohort	None	NS	Only fresh embryo transfer	(Imudia et al., 2012)
	IVF with 1 <sup>st</sup> trimester serum hCG<10 <sup>th</sup> percentile vs IVF with normal serum hCG	46 vs 161	Retrospective cohort	None	18.2 vs 4.2% (NS)		(Haddad et al., 1999)

ART: Assisted Reproductive Technologies, cIVF : conventional *In Vitro* Fertilization, DET: Dual Embryo Transfer, EPE<sub>2</sub>: Elevated Peak serum Estradiol level (>90<sup>th</sup> percentile, 3450-4500 pg/mL), GH: Gestational Hypertension, GIFT: Gamete Intra-Fallopian Transfer, ICSI: Intra-Cytoplasmic Sperm Injection, IVF: *In Vitro* Fertilization (cIVF + ICSI), LGA: Large for Gestational Age, NC: Naturally Conceived, NS: Not Significant, OI: Ovulation Induction, PCOS: Polycystic Ovary Syndrome, PE: PreEclampsia, perc: percentile, SD: Standard Deviation, SET: Single Embryo Transfer, SGA : Small for Gestational Age, WG: Weeks of Gestation.

Overall, most of the studies with good statistical power demonstrate increased rates of placenta-related diseases after any technique of ART. Most of these pathologies have been associated with placental dysfunction and notably abnormal trophoblastic invasion, regardless of potential interference from co-existing maternal risk factors such as BMI, maternal age and fertility status (Thomopoulos *et al.*, 2013). The artificial manipulation of gametes and/or embryos may also trigger anomalies in placental function leading to suboptimal placental performance.

In mammals, the placenta is a pregnancy-specific temporary organ that creates intimate contact between mother and foetus to maintain gestation and foetal well-being thanks to a myriad of functions. The placenta ensures the exchange of gases, nutrients and waste products (Zhang *et al.*, 2008) and, in parallel, produces various hormones and acts as a barrier against various stresses (Huang *et al.*, 2013).

Its development involves a finely-tuned temporal and spatial regulation of trophoblastic invasion, essential for proper future functions of the placenta and foetal growth. Proper trophoblastic invasion is achieved thanks to a molecular crosstalk between the maternal endometrium and the peripheral multipotent cells of the blastocyst, known as trophoblasts (Chelbi *et al.*, 2008). As ART involves hormone-driven changes in the endometrium and embryo manipulations including exposure to artificial culture media, it might logically jeopardize the delicate step of implantation (Chelbi *et al.*, 2008; Denomme *et al.*, 2012; Choux *et al.*, 2015).

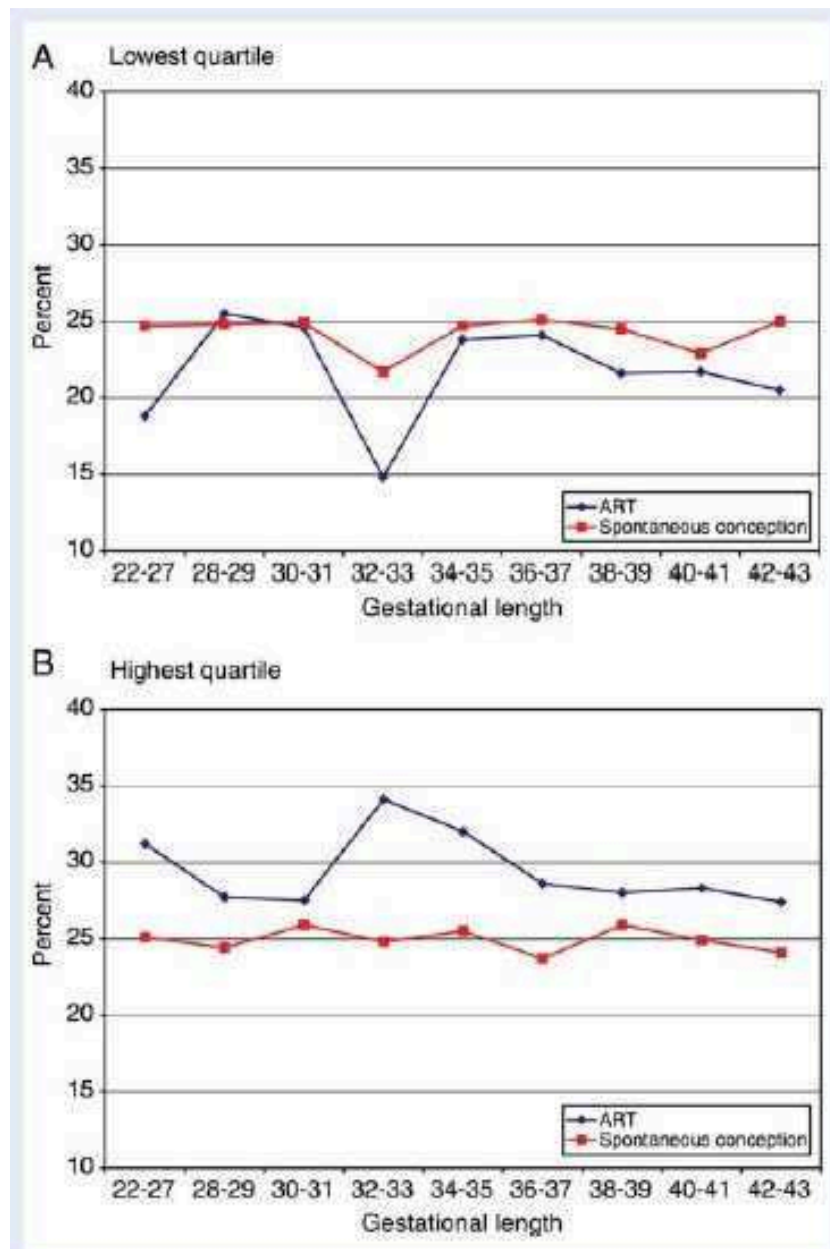
## II. Changes in placenta after ART at various levels: morphologic, microscopic and molecular. Could these modifications be written through epigenetic changes?

### 1) Article 1: The placenta: phenotypic and epigenetic modifications induced by Assisted Reproductive Technologies throughout pregnancy

In the first article of this thesis, after highlighting the major role of the placenta for the well-being of the pregnancy and foetal growth, we reviewed the potential effects of ART reported on animal and human placenta at the macroscopic, ultrastructural and molecular levels and hypothesized that these modifications could be written through epigenetic changes. Here is a summary of the main data of this article with up-to-date information.

### 2) Increased placental weight

Along the same line as the description of the “large baby syndrome” in cattle and sheep by Young *et al.*, in 1998 (Young *et al.*, 1998), enlarged placenta has also been described at birth after ART. Indeed, in humans, enlarged placentas have been observed in complicated pregnancies associated with low birth weight, such as cases of late-onset preeclampsia, foetal death or advanced maternal age (Eskild *et al.*, 2009; Haavaldsen *et al.*, 2011). The same phenomenon is seen in singletons pregnancies resulting from ART. ART pregnancies were overrepresented in the highest placental weight quartile (**Figure 3**) and the placental weight/birthweight ratio was commonly higher while mean birthweight was lower, even after adjusting for potential confounding factors (Haavaldsen *et al.*, 2012).



**Figure 1** The proportion of pregnancies in the lowest (**A**) and highest (**B**) quartile of placental weight in pregnancies with ART or spontaneous conception by length of gestation at delivery. All singleton pregnancies in Norway during 1999–2008 ( $n = 536\,567$ ).

**Figure 3: The placenta is enlarged after ART**  
 From (Haavaldsen *et al.*, 2012)

In animal studies, fewer trophoblastic cells were observed on blastocysts after IVF (Giritharan *et al.*, 2007). The placenta was smaller in early pregnancy, but its weight gradually increased throughout pregnancy to finish bigger than controls at birth (Bloise *et al.*, 2012). In the meantime, foetuses that were smaller at the beginning of gestation reached the same weight as controls by birth (Bloise *et al.*, 2012), meaning that dynamic processes may develop during pregnancy to compensate for primary placental insufficiency. In the mouse model, placental weight near term was found to be higher not only after IVF but also in conceptuses exposed to superovulation, *in vivo* fertilization and embryo transfer and even if they were exposed to embryo transfer alone (de Waal *et al.*, 2015).

### 3) Changes in ultrastructure

At a microscopic level, changes in ultrastructural features have also been reported after ART, including degenerative alterations of the terminal villi with a thicker placental blood barrier (Zhang *et al.*, 2011).

### 4) Blood supply

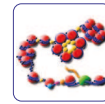
These dynamic processes are also well-illustrated in terms of blood supply. For example, in a bovine model, the density of placenta blood vessels in conceptuses after IVF was lower than controls at the beginning of pregnancy and greater by the end of pregnancy (Miles *et al.*, 2004; Miles *et al.*, 2005). In the mouse model, superovulation of the recipient led to lower birth weight, increased vascular resistance in the placenta and lower microvascular density at E18.5 than in pseudo pregnant dams without superovulation (Weinerman *et al.*, 2017).

### 5) Changes in molecular mechanisms

Other mechanisms evidenced in mouse models, such as increased cell fusion and cell proliferation, revealing enhanced capture of nutrients after ART (Eckert *et al.*, 2012; Sun *et al.*, 2014), illustrate the placenta's ability to adapt to its environment.

Metabolic changes in the placenta after ART are associated with hyper expression of corresponding proteins and genes: upregulation of fusion proteins (Zhang *et al.*, 2008), of an angiogenic transcription factor (Miles *et al.*, 2004), of genes involved in metabolism, immune response, transmembrane signalling and cell cycle control (Zhang *et al.*, 2010; Nelissen *et al.*, 2014). Another study in mice showed that IVF could induce actin cytoskeleton disorganization, disturbed hematopoiesis and vasculogenesis, dysregulated energy and amino acid metabolism, disrupted genetic information processing in extraembryonic tissue, and was altogether responsible for impaired placental formation and function leading to delayed embryonic development and even death (Tan *et al.*, 2016).

These various changes suggest that placenta is affected by ART. In this first article, we hypothesized that ART could affect the delicate step of trophoblastic implantation and that the subsequent suboptimal placenta could adapt throughout pregnancy to compensate for primary defects by various changes such as weight, ultrastructure, metabolic or nutrients exchange. The way to link environmental changes to dynamic phenotypic changes is epigenetics.



REVIEW

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# The placenta: phenotypic and epigenetic modifications induced by Assisted Reproductive Technologies throughout pregnancy

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## Abstract

Today, there is growing interest in the potential epigenetic risk related to assisted reproductive technologies (ART). Much evidence in the literature supports the hypothesis that adverse pregnancy outcomes linked to ART are associated with abnormal trophoblastic invasion. The aim of this review is to investigate the relationship between epigenetic dysregulation caused by ART and subsequent placental response. The dialogue between the endometrium and the embryo is a crucial step to achieve successful trophoblastic invasion, thus ensuring a non-complicated pregnancy and healthy offspring. However, as described in this review, ART could impair both actors involved in this dialogue. First, ART may induce epigenetic defects in the conceptus by modifying the embryo environment. Second, as a result of hormone treatments, ART may impair endometrial receptivity. In some cases, it results in embryonic growth arrest but, when the development of the embryo continues, the placenta could bring adaptive responses throughout pregnancy. Amongst the different mechanisms, epigenetics, especially thanks to a finely tuned network of imprinted genes stimulated by foetal signals, may modify nutrient transfer, placental growth and vascularization. If these coping mechanisms are overwhelmed, improper maternal-foetal exchanges occur, potentially leading to adverse pregnancy outcomes such as abortion, preeclampsia or intra-uterine growth restriction. But in most cases, successful placental adaptation enables normal progress of the pregnancy. Nevertheless, the risks induced by these modifications during pregnancy are not fully understood. Metabolic diseases later in life could be exacerbated through the memory of epigenetic adaptation mechanisms established during pregnancy. Thus, more research is still needed to better understand abnormal interactions between the embryo and the milieu in artificial conditions. As trophoblast cells are in direct contact with the environment, they deserve to be studied in more detail. The ultimate goal of these studies will be to render ART protocols safer. Optimization of the environment will be the key to improving the dialogue between the endometrium and embryo, so as to ensure that placentation after ART is similar to that following natural conception.

**Keywords:** Assisted Reproductive Technologies, Epigenetic, Imprinted gene, Placenta, Pregnancy

## Review

### Introduction

Much evidence in the literature supports the hypothesis that some adverse pregnancy outcomes observed after ART originate from suboptimal placental function caused by abnormal trophoblastic invasion. Indeed in humans, after adjusting for several confounding factors, the risk of

spontaneous abortion is higher in ART cohorts than in spontaneous pregnancies [1–3]. Similarly, in several animal models, more abortions are reported after IVE, culture or superovulation than with natural conception [4–6]. Then, throughout a pregnancy following ART, placental-related defects can also occur [7]. Notably, human studies found an increased risk of gestational hypertension, pre-eclampsia, placenta praevia and placental abruption [7]. In addition, the risks of low birth weight [8] and prematurity [9, 8] were increased after ART. In the same way, intra-uterine growth retardation (IUGR) as well as overgrowth has been described in animals following ART

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procedures [10, 11, 4, 12–18]. Even if co-existing maternal risk factors (such as BMI, maternal age and infertility status) may affect placental development, the artificial manipulation of gametes and/or embryos could also play a role.

The aim of this review was to investigate the phenotypic and epigenetic mechanisms by which ART could interfere with placental formation and function, resulting in placenta-related adverse pregnancy outcomes. The first paragraph will insist on the key role of epigenetics in placental function. Then, the ART-induced placental variations occurring throughout pregnancy will be reported. To finish, the potential long-term effects of these placental modifications and the future research perspectives will be addressed.

### ***Proper epigenetic regulation is essential for a functional placenta***

#### **1. Epigenetics in placental function**

In mammals, the placenta is a pregnancy-specific temporary organ that creates intimate contact between mother and foetus ensuring the maintenance of gestation and foetal well-being by the exchange of gases, nutrients and waste products [19]. It originates from the peripheral multipotent cells of the blastocyst (trophoblast). In humans, placental syncytiotrophoblasts formed by the fusion of cytotrophoblasts constitute the site of exchange between the maternal and foetal circulation. It has specific endocrine functions, such as the production of placental hormones, but it also functions as a barrier, ensuring a stable environment to a foetus deprived of efficient defence mechanisms against various stresses (oxidative, xenobiotic, chemical) [20]. A finely tuned temporal and spatial regulation of trophoblastic invasion is essential for proper future function of the placenta and foetal development [21]. This involves molecular crosstalk between the endometrium and trophoblast [21].

Notably, epigenetic regulation is a significant factor in placental development and adaptive function to environmental stress [22].

Epigenetics may be defined as a set of cell-based molecular mechanisms able to modify gene expression. These mechanisms are heritable through mitosis or even sometimes meiosis and not sustained by DNA sequence variation [23]. Epigenetic regulation controls transcription at two levels: directly on the DNA (through DNA methylation/hydroxymethylation mechanisms) and on the proteins around which the DNA is wrapped to constitute the nucleosomes (histone modifications). Epigenetic regulation also controls translation or mRNA stability by the

expression of non-coding RNAs (such as microRNA, Piwi, and Miwi).

For instance, imprinted genes, which are epigenetically regulated, are abundantly expressed in foetal and placental tissues and are apparently absent in non-placental organisms [24, 25]. It is postulated that genomic imprinting coevolved with placentation or drove the evolution of the placenta [26], sometimes through modifications of retrotransposons [27]. Imprinted genes are expressed in a parent-of-origin manner thanks to epigenetic modifications silencing either the paternal or the maternal allele. These epigenetic modifications (DNA methylation being the most described) are established in a sex-specific manner during gametogenesis on regulatory sequences referred to as imprinting control regions (ICRs). After fertilization, these ICRs act *in cis* to achieve monoallelic expression of most imprinted genes. Up to now, approximately 150 imprinted genes have been identified in mice and humans. In mice, these are under the control of 23 identified ICRs [28–30] (<http://www.geneimprint.com/site/genes-by-species>). Interestingly, they are generally not imprinted in all tissues, and the imprinted pattern can be limited to a precise developmental stage. In addition, the conservation of imprinted status or even the sense of the imprinting (maternal or paternal allele expressed) may vary between mammalian species [28]. Imprinted genes, which represent a very small percentage of genes, appear to play essential roles in embryonic growth and placental development by regulating the transport capacity of the placenta thereby controlling the supply of nutrients [31, 32]. During preimplantation development, genomic imprinting is jeopardized by global DNA demethylation, and some actors such as the complex Zfp57/TRIM28/KAP1 are required to protect epigenetic imprinting marks [33]. Moreover, imprinted genes are functionally haploid by definition and thus potentially more susceptible to mutations and epimutations [34]. Their dysregulation may therefore have major consequences on the placental phenotype with long-term consequences for the developmental programming of adult health and disease [35].

#### **2. Epigenetic modifications in the placenta and adverse pregnancy outcomes**

To function adequately, the developing placenta needs the proper epigenetic regulation of imprinted and non-imprinted genes. Indeed, experimental studies conducted in both humans and animals have clearly shown the importance of epigenetics in the regulation of placental development. For example, drug-induced disruption of DNA methylation was able to inhibit human trophoblastic invasion *in vitro*



by disturbing the expression of epigenetically regulated genes such as E-cadherin [36] as well as the proliferation of trophoblast cells in rat placenta [37]. The deletion of placental-specific *Igf2* in mice consistently led to reduced placental growth and subsequent foetal growth restriction [38]. In addition, numerous findings proved that disturbed placental epigenetic regulation may cause abnormal trophoblastic invasion, which may contribute to the pathophysiology of some spontaneous miscarriages, IUGR and preeclampsia. Indeed, in humans, *DNMT1* expression (DNA methyltransferase 1 involved in DNA methylation maintenance) and global DNA methylation were significantly lower in chorionic villi from early foetus losses than in those harvested following selective pregnancy termination [39].

Moreover, in humans and animals, a great number of associations have been found between IUGR and epigenetic variations of imprinted or non-imprinted genes in placentas. Notably, by analyzing more than 200 human term placentas, Banister and colleagues found that the DNA methylation pattern of 22 loci was highly predictive of IUGR [40]. In mice, induced loss of imprinting and the subsequent overexpression of the imprinted *Phlda2* gene were able to trigger placental and foetal growth retardation in the offspring whereas its deletion caused overgrowth [41]. Similarly, in humans, some authors demonstrated that *PHLDA2* was up-regulated in the placenta in cases of IUGR [42–44] and that its expression level correlated negatively with birth weight [45]. As it is considered a negative growth regulator, the authors suggested that this imprinted gene potentially plays a direct role in the pathophysiology of IUGR.

Other imprinted genes were also up-regulated (*CDKN1C*) or down-regulated (*MEG3*, *GATM*, *ZAC1*, *GNAS*, *MEST*, *IGF2*) in IUGR placentas [42, 46, 47, 44]. Some of these differential expressions were associated with decreased placental methylation, as was the case for *H19/IGF2* ICR1 [48], or loss of imprinting, as was the case for *ZAC1* (= *PLAGL1*) and *H19* differentially methylated regions (DMRs) [42].

In addition, other examples of non-imprinted genes highlight the possibility that foetal growth potential could be negatively impacted by epigenetic dysregulation in the placenta. Ruebner and colleagues pointed out that expression of Syncytin-1, a protein that promotes cellular fusion in the syncytiotrophoblast, was lower in human IUGR placentas than in controls [49]. The same team recently linked decreased expression of this protein to epigenetic hypermethylation of its promoter [50].

In an induced IUGR rat model, Reamon-Buettner and colleagues reported decreased expression and aberrant DNA methylation patterns of the promoter region of the *Wnt2* gene, which is known to be implicated in placental vascularization [51]. In humans, the same pattern was found with lower *WNT2* expression and higher DNA methylation in growth-restricted neonates than in controls [52].

Interestingly, epigenetic changes were also found on repeated sequences. For example, Michels and colleagues found an increased LINE-1 methylation level in placental tissues from low birth weight infants [53]. Other evidences about preeclampsia reinforce the idea that epigenetic disorders may be involved in abnormal trophoblastic invasion. Actually, mice with induced loss of expression of the imprinted *Cdkn1c* gene developed a preeclampsia-like syndrome, with hypertension and proteinuria [54]. Besides, widespread DNA methylation changes were found in placentas of a cohort of patients suffering from early onset preeclampsia but not in gestational age-matched controls [55]. Some of these methylation modifications correlated negatively with expressional changes, especially for genes implicated in angiogenesis (*such as EPAS 1* and *FLT 1*). Moreover, *BHLHE40*, a gene coding for a protein that can prevent trophoblast differentiation exhibited significantly decreased DNA methylation and increased expression in preeclampsia placentas [55]. In addition, the expression of maspin (*SERPINB5*), a serine protease inhibitor and an inhibitor of cell migration [56], which may modify trophoblast cell invasion in the first trimester [57], could also be modified in preeclampsia. In the same family of genes, SERPIN A3 is a specific inhibitor of elastase, which plays a crucial role during the implantation process. SERPIN A3 displayed decreased methylation and increased gene expression in placentas from pregnancies complicated by preeclampsia compared with controls [58], through a complex epigenetic regulation [59]. As for IUGR, several studies highlighted the increased methylation [50] and reduced expression of *syncytin-1*, as well as the down-regulation of *WNT2* in preeclamptic placentas. These modifications were possibly responsible for impaired placental function [60]. Interestingly, epigenetic modifications could also correlate with the severity of the disease. For instance, hypertension tended to be more severe in preeclamptic women with biallelic expression of *H19*, than in women with normally imprinted expression of this gene [61]. Recently, Anton and colleagues demonstrated a correlation between disease severity and alterations in DNA methylation (hypermethylation of *CDH11*, *COL5A1*, *TNF*, hypomethylation of *NCAM1*) in preeclamptic placentas [62].

In summary, there is a wealth of data highlighting the particular role of epigenetics in placental regulation and the potential link between epigenetic dysregulation and adverse pregnancy outcomes. The notion of epigenetic risk emerged in recent decades and a recent meta-analysis confirmed the increased risk of imprinting disorders (such as Beckwith-Wiedemann and Silver-Russel syndromes) after ART [63]. This raised the issue of potential methylation defects associated with ART [64]. Most studies that have examined the methylation status of imprinting genes in foetuses or placentas in animal models or in humans have associated epigenetic anomalies with adverse effects on embryonic development [65].

What follows aims to investigate the placental modifications induced by ART and to understand their link with adverse pregnancy outcomes. The hypothesis is that epigenetic dysregulation could constitute the logical link between environmental changes due to ART, abnormal trophoblastic invasion and subsequent adverse pregnancy outcomes. Indeed, ART, via epigenetic dysregulation, could disturb the dialogue between the embryo and endometrium and cause abnormal trophoblastic invasion, which triggers placental adaptive responses (Fig. 1).

### **ART and trophoblastic invasion disturbances**

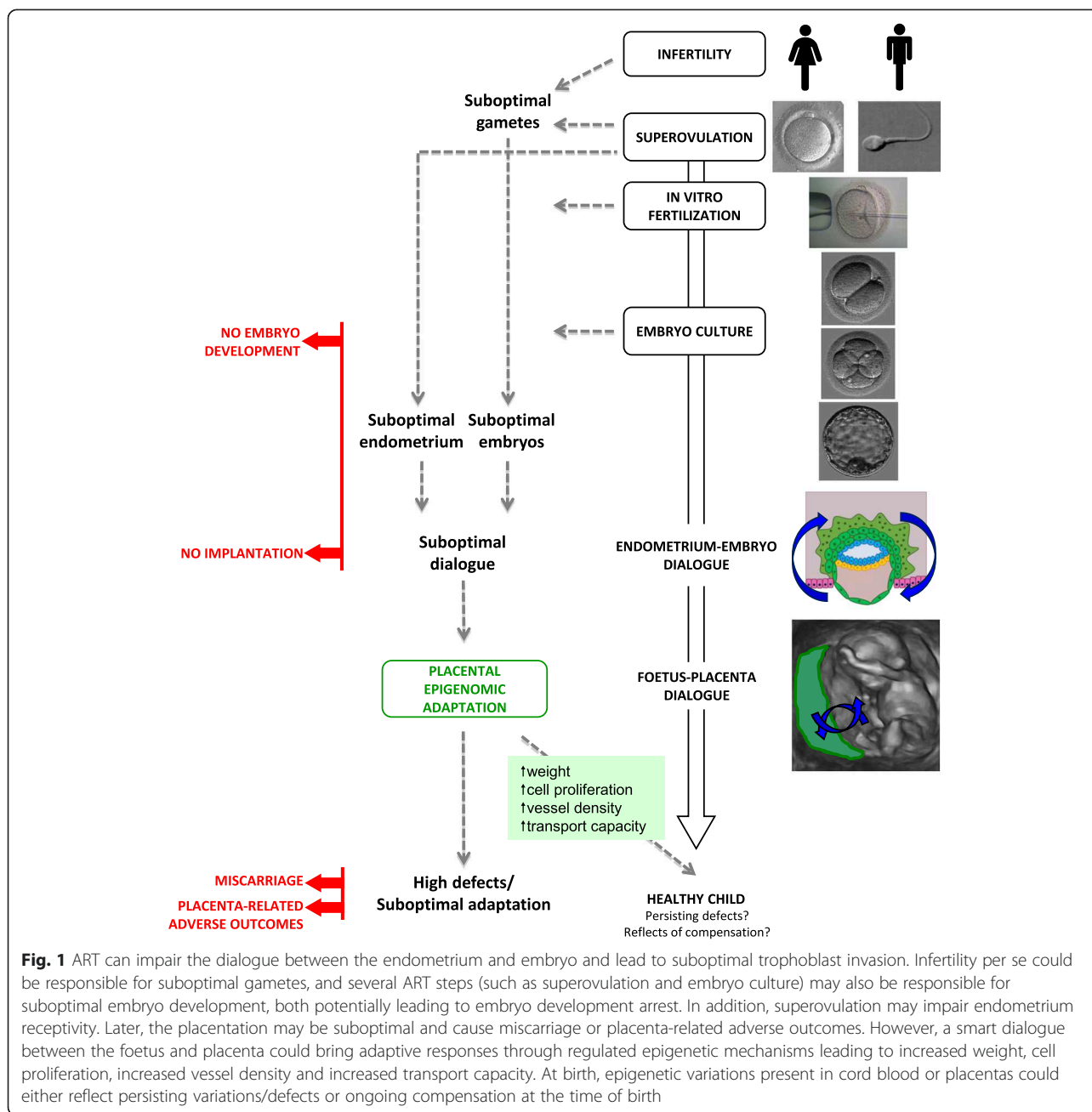
#### **1. ART and the epigenetic status of the conceptus**

In animal models (especially in mice), most studies have shown that ART procedures (such as superovulation and embryo culture), whether isolated or in association, could lead to blastocyst epigenetic defects in several loci (such as *H19*, *Snrpn*, *Peg3*, *Kcnq1ot1* genes as well as repetitive sequences) [66–71].

Moreover, these epigenetic abnormalities were not restricted to the early stages. In mice, several studies reported placenta-specific imprinting defects after implantation, appearing in suboptimal culture conditions, such as in vitro culture associated with in vitro fertilization [5], embryo transfer [72], poorer media [73, 69] or increased oxygen concentration [74] (Table 1). When assessed by transcriptomics, it was clear that the modifications of placental gene expression in mice placenta at mid-gestation were very different depending on the richness of the culture milieu. They were much stronger when simple M16 culture medium was used than when the more complex G1/G2 medium was used [75]. Interestingly, amongst the modified genes, imprinted genes were overrepresented. Recently, Hossain and colleagues found that other aspects of epigenetics could be affected by in vitro

manipulations by observing the down-regulation of miRNAs in bovine placentas from in vitro production (IVF and in vitro culture) compared with those from artificial insemination [76]. Even in human placentas, epigenetic modifications were observed. Indeed, ART was associated with lower DNA methylation levels and higher expression levels of SERPINF1 [77]. This protein is ubiquitously expressed and presents a potent anti-angiogenic activity [78]. Thus, its deregulation may detrimentally affect placentation and foetal development.

Surprisingly, placenta appears to be more susceptible to modifications in DNA methylation and/or expression of imprinted genes at mid-gestation [74, 79, 69, 72] (Table 1). Discussing this observation, Mann and colleagues proposed two scenarios to explain why the defects were apparently restricted to the trophoblast lineage [69]. In the first hypothesis, extra-embryonic cells, in contact with the culture medium, are more severely affected by in vitro culture, which is responsible for a loss of imprinting in mid-gestation placentas. Indeed, trophoblast (TE) cells are directly exposed to the environment. Besides, they are also the first lineage to differentiate in the embryo as trophoblast stem cells, from which the different cell lines of the future placenta will originate [80]. Other studies are in accordance with this hypothesis. Notably, TE cells from blastocysts cultured in vitro showed strong expressional modifications with the activation of stress-related pathways and the down-regulation of genes involved in placentation [81, 82]. Specifically, *Igf2* expression in TE cells was lower after IVF than in controls [81]. In the second hypothesis developed by Mann and colleagues, the embryo could be able to restore a correct imprint thanks to lineage-restricted de novo methylation occurring in inner cell mass (ICM) but not in TE cells. A third hypothesis involves the selection of viable embryos through active selective elimination mechanisms that act to discard embryos with abnormal imprinting before mid-gestation. Indeed, the studied embryos were those that reached this developmental stage. In mice, following ART, an increased number of resorption sites was observed. This number was even higher when the embryos were fertilized and cultured in vitro than when only cultured in vitro. This could indicate that embryos with defective imprinting do not survive and that the effect is cumulative [5, 4]. Reinforcing this idea, Yin et al. showed that mice injected with an inhibitor of DNA methyltransferase 1 (Dnmt1), an enzyme responsible for methylation maintenance, had a smaller number of implanted embryos [39]. Furthermore, at mid-gestation, these embryos had



**Fig. 1** ART can impair the dialogue between the endometrium and embryo and lead to suboptimal trophoblast invasion. Infertility per se could be responsible for suboptimal gametes, and several ART steps (such as superovulation and embryo culture) may also be responsible for suboptimal embryo development, both potentially leading to embryo development arrest. In addition, superovulation may impair endometrium receptivity. Later, the placentation may be suboptimal and cause miscarriage or placenta-related adverse outcomes. However, a smart dialogue between the foetus and placenta could bring adaptive responses through regulated epigenetic mechanisms leading to increased weight, cell proliferation, increased vessel density and increased transport capacity. At birth, epigenetic variations present in cord blood or placentas could either reflect persisting variations/defects or ongoing compensation at the time of birth

a lower global DNA methylation level, which was associated with growth retardation [39]. These results strengthen earlier experimental studies in mice that highlighted the fundamental contribution of DNA methylation enzymes to embryonic development [83].

In summary, these data support the hypothesis that a suboptimal embryo environment induced by ART greatly disturbs the epigenetic status of not only the embryo (eventually causing development arrest) but also the extra-embryonic tissues.

## 2. ART and endometrial receptivity

Apart from modifying the epigenetic status of the conceptus, another way in which ART could alter trophoblastic invasion could be its effect on the endometrium.

Much evidence has linked poor endometrium quality to abnormal early placentation. Even though some genetic causes of endometrial defects leading to recurrent miscarriages have been described [84–86], ovarian stimulation, which is required in most ART procedures, may also be responsible for poorer

**Table 1** Conceptuses and/or placentas in mice: resorption rate, weight, gene expression and/or DNA methylation of imprinted genes

Species	GA study	Control group	Manipulation group	RR	Weight		Gene expression		Methylation		References
					F	P	F	P	F	P	
Mouse	E14	Blastocyst transfer	SO, IVC M16 (1-cell=>blastocyst)	=	=	NA	= <i>Igf2, Grb10, Grb7, H19</i>	NA	= <i>H19</i>	NA	[73]
			SO, IVC M16+FCS (1-cell=>blastocyst)	↑	↓	NA	↓ <i>H19, Igf2, Grb7</i> ↑ <i>Grb10</i>	NA	↑ <i>H19</i>	NA	
Mouse	E18	SO, blastocyst transfer	SO, IVC (1-cell=>morula) 7 % O2, (morula=>blastocyst) 2 % O2, transfer	↑	↓	=	NA	= <i>Slc2a1, Slc2a3, Igf2, Igf2r, H19</i>	NA	NA	[186]
			SO, IVC (1-cell=>blastocyst) 7 % O2, transfer	=	=	=	NA	= <i>Slc2a1, Slc2a3, Igf2, Igf2r, H19</i>	NA	NA	
			SO, IVC (1-cell=>morula) 7 % O2, (morula=>blastocyst) 20 % O2, transfer	=	=	=	NA	= <i>Slc2a1, Slc2a3, Igf2, Igf2r, H19</i>	NA	NA	
Mouse	E12.5	SO, blastocyst transfer	SO, IVF, IVC KSOM/AA, blastocyst transfer	=	↓	=	NA	NA	NA	NA	[4]
			SO, IVF, IVC WM, blastocyst transfer	↑	↓	↓	NA	NA	NA	NA	
			SO, IVF, IVC KSOM/AA, blastocyst transfer	=	↓	↓	NA	NA	NA	NA	
Mouse	E15.5	SO, blastocyst transfer	SO, IVF, IVC KSOM/AA, blastocyst transfer	NA	↓	=	NA	↑ <i>Slc7a3</i> = <i>Igf2, H19, Glut1, Snat1, Snat2, Snat4</i> ↓ <i>Glut3</i>	NA	NA	[10]
			SO, IVF, IVC KSOM/AA, blastocyst transfer	NA	↓	↑	NA	= <i>Snat1, Slc7a3</i> ↓ <i>Igf2, H19, Glut1, Glut3, Snat2, Snat4</i>	NA	NA	
Mouse	E9.5	In vivo fertilization	IVC KSOM/AA (2-cells=>blastocyst)	NA	NA	NA	Monoallelic: <i>H19, Snrpn</i>	Monoallelic: <i>H19, Snrpn, Ascl2, Peg3</i>	= <i>H19, Snrpn</i>	= <i>H19, Snrpn</i>	[69]
			IVC WM (2-cells=>blastocyst)	NA	NA	NA	Monoallelic: <i>H19, Snrpn</i>	Biallelic: <i>H19, Snrpn, Ascl2, Peg3</i>	= <i>H19, Snrpn</i>	Partial LOM: <i>H19, Snrpn</i>	
Mouse	E9.5	In vivo fertilization	SO, blastocyst transfer	NA	NA	NA	Monoallelic: <i>H19, Cdkn1c, Kcnq1, Ascl2, Zim1, Snrpn, Kcnq1ot1, Peg3, Igf2, Mkrn3</i>	Biallelic: <i>H19</i> High levels of misexpression: at least 1/8 IG ↑ <i>Ascl2, = H19</i> ↓ <i>Igf2</i>	NA	NA	[72]
			SO, IVC KSOM/AA (2-cells=>blastocyst), blastocyst transfer	NA	NA	NA	Monoallelic: <i>H19, Cdkn1c, Kcnq1, Ascl2, Zim1, Kcnq1ot1, Peg3, Igf2</i> Low levels of misexpression: <i>Snrpn, Mkrn3</i>	Biallelic: <i>H19</i> ↑ <i>Ascl2, = H19</i> ↓ <i>Igf2</i>	NA	NA	

**Table 1** Conceptuses and/or placentas in mice: resorption rate, weight, gene expression and/or DNA methylation of imprinted genes (Continued)

Mouse	E9.5	In vivo fertilization	SO	NA	NA	NA	Monoallelic: <i>H19, Snrpn, Igf2, Kcnq1ot1</i> = <i>Igf2</i>	Biallelic: <i>H19, Snrpn</i> Monoallelic: <i>Igf2, Kcnq1ot1</i> ↑ <i>Igf2</i>	NA	= <i>H19, Snrpn</i>	[79]
		Blastocyst transfer	SO, blastocyst transfer	NA	NA	NA	Monoallelic: <i>H19, Snrpn, Igf2</i> = <i>Igf2</i>	Biallelic: <i>H19</i> Monoallelic: <i>Snrpn, Igf2</i> ↑ <i>Igf2</i>	NA	= <i>H19, Snrpn</i>	
Mouse	E10.5	SO, blastocyst transfer	SO, IVC (1-cell=>blastocyst) (M16 or sequential G1/G2), blastocyst transfer	↑	NA	NA	NA	↑ <i>H19, Igf2, Zac1, Slc38a4, Cdkn1c, Gtl2, Rian, Dlk1, Nnat, Peg3</i> = <i>Igf2r, Grb10</i> ↓ <i>Dnc, Gatm, Mest</i>	= <i>Igf2, H19</i>	= <i>H19, Igf2, Igf2r, Dlk1-Dio3</i>	[5]
			SO, IVF, IVC (M16 or sequential G1/G2), blastocyst transfer	↑	NA	NA	NA	↑ <i>H19, Igf2, Igf2r, Zac1, Slc38a4, Cdkn1c, Gtl2, Rian, Dlk1, Nnat, Peg3</i> = <i>Grb10, Mest</i> ↓ <i>Dnc, Gatm</i>	= <i>Igf2</i>	= <i>H19, Igf2, Igf2r, Dlk1-Dio3</i>	
Mouse	E14	In vivo fertilization	SO, IVF, IVC, blastocyst transfer	NA	NA	NA	↓ <i>Igf2</i> ↑ <i>H19</i>	↑ <i>Igf2</i> ↓ <i>H19</i>	LOM: <i>H19</i>	LOM: <i>H19</i>	[187]
			SO, IVF, IVC, vitrifying/warming morula, blastocyst transfer	NA	NA	NA	↓ <i>Igf2</i> ↑ <i>H19</i>	↑ <i>Igf2</i>	LOM: <i>H19</i>	LOM: <i>H19</i>	
			SO, IVF, IVC, blastocyst transfer	NA	NA	NA	↑ <i>Igf2</i> ↓ <i>H19</i>	↑ <i>Igf2</i> ↓ <i>H19</i>	LOM: <i>H19</i>	= <i>H19</i>	
Mouse	E10.5	In vivo fertilization	SO, IVF, IVC KSOM/AA 5 % O2, morula/blastocyst transfer	NA	NA	NA	Monoallelic: <i>Igf2, Cdkn1c, Snrpn, Kcnq1ot1</i> Biallelic: <i>H19, Peg3</i>	Biallelic: <i>H19, Snrpn, Peg3, Cdkn1c</i> Monoallelic: <i>Kcnq1ot1</i>	= <i>H19, Snrpn, Peg1, Kcnq1ot1, Dlk1/Gtl2, Peg3</i>	= <i>Snrpn, Kcnq1ot1, Peg1, Dlk1/Gtl2, Peg3</i> ↓ <i>H19</i>	[74]
			SO, IVF, IVC KSOM/AA 20 % O2, morula/blastocyst transfer	NA	NA	NA	Monoallelic: <i>Igf2, Snrpn, Kcnq1ot1, Cdkn1c</i> Biallelic: <i>H19, Peg3</i>	Biallelic: <i>H19, Snrpn, Peg3, Cdkn1c, Kcnq1ot1</i>	= <i>H19, Snrpn, Peg1, Kcnq1ot1, Dlk1/Gtl2, Peg3</i>	= <i>Snrpn, Kcnq1ot1, Peg1, Dlk1/Gtl2</i> ↓ <i>Peg3, H19</i>	

*E* embryonic day, *F* foetus, *FCS* foetal calf serum, *GA* gestational age, *ICSI* intra-cytoplasmic sperm injection, *IVC* in vitro culture, *IVF* in vitro fertilization, *IVPS* in vitro produced with serum, *KSOM/AA* optimal potassium-modified, simplex optimized medium with amino acids, *LOM* loss of methylation, *NA* not analyzed, *OVM* oocyte in vitro maturation, *RR* resorption rate, *P* placenta, *SO* superovulation, *SOF* synthetic oviductal fluid, *mSOF* modified synthetic oviductal serum fluid medium without serum or coculture, *WM* Whitten's medium, ↑: increased, ↓: decreased, =: no significant difference compared with control

endometrium quality. Since the ovary and the uterus share several signalling pathways, and since hormones secreted by the ovary have a direct effect on uterus function, ovarian stimulation probably modifies the uterine environment. This is assessed by studies that demonstrated differential expression of genes in the endometrium between stimulated and natural cycles, with a dose-response effect [87, 88].

In mice, the implantation rate was lower and post-implantation foetal mortality was higher in superovulated recipients than in non-stimulated controls [89]. Similar observations were also reported in humans, with a dose-dependent effect: the risk of spontaneous abortion was significantly higher in women stimulated with high levels of hormones than in those stimulated with lower levels [3]. Besides, high serum estradiol levels at ovulation triggering after controlled ovarian stimulation are associated with placenta-related adverse pregnancy outcomes such as growth restriction or preeclampsia [90, 91].

Other evidences highlight the impact of a suboptimal endometrium induced by ovarian stimulation on placental and foetal growth. Notably, hormones are known to modify birth weight. Indeed, singletons born after IVF have on average a lower birth weight than singletons born after natural cycles with mild stimulation [92]. Moreover, an inverse correlation between birth weight and estradiol levels achieved in case of IVF [93] was found. In mice, the mean weight of foetuses was also lower in stimulated than in non-stimulated recipients [89, 94].

Surprisingly, birth weight was higher in ART-offspring after the transfer of cryopreserved/thawed embryos than with fresh embryos [95, 96]. While it could be hypothesized that this was caused by a direct effect on the embryo, differences in hormonal treatment between the two groups could have an important effect as well. In the first case (cryopreserved embryos), women are not treated with follicle-stimulating hormone (FSH) to induce multifollicular growth, while they are treated in the second case. In natural conception, when two children from the same mother are compared, the second one is usually heavier [97]. However, when the first is born following transfer of a frozen embryo and the second after IVF, the situation is reversed [95]. On average, birth weight following frozen embryo transfer is the same as that following natural conception [98]. The fact that frozen embryos are transferred without controlled ovarian hyperstimulation suggests that the endometrium-embryo dialogue is in this situation closer to the “natural” dialogue and enables normal placentation. It is also possible that freezing

selects embryos with normal epigenetic profiles, by unknown putative mechanisms. However, recently, two different teams highlighted that the risk of large for gestational age and preeclampsia could be increased in frozen embryo cycles compared with fresh cycles or natural conception [99, 100]. Therefore, further studies are needed to determine the impact of the different protocols used in frozen embryo transfer (hormonal treatments used, duration of culture, cryoprotectants, culture media, etc.).

Other data are in keeping with the hypothesis that superovulation and hormone treatment may impair placentation. For example, a recent study examining near-term placentas in superovulated mouse recipients found altered trophoblast differentiation causing a reduced maternal-foetal exchange area [94]. Besides, in humans, pregnancy-associated plasma protein A (PAPP-A) levels in maternal serum were decreased in first-trimester ART pregnancies [101–105]. PAPP-A is known to play a critical role in trophoblastic invasion [106] by contributing to maternal tolerance towards the foetus [107]. Giorgetti et al. confirmed these low levels after ART and further added that maternal serum PAPP-A levels correlated strongly and inversely with estradiol levels at ovulation triggering [108]. Accordingly, PAPP-A values were lower after the transfer of fresh embryos (when ovarian stimulation was used) than after the transfer of frozen embryos or after unstimulated cycles [101, 109].

All these findings highlight a tight relationship between high hormone levels and impaired trophoblastic invasion presumably through decreased endometrium receptivity. Exposing the endometrium to high levels of estradiol and progesterone produced by multiple corpora lutea could possibly render it less efficient for embryo implantation than it is during natural cycles [110]. Thus, ART processes, and especially hormone treatments, may increase the rate of adverse pregnancy outcomes by inducing more trophoblastic invasion defects.

In addition to hormone treatments, infertility per se could involve an altered uterine environment. For example, some authors recently suggested that endometriosis may be accompanied by epigenetic modifications implicated in diminished endometrial receptivity and altered gene expression. Epigenetic modifications on the promoter of a mediator of endometrial receptivity, HOXA10, may be one of the mechanisms involved, as reported in women [111–113] and in several animal models [114, 115].

To summarize, ART, through its negative effect on the endometrium-embryo dialogue, could participate in preventing successful trophoblastic invasion. This could potentially explain the occurrence of adverse pregnancy

outcomes after ART. Depending on the severity of the defects, ART could gradually lead to developmental arrest, miscarriages, preeclampsia or IUGR (Fig. 1). But in most cases, pregnancies obtained after ART are able to continue without obvious immediate adverse outcomes. This sustains the hypothesis that initial defective trophoblastic invasion could trigger placental adaptive responses during pregnancy.

#### **ART and the possible induction of placental adaptive responses**

Nuclear transplantation in animals is known to produce placental phenotypic modifications (such as placentomegaly), to modify placental metabolism and to disturb imprinted gene expression [116, 117]. Given these placental modifications after somatic cell nuclear transfer, we wondered whether ART could trigger placental responses.

##### 1. Phenotypic placental responses

In the literature, several studies in animals showed that a suboptimal placenta is created by in vitro conditions but that counterbalancing mechanisms also occurred. First, a smaller quantity of TE cells developed in mouse blastocysts from in vitro culture than in naturally conceived blastocysts [82]. At later stages (12.5 dpc), IVF embryos and placentas were smaller than those in the control group [4] (Table 1). However, the placenta was slightly larger (+9 %) at 15.5 dpc and to an even greater extent (+25 %) at 18.5 dpc, while foetus weight was 16 % lower at 15.5 dpc but only 9 % lower at 18.5 dpc in the IVF group than in controls [10] (Table 1). At this later stage, cell proliferation was greater in IVF placentas than in controls, in both the labyrinth and spongiotrophoblast layers. By birth, IVF fetuses had reached the same weight as the controls [10]. In the in vitro context, placentas were found to be lighter than control placentas at early gestation and heavier at late gestation. While a larger placenta is not necessarily synonymous of a higher efficiency in nutrient and oxygen transfer, it can in this case probably contribute to a compensatory growth of the foetus, despite initial functional limitations. Similar results were observed in the sheep model: fetuses from in vitro cultured embryos were 60 % smaller than naturally conceived fetuses at day 24 of gestation, whereas no difference was found at later stages [16].

Likewise, in humans, the enlargement of placentas has been observed in complicated pregnancies associated with low birth weight, such as pregnancies with late-onset preeclampsia, foetal death or advanced maternal age [118–120]. Interestingly, the same

phenomenon was seen in singletons from ART. Placentas from ART pregnancies were overrepresented in the highest quartile of weight, and the placental weight/birth weight ratio was commonly higher, while the mean birth weight was lower, even after adjusting for potential confounding factors [121]. This increased placental weight after IVF could be the result of compensatory responses.

##### 2. Mechanisms involved in placental responses

###### • Metabolic pathways

According to Coan and co-workers, the placental phenotype is responsive to nutritional conditions. When foetal nutrient availability is compromised, it adapts to maximize the nutrient transfer capacity [122]. These compensatory mechanisms may start from the blastocyst stage, within extra-embryonic lineages. Actually, using a mouse maternal protein restriction model, some authors demonstrated increased endocytosis, cell proliferation and invasiveness in the trophoblast, which may reveal enhanced nutrient capture [123, 124]. The up-regulated expression of nutrient supply genes such as glucose and system A amino acid transporters was shown in small murine placentas during late gestation, thus reflecting a response to foetal demand signals [122]. The foetus itself plays a role in its own development and growth by sending signals to the placenta, which will respond by regulating genes involved in growth control, specific transport systems and vascularization [125]. These metabolic responses are well-illustrated in IVF studies on animal species [126, 15, 127]. Indeed, at early gestation, bovine conceptuses after IVF and culture displayed placentas with decreased blood vessel density, while at late gestation, placentas had greater blood vessel density [15, 127]. This impaired placental vasculogenesis early in gestation was also reported for sheep embryos developed in vitro [128]. This compensatory process could implicate the angiogenic pathway and particularly an angiogenic transcription factor, peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) protein, which could modulate the density of maternal blood vessels throughout pregnancy [15]. In addition to the gain in vascularization, increasing cell fusion could improve foeto-maternal exchanges. Indeed, two proteins involved in membrane fusion, annexin A3 and  $\alpha$ -SNAP, were found to be up-regulated in human term placentas obtained after ART [19].

Besides, in human placentas after ART, genome-wide mRNA expression revealed the overexpression of genes involved in metabolism, immune response, transmembrane signalling

and cell cycle control [129, 130]. Similarly, transcriptomic data in mouse placental tissues show that IVF techniques trigger the induction of genes involved in cellular proliferation and cell cycle pathways [75].

In summary, the kinetics of placental and foetal growth altered by ART may be linked to modifications in various biological pathways, probably triggering the placental compensation phenomenon. While the complete picture of the systems that regulate this compensation is still blurred, epigenetic changes certainly play a part in the adaptive mechanisms.

- Imprinted gene network

Concerning the regulation of this placental response, one interesting hypothesis is that potential primary dysfunctions of the placenta could be corrected by the imprinted gene network of placental mammals (IGN). The modulation of this network of coordinated imprinted genes (and probably non-imprinted genes), which are involved in growth control and specific placental transport systems, could contribute to the tight regulation of foetal growth during post-implantation development. This was described in mice for *Igf2*, *Zac1* and *H19* [131, 132] and recently in the human placenta for *ZAC1* [133].

To support this hypothesis, in mouse placentas after ART, most genes of the IGN were up-regulated in a coordinated fashion, when compared with the control group [5]. The fact that these genes with placental reciprocal functions were up-regulated after ART despite phenotypically and morphologically normal embryos suggests that placental IGN may participate in the control of normal foetal growth in ART pregnancies. However, the methylation status of their DMRs after ART was either similar to that in controls or only slightly modified during gestation [5, 69]. In the same way, the methylation of repeated sequences (ALUYb8,  $\alpha$ -satellites and LINE-1) were reported to be unchanged after ART [134]. Other epigenetic mechanisms, such as histone modifications, could therefore be involved. In fact, according to Lewis et al., an ancestral imprinting mechanism, restricted to the placenta, is based on histone modifications [135], which may confer the short-term and flexible response implicated in development [136–138].

Regrettably, no evidence is available in animals at birth concerning the occurrence of epigenetic modifications in the placenta. In humans, nothing is certain (Table 2). Three studies that carried out large DNA methylation analyses using arrays

found conflicting data. Indeed, the first published study described quantitative differences in global DNA methylation (briefly with a higher and a lower degree of DNA methylation in post-IVF cord blood and placental samples, respectively) and for several imprinted genes [77] (Table 2). In contrast, two recent studies reported either opposite cord blood findings [139] or none variability in DNA methylation at 25 imprinted DMRs [134] (Table 2). However, the three studies are not comparable regarding the sample size (10 individuals versus 73), the mode of reproductive treatment (IVF versus unspecified ART) and the method used.

Moreover, other studies focusing on the DNA methylation of specific imprinted genes also generated contradictory results. Indeed, although some authors reported no epigenetic changes after ART [140, 141], several authors reported variations in methylation levels in both cord blood and/or placentas for a number of imprinted genes such as *MEST* [142, 143], *H19* [144, 142, 145], *KCNQ1OT1* [146] or *SNRPN* [142]. However, none of them agreed on the changes in DNA methylation and these variations were mild (from 0.6 to 4.5 % differential methylation levels) (Table 2). Once again, these studies are difficult to compare given the limitations similar to those mentioned above. However, most studies focused on normal pregnancy, thus excluding placenta-related adverse pregnancy outcomes (such as preeclampsia, hypertension, some IUGR) and therefore possibly ignoring major differences.

Concerning the expression analysis of imprinted genes, conflicting results were also reported. Dysregulation mainly took place in the placenta and only for three imprinted genes (*H19*, *IGF2*, *MEST*) [77, 144, 145] (Table 2).

Finally, these minimal expressional changes at term compared with more significant changes during pregnancy in animals could reflect the remains of defects that were partially compensated during prenatal life or even methylation allelic polymorphisms (placental epipolymorphism [147]).

Thus, epigenetic “defects” in animals’ placentas after in vitro manipulations are found in most studies. Most authors consider this variety of placental phenotypes triggered by ART to originate from epigenetic errors at imprinted genes [74], but should we really consider these epigenetic modifications as “errors” or should we regard them as smart adaptation mechanisms developed by the placenta? From the results



**Table 2** Effects of ART on imprinted genes and retrotransposable element expression and methylation in chorionic villous samples from abortion, peripheral blood, cord blood and placenta

Control group	Manipulation group	Gene	Sample	Technique for expression	Results of expression analysis		Technique for methylation	Results of methylation analysis		References
					Trends	Fold change		Trends	Differential methylation level	
30 NC	18 IVF or ICSI	<i>KCNQ1OT1</i>	CPB	NA			MS-PCR	MS-PCR: hypoM (3/12)		[188]
			CB				MSED-qPCR	MSED-qPCR: =		
			P					=		
13 NC	10 IVF	<i>MEST</i>	CB	RT-qPCR	=		Methylation array	?	21.8 %	[77]
		<i>SLC22A2</i>	CB		=			↓	3.0 %	
		<i>PEG10</i>	CB		=			↓	4.2 %	
		<i>PEG3</i>	CB		=			↓	5.2 %	
		<i>GNAS</i>	CB		=			↓	3.0 %	
		<i>NNAT</i>	CB		=			↓	1.6 %	
		<i>PEG3</i>	P		=			↑	6.7 %	
		<i>MEST</i>	P		↑	2.09-fold		↓	1.9 %	
		<i>SLC22A2</i>	P		=			↓	7.3 %	
77 NC	35 IVF	<i>MEST</i>	MPB/CB	NA			SIRPH	↑	MBP: 2.0 %, CB: 3.0 %	[143]
		<i>MEST</i>	ACM					=		
		<i>KCNQ1OT1, H19, SNRPN, GRB10, DLK1/MEG3 IG-DMR, GNAS NESP55, GNAS NESPas, GNAS XL-alpha-s, GNAS Ex1A</i>	MPB/CB					=		
	77 ICSI	<i>MEST, KCNQ1OT1, H19, SNRPN, GRB10, DLK1/MEG3 IG-DMR, GNAS NESP55, GNAS NESPas, GNAS XL-alpha-s, GNAS Ex1A</i>	MPB/CB/ACM					=		
77 ICSI	35 IVF	<i>MEST</i>	MPB/CB					↑	MBP: 3.0 %, CB: 3.0 %	
		<i>MEST</i>	ACM					=		
		<i>KCNQ1OT1, H19, SNRPN, GRB10, DLK1/MEG3 IG-DMR, GNAS NESP55, GNAS NESPas, GNAS XL-alpha-s, GNAS Exon1A</i>	MPB/CB					=		

**Table 2** Effects of ART on imprinted genes and retrotransposable element expression and methylation in chorionic villous samples from abortion, peripheral blood, cord blood and placenta (Continued)

29 NC	24 IVF, 14 ICSI, 4 IVF or ICSI	<i>KCNQ1OT1</i>	CVS	NA			Bisulphite pyrosequencing	↓	4.0 %	[146]
		<i>H19, MEG3, MEST, NESPS5, PEG3, SNRPN</i>	CVS					=		
12 NC	45 ART	<i>H19</i>	CB	RT-qPCR	=		Parental allele-specific methylation	=		[145]
		<i>IGF2R</i>	CB		↓	0.61-fold		=		
		<i>H19</i>	P		↓	0.72-fold		↑ LOI		
		<i>IGF2</i>	P		↓	0.52-fold		NA		
		<i>IGF2R</i>	P		=			=		
12 NC	32 IVF, 45 ICSI	<i>H19</i>	P	NA			MS-SNuPE	=		[141]
30 NC	61 ART	<i>H19</i>	CB	NA			COBRA + sequencing	=		[140]
59 NC	59 IVF	<i>KCNQ1</i>	CB	NA <sup>a</sup>			Bisulfite pyrosequencing	↑	0.6 %	[142]
		<i>MEST, GRB10, H19, IGF2 DMRO, SNRPN</i>	CB					=		
		<i>SNRPN</i>	P					↑	1.7 %	
		<i>MEST</i>	P					↓	3.4 %	
		<i>H19</i>	P					↓	1.3 %	
		<i>GRB10, IGF2 DMRO, KCNQ1</i>	P					=		
27 NC	27 OI	<i>KCNQ1</i>	CB					↑	1.3 %	
		<i>SNRPN</i>	CB					↑	2.1 %	
		<i>GRB10, MEST, H19, IGF2DMRO</i>	CB					=		
		<i>SNRPN</i>	P					↑	2.1 %	
		<i>H19</i>	P					↓	4.5 %	
		<i>KCNQ1, GRB10, MEST, IGF2 DMRO</i>	P					=		
35 NC	5 IVF, 30 ICSI	<i>MEST</i>	P	RT-qPCR	=		Bisulfite pyrosequencing	↓	ND	[144]
		<i>MEG3</i>	P		NA			↓	ND	
		<i>H19</i>	P		↑	1.3-fold		↓	ND	
								( <i>H19</i> CTCF6)		
		<i>IGF2</i>	P		=			NA		
		<i>PEG3, SNRPN, KCNQ1OT1, IG-DMR</i>	P		NA			=		

**Table 2** Effects of ART on imprinted genes and retrotransposable element expression and methylation in chorionic villous samples from abortion, peripheral blood, cord blood and placenta (*Continued*)

121	NC	73	ART	ALU-Yb8, LINE-1	P/CB	NA	NA	Bisulfite pyrosequencing	=		[134]
				<i>DIRAS3, NAP1L5, ZAC1, IGF2R, FAM50B, MEST, GRB10, PEG10, PEG13, INPP5Fv2, H19, KCNQ1OT1, RB1, MEG3, SNRPN, ZNF597, ZNF331, C19MC, PEG3, MCTS2, NNAT, L3MTBL, NESP, GNAS XL, GNAS Ex1A</i>	P/CB			Methylation array	=		
23	NC	73	ART	<i>PHLDA2, GTL2, H19, ZNF331, ZNF597, C19MC, FAM50B, MEST, HYMAI, ZAC1, IGF2, KCNQ1OT1</i>	P	Sequenom iPLEX assay	Monoallelic				
8	NC	10	IVF	<i>GNAS</i> (2 sites), <i>PLAGL1, ZIM2, DIRAS3</i>	CB			Methylation array	↑	ND	[139]

ACM amnion/chorion membranes, ART assisted reproductive technologies, CB cord blood, COBRA combined bisulfite restriction analysis, CPB child peripheral blood, CVS chorionic villous samples, *hypoM* hypomethylation, ICSI intra-cytoplasmic sperm injection, IVF in vitro fertilization, LOI loss of imprinting, MPB maternal peripheral blood, MSED-qPCR methylation-sensitive enzymatic digestion associated with quantitative PCR method, MS-PCR methylation-specific PCR, MS-SNuPE methylation-sensitive single nucleotide primer extension, NA not analyzed, NC naturally conceived, ND not documented, OI ovulation induction, P placenta, RT-qPCR quantitative reverse transcription PCR, SIRPH single nucleotide primer extension assays in combination with ion pair reverse phase high performance liquid chromatography separation techniques, ↑: increased, ↓: decreased, =: no significant difference compared with control

<sup>a</sup>Analysed only on a subset of individuals with outrange methylation levels for three imprinted genes (*H19, KCNQ1, SNRPN*) but no comparisons between conception groups

above, we can postulate that these “defects” are not all harmful for the embryo and that some could be considered compensatory mechanisms. Indeed, they reflect the balance between members of the IGN in the placenta. Biallelic expression as well as the loss of imprinting of parts of the IGN in the placenta could constitute a major compensatory mechanism to allow the developing foetus to cope with a changing or adverse environment. In response to certain stress factors that modify the early environment of the embryo, the placenta could amplify these compensatory mechanisms up to a certain point. In most cases, efficient compensation ensures normal foetal growth up to term. When the compensation is unbalanced, compensation fails and pathological features such as miscarriages, low birth weight or preeclampsia could occur. However, what remains to be determined is whether this compensation step per se could be a risk factor for certain diseases later in life.

#### **Potential long-term effects of ART-related compensation during pregnancy**

These modified maternal-foetal interactions, here after ART, might have consequences for outcomes in infancy and even in adulthood, especially by inducing metabolic and cardiovascular conditions [148–152]. For instance, in humans, new-borns that are either too small or too big may be vulnerable to heart disease, hypertension, type II diabetes and obesity [153–155]. In addition, the size and shape of the placenta have been related to life expectancy in men [156] and their risk for coronary heart disease [157]. Similarly, a high placenta/foetus weight ratio, considered a marker of intra-uterine stress, has been associated with hypertension later in life [158].

As mentioned above, these phenotype modifications of the foetus and placenta are found in ART pregnancies. Thus, the modified intra-uterine environment after ART may be one cause of late-onset diseases [159]. Indeed, although the majority of ART children are healthy, the available data about long-term follow-up of ART children revealed cardiovascular and metabolic risk factors [159]. Notably, children born after ART may exhibit increases in peripheral adipose tissue mass, in systolic and diastolic blood pressure, in fasting glucose levels and IGF-I and IGF-II levels as well as changes in the lipid profile [160–164]. In addition, transcriptomic data at birth revealed activation of metabolic pathways implicated in chronic disorders such as obesity and type II diabetes [77]. However, further large longitudinal studies are needed to confirm these poor outcomes.

Portha and colleagues proposed that the link between the prenatal environment and adverse long-term effects could

be written through epigenetic modifications of the conceptus. These plastic responses to the early environment could be kept in memory throughout life, due to epigenetic changes such as DNA methylation and histone modifications [165]. We can postulate that ART could trigger similar processes.

Nevertheless, in humans, there is no evidence of epigenetic changes persisting into childhood. Indeed, in children conceived after IVE, reassuring data have been reported for DNA methylation for four imprinted genes and even on a global scale [166, 167]. Only one recent study observed that some epigenetic errors can still be observed during childhood, though this concerned only the imprinted gene *SNRPN* [168] whose DNA methylation levels were not found to be modified at birth after ART in either cord blood or in the placenta. However, the heterogeneity of biological samples (blood or buccal cells), age range, type of reproductive technique and the analysis of methylation could hide potential underlying differences.

Another hypothesis might reside in tissue-specific epigenetic modifications. This could explain the absence of DNA methylation variations in blood and buccal cells. Therefore, studying other tissues may reveal defects linked to specific metabolic conditions. Notably, Scherrer's team found increased DNA methylation on the promoter of the gene encoding eNOS (NO synthase) in vascular tissues in mice obtained after ART. This resulted in reduced plasma NO concentrations, increased blood pressure and a shorter lifespan [169].

It is also interesting to consider that tissue-specific epimutations for *H19*, *Snrpn* and *Peg3* genes were described in individual mice generated by ART (ICSI or superovulation) [170].

#### **Ways for medical improvement and future research**

**Ways to improve actual ART protocols** Finally, as placental defects seem to originate from an altered endometrium-embryo dialogue, optimization of the environment during ART is a cornerstone and may improve early placentation. Hence, several simple and practical improvements can be proposed. First, it is possible to optimize the quality of oocytes and the endometrial milieu by using lower doses of hormones. Second, the culture media must be optimized to limit trophoblast cell stress. Even though the parameters of this optimization are far from being mastered, it has been clearly shown that specific culture media generate a lower degree of stress for the embryo [5, 70]. Third, the embryo and endometrium should be better synchronized either by transferring blastocyst-stage embryos (even if extended embryo culture may have per se an impact the epigenetic regulation) and/or by developing molecular diagnostic tests (for example transcriptomic, lipidomic and proteomic profiles) to assess the quality of the endometrium in order to target the best

timing of endometrial receptivity [171]. Fourth, another practice recently developed by some teams, could be to freeze all embryos and transfer them during subsequent cycles with an optimally prepared endometrium [172]. However, the endometrial tests and the fourth solution need to add an embryo cryopreservation step. Recent data reported poorer obstetrical outcomes after frozen embryo cycles (reported above) and a potential negative impact of the cryopreservation itself on the regulation of DNA methyltransferases in preimplantation frozen/thawed embryos [173]. Thus, further studies are required before these strategies can be applied safely.

Another way to improve the chances of success could be post-natal correction. Since imprinted genes in the placenta appear to be major operators in regulating foetal growth, further research is needed to better understand the link they may have with future disease. All in all, imprinted genes could eventually be used as sensors to predict and better prevent diseases later in life. Interestingly, some studies suggest that customized interventions might be implemented to correct effects on phenotypic changes [153]. One example is the post-natal administration of leptin in rats, which was able to reverse the adverse effects of mother-undernutrition: the offspring phenotype as well as the expression and methylation of several hepatic genes were corrected [174]. One other example is the post-natal administration of butyrate (histone deacetylase inhibitor) in the mouse model, which normalized both DNA methylation of the promoter of the *eNOS* gene and vascular function [169]. Further studies in animals are needed to better understand tissue-specific epigenetic regulation in ART. Thus, screening for epigenetic markers during early life could be used to identify more vulnerable patients and to define an appropriate treatment to potentially correct various epigenetic defects.

#### **Future research to assess the impact of ART on health**

More research is needed to better understand the disturbed interactions between the embryo and the milieu, especially in artificial conditions. New insights about the regulation of actors involved in the protection/maintenance of DNA methylation at imprinted genes in a context of ART are now necessary [33]. Moreover, to our knowledge, epigenetic defects have not been studied separately in TE and ICM cells so far. Nonetheless, knowing whether epigenetic dysregulation occurs in all blastocyst cells or only in TE cells could lead to better understanding of the mechanisms implicated in placental defects caused by ART. Knowledge of such mechanisms would be important to evaluate possible consequences for the developing individual soon after birth or even later in life.

Furthermore, although placental compensation enables mice to reach a normal birth weight [10], evidence in humans shows that ART pregnancies still carry a higher

risk of placenta-related adverse pregnancy outcomes [7]. These differences may stem from overwhelmed compensation mechanisms, which, in certain cases, are not fully successful. Several potential reasons may explain this limited correction in humans as compared with mice. First, although placentation is haemochorial in both humans and mice [175, 176], their placentas are not organized in the same way (labyrinth and spongiotrophoblast in mice versus villous trophoblast in humans) and differ in their morphogenesis and exchange functions [175, 177]. Second, in human ART, the cumulative effects are possibly at their utmost point because the standard method is to transfer fresh embryos from a superovulated cycle, which is not performed in mice because pseudopregnant females are used. The effects observed in animal models are therefore possibly exacerbated in humans. Third, contrary to animal models, parental infertility is the major reason why ART is used in humans, and this infertility may be partly responsible for the epigenetic disorders and abnormal placentation leading to maternal pathologies, such as abruptio placentae and preeclampsia [178–180]. Therefore, any extrapolation of animal studies to humans should be done with caution.

Moreover, concerning the methodology, most epigenetic studies have addressed the effects of ART stressors on DNA methylation at the individual gene level and often analyze one or few CpG. Thus, genome-wide as well as gene-specific approaches that can target regulatory regions (promoters, enhancers, gene body, or elsewhere) and assess functional significance is now needed. High-throughput tools, which are becoming available, may be applied more widely to study the epigenomic changes associated with ART. Otherwise, in most studies, only overall expression and methylation levels are examined (Tables 1 and 2). Although it could be valuable, monoallelic expression of imprinted genes is hard to perform, given the need for informative SNPs in parents to perform this analysis.

From a global DNA methylation point of view, placenta tissue has been shown to display a very low DNA methylation profile compared with other somatic tissues [181]. More recently, human studies on placenta samples using high-throughput tools (methylome) revealed that placenta presents large partially methylated domains (PMD) which are stable during pregnancy [182]. This unique property of the placenta might contribute to the regulation of the expression of key genes important for foetal development. Besides, in placenta samples, the genes enriched in the highly methylated regions (HMD) are involved in defence responses. The review that we present here focuses on imprinted genes, but research aiming to delineate the variations that exist at such loci, between placenta from ART and spontaneous pregnancies, would help us to understand how this alternative epigenetic

mechanism may contribute to placental remodelling and pregnancy outcomes.

In addition, to date, no study has focused on histone modifications in ART placentas, although higher concentrations of H3K4 trimethylation have been found in mouse blastocysts cultured *in vivo* than *in vitro* [183]. Recently, Court and colleagues suggested that placental-specific imprinted loci could be imprinted by an epigenetic mechanism, such as histone modification, independent of germline methylation [30]. Furthermore, other interesting data about miRNAs indicate that they also deserve to be studied in more detail [76, 184]. Studies on combinations of epigenetic factors would also bring additional knowledge about the respective roles of the different epigenetic alterations after ART.

Besides, since gene expression and DNA methylation are sexually dimorphic in male and female placentas it is also important for future epigenetic placental studies to take into account the sex of the foetuses [185]. For example, a study that investigated the epigenetic variations of *ZAC1* in cases of IUGR revealed down-regulated expression in placentas from girls but not boys [133].

Moreover, the link between placental growth and epigenetics was not investigated. It would be interesting to carry out studies comparing placental development during the early steps of foetal life with placental epigenetic results at birth to unravel the sequence of epigenetic events and distinguish between causal changes and the resulting epigenetic landscape.

## Conclusions

Much evidences support the hypothesis that suboptimal trophoblastic invasion due to a disturbed dialogue during the early phases of placentation could potentially explain the higher frequency of adverse pregnancy outcomes, such as miscarriages or preeclampsia, associated with ART. The dialogue between the endometrium and embryo is a crucial step to achieve successful trophoblastic invasion, ensuring a non-complicated pregnancy and the development of healthy offspring. This dialogue seems to be disturbed by ART, either by impairing endometrial receptivity or by modifying the early steps in the epigenetic development of the embryo. But this initially disturbed placentation also gives rise to a smart dialogue between the foetus and placenta, which may bring adaptive responses, notably through epigenetic mechanisms. Indeed, a coordinated group of genes called the imprinted gene network, stimulated by foetal signals, may modify nutrient transfer as well as placental growth and vascularization.

If these mechanisms are overwhelmed, improper maternal-foetal exchanges could occur, potentially leading to abortion or adverse pregnancy outcomes. Fortunately, in most cases, successful adaptation enables normal progress of the pregnancy and healthy offspring. However,

these adaptation mechanisms *per se* could have adverse effects later in life. More research is thus needed to assess the real impact of ART on future health. The better understanding of the placental mechanisms triggered by ART will aim *in fine* to render the ART protocols safer.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

CC and PF played a role in the conception of the review. CC, VC and PF took part in the collection and assembly of data. CC, VC and PF carried out data analysis and interpreted the findings. CC, VC, CB, DV, PS and PF drafted the manuscript. CC, DV and PF wrote the critical discussion. All authors read and approved the final manuscript.

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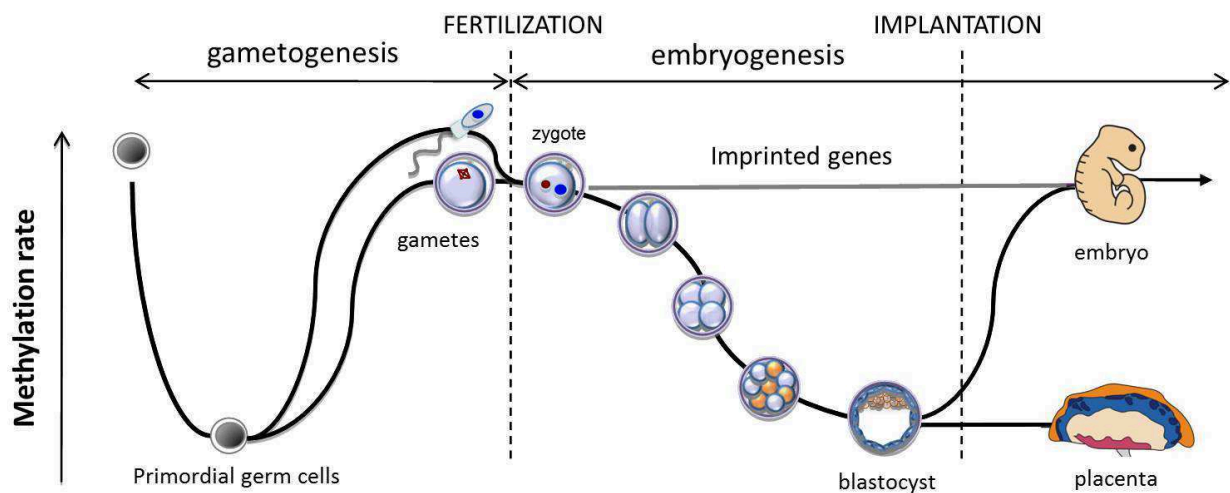


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### III. ART and epigenetic modifications

The modifications, supported by the placenta throughout pregnancy after ART, could be written through epigenetic changes. As ART occurs specifically during preimplantation development, when important epigenetic events happen, the scientific community has naturally become interested in a potential ART-induced epigenetic vulnerability (**Figure 4**).



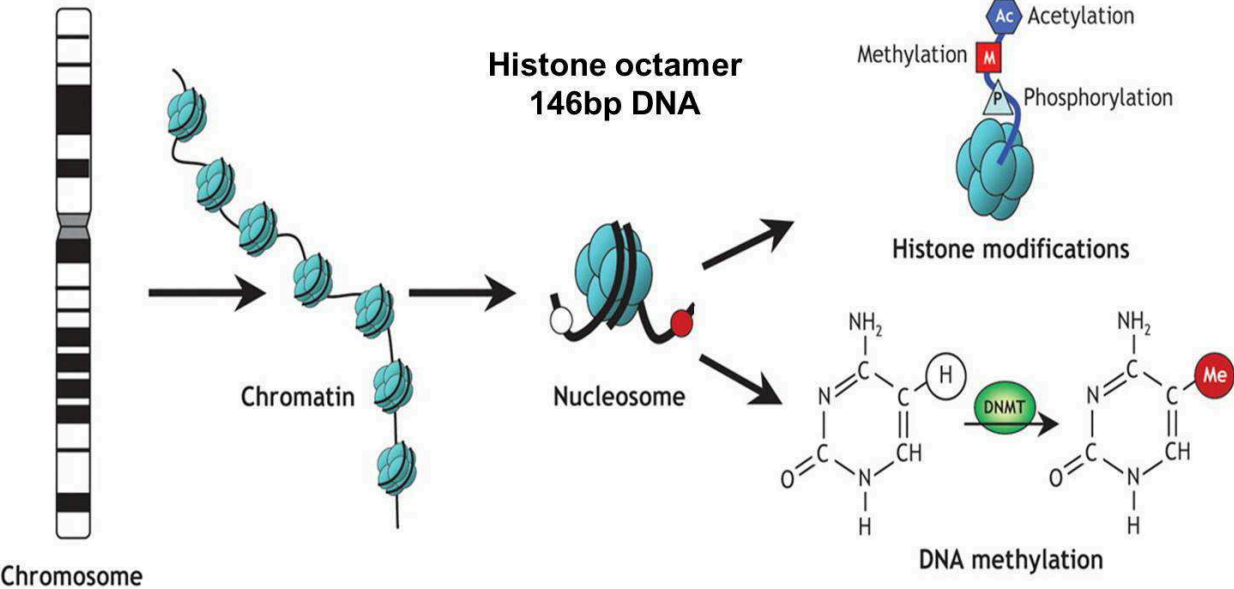
**Figure 4: Preimplantation development of the embryo is a sensitive period when important epigenetic events take place**

From (Fauque, 2009). Soon after the fertilization, a global wave of demethylation occurs, resulting in an embryo with multipotent cells. Then, specific remethylation is necessary for the specialization of cells and the organization of the structure of the embryo. During the whole process, mechanisms are required to maintain the methylation of the imprinted genes.

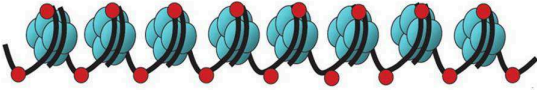
#### 1) Epigenetics: general information

Epigenetics may be defined as “the study of heritable changes in genome function that occur without a change in DNA sequence” (*The Epigenome Network of excellence*) (Portha *et al.*, 2014). These mechanisms are heritable through mitosis and not sustained by DNA sequence variation (Nelissen *et al.*, 2011). Epigenetic regulation controls transcription at two levels: directly on the DNA (through DNA

methylation) and on the proteins around which the DNA is wrapped to constitute the nucleosome (histone modifications). The chromatin is the substrate on which epigenetic information is written (**Figure 5**).

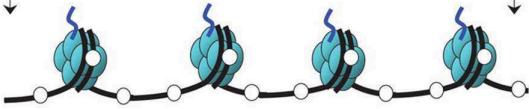


« OFF »



DNA: methylated  
Histones: lysine9 et lysine27 methylated  
Chromatin: compacted

« ON »



DNA: demethylated  
Histones: acetylated, lysine4 methylated  
Chromatin: relaxed

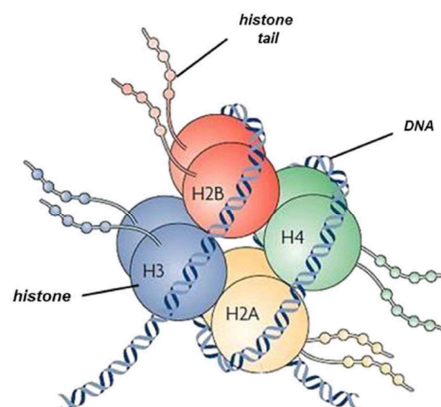
**Figure 5: The chromatin is the substrate on which epigenetic information is written**  
 Epigenetics controls transcription at two levels: directly on the DNA through DNA methylation, and on the proteins around which the DNA is wrapped called histones. Basically, when DNA is methylated, lysine 9 of histone H3 is di- or tri- methylated, chromatin is compacted and transcription is blocked. On the contrary, when DNA is demethylated, Lysine 9 of histone H3 is acetylated and lysine 4 of histone H3 is di- or tri- methylated, chromatin is relaxed and transcription is facilitated.

## **DNA methylation**

The best known epigenetic mark is DNA methylation which consists in the addition of a methyl group on a cytosine (thus forming a 5-methylcytosine), usually when the cytosine is followed by a guanine, thus forming a CpG dinucleotide (Illingworth *et al.*, 2009). The mammalian genome contains low levels of CpG except at 'CpG islands' which often coincide with promoter regions. The methylation of these promoter regions often correlates with their silencing (Illingworth *et al.*, 2009; Cedar *et al.*, 2012; Jones, 2012). Proper regulation of DNA methylation is crucial, as it is implicated in important functions such as cell differentiation, allele-specific expression of imprinted genes, control of transposable elements (Bourc'his *et al.*, 2004) or X-chromosome inactivation in females. DNA methylation is implemented by DNA methyltransferases (DNMTs), which are highly conserved across the mammalian species (Goll *et al.*, 2005). DNMT3A, DNMT3B and their cofactor DNMT3L are called *de novo* DNMTs, even if growing evidence demonstrate that there could also be implicated in methylation maintenance, similar to DNMT1 which is known as the maintenance DNMT (Portha *et al.*, 2014).

## **Histone modifications**

In eukaryotes, DNA is wrapped around and octamer of histones to form a nucleosome, the basic unit of chromatin. (Kouzarides, 2007). Each nucleosome is constituted by a DNA strand and 2 copies of each histone (H2A, H2B, H3 and H4) (**Figure 6**).

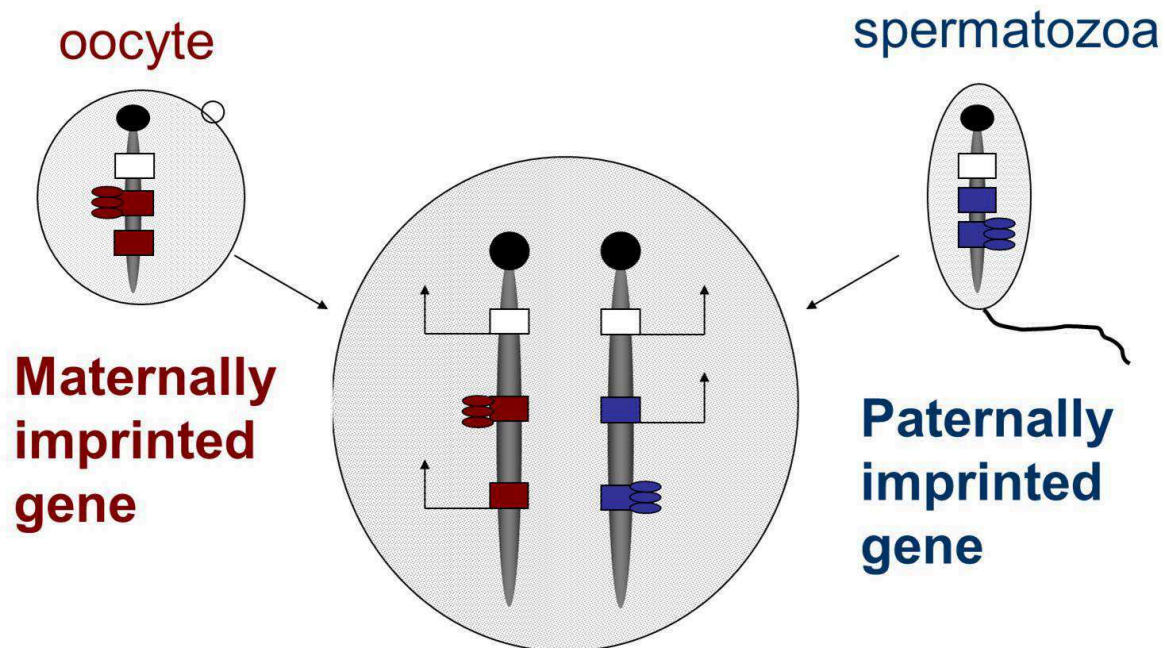


**Figure 6:** A nucleosome is constituted by an octamer of histones and a DNA strand  
From (Portha *et al.*, 2014)

The degree of compaction of the DNA-histone complex participates in gene expression control. The compact configuration is maintained thanks to either the electrostatic bond between positively charged histones and negatively charged DNA, or by covalent post-translational modifications present on amino-terminal tail of each histone protein (Portha *et al.*, 2014). These modifications called ‘the histone code’, together with the degree of compaction of the nucleosomes, lead to changes in regulation of DNA transcription, replication, recombination and repair. For example, acetylation of the lysine 9 of histone H3 (H3K9ac) neutralizes the positive charge of the histone H3, decreasing the histone’s affinity to bind DNA, resulting in a more “relaxed” chromatin state which is permissive to gene expression. More complex than acetylation, histone methylation can either be a permissive or a repressive mark according to the residuals location on the histone tail. Though tri methylation of lysine 4 on histone H3 (H3K4me3) is permissive, methylation of lysine 9 on histone H3 is repressive when located in the promoters regions (Portha *et al.*, 2014).

## 2) Imprinted genes

Epigenetics controls the regulation of imprinted genes, which are essential in regulating placental and foetal growth and development through the control of nutrient exchange, metabolic processes and placental signalling (Novakovic *et al.*, 2012; Choux *et al.*, 2015; Moore *et al.*, 2015). Imprinted genes are expressed in a parent-of-origin manner thanks to epigenetic modifications which silence either the paternal or the maternal allele (**Figure 7**).



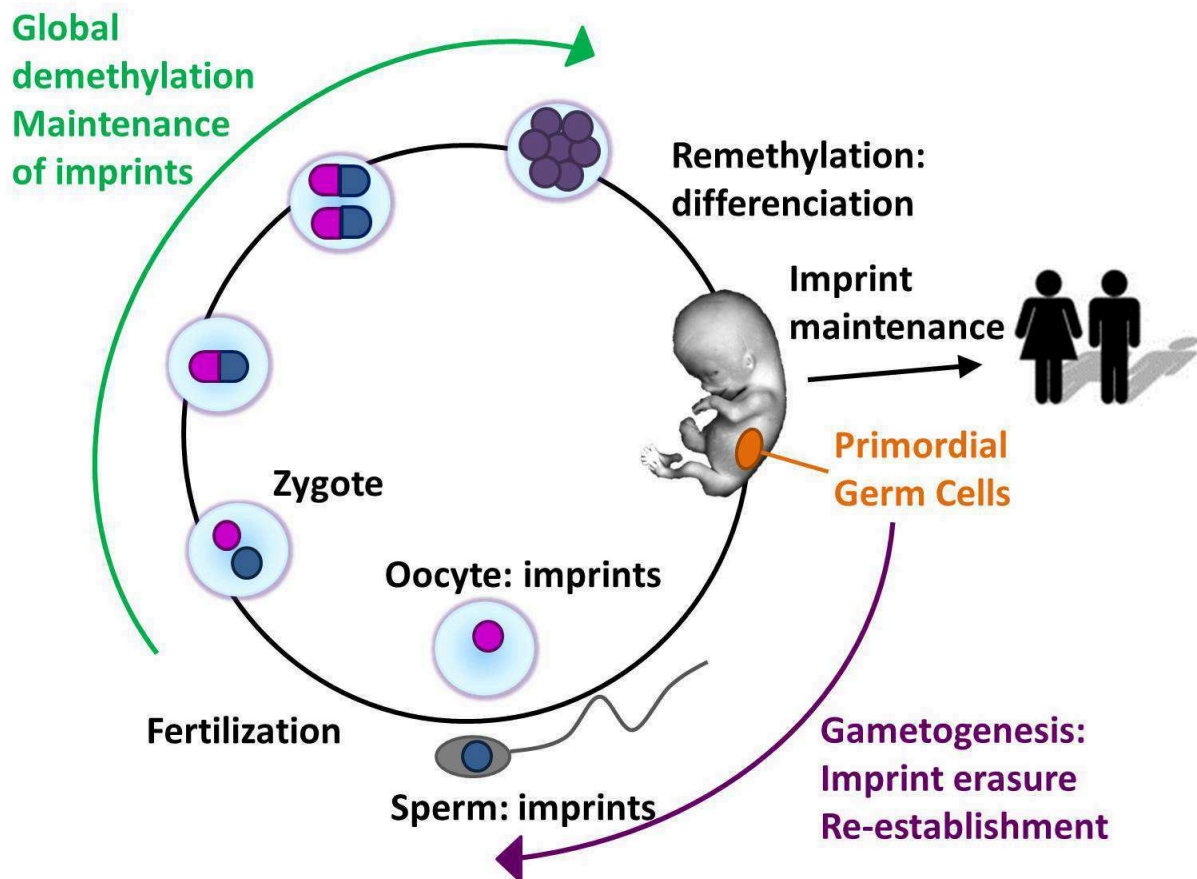
**Figure 7: Parental imprinting**

From (Fauque, 2009). Contrary to other genes that are expressed on both alleles, imprinted genes are expressed in a parent-of-origin manner so that either the paternal or the maternal allele is silenced.

These epigenetic modifications are established on regulatory sequences referred to as imprinting control regions (ICRs) in a sex-specific manner during gametogenesis. After fertilization, these ICRs acts in *cis* to achieve monoallelic expression of most imprinted genes. Preimplantation development is a sensitive period for imprinting because of the global wave of demethylation of the genome, making the role of some entities essential for the protection of epigenetic imprinting marks (Messerschmidt, 2012).

Indeed, the periconception window encompasses gametogenesis, fertilization and early embryogenesis, and usually comprises key epigenetic rearrangements (erasure and reinstallation of epigenetic marks) in two phases. The first phase is characterized by genomic imprinting in the gamete. For this purpose, epigenetic marks (like methylation) of ICRs are applied differentially to the germinal cells according to the environmental somatic context (i.e. being either in a testis or in an ovary). Following this period, the male and female gametic epigenomes are not equivalent at least for

the imprinted genes (less than 0.5% of the genes). Then, the second phase is a genome-wide wave of demethylation occurring after fertilization (**Figure 8**). Since parental imprinting is preserved for many imprinted genes throughout development, the methylation marks of ICRs have to be 'protected', again by mechanisms that are largely unknown to date (Reik *et al.*, 2001; Dolinoy *et al.*, 2007).



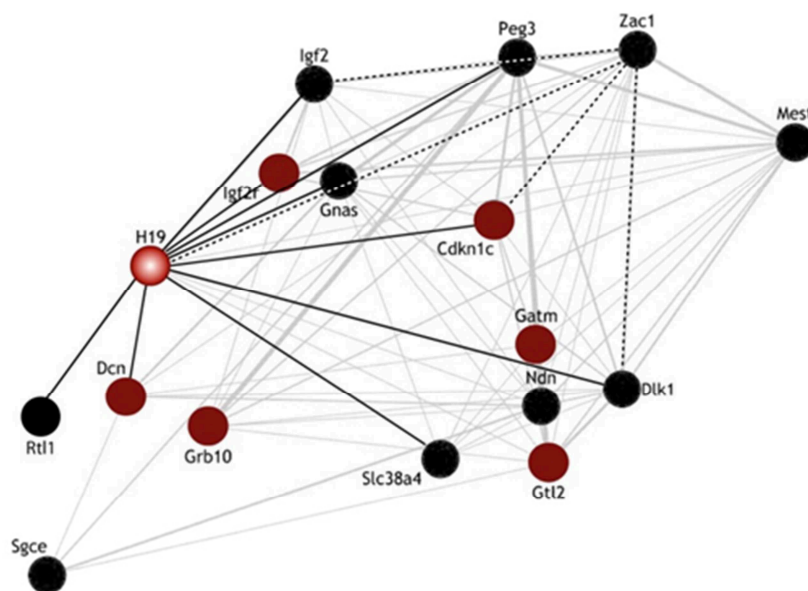
**Figure 8: Epigenetic reprogramming**

Adapted from (Dolinoy *et al.*, 2007). When the fertilization occurs, oocyte and spermatozoa are well imprinted, with maternal and paternal imprint, respectively. Soon after fertilization, a genome-wide wave of demethylation occurs in the global genome, except for the imprinting genes. This global demethylation is followed by selective remethylation that enables cell differentiation. The imprint is maintained throughout life, except in the primordial germ cells. Indeed, during the gametogenesis, imprints present on the maternal and maternal chromosomes are erased and primary imprints that reflect the sex of the individual are established.



Imprinted genes are functionally haploid by definition and thus potentially more susceptible to mutations and epimutations (Fowden *et al.*, 2011). Given their important role in foetal and placental growth, dysregulation may have major consequences on placental phenotype, pregnancy and neonatal outcomes (Varmuza *et al.*, 2015). For example, deregulation of the *H19/IGF2* imprinted region can cause imprinting diseases such as Beckwith-Wiedemann and Silver-Russel syndromes.

It appears that some imprinted genes may be organized as a network of genes with reciprocal functions, coordinated in order to tightly regulate foetal and placental growth during pregnancy (**Figure 9**). This Imprinting Gene Network (IGN) was first described in mice for genes such as *Igf2*, *Cdkn1c* and *H19* (Varrault *et al.*, 2006), and more recently in the human placenta (Iglesias-Platas *et al.*, 2014), converging evidence indicating that *ZAC1* (= *PLAGL1*) could act as a transcription factor and thus be an upstream regulator for this network of genes. It has been hypothesized that this set of genes could be upregulated in a coordinated fashion after ART, the final goal being to correct primary dysfunctions of the placenta (Fauque *et al.*, 2010).



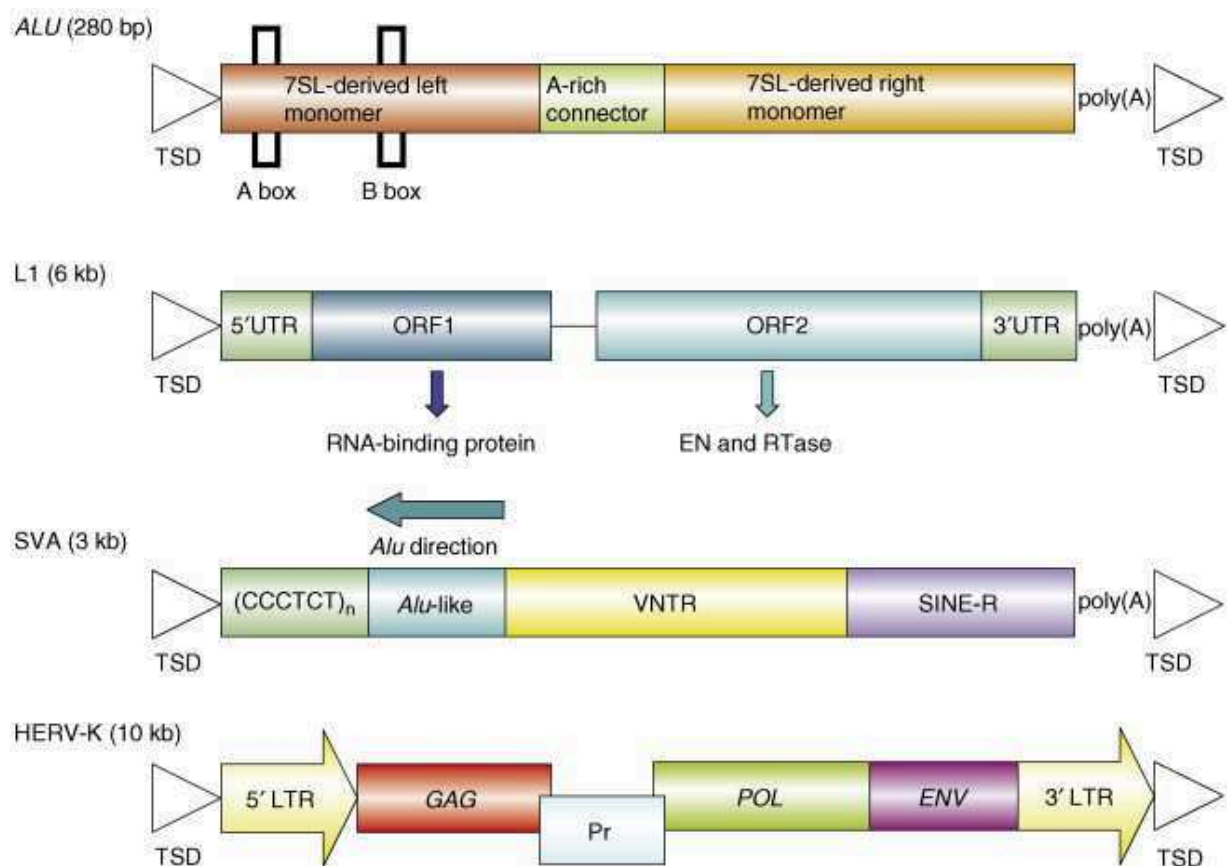
**Figure 9: The imprinting gene network**

From (Gabory *et al.*, 2009). Maternally and paternally expressed genes are shown in red and black, respectively. Grey, black and dotted black lines represent connections between genes that have already been demonstrated.

### 3) Transposable elements

TEs are repeated sequences, representing around 50% of the human genome, that are potentially harmful if not properly controlled, notably by epigenetic mechanisms (Fauque *et al.*, 2014).

Human endogenous retroviruses (HERVs) are mostly immobile remnants of ancient elements, but Long and Short Interspersed Nucleotide Elements (LINE and SINES) can still generate de novo transposition events. The structures of each class of human transposon are depicted in **Figure 10**.



**Figure 10: Structure of human transposable elements**

From (Mills *et al.*, 2007). L1: Line-1; LTR: long terminal repeat; ORF: open reading frame; Pr: protease; SINE-R: short interspersed repetitive element-R; SVA: SINE/VNTR/ALU; TSD: target site duplication; UTR: untranslated region; VNTR: variable number of tandem repeats

Some studies have highlighted that preimplantation embryos may be particularly prone to transcriptional TE activation (Kano *et al.*, 2009) and retrotransposition (van den Hurk *et al.*, 2007). In the placenta, some specific copies of the human retroviruses ERVFRD and ERVW have been domesticated for their ability to produce syncytins, which are fusogenic retroviral envelope proteins. Notably, ERVW-1 and ERVFRD-1 encode envelope glycoproteins syncytin-1 and -2, respectively. They are known to be involved in cell fusion, particularly the fusion of cytotrophoblasts, a necessary step in the formation of the syncytiotrophoblast, which is a placental exchange area between mother and foetus (Bolze *et al.*, 2017). Altogether, there is strong evidence to suggest that TEs are involved in placental functions and subsequent foetal growth and development.

#### 4) Epigenetic dysregulation of IGs and TEs in the placenta is responsible for placenta-related adverse pregnancy outcomes

In this first article of this thesis, we described the associations between the epigenetic dysregulation of IGs and TEs and adverse pregnancy outcomes such as preeclampsia and IUGR. For example, some imprinted genes such as *ZAC1* (= *PLAGL1*), *MEST* and *IGF2* were found to be downregulated in IUGR placentas (McMinn *et al.*, 2006; Koukoura *et al.*, 2011). Other studies also evidenced decreased placental DNA methylation and/or loss of imprinting at *H19* DMR in IUGR (Diplas *et al.*, 2009; Bourque *et al.*, 2010).

Concerning TEs, reduced expression of syncytin-1, a protein produced by the retrovirus ERVW-1 that promotes cellular fusion in the syncytiotrophoblast, was found in human IUGR and preeclampsia placentas (Ruebner *et al.*, 2010). Interestingly, this was associated with DNA hypermethylation of its ERVW-1 promoter (Ruebner *et al.*, 2013). More recently, a review summarized the potential consequences of dysregulation of syncytin functions on human placenta pathologies such as preeclampsia, IUGR and gestational trophoblastic diseases (Bolze *et al.*, 2017).

This wealth of data stresses the importance of epigenetics in regulating placental function and the potential link between epigenetic dysregulation and adverse pregnancy outcomes. As ART has been linked to adverse pregnancy outcomes, as it

takes place during the sensitive period of preimplantation when important epigenetic regulation take place, and as it has been associated with increased risk of imprinting disorders (Lazaraviciute *et al.*, 2014), it raises the issue of a potential epigenetic risk induced by ART.

5) ART has been associated with epigenetic modifications in embryos and placenta

In article 1, we extensively reviewed the articles dealing with the epigenetic modifications after ART.

**Animal models**

ART are used on isolated gametes and embryos during the periconception period, which is characterized by acute epigenetic sensitivity. Today, following one of the first studies on the imprinted gene *H19* in the mouse model published by our group in 2007 (Fauque *et al.*, 2007), it has been clearly demonstrated that ART can trigger epigenetic errors in the embryo at the blastocyst stage in several loci such as *H19*, *Snrpn*, *Peg3*, *Kcnq1ot1* genes and repetitive sequences (**Table 3**) as well as later during pregnancy in various species (**Table 4**) including human embryos (Chen *et al.*, 2010; Santos *et al.*, 2010; Ibalá-Romdhane *et al.*, 2011; van Montfoort *et al.*, 2012). Apparently, from mid-gestation, the placenta appears more prone to modifications although the embryo seems rather preserved (Mann *et al.*, 2004). Placenta-specific imprinting defects are demonstrated after IVF and appear to be more severe in suboptimal conditions, suggesting a dose-dependent effect.

These ART-induced epigenetic alterations of embryos could originate from subtle alterations in gamete physiology due to the artificial context associated with ART procedures (ovarian stimulation, *in vitro* fertilization, sperm/ovum manipulations) or a context of infertility and/or the *in vitro* environment of the embryo (culture conditions, culture media components), which is at best a poor substitute for the 'natural' environment.

**Table 3: Effects of ART on imprinted genes and repeated sequence expression and methylation in mouse blastocysts**

Control group	Manipulation group	Gene	Technique for expression	Results of expression analysis	Technique for methylation	Results of methylation analysis	References
<i>In vivo</i> fertilization	SO, IVC KSOM/AA (2-cells=>blastocyst)	<i>H19</i>	Allele-specific RT-qPCR	Monoallelic	MS-PCR	No LOM	(Doherty <i>et al.</i> , 2000)
	SO, IVC WM (2-cells=>blastocyst)			Biallelic		Paternal allele LOM	
	SO, IVC KSOM/AA (2-cells=>blastocyst)	Monoallelic		NA			
	SO, IVC WM (2-cells=>blastocyst)	Monoallelic		NA			
<i>In vivo</i> fertilization	SO, IVC KSOM/AA (2-cells=>blastocyst)	<i>H19</i>	Allele-specific RT-qPCR	=	Cloning-sequencing	Paternal allele LOM	(Mann <i>et al.</i> , 2004)
		<i>Snrpn</i>		=		Maternal allele LOM	
		<i>Peg3</i>		=		NA	
	SO, IVC WM (2-cells=>blastocyst)	<i>H19</i>		Biallelic		Paternal allele LOM	
		<i>Snrpn</i>		=		Maternal allele LOM	
		<i>Peg3</i>		=		NA	
SO (2-cells=>blastocyst)	SO, IVF, IVC (2-cells=>morula or blastocyst)	<i>Igf2, H19</i> <i>Cdkn1c, Slc22a1l</i>	Allele-specific RT-qPCR	Aberrant =	NA	NA	(Li <i>et al.</i> , 2005)
<i>In vivo</i> fertilization	SO	<i>H19</i>	RT-qPCR	↓	Cloning-sequencing	No LOI	(Fauque <i>et al.</i> , 2007)
	SO, IVC (1-cell=>blastocyst)	<i>H19</i>		↓		No LOI	
	SO, IVF, IVC (=>blastocyst)	<i>H19</i>		↓		LOI	
<i>In vivo</i> fertilization	SO (low and high hormone dosages=>blastocyst)	<i>Snrpn</i>	NA	NA	Cloning-sequencing	Maternal allele LOM, dose-dependent	(Market-Velker <i>et al.</i> , 2010)
		<i>Peg3</i>		NA		Maternal allele LOM, dose-dependent	
		<i>Kcnq1ot1</i>		NA		Maternal allele LOM, dose-dependent	
		<i>H19</i>		NA		Maternal allele gain of methylation, dose-dependent Paternal allele LOM, dose-dependent	
<i>In vivo</i> fertilization	IVC (2-cells=>blastocyst) 6 different media systems	<i>H19</i>	Allele-specific RT-qPCR	Biallelic (all media)	Cloning-sequencing	Paternal allele LOM with 3/6 media	(Market-Velker <i>et al.</i> , 2010)
		<i>Snrpn</i>		Monoallelic		Maternal allele LOM with 4/6 media	
		<i>Peg3</i>		Monoallelic		Maternal allele LOM with 5/6 media	
	SO (Low hormone dosage), IVC (2-cells=>blastocyst) 6 different media systems	<i>H19</i>		Biallelic (all media)		NA	
		<i>Snrpn</i>		Monoallelic		NA	
		<i>Peg3</i>		Monoallelic		NA	
<i>In vivo</i> fertilization	SO (low hormone dosage)=> blastocyst	<i>IAP</i>	NA		Cloning -sequencing	=	(Liang <i>et al.</i> , 2013)
	SO (high hormone dosage)=> blastocyst	<i>Line-1</i>				=	
		<i>IAP</i>				=	
		<i>Line-1</i>				↓ 17%	

<i>In vivo</i> fertilization	IVC (8-cells=>blastocyst)	<i>Grb10</i>	RT- fluorescent qPCR	=	Cloning- sequencing	↓	(Yao et al., 2017)
	Vitrified-warmed + IVC (8-cells=>blastocyst)			↓		↓	
IVC	Vitrified-warmed + IVC (8-cells=>blastocyst)			↓		NA	
IVC (1cell=> blastocyst)	SO, IVC(1-cell=> blastocyst)	<i>Grb10</i>	RT- fluorescent qPCR	↓	Cloning- sequencing	↑	(Chen <i>et al.</i> , 2018)
		<i>H19</i>		↑		NA	
		<i>Igf2</i>		↑		NA	
		<i>Snrpn</i>		=		NA	
	SO, IFV, IVC(1-cell=> blastocyst)	<i>Grb10</i>		↓		↑	
		<i>H19</i>		↑		NA	
		<i>Igf2</i>		=		NA	
		<i>Snrpn</i>		=		NA	

IAP: intracisternal A-particle, IVC: *In Vitro* Culture, IVF: *In Vitro* Fertilization, KSOM/AA: optimal potassium-modified, simplex optimized medium with aminoacids, LOI: Loss Of Imprinting, LOM: Loss Of Methylation, MS-PCR: Methylation-Specific PCR, NA: Not Analysed, RT-qPCR: quantitative Reverse Transcription PCR, SO: SuperOvulation, WM: Whitten's Medium, ↑: increased, ↓: decreased, =: no significant difference compared with control.

**Table 4: Conceptuses and/or placentas in different animal species: resorption rate, weight, gene expression and/or DNA methylation of imprinted genes and genes involved in foeto-placental functions**

Species	GA study	Control group	Manipulation group	RR	Weight		Gene expression		Methylation		References
					F	P	F	P	F	P	
Mouse	E14	Blastocyst transfer	SO, IVC M16 (1-cell=>blastocyst)	=	=	NA	= <i>Igf2, Grb10, Grb7, H19</i>	NA	= <i>H19</i>	NA	(Khosla <i>et al.</i> , 2001)
			SO, IVC M16+FCS (1-cell=>blastocyst)	↑	↓	NA	↓ <i>H19, Igf2, Grb7</i> ↑ <i>Grb10</i>	NA	↑ <i>H19</i>	NA	
Cow	E222	SO, blastocyst transfer	OVM, IVF, IVC, blastocyst transfer	NA	↑	↑	NA	= <i>PPARY, VEGF</i>	NA	NA	(Miles <i>et al.</i> , 2004)
Cow	E70	SO, blastocyst transfer	OVM, IVF, IVC IVPS, blastocyst transfer	NA	=	=	NA	= <i>VEGF, PPARY</i>	NA	NA	(Miles <i>et al.</i> , 2005)
			OVM, IVF, IVC mSOF, blastocyst transfer	NA	=	↑	NA	= <i>PPARY</i> ↓ <i>VEGF</i>	NA	NA	
Cow	E80	Artificial insemination with frozen-thawed semen	OVM, IVF with frozen-thawed spermatozoa, IVC, blastocyst transfer	NA	↑	NA	NA	NA	NA	= (genome-wide 5mC content)	(Hiendleder <i>et al.</i> , 2004)
Mouse	E18	SO, blastocyst transfer	SO, IVC (1-cell=>morula) 7%O <sub>2</sub> , (morula=>blastocyst) 2%O <sub>2</sub> , transfer	↑	↓	=	NA	= <i>Slc2a1, Slc2a3, Igf2, Igf2r, H19</i>	NA	NA	(Feil <i>et al.</i> , 2006)
			SO, IVC (1-cell=>blastocyst) 7%O <sub>2</sub> , transfer	=	=	=	NA	= <i>Slc2a1, Slc2a3, Igf2, Igf2r, H19</i>	NA	NA	
			SO, IVC (1-cell=>morula) 7%O <sub>2</sub> , (morula=>blastocyst) 20%O <sub>2</sub> , transfer	=	=	=	NA	= <i>Slc2a1, Slc2a3, Igf2, Igf2r, H19</i>	NA	NA	
Mouse	E12.5	SO, blastocyst transfer	SO, IVF, IVC KSOM/AA, blastocyst transfer	=	↓	=	NA	NA	NA	NA	(Delle Piane <i>et al.</i> , 2010)
			SO, IVF, IVC WM, blastocyst transfer	↑	↓	↓	NA	NA	NA	NA	
		SO, IVF, IVC KSOM/AA, blastocyst transfer	=	↓	↓	NA	NA	NA	NA		

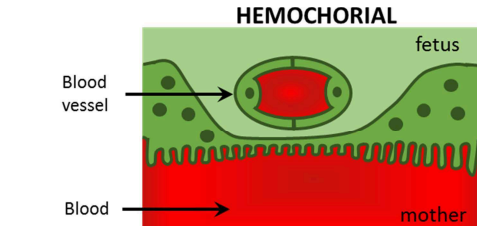
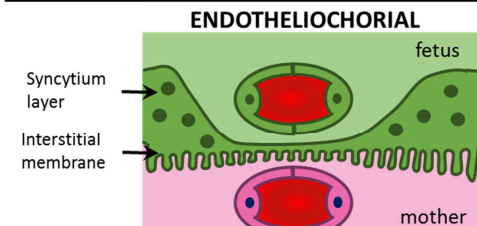
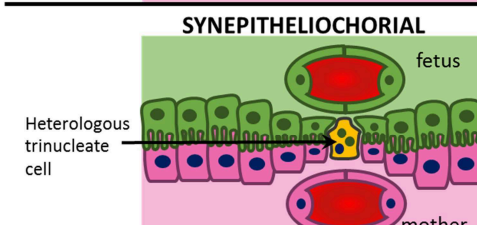
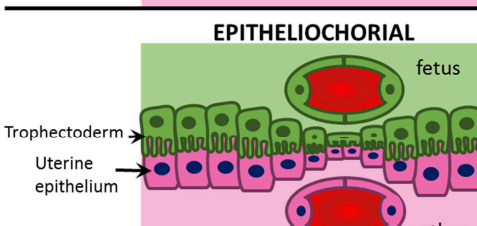
Mouse	E15.5	SO, blastocyst transfer	SO, IVF, IVC KSOM/AA, blastocyst transfer	NA	↓	=	NA	↑ <i>Slc7a3</i> = <i>Igf2, H19, Glut1, Snat1, Snat2, Snat4</i> ↓ <i>Glut3</i>	NA	NA	(Bloise <i>et al.</i> , 2012)
	E18.5	SO, blastocyst transfer	SO, IVF, IVC KSOM/AA, blastocyst transfer	NA	↓	↑	NA	= <i>Snat1, Slc7a3</i> ↓ <i>Igf2, H19, Glut1, Glut3, Snat2, Snat4</i>	NA	NA	
Sheep	E20, 22, 24, 26, 28	<i>In vivo</i> fertilization	OVM, IVF, IVC SOF, blastocyst transfer	↑	E24:↓ Late:=	NA	NA	E20: ↓ <i>DNMT1, IGF2, MEST, H19, CDKC1C</i> E22: ↓ <i>DNMT1</i> Term: = <i>DNMT1</i>	NA	E22: <i>H19</i> LOM	(Ptak <i>et al.</i> , 2013)
Mouse	E9.5	<i>In vivo</i> fertilization	IVC KSOM/AA (2-cells=>blastocyst)	NA	NA	NA	Monoallelic: <i>H19, Snrpn</i>	Monoallelic: <i>H19, Snrpn, Ascl2, Peg3</i>	= <i>H19, Snrpn</i>	= <i>H19, Snrpn</i>	(Mann <i>et al.</i> , 2004)
			IVC WM (2-cells=>blastocyst)	NA	NA	NA	Monoallelic: <i>H19, Snrpn</i>	Biallelic: <i>H19, Snrpn, Ascl2, Peg3</i>	= <i>H19, Snrpn</i>	Partial LOM: <i>H19, Snrpn</i>	
Mouse	E9.5	<i>In vivo</i> fertilization	SO, blastocyst transfer	NA	NA	NA	Monoallelic: <i>H19, Cdkn1c, Kcnq1, Ascl2, Zim1, Snrpn, Kcnq1ot1, Peg3, Igf2, Mkrn3</i>	Biallelic: <i>H19</i> High levels of misexpression: at least 1/8 IG ↑ <i>Ascl2</i> , = <i>H19</i> ↓ <i>Igf2</i>	NA	NA	(Rivera <i>et al.</i> , 2008)
			SO, IVC KSOM/AA (2-cells=>blastocyst), blastocyst transfer	NA	NA	NA	Monoallelic: <i>H19, Cdkn1c, Kcnq1, Ascl2, Zim1, Kcnq1ot1, Peg3, Igf2</i> Low levels of misexpression: <i>Snrpn, Mkrn3</i>	Biallelic: <i>H19</i> ↑ <i>Ascl2</i> , = <i>H19</i> ↓ <i>Igf2</i>	NA	NA	
Mouse	E9.5	<i>In vivo</i> fertilization	SO	NA	NA	NA	Monoallelic: <i>H19, Snrpn, Igf2, Kncq1ot1 = Igf2</i>	Biallelic: <i>H19, Snrpn</i> Monoallelic: <i>Igf2, Kncq1ot1</i> ↑ <i>Igf2</i>	NA	= <i>H19, Snrpn</i>	(Fortier <i>et al.</i> , 2008)
		Blastocyst transfer	SO, blastocyst transfer	NA	NA	NA	Monoallelic: <i>H19, Snrpn, Igf2 = Igf2</i>	Biallelic: <i>H19</i> Monoallelic: <i>Snrpn, Igf2</i> ↑ <i>Igf2</i>	NA	= <i>H19, Snrpn</i>	
Mouse	E10.5	SO, blastocyst transfer	SO, IVC (1-cell=>blastocyst) (M16 or sequential G1/G2), blastocyst transfer	↑	NA	NA	NA	↑ <i>H19, Igf2, Zac1, Slc38a4, Cdkn1c, Gtl2, Rian, Dlk1, Nnat, Peg3 = Igf2r, Grb10</i> ↓ <i>Dnc, Gatm, Mest</i>	= <i>Igf2, H19</i>	= <i>H19, Igf2, Igf2r, Dlk1-Dio3</i>	(Fauque <i>et al.</i> , 2010)



			SO, IVF, IVC (M16 or sequential G1/G2), blastocyst transfer	↑	NA	NA	NA	↑ <i>H19, Igf2, Igf2r, Zac1, Slc38a4, Cdkn1c, Gtl2, Rian, Dlk1, Nnat, Peg3</i> = <i>Grb10, Mest</i> ↓ <i>Dnc, Gatm</i>	= <i>Igf2</i>	= <i>H19, Igf2, Igf2r, Dlk1-Dio3</i>	
Mouse	E14	<i>In vivo</i> fertilization	SO, IVF, IVC, blastocyst transfer	NA	NA	NA	↓ <i>Igf2</i> , ↑ <i>H19</i>	↑ <i>Igf2</i> ↓ <i>H19</i>	LOM: <i>H19</i>	LOM: <i>H19</i>	(Wang <i>et al.</i> , 2010)
			SO, IVF, IVC, vitrifying/warming morula, blastocyst transfer	NA	NA	NA	↓ <i>Igf2</i> , ↑ <i>H19</i>	↑ <i>Igf2</i>	LOM: <i>H19</i>	LOM: <i>H19</i>	
		SO, IVF, IVC, blastocyst transfer	NA	NA	NA	↑ <i>Igf2</i> , ↓ <i>H19</i>	↑ <i>Igf2</i> ↓ <i>H19</i>	LOM: <i>H19</i>	= <i>H19</i>		
Mouse	E10.5	<i>In vivo</i> fertilization	SO, IVF, IVC KSOM/AA 5%O <sub>2</sub> , morula/blastocyst transfer	NA	NA	NA	Monoallelic: <i>Igf2, Cdkn1c, Snrpn, Kcnq1ot1</i> Biallelic: <i>H19, Peg3</i>	Biallelic: <i>H19, Snrpn, Peg3, Cdkn1c</i> Monoallelic: <i>Kcnq1ot1</i>	= <i>H19, Snrpn, Peg1, Kcnq1ot1, Dlk1/Gtl2, Peg3</i>	= <i>Snrpn, Kcnq1ot1, Peg1, Dlk1/Gtl2, Peg3</i> ↓ <i>H19</i>	(de Waal <i>et al.</i> , 2014)
			SO, IVF, IVC KSOM/AA 20%O <sub>2</sub> , morula/blastocyst transfer	NA	NA	NA	Monoallelic: <i>Igf2, Snrpn, Kcnq1ot1, Cdkn1c</i> Biallelic: <i>H19, Peg3</i>	Biallelic: <i>H19, Snrpn, Peg3, Cdkn1c, Kcnq1ot1</i>	= <i>H19, Snrpn, Peg1, Kcnq1ot1, Dlk1/Gtl2, Peg3</i>	= <i>Snrpn, Kcnq1ot1, Peg1, Dlk1/Gtl2, Peg3</i> ↓ <i>Peg3, H19</i>	
Mouse	E18.5	<i>In vivo</i> fertilization	SO, IVF, IVC KSOM/AA 5%O <sub>2</sub> , morula/blastocyst transfer	NA	↓	↑	Monoallelic: <i>Igf2, Cdkn1c</i> (liver) Biallelic: <i>H19</i> (liver), <i>Peg3, Kcnq1ot1, Snrpn</i> (brain)	Monallelic: <i>Igf2, Cdkn1c, Snrpn</i> Biallelic: <i>H19, Peg3, Kcnq1ot1</i>	↓ <i>H19/Igf2</i> (brain, liver) = <i>Snrpn, Peg3, Kcnq1ot1</i> (brain, liver)	↓ <i>H19/Igf2, Snrpn, Peg3, Kcnq1ot1</i>	(de Waal <i>et al.</i> , 2015)
			SO, blastocyst transfer with NSET	NA	=	↑	NA	NA	NA	= <i>H19/Igf2, Peg3</i>	
			Blastocyst transfer with NSET	NA	=	↑	NA	NA	NA	= <i>H19/Igf2, Peg3</i>	

E: Embryonic day, F: Foetus, FCS: Foetal Calf Serum, GA: Gestational Age, ICSI: Intracytoplasmic Sperm Injection, IVC: *In Vitro* Culture, IVF: *In Vitro* Fertilization, IVPS: *In Vitro* Produced with Serum, KSOM/AA: optimal potassium-modified, simplex optimized medium with aminoacids, LOM: Loss Of Methylation, NA: Not Analysed, NSET: non-surgical embryo transfer device, OVM: Oocyte *in Vitro* Maturation, RR: Resorption Rate, P: Placenta, SO: SuperOvulation, SOF: Synthetic Oviductal Fluid, mSOF: modified Synthetic Oviductal serum Fluid medium without serum or coculture, WM: Whitten's Medium, ↑: increased, ↓: decreased, =: no significant difference compared with control.

However, most of these studies have been conducted in mice models and the differences between mouse and human placentas prevent to draw reliable conclusions about what happens in humans. Indeed, the organization of the placenta differs from one species to another (**Figure 11**). In humans, a continuous layer of placental syncytiotrophoblasts formed by the fusion of cytotrophoblasts constitute the interface and site of exchange between maternal and foetal circulation.

	Species	Placental form	Structure
<b>HEMOCHORIAL</b> 	Human	Discoid	Villi
	Mouse	Discoid	Labyrinth
<b>ENDOTHELIOCHORIAL</b> 	Cat	Zonary	Labyrinth
	Dog	Zonary	Labyrinth
<b>SYNEPITHELIOCHORIAL</b> 	Cow	Cotyledonary	Villi
	Sheep	Cotyledonary	Villi
<b>EPITHELIOCHORIAL</b> 	Horse	Diffuse	Villous
	Pig	Diffuse	Folded

**Figure 11: Functional and morphological differences between mammal placentas**

Adapted from (Cornelis *et al.*, 2013). This figure gives an overview of the diversity of placental phenotypes between mammal species. The four major placental types are classified from top to bottom in the order of decreasing degrees of invasiveness. The most invasive placenta is the hemochorial one, in which the syncytiotrophoblast layer is indirect contact with maternal blood. Humans and mice are two species with such placentas but mice have a specific hemo-trichorial placenta. In endotheliochorial placentation, the syncytium layer is not in direct contact with maternal blood but is in contact with the maternal blood vessel. Synepitheliochorial placentation is characterised by cell fusion between a uterine epithelial cell and trophoblast cells, thus creating a heterologous trinucleate cell. Finally, epitheliochorial placentation is non-invasive, with the trophoctoderm being simply in contact with intact uterine epithelium.

## ***Human studies***

In this review, we also described the studies in human assessing the potential impact of ART on the epigenetic control of IGs and TEs (**Table 5**).

However, most human studies are heterogeneous, pooling different types of ART, different culture media, different duration of embryo culture, not adjusting for potential confounding factors and thus reporting conflicting results. Moreover, although some studies reported subtle differences in the methylation or expression levels of IGs in cord blood or placenta in ART-induced compared to natural conception pregnancies (Tierling *et al.*, 2010; Turan *et al.*, 2010; Zechner *et al.*, 2010; Rancourt *et al.*, 2012; Nelissen *et al.*, 2013), most of these have focused either on cord blood or placenta and have rarely combined expression and methylation analyses. None of these studies have focused on several classes of transposable elements.

The main concern is that very few studies have investigated the differences between ART techniques, and most of articles pool different ART techniques without specifying if the group includes intra-uterine insemination or ovulation induction (Turan *et al.*, 2010; Zechner *et al.*, 2010; Shi *et al.*, 2011) or if the IVF group includes fresh as well as frozen embryo transfers. However, as it has been specified above, different ART techniques could have various consequences on pregnancy (**Table 2**), and thus potentially on epigenetic regulation, which underlines the importance of studying each ART technique specifically.

Another main difference that prevents us from generalizing the results of animal models to humans is infertility itself. Indeed, a recent study has demonstrated that infertility *per se* could modify placental gene expression (Litzky *et al.*, 2017).

**Table 5: Effects of ART on imprinted genes and retrotransposable element expression and methylation in chorionic villous samples from abortion, peripheral blood, cord blood and placenta in human**

Control group	Manipulation group	Gene	Sample	Results of expression analysis		Results of methylation analysis		References
				Trends	Fold change	Trends	Differential methylation level	
30 NC	18 IVF or ICSI	KCNQ10T1	CPB	NA		MS-PCR: hypoM (3/12)		(Gomes <i>et al.</i> , 2009)
			CB			MSED-qPCR: =		
			P			=		
13 NC	10 IVF	MEST	CB	=		↑	21.8%	(Katari <i>et al.</i> , 2009)
		SLC22A2	CB	=		↓	3.0%	
		PEG10	CB	=		↓	4.2%	
		PEG3	CB	=		↓	5.2%	
		GNAS	CB	=		↓	3.0%	
		NNAT	CB	=		↓	1.6%	
		PEG3	P	=		↑	6.7%	
		MEST	P	↑	2.09	↓	1.9%	
SLC22A2	P	=		↓	7.3%			
77 NC	35 IVF	MEST	MPB / CB	NA		↑	MBP : 2.0%, CB : 3.0%	(Tierling <i>et al.</i> , 2010)
		MEST	ACM			=		
	KCNQ10T1, H19, SNRPN, GRB10, DLK1/MEG3 IG-DMR, GNAS NESP55, GNAS NESPas, GNAS XL-alpha-s, GNAS Ex1A	MPB / CB	=					
77 ICSI	35 IVF	MEST, KCNQ10T1, H19, SNRPN, GRB10, DLK1/MEG3 IG-DMR, GNAS NESP55, GNAS NESPas, GNAS XL-alpha-s, GNAS Ex1A	MPB / CB / ACM	NA		=	MBP : 3.0%, CB : 3.0%	(Tierling <i>et al.</i> , 2010)
		MEST	MPB / CB			↑		
		MEST	ACM			=		
77 ICSI	35 IVF	KCNQ10T1, H19, SNRPN, GRB10, DLK1/MEG3 IG-DMR, GNAS NESP55, GNAS NESPas, GNAS XL-alpha-s, GNAS Exon1A	MPB / CB	NA		=	MBP : 3.0%, CB : 3.0%	(Tierling <i>et al.</i> , 2010)
		MEST	MPB / CB			↑		
		MEST	ACM			=		
29 NC	24 IVF, 14 ICSI, 4 IVF or ICSI	KCNQ10T1	CVS	NA		↓	4.0%	(Zechner <i>et al.</i> , 2010)
		H19, MEG3, MEST, NESP55, PEG3, SNRPN	CVS			=		
12 NC	45 ART	H19	CB	=		=		(Turan <i>et al.</i> , 2010)
		IGF2R	CB	↓	0.61	=		
		H19	P	↓	0.72	↑ LOI		
		IGF2	P	↓	0.52	NA		
		IGF2R	P	=		=		

12 NC	32 IVF, 45 ICSI	H19	P		=		(Wong <i>et al.</i> , 2011)	
30 NC	61 ART	H19	CB	NA	=		(Shi <i>et al.</i> , 2011)	
59 NC	59 IVF	KCNQ1	CB	NA	↑	0.6%	(Rancourt <i>et al.</i> , 2012)	
		MEST, GRB10, H19, IGF2 DMR0, SNRPN	CB		=			
		SNRPN	P		↑	1.7%		
		MEST	P		↓	3.4%		
		H19	P		↓	1.3%		
		GRB10, IGF2 DMR0, KCNQ1	P		=			
27 NC	27 OI	KCNQ1	CB	NA	↑	1.3%	(Rancourt <i>et al.</i> , 2012)	
		SNRPN	CB		↑	2.1%		
		GRB10, MEST, H19, IGF2DMR0	CB		=			
		SNRPN	P		↑	2.1%		
		H19	P		↓	4.5%		
		KCNQ1, GRB10, MEST, IGF2 DMR0	P		=			
35 NC	5 IVF, 30 ICSI	MEST	P	=	↓	ND	(Nelissen <i>et al.</i> , 2013)	
		MEG3	P	NA	↓	ND		
		H19	P	↑ 1.3	↓	ND		
		IGF2	P	=	NA	(H19 CTCF6)		
		PEG3, SNRPN, KCNQ1OT1, IG-DMR	P	NA	=			
121 NC	73 ART	ALU-Yb8, LINE-1	P / CB	NA	=		(Camprubi <i>et al.</i> , 2013)	
		DIRAS3, NAP1L5, ZAC1, IGF2R, FAM50B, MEST, GRB10, PEG10, PEG13, INPP5Fv2, H19, KCNQ1OT1, RB1, MEG3, SNRPN, ZNF597, ZNF331, C19MC, PEG3, MCTS2, NNAT, L3MTBL, NESP, GNAS XL, GNAS Ex1A	P / CB	NA	=			
23 NC	73 ART	PHLDA2, GTL2, H19, ZNF331, ZNF597, C19MC, FAM50B, MEST, HYMAI, ZAC1, IGF2, KCNQ1OT1	P	Monoallelic				
8 NC	10 ART	GNAS, PLAGL1, ZIM2, DIRAS3	CB	NA	↑		(Melamed <i>et al.</i> , 2015)	
49 NC	66 ART	GRB10, H19, MEST, SNRPN	P	NA	=		(Song <i>et al.</i> , 2015)	
14 NC	17 IVF	H19	P		↑	1.79	(Sakian <i>et al.</i> , 2015)	
		IGF2			↓	0.84		
	14 ICSI	H19	P		↑	1.93		
		IGF2			↓	0.74		
17 IVF	14 ICSI	H19	P		=			
		IGF2			=			
66 NC	67 IVF	LINE-1	CB	NA	=		(Vincent <i>et al.</i> , 2016)	
		PLAGL1	CB	↓	0.65	↑		2.01%
		KCNQ1OT1	CB	↓	0.79	=		
		CDKN1C, IGF2	CB	=		NA		
		KCNQ1OT1 (=KvDMR1), PEG10, PLAGL1	P	=		=		

		LINE-1	P	NA	=		
		LINE-1	CB	NA	=		
		<i>PLAGL1</i>	CB	↓	0.74	=	
		<i>KCNQ1OT1</i>	CB	=		=	
		<i>CDKN1C, IGF2</i>	CB	=		NA	
		<i>KCNQ1OT1 (=KvDMR1), PEG10, PLAGL1</i>	P	=		=	
		LINE-1	P	NA		=	
65 NC	126 IVF/ICSI	LINE-1	P	NA	↓	0.02%	(Ghosh <i>et al.</i> , 2017)
	39 ICSI		P		↓	0.03%	
	87 IVF		P		↓	0.02%	
	73 20%O2		P		↓	0.02%	
	53 5%O2		P		=		
	90 fresh		P		↓	0.02%	
	36 frozen		P		=		
53 NC	34 IVF	<i>ATG4C</i>	CB	NA	↑	0.32%	(El Hajj <i>et al.</i> , 2017)
		<i>BAZ2B</i>	CB		=		
		<i>SNORD 1</i>	CB		=		
	41 ICSI	<i>ATG4C</i>	CB		↑	0.36%	
		<i>BAZ2B</i>	CB		=		
		<i>SNORD 1</i>	CB		=		

ACM: Amnion / Chorion Membranes, ART: Assisted Reproductive Technologies, CB: Cord Blood, COBRA: Combined Bisulfite Restriction Analysis, CPB: Child Peripheral Blood, CVS: Chorionic Villous Samples, hypoM : hypomethylation, ICSI: Intra-Cytoplasmic Sperm Injection, IVF: *In Vitro* Fertilisation, LOI: Loss Of Imprinting, MPB: Maternal Peripheral Blood, MSED-qPCR : Methylation-Sensitive Enzymatic Digestion associated with quantitative PCR method, MS-PCR: Methylation-Specific PCR, MS-SNuPE: Methylation-Sensitive Single Nucleotide Primer Extension, NA: Not Analysed, NC: Naturally Conceived, ND : Not Documented, OI: Ovulation Induction, P: Placenta, RT-qPCR: quantitative Reverse Transcription PCR, SIRPH: Single nucleotide primer extension assays in combination with ion pair Reverse Phase High performance liquid chromatography separation techniques, †: increased, ‡: decreased , =: no significant difference compared with control, \*analysed only on a subset of individuals with outrange methylation levels for 3 imprinted genes (*H19, KCNQ1, SNRPN*) but no comparisons between conception groups.

In addition, most studies are interested in DNA methylation and expression but, to the best of our knowledge, very few studies have investigated the other factors of epigenetic regulation and no studies have explored histone regulation in humans after ART.

Indeed, most studies focusing on histone regulation have been conducted in mouse models and mainly in pre-implantation embryos. For example, a study profiling epigenetic modification at the *Mest* and *H19* loci in mouse blastocysts found an increased abundance of permissive histone marks and a decrease in repressive histone modifications (Jahangiri *et al.*, 2014). Similarly, altered methylation of histones and DNA at the *H19/Igf2* region has also been shown in embryonic stem cells from mice pre-implantation embryos (Li *et al.*, 2005).

All in all, most of the authors agree that ART has an effect on epigenetic control, but without determining the exact cause and mechanisms of these changes or how to avoid them. Thus we considered that it could be valuable to study methylation, expression and histone modifications in IGs and TEs in human placenta and cord blood in humans after ART.

#### 6) Long-term consequences

The numerous phenotypic, ultrastructural, metabolic and epigenetic modifications affecting placenta after ART raise the issue of the potential long-term consequences of such changes. Indeed, as stated by the DOHaD (Developmental Origin of Health and Diseases), modified maternal-foetal interactions *in utero* might have consequences for outcomes in infancy and even in adulthood (Gillman, 2005; Barker, 2007). For example, the size and shape of the placenta have been related to life expectancy in men (Barker *et al.*, 2011) and their risk for coronary heart diseases

(Eriksson *et al.*, 2011). In addition, in humans, new-borns that are either too small or too big may be vulnerable to heart disease, hypertension, type 2 diabetes and obesity (Simmons *et al.*, 2001; Gluckman *et al.*, 2008; Simmons, 2009). Although the majority of these children are healthy, the available data about long-term follow-up revealed cardiovascular and metabolic risk factors (Hart *et al.*, 2013). Notably, they may exhibit increases in peripheral adipose tissue mass, in blood pressure, in fasting glucose levels, and IGF-I and -II as well as changes in the lipid profile (Ceelen *et al.*, 2007; Miles *et al.*, 2007; Ceelen *et al.*, 2008; Ceelen *et al.*, 2009; Sakka *et al.*, 2010; Vrooman *et al.*, 2017). A recent review confirmed the increased risk of cardiometabolic diseases in individuals conceived by ART (Vrooman *et al.*, 2017).

Portha *et al.* proposed that the link between the prenatal environment and adverse long-term effects could be written through epigenetic modifications of the conceptus (Portha *et al.*, 2014). Epigenetic marks are dynamic and altered by the prenatal environment. This developmental plasticity aims to tune the individual's gene expression to suit its current environment. However, if the resulting phenotype does not match with the post-natal environment, disease may result, with a level of susceptibility that increases according to the degree of mismatch (Portha *et al.*, 2014; Barouki *et al.*, 2018).

All in all, this comprehensive review of the current literature regarding the potential impact of ART on the epigenetic control of IGs and TEs raise three main concerns, which inspired the three main objectives of our work.



#### IV. Objectives

1/ Numerous animal studies have proven the impact of ART on epigenetic regulation but human placenta has very different anatomy and functioning and is not comparable to any animal placenta. Studies on human placenta exist but most of them suffer from heterogeneous design that prevents the author from drawing solid conclusions. Moreover, the regulation of TEs after ART remains little explored so far. Thus, the first objective of this work was to address the effects of ART on the epigenetic setting of the conceptus. By including a very homogeneous cohort with exhaustive biological and clinical data, we studied various IGs either maternally (*KCNQ1OT1* and *SNURF*) or paternally imprinted (*H19/IGF2*), and four TE families (*AluYa5*, *LINE-1Hs*, *ERVFRD-1* and *ERVW-1*) in placenta and cord blood. We also addressed the expression of genes associated with the same DMRs (*KCNQ1*, *SNRPN* and *H19*, respectively) and TEs (*AluYa5*, *LINE-1 ORF2*, *syncytin-2* and *syncytin-1*, respectively).

2/ Some studies in the mouse model have investigated the regulation of histones in the context of ART but, to the best of our knowledge, this issue have not been studied in humans. Thanks to high quality placenta samples, and by studying both active and repressive histone marks, we expect to better understand the histone regulation in placenta after ART and its link with DNA methylation and expression.

3/ Animal studies have shown that each step of ART - hormone treatments, gamete and embryo manipulation and even embryo transfer alone - can disturb the epigenetic regulation. However, in humans, as studies usually pool different techniques and groups of patients and as the infertility factor has to be taken into account, it is difficult to unravel the exact causes and consequences of each manipulation. By including different groups of ART patients (fresh embryo transfer after ovarian hyperstimulation, frozen-thawed embryo transfer, different times of embryo culture) and even different groups of natural pregnancy patients (stratified on

the time to pregnancy, from fertile patients to infertile), we have intended to better understand which steps were more at risk of epigenetic changes and the potential ways to counterbalance these effects.

*In fine*, the objectives of this work are to comprehensively describe the epigenetic modifications of some imprinted genes and transposable elements induced by ART in the placenta and cord blood at birth. We will also address the potential effects and/or consequences of these modifications on a clinical basis, on the obstetrical and perinatal outcomes and even on long-term effects. In the end, by studying different techniques of ART and even fertile patients with longer or shorter time to pregnancy, the aim is to bring a better understanding of the causes of these dysregulations (infertility, hormonal treatments, gamete and embryo manipulation...). In the future, the complete understanding of the causes and consequences of ART on epigenetic regulation will enable to minimize these variations in the interest of full epigenetic safety after ART.

***MATERIALS  
AND  
METHODS***

## MATERIALS AND METHODS

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### I. Conçue Cohort

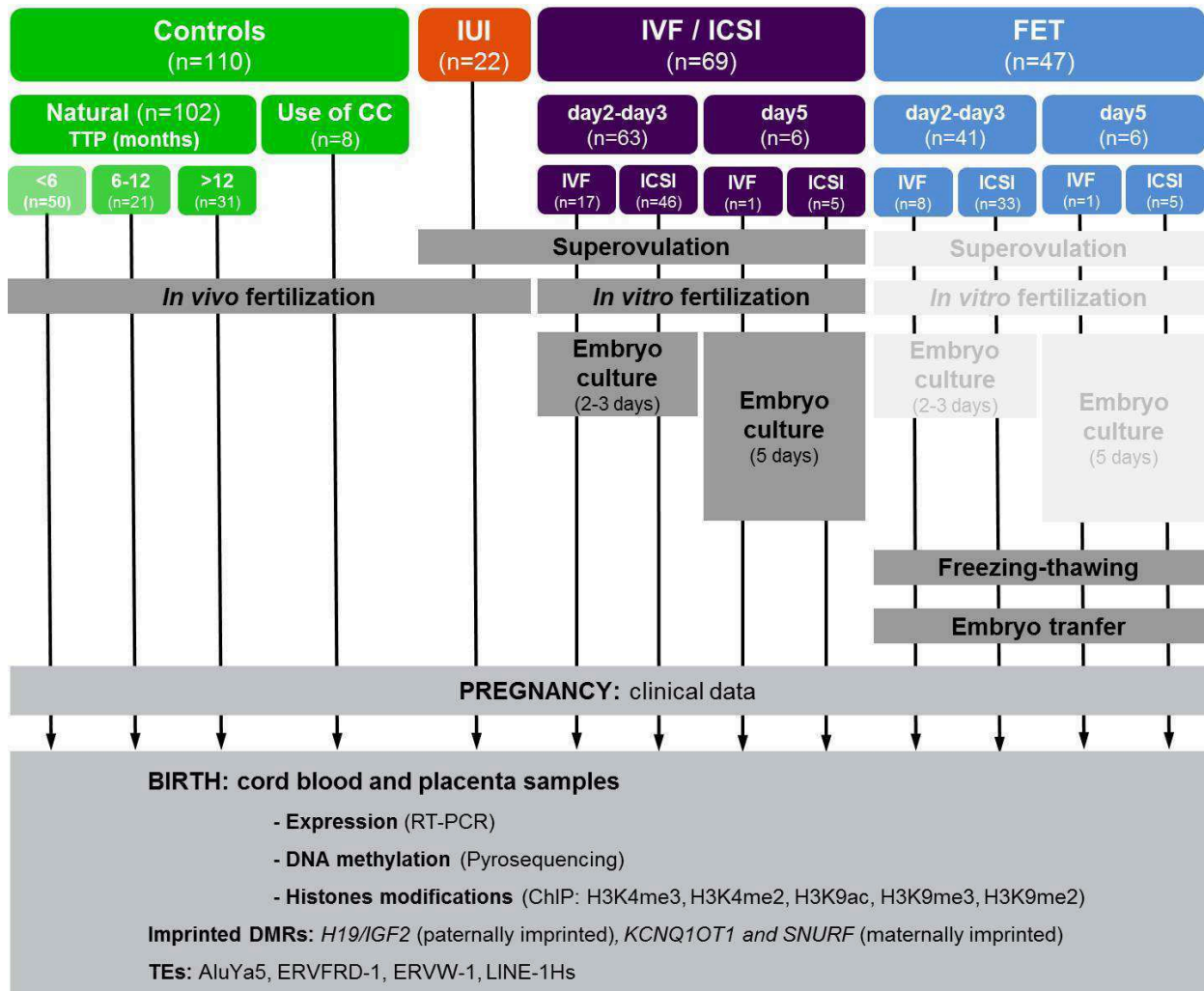
Patients from “Conçue’s cohort” were prospectively included from 1<sup>st</sup> January 2013 to 31<sup>st</sup> October 2018 in the department of Obstetrics, Gynecology and Reproductive Biology at Dijon University Hospital, France.

At the time of conception, the mothers’ and fathers’ ages ranged from 20 to 43 and 18 to 50 years, respectively. Exclusion criteria were: pregnancy resulting from oocyte and sperm donation; fetuses with an abnormal karyotype; and maternal neurological, cardiac or pulmonary disorders, diabetes, hypertension HIV, hepatitis B or C infections.

All women had given written informed consent in accordance with the declaration of Helsinki. The study was approved by the Institutional Review Board and the ethic Committee of Dijon University Hospital (*Comité de Protection des Personnes [CPP] Est I, n°2012-A01010-43*)

**Figure 12** presents the flowchart of the cohort.

A total of 248 women were included. The characteristics of the cohort are presented in **Annex 1**.



**Figure 12: Flowchart of Conçue's cohort**

Controls were singleton pregnancies achieved following natural conception. They were further divided according to the TTP.

IVF/ICSI patients were those with singleton pregnancies achieved following fresh embryo transfer.

FET patients were those with singleton pregnancies achieved following frozen-thawed embryo transfer. These patients were conditioned in artificial cycles with a sequential protocol with a first phase with oestrogens alone started on day-2 of the menstrual cycle. Uterus was assessed with transvaginal ultrasound at day-18; if endometrium >7mm, a second phase with estrogens + intravaginal progesterone was started and pursued until 12WG.

IVF, ICSI and freezing/thawing protocols were standardized (Desch *et al.*, 2017).

FET: frozen-thawed embryo transfer; ICSI: Intra-cytoplasmic sperm injection; IUI: Intra-uterine insemination; IVF: *In vitro* fertilization; TTP: Time to pregnancy

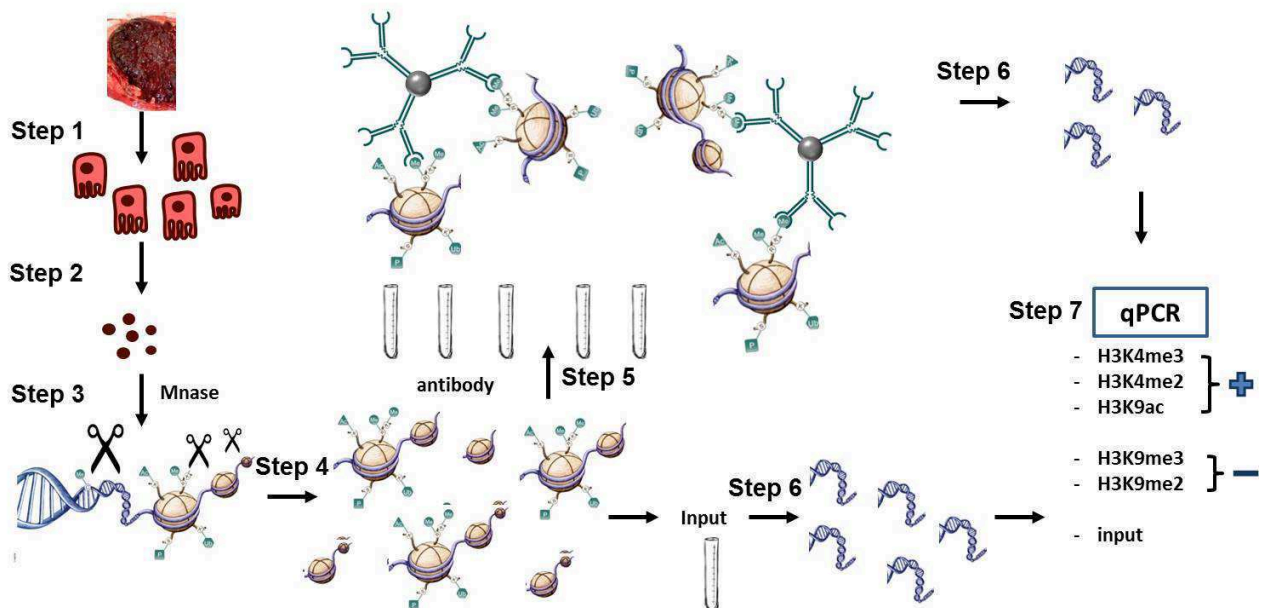
## II. Methylation and expression analyses

For clinical data collection, samples preparation, quantitative DNA methylation analyses and expression analyses, see Materials and methods in Article 2.

## III. Post-translational histone modifications analyses by Chromatin ImmunoPrecipitation (ChIP)

For the three DMRs previously analysed (*H19/IGF2*:IG-DMR, *KCNQ1OT1*:TSS-DMR, and *SNURF*:TSS-DMR, we quantified specifically three activating histone marks (H3K4me2/3: di/trimethylation of lysine 4 of histone H3 and H3K9ac: acetylation of lysine 9 of histone H3) (Umlauf *et al.*, 2004) and two inhibiting histone marks (H3K9me2/3: di/trimethylation of lysine 9 of histone H3) (Monk *et al.*, 2006; Jahangiri *et al.*, 2014).

ChIP was performed as previously described, with some adaptations (Umlauf *et al.*, 2004; Umlauf *et al.*, 2004; Monk *et al.*, 2006) (**Figure 13**).



**Figure 13:** Technique for Chromatin ImmunoPrecipitation (ChIP)

### ***Preparation of chromatin from placenta samples***

**Step 1:** Approximately 2 grams of frozen placenta was rinsed two times in cold PBS and placed in lysis tubes (Zymo Research BashingBeads Lysis Tubes - 0.5 mm) containing 1 mL buffer I (0.5 M Tris-HCl pH 7.5, 0.5 M KCl, 2.5 M NaCl, 0.5 M MgCl<sub>2</sub>, 25 mM EGTA, 0.3 M sucrose, 0.5 mM DTT, 0.1 mM PMSF, 3.6 ng/mL aprotinin, 5 mM sodium butyrate) and subject to three intervals of agitation (90 sec, 5000 rpm) using a Precellys24 homogenizer (Bertin technologies) with 5 minutes on ice between each agitation cycle.

**Step 2:** The cell suspension was then placed in 7 mL of buffer II (buffer I with NP40 at a final concentration of 0.2%) to purify nuclei by centrifugation at 8500 rpm/12720g for 20 minutes with low acceleration and low deceleration on a sucrose gradient (8 mL from the previous step carefully placed on 25 mL of buffer III (0.5 M Tris-HCl pH 7.5, 0.5 M KCl, 2.5 M NaCl, 0.5 M MgCl<sub>2</sub>, 25 mM EGTA, 1.2 M sucrose, 0.5 mM DTT, 0.1 mM PMSF, 3.6 ng/mL aprotinin, 5 mM sodium butyrate) in Sorvall™ RC 6 Plus Centrifuge (ThermoScientific™). The nuclear pellet was resuspended in digestion buffer (0.32 M sucrose, 50 mM Tris-HCl pH 7.5, 4 mM MgCl<sub>2</sub>, 1mM CaCl<sub>2</sub>, 0.1 mM PMSF, 5mM sodium butyrate) to 0.4 mg DNA/mL (Quantification by absorbance). Aliquots of 500 µL were distributed in 1.5 mL tubes.

**Step 3:** Micrococcal nuclease (Nuclease S7 15 IU/µL, Roche; final concentration of 30 mIU/µL) was used to digest the chromatin to yield fragments one to five nucleosomes in length, which typically presented an incubation time of 3 minutes at 37°C. Digestion was stopped by adding 0.5 M EDTA at a final concentration of 20 mM and cooling on ice. After centrifugation (10 min, 15800g, 4°C), the supernatant was designated fraction S1. The pellet was resuspended in 500µL lysis buffer (1 mM Tris-HCl pH 7.5, 0.2 mM EDTA, 0.2 mM PMSF, 5 mM sodium butyrate) and left 20-30 minutes on ice and subjected to a second centrifugation step (10 min, 15800g, 4°C), the supernatant of which was designated fraction S2.

### ***Immunoprecipitation of fresh chromatin***

**Step 4:** For ChIP, we used antibodies directed against H3K4me3 (Diagenode C15410003-50), H3K4me2 (Millipore 07-030), H3K9ac (Cell Signaling 9649S), H3K9me3 (Abcam AB8898), H3K9me2 (Diagenode C15410060) and a negative control (mock precipitation with mouse IgG Millipore 12-371).

Chromatin was quantified by absorbance. For each condition, 4 µg of chromatin was used (constituted of 75% S1 and 25% S2) and suspended in incubation buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 20 mM sodium butyrate, 5 mM EDTA, 0.1 mM PMSF) in a total volume of 500 µL for each condition. We precleared chromatin by agitating overnight at 4 °C with 4% Dynabeads ® Protein G for immunoprecipitation (Invitrogen) washed three times in PBS-BSA 5%. In parallel, antibodies were combined to Dynabeads ® Protein G for immunoprecipitation (Invitrogen), each antibody being agitated overnight in 250 µL of PBS-BSA 5% containing 16% of beads previously washed three times in PBS-BSA 5%.

The following day, beads were removed from precleared chromatin and antibodies-beads complexes were washed two times in PBS-BSA 5%.

**Step 5:** ChIP was then carried out for 4h at 4 °C.

The antibody-chromatin complexes were subsequently washed three times with each buffer A (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 5 mM sodium butyrate, 75 mM NaCl), B (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 5 mM sodium butyrate, 125 mM NaCl) and C (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 5 mM sodium butyrate, 175 mM NaCl) to ensure only the fraction of chromatin linked to the antibodies was retained. Elution was performed in 400 µL of elution buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM EDTA, 1% SDS).

**Step 6:** After a digestion with proteinase K (100 µg/mL) for 1 hour at 65 °C, DNA was obtained from the input and bound fractions with Nucleospin gel and PCR clean-up (Macherey-Nagel), according to the manufacturer's protocol (protocol for samples SDS rich for bound fractions) with a final elution with 40 µL of water.

### ***Quantification of immunoprecipitated chromatin***

**Step 7:** Levels of immunoprecipitated chromatin at each region of interest were determined by quantitative real-time PCR amplification with the QuantStudio™ 5 Real-Time PCR system (Applied Biosystems™), using the SYBR™ Green PCR Master Mix (Applied) (see **Table 6**). Data were analysed with QuantStudio™ Design & Analysis Software (v1.3.1). Each PCR was run in triplicate and level of immunoprecipitation was quantified as a percentage of total input material as follows: % of input =  $2^{(-\Delta Ct)}$  where  $\Delta Ct$  is the difference in mean Ct triplicate between the DNA of interest and the DNA of the input.



To overcome the inherent variability of different immunoprecipitations, precipitation levels obtained at the region of interest were normalised to the level obtained for positive control intervals. Interrogation of placenta CHIP-seq datasets in the Genome Data viewer function in the GEO data repository revealed that the promoter of KLF10 was enriched for the permissive histones marks H3K4me3, H3K4me2 and H3K9ac and was selected as a control region. For a control of repressed chromatin, we selected a heterochromatic satellite region on chromosome 4 which is ubiquitously associated with both H3K9me3 and H3K9me2 (**Table 6**). The figures present the ratio between the % of input obtained at the region of interest and the % of input obtained at the control region.

**Table 6: Primers for qRT-PCR ChIP**

Region	Data base Reference	Sequence Number Nucleotide position	Primers	Product Reaction temperature
<b>Housekeeping genes</b>				
<i>GAPDH</i>	Ensembl	Ensembl ENSG00000111640 hg38: chr12:6,533,927-6,538,374 forward strand	F: CAATCCCCATCTCAGTCGT R: GCAGCAGGACACTAGGGAGT	89 pb 60°C
<i>KLF10</i>	Ensembl	Ensembl ENSG00000155090 hg 38: chr8:102,648,779-102,655,902 reverse strand	F: GACAAGACCAGGCGAGGAAG R: GCCAACCATGCTCAACTTCG	89 pb 60°C
<i>RPL19</i>	Ensembl	Ensembl ENSG00000108298 hg 38: chr17:39,200,283-39,204,727 forward strand	F: ATGAGTGAGTTTGTGGCAGA R: CAGCGAAAGGAAAGAGCTCGCTC	132 pb 60°C
<i>SAT2</i> chr1	NCBI Alexiadis <i>et al.</i> , 2017	X72623	F: CATCGAATGGAATGAAAGGAGTC R: ACCATTGGATGATTGCAGTCAA	160 pb 60°C
<i>SAT<math>\alpha</math></i> chr4	NCBI Alexiadis <i>et al.</i> , 2017	M38467	F: CTGCACTACCTGAAGAGGAC R: GATGGTTCAACTCTTACA	139 pb 60°C
<b>Repeats</b>				
ERVFRD-1 LTR	Ensembl	ENSG00000244476 hg38: chr6:11,102,489-11,111,732 reverse strand	F: GGTGCAGTGA CTGGATAACC R: AACCACGTACGAGGGTTGAA	90 pb 60°C
ERVW-1 LTR	Ensembl	ENSG00000242950 hg38: chr7:92,468,380-92,477,986 reverse strand	F: TTTTCTGGGGAAGCCGAGG R: ATGGGCGACTGTTGAGACTT	110 pb 60°C
ERVW-1 IN	Ensembl	ENSG00000242950 hg38: chr7:92,468,380-92,477,986 reverse strand	F: CTCCTCTTGGACCCTGTATCTTTA R: GCATCTTGGCTCCATTTGAGTTT	91 pb 60°C
<b>Imprinted genes</b>				
<i>H19/IGF2: IG-DMR</i>	UCSC	hg38: chr11:1,997,582-2,003,510	F: AGCTGTGCTCTGGGATAGATG R: ATGATCACAGTGTGTTCCACC	60 pb 60°C
<i>KCNQ1OT1: TSS-DMR</i>	UCSC	hg38 : chr11:2,698,718-2,701,029	F: ATTTCCGACTCCGGTCCCAA R: CATCGTGGTTCTGAGTCCGC	94 pb 60°C
<i>SNURF: TSS-DMR</i>	UCSC	hg38 : chr15:24,954,857-24,956,829	F: CTGTGCTACTGCCCTTCTG R: GGAGTGACTAAGGACGCTGAATG	68 pb 60°C

4.5  $\mu$ L 2X SYBR<sup>TM</sup> Green PCR Master Mix (Applied) was used with 0.1  $\mu$ L primers (0.1  $\mu$ g/ $\mu$ L), 0.4  $\mu$ L water and 5  $\mu$ L DNA (diluted 1/40), for a final volume of 10  $\mu$ L. Amplification was performed in triplicate using QuantStudio<sup>TM</sup> 5 Real-Time PCR system (Applied Biosystems<sup>TM</sup>) with the following conditions: 10 min denaturation phase at 95°C, followed by 40 cycles of two steps: 15 s denaturation at 95°C and 1 min annealing/extension at 60

#### IV. Statistical analyses

Categorical variables were expressed as numbers (percentages) and compared using the Chi-2 test or Fisher exact test when appropriate. Continuous variables were expressed as means  $\pm$  standard deviations (SD) or medians and interquartile ranges [IQR], and compared using the Student or Mann-Whitney test, as appropriate. Correlations between continuous variables were estimated using Spearman's rank correlation coefficients. Birth weights were normalized by conversion to z-scores calculated using normal birthweight curves of our population accounting for gestational age and new-born's sex (Rousseau *et al.*, 2008). Placental weights were also converted into z-scores according to gestational age and new-born's sex (Thompson *et al.*, 2007). As recommended by Hogg *et al.* (Hogg *et al.*, 2014), multivariate analyses were adjusted for gestational age at delivery, the new-born's sex, parity and maternal age. Multiple regression linear models were used, after log-transformation of the dependent variable in the absence of normal distribution. All statistical analyses were performed with SAS software, version 9.4 (SAS Institute Inc, USA). A two-tailed  $P < 0.05$  was considered significant.

# ***RESULTS***

# RESULTS

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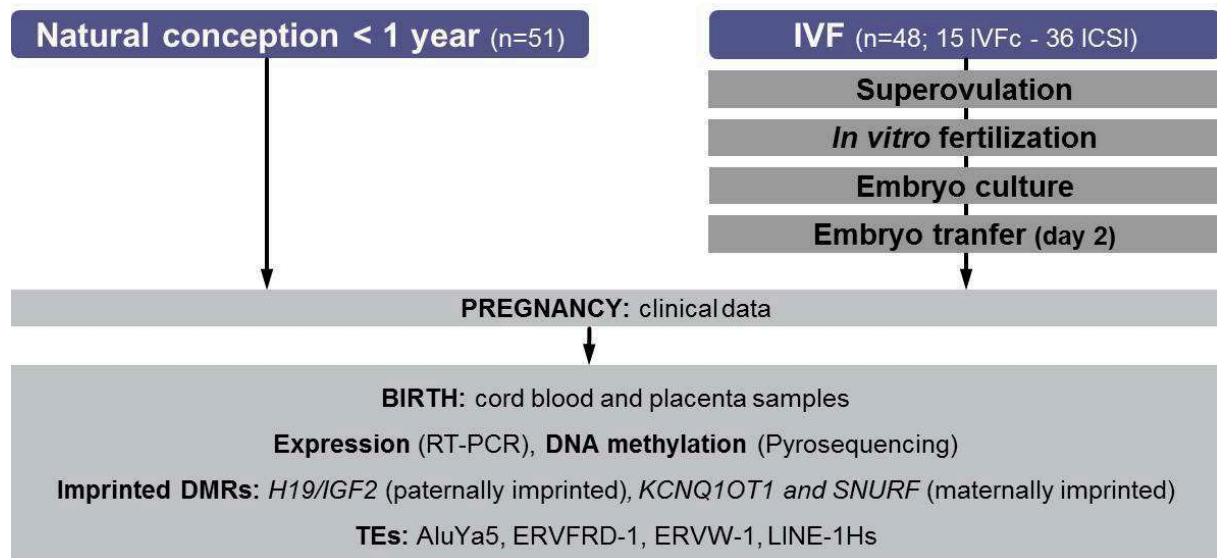
## I. METHYLATION AND EXPRESSION

### 1) Introduction: the impact of ART on methylation and expression of IGs and TEs

We hypothesized that ART could affect the settlement of important developmental processes through epigenetic changes of the conceptus, thus potentially triggering placenta-related adverse outcomes (Choux *et al.*, 2015). Although studies on animal models have proven the link between IVF and epigenetic dysregulation (**Tables 3 and 4**), the human placenta has very different anatomy and functioning and is not comparable to any other animal placenta. Because most studies on human placenta suffer from heterogeneous design that prevents the author from drawing solid conclusions and the regulation of TEs after ART remains little explored so far, the first objective of this work was to investigate whether ART could trigger epigenetic modifications of IGs and TEs in placenta and potentially in cord blood in human.

2) **Article 2:** “The epigenetic control of transposable elements and imprinted genes in new-borns is affected by the mode of conception: ART versus spontaneous conception without underlying infertility”

This study, whose design is presented in **Figure 14**, revealed the occurrence of changes in the epigenetic regulation of several IGs and TEs in placenta, which could serve as proof of some compensation mechanisms developed after ART, possibly due to some prior dysfunctions at the beginning of the pregnancy.



**Figure 14: Design of the first study (Article 2)**

In this study, we compared a very homogeneous cohort of patient with singleton pregnancy achieved following IVF/ICSI with fresh embryo transfer at day-2 and controls with singleton pregnancy obtained within one year after stopping contraception. The prospective design enabled to obtain exhaustive biological and clinical data

Indeed, the hypomethylation of 2 IGs which are part of the IGN (*H19/IGF2* and *KCNQ1OT1*) clearly illustrates the plasticity of the placenta, the upregulation of some genes probably used to compensate the failure of other mechanisms. Besides, the lack of expressional differences at birth and the negative correlation found between H19 expression and gestational age at delivery could witness the dynamic adaptation

occurring throughout pregnancy. Indeed, as the placenta undergoes rapid epigenomic changes, a placenta collected at birth may not reflect the changes occurring throughout pregnancy (Barouki *et al.*, 2018). Based on animal studies that demonstrated dynamic changes throughout pregnancy (Fortier *et al.*, 2008; Fortier *et al.*, 2014), we can postulate that, depending on the severity of the primary injury and the efficacy of compensation mechanisms involved, modifications in levels of expression may have return to normal at birth or persist.

Moreover, concerning retroviruses, DNA methylation and expression level of ERVFRD-1 in placenta was lower in the IVF/ICSI group than in the control group; expression of ERVFRD-1 in placenta correlated positively with birth weight and placental weight only in the control group and the expression level of ERVFRD-1 in placenta correlated negatively with the gestational age at delivery in the IVF/ICSI group. Altogether, these data suggest that compensatory mechanisms implicating syncytins, such as an increase in cell fusion in placenta, could play a fundamental role in placental physiology, could be promoted in IVF/ICSI by hypomethylation of ERVFRD-1 promotor and could be exhausted by the end of the pregnancy following ART.

In the light of these findings, seeing as TEs and IGs are known to be involved in foetal and placental development, their dysregulation by ART could partially explain the increased rate of placenta-related adverse pregnancy outcomes following ART. To our knowledge, this is the first report of the impact of ART on the regulation of syncytins and the first study to address both the methylation and expression of TEs and IGs in human cord blood and placenta at birth.

# The epigenetic control of transposable elements and imprinted genes in newborns is affected by the mode of conception: ART versus spontaneous conception without underlying infertility

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**STUDY QUESTION:** Do assisted reproductive technologies alter DNA methylation and/or transcription of transposable elements and imprinted genes in cord blood and placenta?

**SUMMARY ANSWER:** After ART, DNA methylation and/or transcription changes of some transposable elements and imprinted genes were found in placenta samples while transcription modifications for some transposable elements were also discovered in cord blood.

**WHAT IS KNOWN ALREADY:** Recent studies have confirmed the increased risk of placenta-related adverse pregnancy outcomes and the excess of imprinted disorders with abnormal methylation patterns after ART, which raises the issue of a potential ART-induced epigenetic risk.

**STUDY DESIGN, SIZE, DURATION:** A total of 51 IVF/ICSI (15 conventional and 36 ICSI) singleton pregnancies were prospectively included from January 2013 to April 2015 and compared to 48 spontaneously conceived singleton pregnancies.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** The DNA methylation and transcription of three imprinted loci (*H19/IGF2*, *KCNQ1/OT1* and *SNURF* DMRs) and four transposon families (LINE-1, ERVFRD, AluYa5 and ERVW) in cord blood and placenta obtained at birth were assessed by pyrosequencing and quantitative RT-PCR, respectively. All data were adjusted for gestational age at delivery, sex of the newborn, parity and maternal age.

**MAIN RESULTS AND THE ROLE OF CHANCE:** DNA methylation levels of *H19/IGF2*, *KCNQ1/OT1*, LINE-1Hs and ERVFRD-1 were significantly lower in IVF/ICSI placentas than in control placentas, while there was no difference for cord blood. Moreover, the expression of ERVFRD-1 and LINE-1 ORF2 in cord blood and ERVFRD-1 in placenta was lower in the IVF/ICSI group than in controls. The expression of ERVFRD-1 in placenta correlated positively with birth weight and placenta weight, but only in the control group, thus pointing to the potential deregulation of syncytin function after ART.

**LARGE SCALE DATA:** N/A.



**LIMITATIONS, REASONS FOR CAUTION:** The control group of fertile couples having conceived within 1 year prevented us from deciphering the distinct roles of ART and infertility.

**WIDER IMPLICATIONS OF THE FINDINGS:** These novel findings of ERVFRD (syncytin-2) expression correlating with birth weight and placenta weight suggest that more research on syncytins and pregnancy-associated diseases could lead to them being used as biomarkers or even as therapeutic targets. The epigenetic modifications in placenta for sequences involved in foetal development raise the question of their potential effects on pregnancy and future life. These results should encourage us to analyse the exact causes and consequences of epigenetic changes and strive to minimize these variations in the interests of epigenetic safety after ART.

**STUDY FUNDING/COMPETING INTEREST(S):** The study was funded by a grant from Besançon and Dijon University Hospitals. The authors have no conflicts of interest to declare.

**Key words:** assisted reproductive technologies / imprinted genes / DNA methylation / birth / transposable elements

## Introduction

It is currently estimated that more than 5 million children have been born by assisted reproductive technologies worldwide (Hyrapetian et al., 2014). Since almost 10% of couples are infertile (Boivin et al., 2007), providing them with safe techniques to reach parenthood is a public health issue. However, ART has been associated with increased incidence of placenta-related adverse pregnancy outcomes, such as preeclampsia, intrauterine growth restriction (IUGR) and placenta praevia, even in singleton pregnancies of nulliparous women (Qin et al., 2016; Zhu et al., 2016). Adverse perinatal outcomes, a higher risk of major malformations and imprinting disorders have also been reported in children born following ART (Hansen et al., 2005; Pinborg et al., 2013; Lazaraviciute et al., 2014). These issues combined raise the question of a potential ART-induced epigenetic risk.

ARTs are used in the context of gametogenesis and preimplantation embryogenesis, which coincide with the establishment of genomic imprinting and transposable element (TE) control, through DNA methylation and other epigenetic modifications. Imprinted genes (IGs) are essential for regulating embryonic and placental development, through the control of nutrient exchange, metabolic processes and placental signalling (Choux et al., 2015; Moore et al., 2015). TEs are repeated sequences, representing around 50% of the human genome, and are potentially harmful if not properly controlled (Fauque and Bourc'his, 2014). Human Endogenous Retroviruses (HERVs) are mostly immobile remnants of ancient elements but Long and Short Interspersed Nucleotide Elements (LINE and SINEs) can still generate *de novo* transposition events. In all cases, TEs can have important functions as cis regulatory modules (enhancers or promoters) for nearby genes (Hu et al., 2017). Some studies have highlighted that preimplantation embryos could be particularly prone to transcriptional TE activation (Kano et al., 2009) and retrotransposition (van den Hurk et al., 2007). LINE-1 loses methylation and gains expression in the placenta between the first and the third trimester (He et al., 2014). Even more strikingly, specific ERVW and ERVFRD copies have been domesticated for their ability to produce fusogenic retroviral envelope proteins, the syncytins, which are essential for the formation of the syncytiotrophoblast (Bolze et al., 2017). Altogether, there is strong evidence to suggest that TEs could be involved in placental functions and subsequent control of foetal growth and development.

We hypothesized that ART could affect the settlement of important developmental processes through epigenetic changes (Choux et al., 2015),

thus triggering placenta-related adverse outcomes with potential long-term effects. This hypothesis was recently confirmed in mice (De Waal et al., 2015). However, although some studies have reported subtle differences in the methylation or expression levels of IGs in human cord blood or placenta in ART-induced compared with spontaneous conception pregnancies (Tierling et al., 2010; Turan et al., 2010; Zechner et al., 2010; Rancourt et al., 2012; Nelissen et al., 2013; Vincent et al., 2016), most of these have focused either on cord blood or placenta and have rarely reported combined expression and methylation analyses. Moreover, few studies have been interested in comparing conventional IVF to IVF with ICSI (Wong et al., 2011; Sakian et al., 2015; Vincent et al., 2016).

To conclusively address the effect of ART on the epigenetic setting of the conceptus, we conducted a prospective study in which IGs and TEs were analysed for their DNA methylation and transcriptional levels in cord blood and placenta at birth, and compared the findings in singleton pregnancies obtained after IVF with those obtained following spontaneous conception pregnancies. We found methylation and/or transcription changes of some TEs and IGs in ART placenta samples, which could indicate an altered placental epigenetic regulation resulting from ART.

## Materials and Methods

### Study population

Patients were prospectively included from 1 January 2013 to 30 April 2015 in the Department of Obstetrics, Gynaecology and Reproductive Biology at Dijon University Hospital, France. Controls were singleton pregnancies achieved following spontaneous conception within 1 year after stopping contraception. IVF/ICSI patients were those with singleton pregnancies achieved following fresh embryo transfer after 2 days of *in vitro* culture. ICSI and IVF protocols were standardized (Desch et al., 2017). At the time of conception, the mothers' and fathers' ages ranged from 20 to 43 and 18 to 50 years, respectively. Exclusion criteria were: pregnancies resulting from oocyte or sperm donation; fetuses with an abnormal karyotype; and maternal neurological, cardiac or pulmonary disorders, diabetes, hypertension, HIV or hepatitis B or C infections.

### Clinical data collection

Clinical data concerning the mother's and father's history and treatments were collected prospectively during the ART procedures, throughout the pregnancy and after birth. Importantly, diseases occurring during

pregnancy, as well as the characteristics of the placenta and the newborn at birth (weight, birth defects and neonatal data) were exhaustively recorded.

## Sample preparation

Biological samples were collected within 15 min after delivery. Placenta samples (1 cm<sup>3</sup>) were extracted from the foetal side near the umbilical cord insertion point, and washed twice in 0.9% NaCl before being fragmented and immersed in RNAlater RNA Stabilization Reagent<sup>®</sup> (Qiagen, USA) until RNA or DNA extraction. Briefly, RNA was extracted from approximately 100 mg of placenta using TRI Reagent<sup>®</sup> (Sigma-Aldrich, USA), according to the manufacturer's protocol with an additional DNase digestion step (Ambion<sup>®</sup> TURBO DNA-free™, Invitrogen, USA). Blood RNA was extracted using the PAXgene Blood RNA kit<sup>®</sup> (Qiagen) from cord blood collected in PAXgene blood RNA Tubes<sup>®</sup> (PreAnalytiX, Switzerland), according to the manufacturer's protocol, which includes a DNase digestion step. DNA was extracted from umbilical cord blood and placenta samples using a salting out method (Bruno et al., 2015).

## Quantitative DNA methylation analyses

Three differentially methylated regions (DMRs) of IGs (*H19/IGF2*:IG-DMR, *KCNQ1/OT1*:TSS-DMR and *SNURF*:TSS-DMR, named according to the recommendations for nomenclature; Monk et al., 2016) and four TE families (AluYa5, LINE-1Hs, ERVFRD-1 and ERVV-1) were studied by pyrosequencing. The DNA methylation assays investigated 3, 9, 7, 3, 3, 6 and 3 CpG sites, respectively (Table S1); each one included a conversion bisulphite treatment control and was tested with a DNA methylation scale (0–25–75–100%) obtained with EpiTect<sup>®</sup> Control bisulphite-converted unmethylated/methylated DNA (human). Primers are available in Table S1. Bisulphite conversion of genomic DNA and pyrosequencing analysis were performed as previously described (Bruno et al., 2015). Briefly, genomic DNA (750 ng) was modified using the EpiTect Bisulfite Kit<sup>®</sup>

(Qiagen). Bisulphite-treated DNA (37.5 ng) was used as the template for PCR amplification. Pyrosequencing reactions were performed in a PyroMark Q24 MDx<sup>®</sup> system (Pyrosequencing AB, Sweden) with the PyroGold Reagents kit<sup>®</sup> (Qiagen). The DNA methylation level was calculated as the ratio of the C to T peaks at a given CpG site using PyroMark<sup>®</sup> Q24 Software v.2.0.6 (Qiagen). To overcome potential between-plate variability, a common control was placed in each plate of pyrosequencing and the other DNA methylation values were normalized in accordance with this control.

## Expression analysis

Real time-PCR was used to study the expression of genes associated with three DMRs (*H19* for *H19/IGF2* DMR, *KCNQ1* for *KCNQ1/OT1* DMR and the common *SNRPN* and *SNURF* exons for *SNURF* DMR) and three TEs (the LINE-1 protein, ORF2; the envelope proteins of the two retroviruses ERVFRD-1 and ERVV-1, syncytin-2 and -1, respectively, and the sequence AluYa5). Expression was normalized on three housekeeping genes (*GAPDH*, *SDHA* and *TBP*).

cDNA was synthesized using Maxima Reverse Transcriptase (Thermo Scientific<sup>®</sup>). The level of expression of each IG or TE (X) was normalized to the geometric mean of expression levels of three housekeeping genes, according to the formula:  $X/\text{geometric mean}(R1, R2, R3) = 2^{(\text{Ct}[X] - \text{arithmetic mean}[\text{Ct}(R1), \text{Ct}(R2), \text{Ct}(R3)])}$ , where Ct is the threshold cycle, and R1, R2, R3 are the housekeeping genes. Data were analysed with Bio-Rad CFX Manager (Version 3.0.1224.1015). Primers and conditions are listed in Table SII.

## Statistical analyses

Categorical variables were expressed as numbers (percentages) and compared using the  $\chi^2$  or Fisher exact test when appropriate. Continuous variables were expressed as means  $\pm$  standard deviations (SD) or medians and interquartile ranges [IQR], and compared using the Student or Mann–Whitney test, as

**Table 1** Characteristics of the population by type of conception.

	Spontaneous (n = 48)	IVF/ICSI (n = 51)	IVF (n = 15)	ICSI (n = 36)
Maternal characteristics				
Age (years)	29.4 $\pm$ 4.0	31.1 $\pm$ 5.3	29.77 $\pm$ 5.0	31.7 $\pm$ 5.4
Pre-pregnancy BMI (kg/m <sup>2</sup> )	22.4 $\pm$ 4.1	23.3 $\pm$ 4.1	24.04 $\pm$ 4.9	23.1 $\pm$ 3.8
Pre-pregnancy parity	1 [0–1] <sup>a,b</sup>	0 [0–1] <sup>a</sup>	0 [0–1] <sup>b</sup>	0 [0–0.5] <sup>b</sup>
Tobacco smoking	4 (8.3)	6 (11.8)	2 (13.3)	4 (11.1)
Paternal characteristics				
Age (years)	31.9 $\pm$ 5.2	33.7 $\pm$ 5.7	32.7 $\pm$ 5.9	32.7 $\pm$ 5.9
Tobacco smoking	16 (33.3)	16 (31.4)	6 (40.0)	10 (27.8)
Birth characteristics				
Birth weight (grams)	3339.3 $\pm$ 484.4	3222.8 $\pm$ 491.2	3351.7 $\pm$ 649.2	3169.0 $\pm$ 407.4
z-score of birth weight	0.13 $\pm$ 1.06	−0.03 $\pm$ 1.02	0.39 $\pm$ 1.24	−0.21 $\pm$ 0.86
Term (weeks of gestation)	39.9 $\pm$ 1.4	39.4 $\pm$ 1.8	39.3 $\pm$ 2.0	39.5 $\pm$ 1.7
Placenta weight (grams)	526.1 $\pm$ 111.0	528.3 $\pm$ 126.0	542.3 $\pm$ 129.1	522.5 $\pm$ 126.0
z-score of placenta weight	−1.09 $\pm$ 0.82	−1.01 $\pm$ 0.88	−0.88 $\pm$ 0.86	−1.06 $\pm$ 0.90
Placenta weight/birth weight	0.16 $\pm$ 0.02	0.16 $\pm$ 0.03	0.16 $\pm$ 0.03	0.16 $\pm$ 0.03
Sex ratio M/F [95% CI]	0.78 [0.64–0.92]	1.04 [0.90–1.18]	0.88 [0.62–1.13]	1.12 [0.95–1.28]
C-section	1 (2.1)	7 (13.7)	2 (13.3)	5 (13.9)

Quantitative data: mean  $\pm$  SD or median [interquartile range], qualitative data: n (%), <sup>a</sup>P-value = 0.009 for comparison IVF/ICSI vs. Spontaneous, <sup>b</sup>P-value = 0.03 for comparison IVF vs. ICSI vs. Spontaneous, BMI, body mass index.

appropriate. Correlations between continuous variables were estimated using Spearman's rank correlation coefficients. Birth weights were converted into z-scores calculated using normal birth weight curves of our population accounting for gestational age and sex of the newborn (Rousseau et al., 2008). Placental weights were also converted into z-scores according to gestational age and sex of the newborn (Thompson et al., 2007). As recommended (Hogg et al., 2014), multivariate analyses were adjusted for gestational age at delivery, sex of the newborn, parity and maternal age. Multiple regression linear models were used, after log-transformation of the dependent variable in the absence of normal distribution. All statistical analyses were performed with SAS software, v9.4 (SAS Institute Inc, USA). A two-tailed  $P < 0.05$  was considered significant.

## Ethical approval

All women had given written informed consent in accordance with the Declaration of Helsinki. The study was approved by the Institutional Review Board and the Ethics Committee of Dijon University Hospital (Comité de Protection des Personnes [CPP] Est I, n°2012-A01010-43).

## Results

The demographic and obstetrical characteristics of the 99 pregnancies included are summarized in Table I.

### DNA methylation of IG DMRs and TEs was decreased in the placenta of IVF/ICSI pregnancies

We studied two maternally inherited DMRs associated with the *KCNQ1OT1* and *SNURF* loci, and one paternally methylated DMR at the *H19/IGF2* locus. For TEs, we included two retroviruses (ERVFRD-I and ERVW-1), whose involvement in placental function has been demonstrated (Bolze et al., 2017), LINE-1 elements and SINES AluYa5, the subfamily responsible for the majority of current Alu retrotransposition activity (Comeaux et al., 2009).

We addressed the potential link between placental DNA methylation and clinical outcomes. In the whole study population, placental weight correlated positively with ERVFRD-I methylation ( $r = 0.30$ ,  $P = 0.0025$ ; Fig. S1A) while birth weight correlated positively with ERVFRD-I and *SNURF* methylation ( $r = 0.23$ ,  $P = 0.0259$  and  $r = 0.20$ ,  $P = 0.0481$ , respectively; Fig. S1B and C).

The variability of DNA methylation was greater in both the cord blood and placenta of IVF/ICSI pregnancies than in samples from spontaneous pregnancies, as there were more outliers (<5%) in the ART group for *H19/IGF2* DNA methylation in placenta (Table SIII). DNA methylation of the studied IG DMRs and TEs in cord blood was not significantly different between groups (Fig. 1A, Table SIV). By contrast, DNA methylation levels of LINE-1Hs, ERVFRD-I, *H19/IGF2* and *KCNQ1OT1* in placenta were lower in IVF/ICSI patients than in controls, even after adjustments (Fig. 1B, Table SIV). For these regions, DNA methylation was globally lower in the IVF/ICSI group but some CpG sites seemed more affected than others (Fig. S2). These differences were maintained after exclusion of the 11 IVF/ICSI patients and the four controls in which any pregnancy-associated disease had occurred: LINE-1Hs ( $P = 0.018$ ), ERVFRD-I ( $P = 0.047$ ), *H19/IGF2* ( $P = 0.020$ ) and *KCNQ1OT1* ( $P = 0.047$ ). In addition, the effects of the four cases of 'vanishing twin' (two embryos implanted with one live

birth only) and of the incidence of C-section were tested and did not modify these outcomes (data not shown).

Sex of the newborn had no effect on DNA methylation of any studied sequences, in cord blood or in placenta (Table SV). The ART technique (IVF or ICSI) did not significantly modify DNA methylation in cord blood (Fig. 2A, Table SVI). In contrast, in placenta, after adjustment for gestational age at delivery, sex of the newborn, parity and maternal age, the DNA methylation of *H19/IGF2* DMR was lower in IVF than in ICSI pregnancies (Fig. 2B, Table SVI).

### Expression of ERVFRD-I was decreased in cord blood and placenta of IVF/ICSI pregnancies

To demonstrate the role of the studied IGs and TEs in the placental physiology, we studied the relationship between placenta weight, birth weight and gestational age at delivery and gene expression. Birth weight and placenta weight correlated positively with the expression of ERVFRD-I in placenta (Fig. 3A and B) in the spontaneous conception group only (no correlation in the IVF/ICSI group; Fig. 3C and D). The gestational age at delivery correlated negatively with *H19* expression in cord blood ( $r = -0.26$ ;  $P = 0.0125$ ; Fig. S3A), this correlation was even stronger in the IVF/ICSI group ( $r = -0.43$ ,  $P = 0.003$ ; Fig. S3B). The gestational age at delivery also correlated negatively with ERVFRD-I expression in placenta ( $r = -0.31$ ,  $P = 0.0292$ ; Fig. S4) in the IVF/ICSI group only.

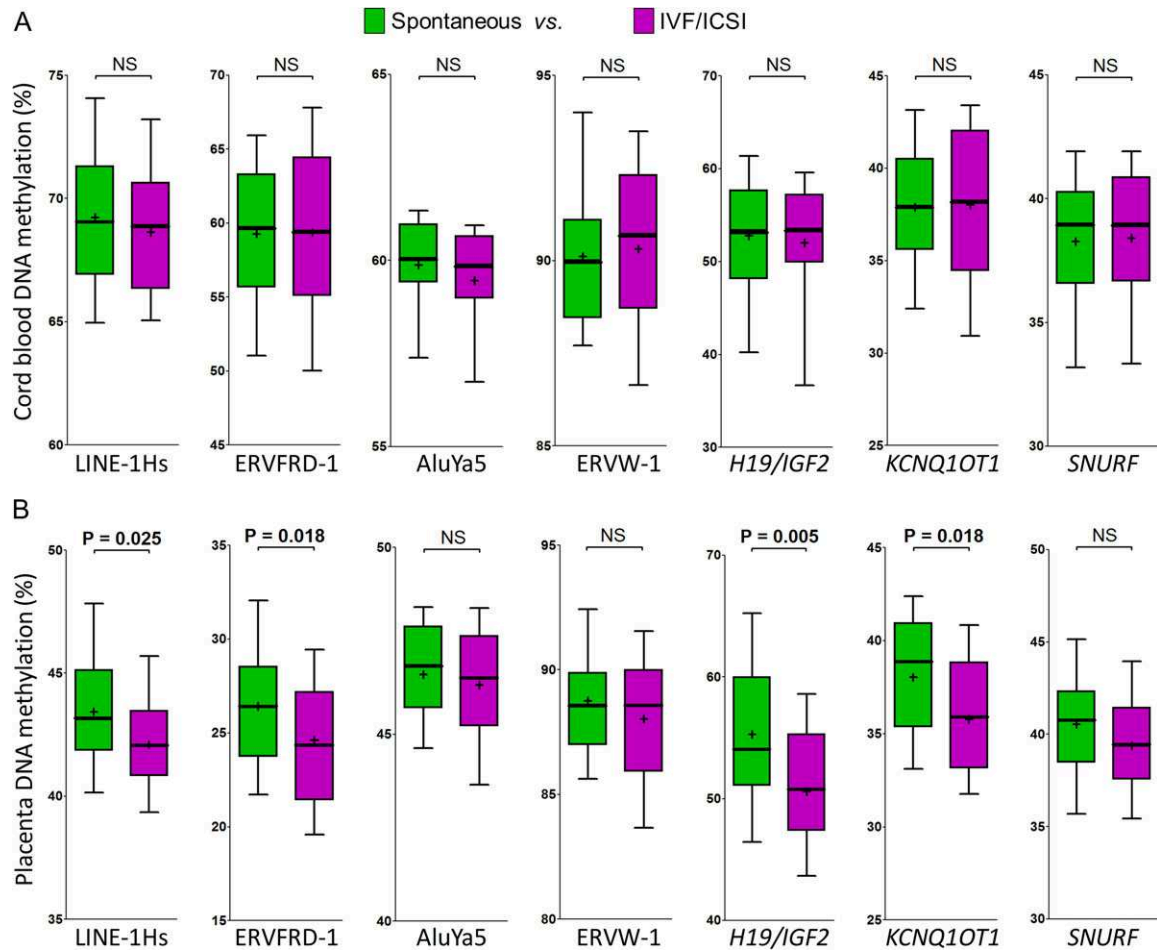
For the expression of some TEs, the percentage of outliers was higher in cord blood and placenta samples from IVF/ICSI pregnancies than in samples from spontaneous pregnancies (Table SIII). Expression levels of LINE-1 ORF2 and ERVFRD-I in cord blood were significantly lower in IVF/ICSI than in controls (Fig. 4A, Table SVII). The expression of other sequences was not different (Table SVII). The expression level of ERVFRD-I in placenta was also lower in IVF/ICSI than in controls (Fig. 4B, Table SVII) and this difference increased after the exclusion of women who experienced any pregnancy-associated disease ( $P = 0.002$ ). In addition, the cases of 'vanishing twin' and C-section had no effect on expression outcomes (data not shown).

While the median relative expression of ERVFRD-I was higher in the cord blood of boys than in the cord blood of girls (1.22 [0.83–1.62] vs. 0.91 [0.72–1.32],  $P = 0.008$ ; Fig. S5A), significant differences according to sex were not observed in the placenta (Fig. S5B). The expression of LINE-1 ORF2 in cord blood was lower in IVF than in ICSI pregnancies (0.59 [0.55–0.64] vs. 0.89 [0.66–1.18],  $P = 0.016$ ; Fig. S6A) but we did not observe any significant difference in placental expression according to the ART mode (Fig. S6B).

## Discussion

By applying a robust methodology that included adjustment for potential confounding factors, our study revealed the occurrence of changes in the epigenetic regulation of IGs and TEs after ART, specifically lower DNA methylation for *H19/IGF2* DMR, *KCNQ1OT1* DMR, ERVFRD-I and LINE-1 in placenta and lower ERVFRD-I expression level in placenta and cord blood. To the best of our knowledge, this is the first report of altered regulation of TEs after ART.

Although the effect of ART on the regulation of IGs has been widely studied, the results are conflicting. Our findings show the absence of

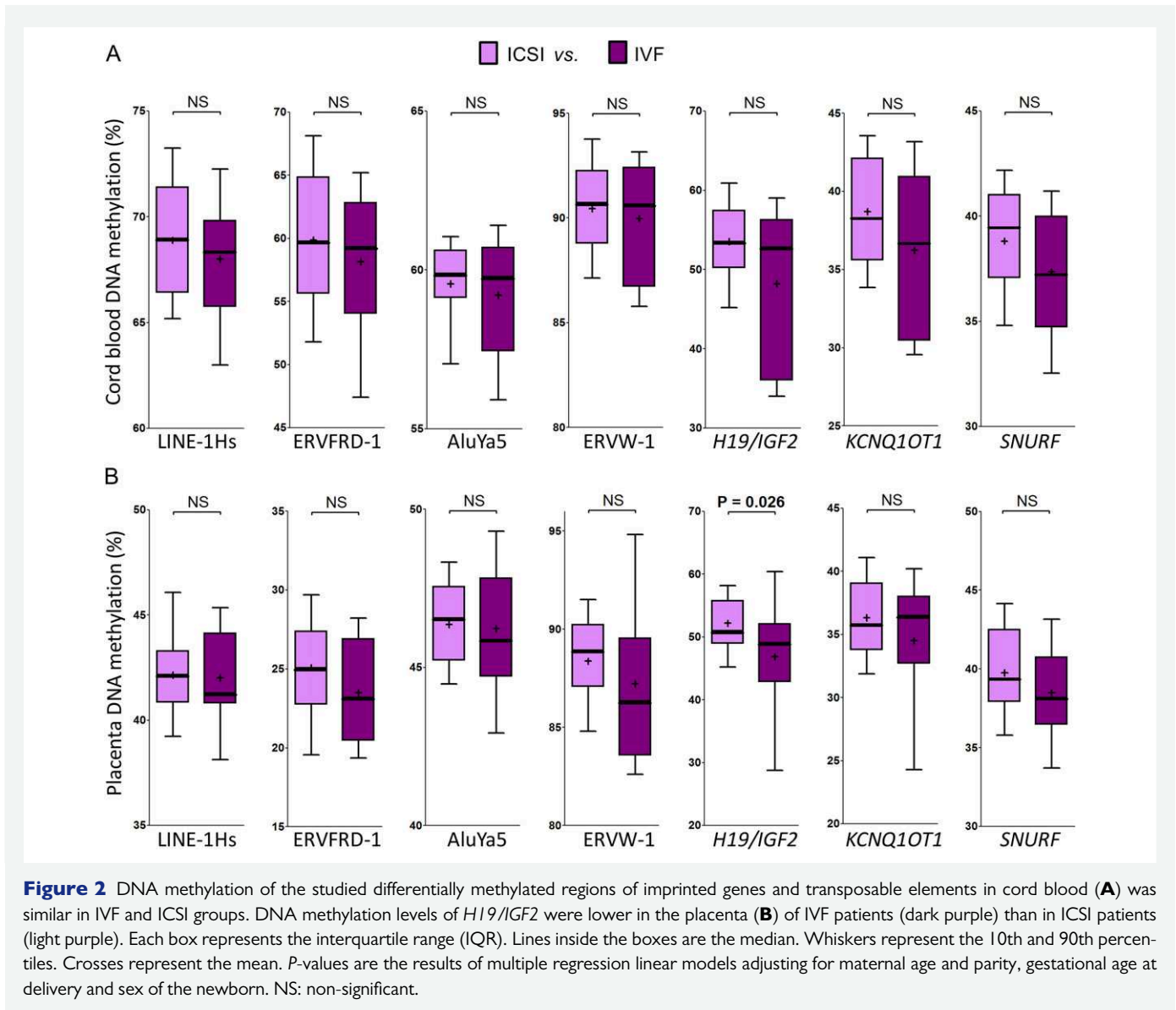


**Figure 1** DNA methylation of the studied differentially methylated regions of imprinted genes and transposable elements in cord blood (**A**) was similar in both groups. DNA methylation levels of LINE-1Hs, ERVFRD-1, *H19/IGF2* and *KCNQ1OT1* were lower in the placenta (**B**) of IVF/ICSI patients (purple) than in the spontaneous conception group (green). Each box represents the interquartile range (IQR). Lines inside the boxes are the median. Whiskers represent the 10th and 90th percentiles. Crosses represent the mean. *P*-values are the results of multiple regression linear models adjusting for maternal age and parity, gestational age at delivery and sex of the newborn. NS: non-significant.

any effect of ART on the methylation status of IGs in cord blood, which is consistent with the majority of studies performed in mice (Fauque *et al.*, 2010; De Waal *et al.*, 2014) and humans (Tierling *et al.*, 2010; Turan *et al.*, 2010; Shi *et al.*, 2011; Camprubi *et al.*, 2013; Vincent *et al.*, 2016), with the exception of one study that found a slight but significant increase in *KCNQ1OT1* methylation in IVF cord blood compared with controls (Rancourt *et al.*, 2012). However, in the placenta, we found that methylation of IGs was altered after ART, and in a locus-specific manner: *KCNQ1OT1* and *H19/IGF2* DMRs were less methylated in IVF/ICSI versus spontaneous conception, while *SNURF* DMR methylation may be considered as similar, after adjustments for confounding factors. Interestingly, De Waal *et al.* (2015) reported similar findings in mice analysed near term after IVF. Liver and brain foetal tissues displayed normal methylation profiles, while placentas were hypomethylated at the *H19/IGF2* DMR but not at the *Snrpn* DMR. Other studies at the early stages of gestation have also reported IVF-induced *H19* placental hypomethylation (Fauque *et al.*, 2010; Wang *et al.*, 2010; De Waal *et al.*, 2014) with normal *Snrpn*

methylation (De Waal *et al.*, 2014). In humans, most studies using bisulphite pyrosequencing agree with ours concerning placental *H19/IGF2* hypomethylation in the IVF/ICSI group (Rancourt *et al.*, 2012; Nelissen *et al.*, 2013), unlike two studies that used other techniques (Wong *et al.*, 2011; Camprubi *et al.*, 2013). Although several studies, like ours, found no difference in placental *SNURF* methylation (Camprubi *et al.*, 2013; Nelissen *et al.*, 2013), others reported increased DNA methylation in the IVF group (Rancourt *et al.*, 2012). Moreover, although our findings of lower placental DNA methylation of *H19/IGF2* in IVF compared with ICSI have not been evidenced by other studies (Wong *et al.*, 2011; Nelissen *et al.*, 2013; Sakian *et al.*, 2015), other works suggest that more changes occur in IVF than in ICSI in cord blood for *PLAGL1* and *MEST* (Tierling *et al.*, 2010; Vincent *et al.*, 2016). This highlights the need for further study in comparing both techniques.

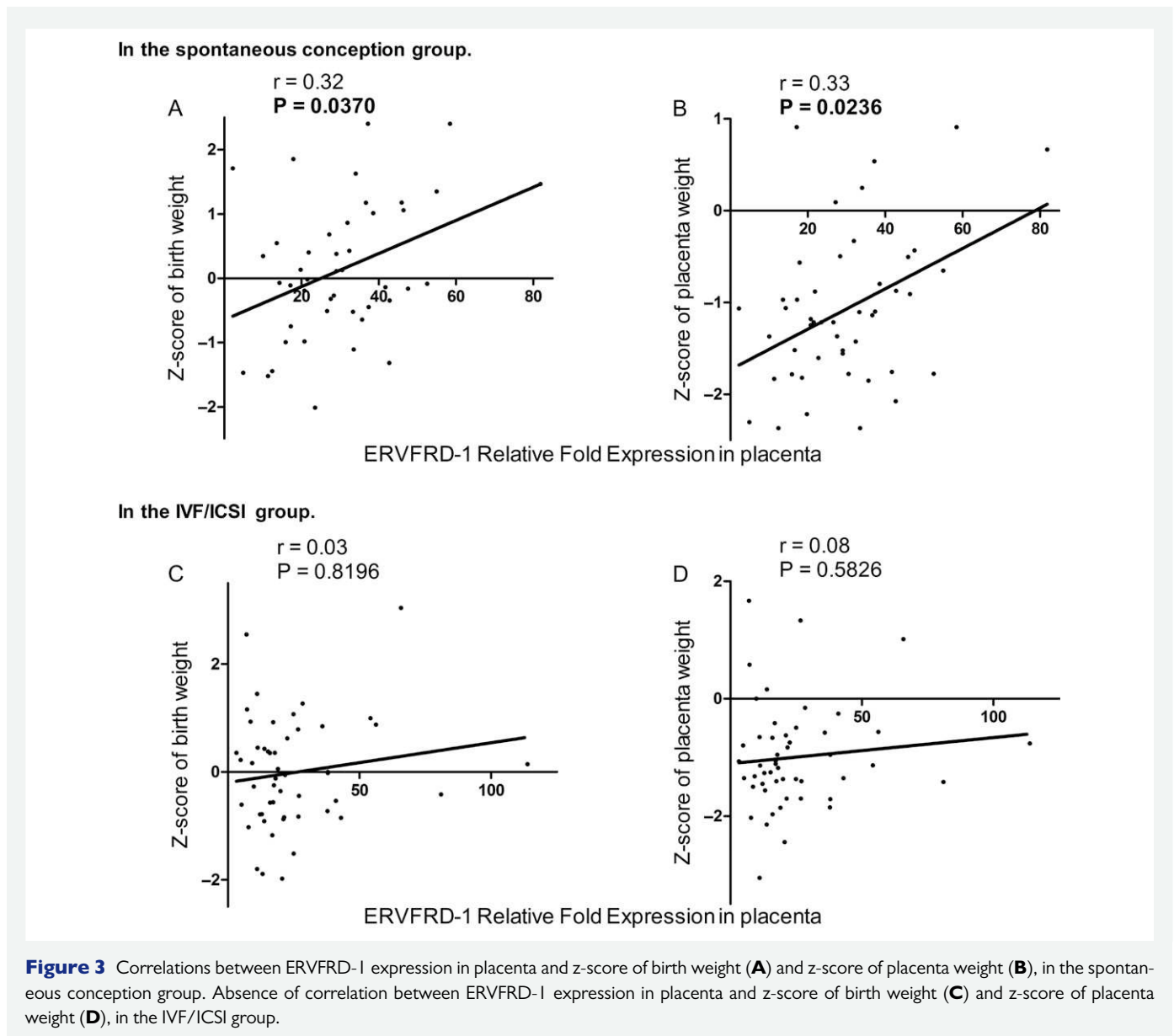
These differences between studies can be explained by the different methodologies used: population samples, merging of different ART techniques and protocols, and the lack of adjusted analyses are



limitations of these studies (Lazaraviciute et al., 2014). By contrast, our monocentric and prospective design ensures high consistency in laboratory techniques, accurate data collection and standardized samples. Furthermore, the two cohorts were very homogeneous, as the IVF/ICSI group included only singleton pregnancies achieved following the transfer of fresh 2-day-old embryos cultured in a unique medium and the control group included only spontaneous pregnancies achieved within 1 year. The statistical analysis adjusted for various confounding factors such as gestational age at delivery, sex of the newborn, maternal age and parity.

The existence of an imprinted gene network (IGN) could also explain the heterogeneity of the results reported (Varrault et al., 2006; Fauque et al., 2010; Iglesias-Platas et al., 2014). The IGN includes a myriad of genes with similar roles controlling essential functions such as foetal nutrition and growth. Thanks to its genetic 'redundancy' (Yuen and Robinson, 2011), the failure of some IGs can be compensated by the expression of others. We recently hypothesized that, in response to primary dysfunctions mediated by ART, the placenta

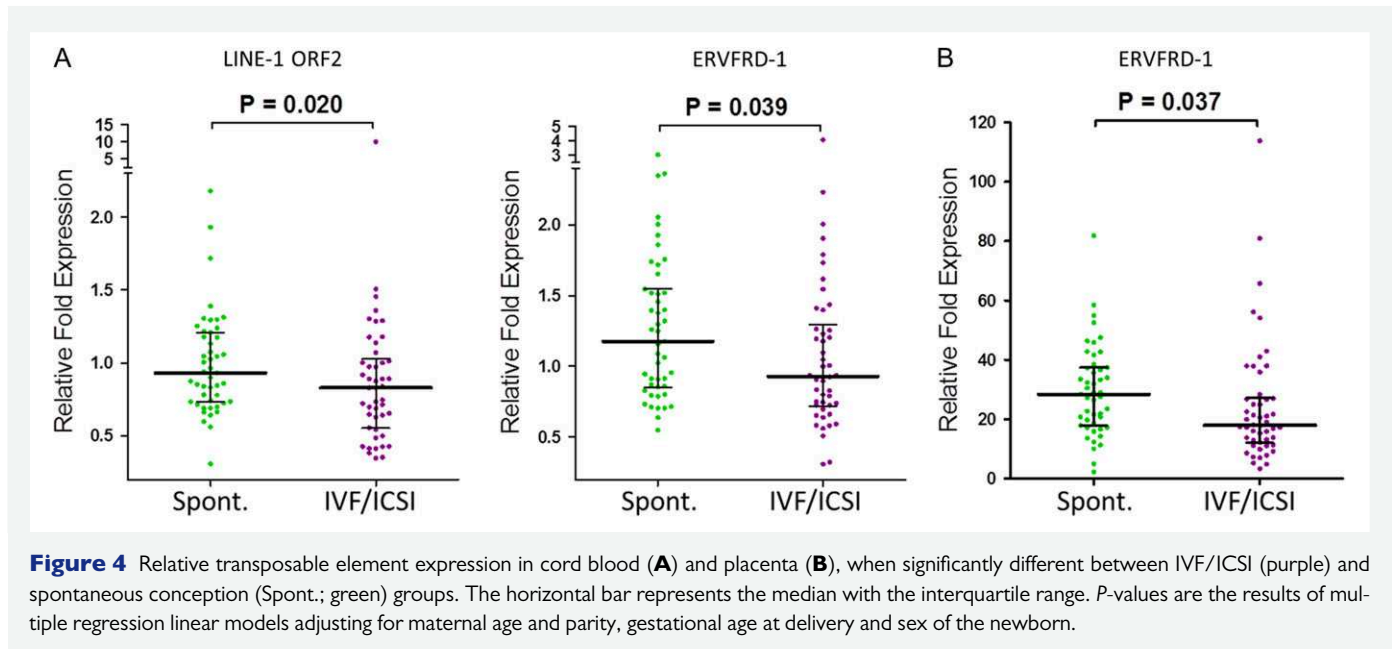
could adapt throughout the pregnancy, with the adaptation being mediated by epigenetic modifications, especially in the IGN (Choux et al., 2015). The increased variation after IVF/ICSI found in our study and in others (Camprubi et al., 2013; Melamed et al., 2015) clearly illustrate this plasticity. The similarity of birth weight in the two groups and the lack of DNA methylation or expression differences in IGs in cord blood also suggest that the foetus is protected against major modifications. In addition, despite DNA methylation changes at *H19/IGF2* and *KCNQ1OT1* DMRs, the lack of transcriptional modifications of *H19* and *KCNQ1* in the placenta at birth and the negative correlation between *H19* expression and gestational age at delivery strengthens the hypothesis of dynamic adaptation throughout pregnancy. The sequence of events is better demonstrated in animal studies. Fortier's team investigated the effects of superovulation in mice on the expression of *Igf2* in the placenta and observed a significant increase at E9.5, which became non-significant at E14.5 and disappeared near term (Fortier et al., 2008, 2014). We can postulate that levels of expression may have returned to normal at birth or some modifications may



persist depending on the severity of the primary injury and on the efficacy of the adaptation mechanisms.

Our novel findings concerning the expression and methylation of the retrovirus ERVFRD-1 indicate that IGN may not be the only pathway involved in placental adaptation. Indeed, human endogenous retroviruses ERVW-1 and ERVFRD-1 encode envelope glycoproteins called syncytin-1 and syncytin-2, respectively. They are known to be involved in cell fusion, particularly the fusion of cytotrophoblasts, a necessary step in the formation of a placental exchange area called the syncytiotrophoblast. Their immunosuppressive properties could also contribute to maternal tolerance towards the foetus (Tolosa *et al.*, 2012; Lokossou *et al.*, 2014). Furthermore, they are involved in pregnancy-associated diseases: the level of syncytin-2 in exosomes from maternal blood is decreased in preeclampsia (Vargas *et al.*, 2014) and placental expression of syncytin-1 and -2 is decreased in IUGR and preeclampsia (Bolze *et al.*, 2017). Interestingly, ovarian stimulation could affect their

regulation, as syncytin-1 has been shown to be up-regulated by progesterone (Noorali *et al.*, 2009). In our study, ERVFRD-1 methylation was decreased in placenta after IVF/ICSI while its expression was decreased in both cord blood and in placenta. Furthermore, the expression of ERVFRD-1 in placenta correlated positively with birth weight and placenta weight, but only in the control group. It can be argued that foetal growth could be linked to placental ERVFRD-1 expression in normal pregnancies, while syncytin-2 regulation could be impaired after IVF/ICSI. This hypothesis is strengthened by the negative correlation between the expression level of ERVFRD-1 in placenta and the gestational age at delivery for IVF/ICSI pregnancies, which shows that, as the pregnancy progresses, the level of syncytin-2 decreases. Altogether, these data suggest that compensatory mechanisms implicating syncytins, such as an increase in cell fusion mediated by ERVFRD-1 in placenta, play a fundamental role in placental physiology, and may be promoted in IVF/ICSI by hypomethylation of its



promotor although they are possibly exhausted at the end of the pregnancy following ART.

For future research, we should broaden the vision of epigenetic regulation in the placenta, which has been limited to DNA methylation in our study. Further studies are needed to assess the role of other actors such as small RNAs and histones modifications, whose implication on placental physiology has been demonstrated (Kohan-Ghadr et al., 2016). Further studies should also focus on an infertile population as a control group. Indeed, since infertility alone might also trigger epigenetic changes (Litzky et al., 2017), the control group of fertile couples having conceived within 1 year prevented us from deciphering the distinct roles of ART and infertility. As studies have demonstrated a link between maternal diet and modifications of the offspring's epigenome (van Dijk et al., 2016), a better control of factors such as parental diet or prenatal care would also be valuable.

To conclude, although syncytins were already known to be involved in placental physiology and pathologies, to our knowledge, this is the first report of positive correlations between syncytin-2 expression in placenta and birth/placenta weight and of ART affecting syncytin-2 regulation. More research about the link between syncytins and pregnancy-associated diseases such as IUGR and preeclampsia could lead to them being used as biomarkers or even as therapeutic targets. This is also the first study to address both the methylation and expression of TEs and IGs in cord blood and placenta at birth. The slight but significant differences between the groups for some placental TEs and IGs methylation suggest that ART modifies placental epigenetic regulation. As TEs and IGs are known to be involved in foetal and placental development, their dysregulation could contribute to explain the increased rate of placenta-related adverse pregnancy outcomes after ART. Even though all of the newborns were healthy and no transcriptional differences were evidenced in IGs at birth, the repercussions of the original injury could be mitigated by compensatory epigenetic mechanisms mediated by the placenta throughout pregnancy. More research is needed to reveal the sequence of events occurring during pregnancy. Since the intrauterine environment could affect the

individual later in life (Developmental Origins of Health and Disease—DOHaD—theory) (Barker, 2007), there is the question of the potential long-term effects of these epigenetic modifications. All in all, these results should encourage us to analyse the exact causes and consequences of epigenetic changes and strive to minimize these variations in the interest of epigenetic safety after ART.

## Supplementary data

Supplementary data are available at *Human Reproduction Online*.

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## Authors' roles

P.F. and C.C. were the principal investigators and take primary responsibility for the paper. P.F., C.C. and C.B. were responsible for the study design. C.C., V.C., J.B., P.S., M.L. and C.B. recruited the patients. C.C., V.C., C.C. and C.B. were involved in experiments. P.F., C.B., D.B. and C.C. coordinated the research. C.B. performed the statistical analyses. C.C. and P.F. drafted the manuscript.

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## Conflict of interest

None declared.

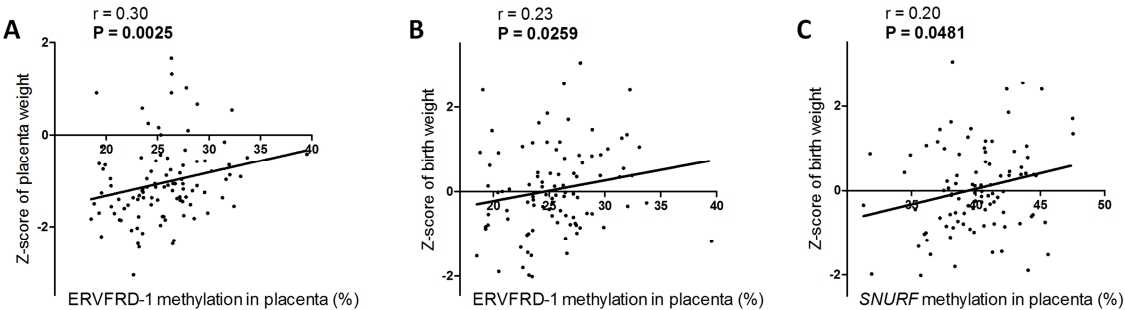
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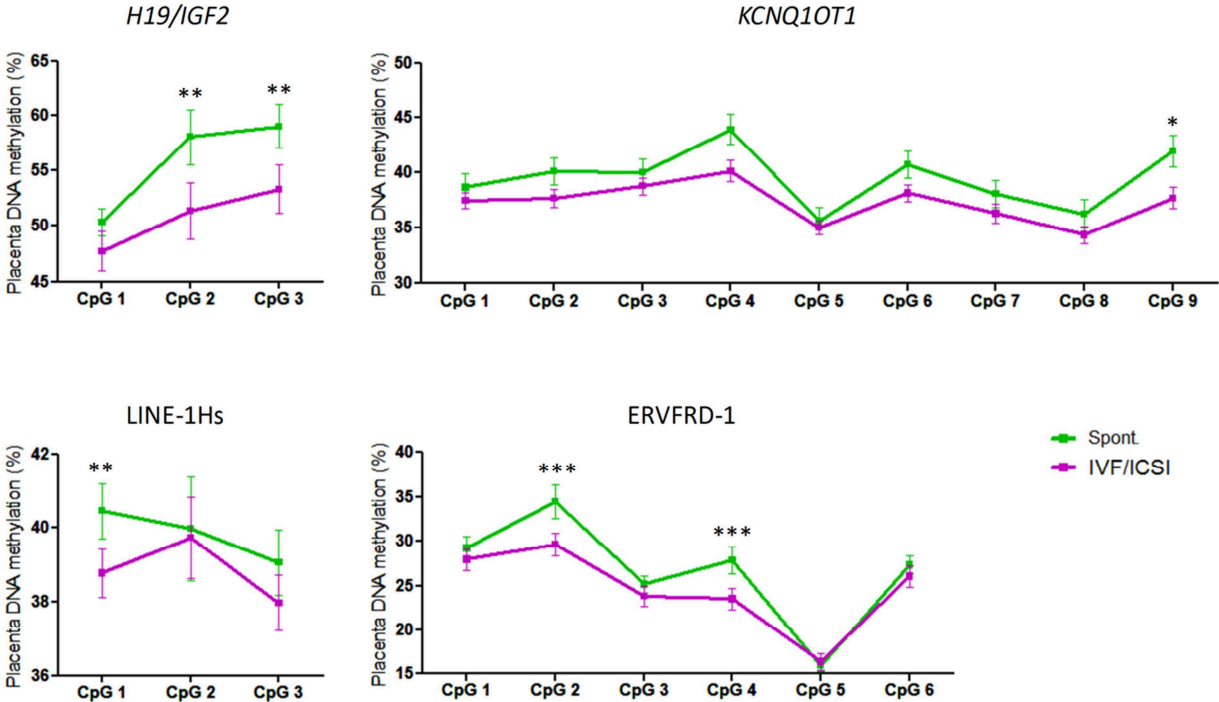


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# Supplementary material



**Figure S1.** In the whole study population. Correlations between z-score of placenta weight and ERVFRD-1 methylation in placenta (A). Correlations between z-score of birth weight and ERVFRD-1 (B) and SNURF (C) methylation in placenta.



**Figure S2:** DNA methylation was globally lower in the IVF/ICSI group (purple) than in the control group (green) but some CpG sites of the region seemed to be more affected than others. For each CpG, data are expressed as mean  $\pm$  95% CI, \*:  $P < 0.05$ , \*\*:  $P < 0.01$ , \*\*\*:  $P < 0.001$ , after adjustment for maternal age and parity, gestational age at delivery and method of conception, and after applying a Bonferroni test correction taking into account the multiple CpG analysed.

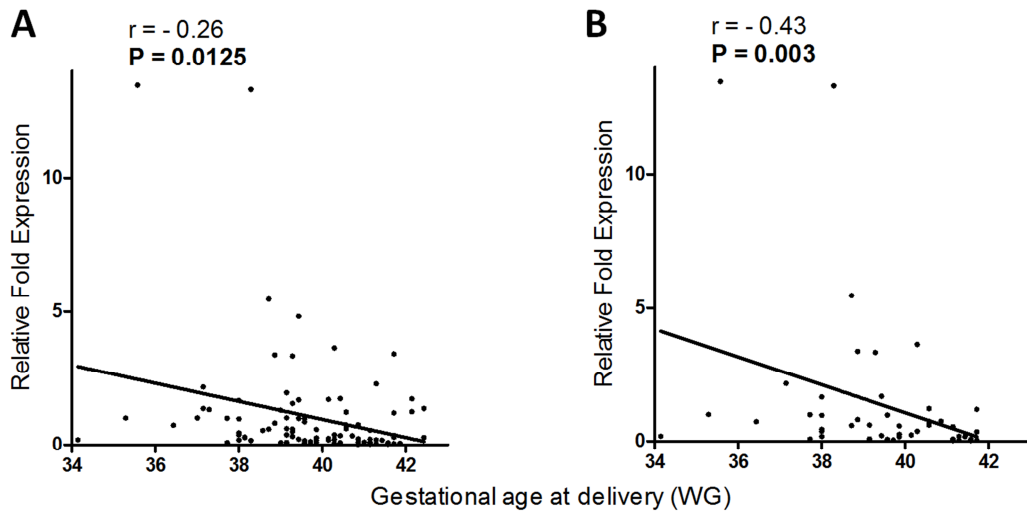


Figure S3: Correlation between gestational age at delivery and *H19* expression in cord blood, in the whole population (A) and in the IVF/ICSI group (B).

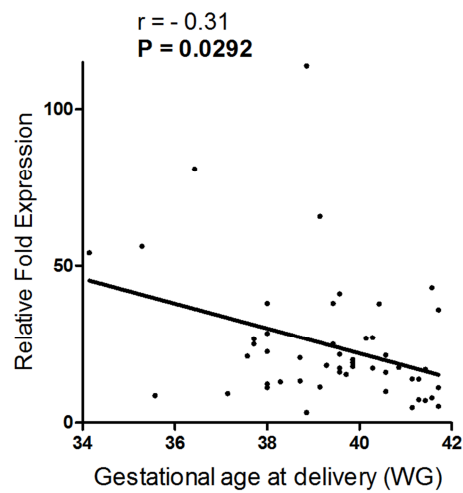
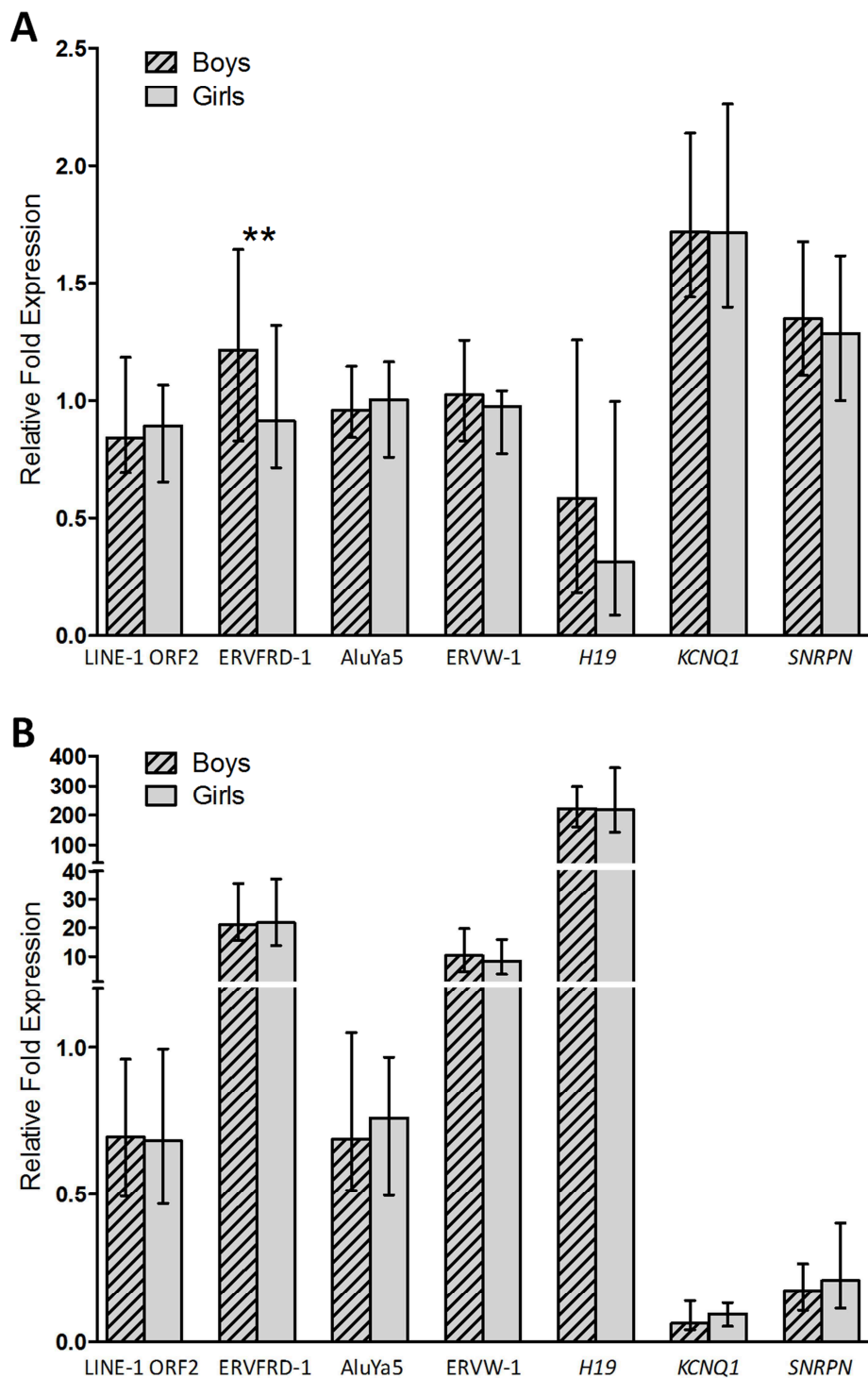
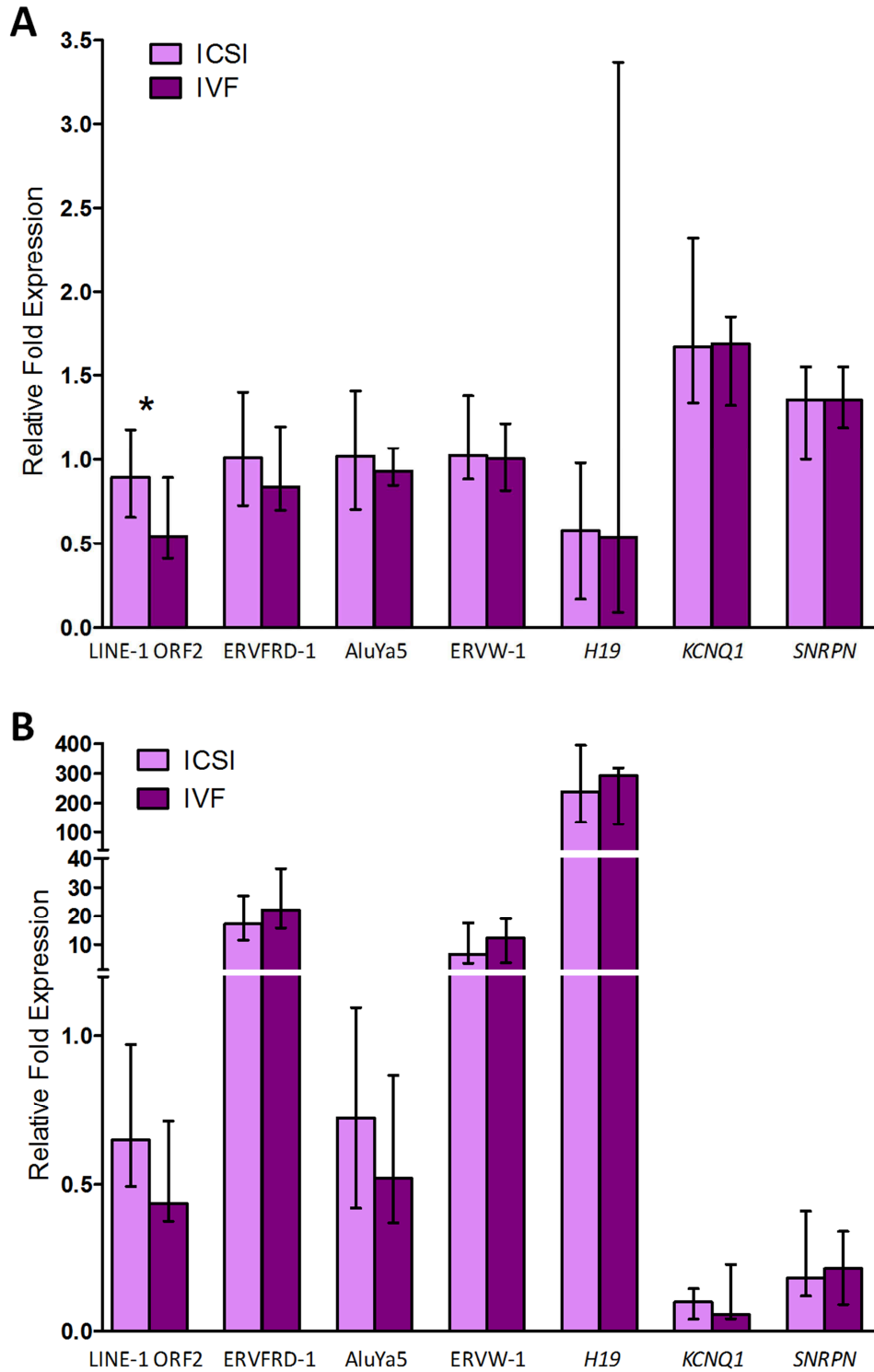


Figure S4: In the IVF/ICSI group. Correlation between gestational age at delivery and ERVFRD-1 expression in placenta.



**Figure S5. Comparison of expression in cord blood (A) and in placenta (B) between boys and girls.** Values are shown as median  $\pm$  interquartile range. \*\*:  $P < 0.01$  after adjustment for maternal age and parity, gestational age at delivery and method of conception.



**Figure S6. Comparison of expression in cord blood (A) and in placenta (B) between ICSI and IVF.** Values are shown as median  $\pm$  interquartile range. \*:  $P < 0.05$  after adjustment for maternal age and parity, gestational age at delivery and sex of the new-born.

**Table S1: Primers for pyrosequencing**

Regions	Data base	Accession number or nucleotide position	PCR primers	Product length PCR reaction Hybridation temperature	Sequence analysed Dispensation order CpG sites
<b>Repeats</b>					
<b>AluYa5</b>	NCBI	AluYa5 19.3	F: biot-GGAGGTTGAGGTAGGAGAATG	92 bp	RCCTCCCRAATTCACRCCATTCT
			R: CTCTATCCCCAACTAAAATACAATAAC	A	CGACTACCAGAAGTCATCGACA
			seq: ACTCACTACAACTCC	56°C	3 (3-5)
<b>ERVFRD-1</b>	Ensembl	ENSG00000244476 reverse strand hg38: chr 6:11,102,722-11,111,965	F: GTATTTGGATTTTTTAAATGGTGTAGTGA	236 bp	TTTTTAYGTTTTGTTTTTYYGGTGGAGGYGTTTAAATTTTYGTAYGTGGTTTTYGTTT
			R: biot-TTCCCACCCCTACAAACCA	A	ACTTCTGATCGTTTAGTTTCGTGATGTCGTTGATTCGTGATCGTAGTTTCG
			seq: ATTTTTTTTAGTGGTAAGATA	56°C	6 (2-7)
<b>ERVW-1</b>	Ensembl	ENSG00000242950 reverse strand hg38: chr7:92,468,380-92,477,986	F: TGTTAGGTGTATTAGGTATTGGTATTG	326 bp	AAYGGTYGTYGATTTTTTGGT
			R: biot-AAATCCAATATTTCCAACTCCTACTCT	A	TATCAGGTCAGCTCGATT
			seq: GAATTAATGGGGATATGG	56°C	3 (2-4)
<b>LINE-1Hs</b>	NCBI	X58075 reverse strand	F: TTTTGAGTTAGGTGTGGGATATA	154 bp	TTYGTGGTYGYGTTT
			R: biot- AAACCCAAAAATCAAAAAATCCCTTTCC	B	GCTCGTGTAGTCAGTCG
			seq: AGGTGTGGGATATAGT	56°C	3 (1-3)
<b>Imprinted genes DMRs</b>					
<b>H19/IGF2 CTCF3</b>	NCBI	hg38: chr11:1,997,582-2,003,510 Boissonnas <i>et al.</i> 2010	F: TTGGTAGGTATAGAAATTGGGG	214 bp	GAYGTTTTTTTTTTGTTTTATTATTYGGATGGTATAGAATYGG
			R: biot-ACACCTAACTTAAATAACCCAAAA	A	AGTATCGCTTTTTGTTTATGATCGATGTATAGTATC
			seq: GTAGTATATGGGTATTTGTG	58°C	3 (2-4)
<b>KCNQ10T1</b>	UCSC	hg38 : chr11:2,698,718-2,701,029 Rancourt <i>et al.</i> 2012	F: ATTTTAGGGGGTGTAGTGGTA	259 bp	GTAGYGYGAGGGYGTTTYGYGTTTGTAGYGTTYGGTYGGTTYGT
			R: biot-ACTTTTATAACCCAACTTTTATCCC	A	AGCTATGTCAGTCGATGGTCAGTTCAGTCGTTGATGTCAGTCAGTCAGGTCG
			seq: AGGTTATTTATTTGGTAAAGG	56°C	9 (2-11)
<b>SNURF</b>	UCSC	hg38 : chr15:24,954,857-24,956,829 Rancourt <i>et al.</i> 2012	F: GGGAGGGAGTTGGGATTTTTG	220 bp	YGGTAAATAAGTAYGTTTGYGYGGTYGTAGAGGTAGGTTGGYGYGTA
			R: biot-AAACCACCCACACAACTAACCTTACCC	A	ATCGCTAATAGTGATCGTTAGTCAGTCAGTCGTTAGTAGTAGTCAGTCGCT
			seq: AGTTGGGATTTTTGTATTG	56°C	7 (1-7)

PCR reaction and pyrosequencing conditions "A": Pyromark PCR Master Mix 2x 12.5 µL, CoralLoad concentrate 2.5 µL, Primer A 0.2 µM, Primer B 0.2 µM, Water 8 µL, Bisulphite-treated DNA 1 µL. PCR purification: 15 µL PCR product, 40 µL Binding Buffer, 29 µL Water, 1 µL Streptavidin Sepharose High Performance Beads, GE Healthcare, Life Sciences®.

PCR reaction and pyrosequencing conditions "B": Pyromark PCR Master Mix 2x 25 µL, CoralLoad concentrate 5 µL, MgCl2 4 µL, Primer A 0.2 µM, Primer B 0.2 µM, Water 13 µL, Bisulphite-treated DNA 1 µL. PCR purification: 46 µL PCR product, 38 µL Binding Buffer, 2 µL Streptavidin Sepharose High Performance Beads, GE Healthcare, Life Sciences®.

**Table SII: Primers for qRT-PCR**

Region	Data base Reference	Sequence Number	Primers	Product Reaction temperature
<b>Housekeeping genes</b>				
<i>GAPDH</i>	Ensembl	ENSG00000111640 hg38: chr12:6,533,927-6,538,374	F: TGCACCACCAACTGCTTAGC	87 pb
			R: GGCATGGACTGTGGTCATGAG	60°C
<i>SDHA</i>	Ensembl Nelissen et al., 2013	ENSG00000073578 hg 38: chr5:218,241-256,700 forward strand	F: CGAGCTGCATTTGGCCTTTC	125 pb
			R: TCCTCCATGTTCCCGAGAGC	60°C
<i>TBP</i>	Ensembl Nelissen et al., 2013	ENSG00000112592 hg38: chr6:170,554,302-170,572,870 forward strand	F: ACCTTACGCTCAGGGCTTGG	101 pb
			R: GCTGTGGGGTCAGTCCAGTG	60°C
<b>Repeats</b>				
<i>AluYa5</i>	NCBI	AluYa5 19.3	F: GGTGGCTCACGCCTGTAAT	103 pb
			R: AGACGGGGTTTCACCGTTTT	60°C
<i>ERVFRD-1 (syncytin-2)</i>	Ensembl De Parseval et al., 2003	ENSG00000244476 hg38: chr6:11,102,489-11,111,732 reverse strand	F: GCCTGCAAATAGTCTTCTTT	114 pb
			R: ATAGGGGCTATTCCCATTAG	60°C
<i>ERVW-1 (syncytin-1)</i>	Ensembl	ENSG00000242950 hg38: chr7:92,468,380-92,477,986 reverse strand	F: AGTGCCCTATGACCATCT	232 pb
			R: ATCTTGCAAGGTGACCAGGG	61°C
<i>LINE-1 ORF2</i>	NCBI	AH002566.2	F: ATCTGAGAACGGCAGACA	76 pb
			R: CCTCCAGTTAGGCTGCTC	60°C
<b>Imprinted genes</b>				
<i>H19</i>	Ensembl	ENST00000414790 hg38: chr11:1,995,163-1,997,875 reverse strand	F: ATCGGTGCCTCAGCGTTC	192 pb
			R: AGAAACAGACCCGCTTCTTG	60°C
<i>KCNQ1</i>	Ensembl	ENSG00000053918 hg38: chr11:2,444,684-2,849,109 forward strand	F: GTTCTTCGGGACGGAGTACG	233 pb
			R: GGTCGACGTGTAGCATCCTC	60°C
<i>SNURF/SNRPN</i>	Ensembl	ENSG00000128739 hg38: chr15:24,823,637-24,978,723 reverse strand	F: GGCCGAATCTTCATTGGCAC	145 pb
			R: GCAACACCAGACCCAAAACC	60°C

Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific®) was used with 1 ng cDNA and 0.3 µM of each primer, for a final volume of 10 µL. Amplification was performed in triplicate using CFX96™ (Bio-Rad®) with the following conditions: 10 min denaturation phase at 95°C, followed by 40 cycles of two steps: 10 s denaturation at 95°C and 30 s annealing/extension at a temperature depending on the primers used.

**Table SIII. Comparisons of 5% and 95% outliers between groups.**

	Outliers 5%, n (%)			Outliers 95%, n (%)		
	Spont. (n = 48)	IVF/ICSI (n = 51)	P-value	Spont. (n = 48)	IVF/ICSI (n = 51)	P-value
<b>Methylation (cord blood)</b>						
LINE-1Hs	3 (6.3)	3 (6.0)	1	3 (6.3)	0 (0)	0.114
ERVFRD-1	2 (4.2)	5 (10.0)	0.436	2 (4.2)	6 (12.0)	0.269
AluYa5	2 (4.2)	3 (6.0)	1	3 (6.3)	2 (4.0)	0.674
ERVW-1	3 (6.3)	8 (16.0)	0.126	2 (4.2)	1 (2.0)	0.613
<i>H19/IGF2 CTCF3</i>	2 (4.2)	5 (10.0)	0.436	2 (4.2)	3 (6.0)	1
<i>KCNQ1OT1</i>	3 (6.3)	5 (10.0)	0.715	3 (6.3)	2(4.0)	0.674
<i>SNURF</i>	2 (4.2)	2 (4.0)	1	3 (6.3)	2(4.0)	0.674
<b>Methylation (placenta)</b>						
LINE-1Hs	2 (4.2)	2 (3.9)	1	2 (4.2)	0 (0)	0.233
ERVFRD-1	2 (4.2)	3 (5.9)	1	3 (6.3)	1 (2.0)	0.352
AluYa5	3 (6.3)	5 (9.8)	0.716	3 (6.3)	4 (7.8)	1
ERVW-1	2 (4.2)	6 (11.8)	0.270	2 (4.2)	1 (2.0)	0.761
<i>H19/IGF2 CTCF3</i>	3 (6.3)	10 (19.6)	<b>0.049</b>	2 (4.2)	0 (0)	0.233
<i>KCNQ1OT1</i>	2 (4.2)	6 (11.8)	0.27	2 (4.2)	0 (0)	0.233
<i>SNURF</i>	3 (6.3)	4 (7.8)	1	2 (4.2)	0 (0)	0.233
<b>Relative Fold Expression (cord blood)</b>						
LINE-1 ORF2	3 (6.4)	12 (26.1)	<b>0.01</b>	2 (4.3)	1 (2.2)	1
ERVFRD-1	2 (4.3)	11 (23.9)	<b>0.006</b>	2 (4.3)	1 (2.2)	1
AluYa5	2 (4.3)	7 (15.2)	0.091	2 (4.3)	10 (21.7)	<b>0.012</b>
ERVW-1	2 (4.3)	0 (0)	0.495	3 (6.4)	7 (15.2)	0.198
<i>H19</i>	4 (8.5)	5 (10.9)	0.740	2 (4.3)	6 (13.0)	0.158
<i>KCNQ1</i>	2 (4.3)	2 (4.3)	1	3 (6.4)	2 (4.3)	1
<i>SNRPN</i>	3 (6.4)	3 (6.5)	1	2 (4.3)	5 (10.9)	0.267
<b>Relative Fold Expression (placenta)</b>						
LINE-1 ORF2	2 (4.3)	6 (12.0)	0.270	2 (4.3)	6 (12.0)	0.27
ERVFRD-1	2 (4.3)	9 (18.0)	<b>0.033</b>	3 (6.4)	4 (8.0)	1
AluYa5	2 (4.3)	12 (24.0)	<b>0.006</b>	2 (4.3)	4 (8.0)	0.678
ERVW-1	3 (6.4)	5 (10.0)	0.716	3 (6.4)	2 (4.0)	0.671
<i>H19</i>	2 (4.3)	6 (12.0)	0.270	3 (6.4)	1 (2.0)	0.352
<i>KCNQ1</i>	1 (2.1)	3 (6.1)	0.618	3 (6.4)	8 (16.3)	0.136
<i>SNRPN</i>	3 (6.4)	4 (8.0)	1	2 (4.3)	2 (4.0)	1

The thresholds to calculate the percentage of outliers were calculated on the spontaneous population, Significant differences in bold, IVF/ICSI: *In vitro* Fertilization group, Spont.: Spontaneous conception group



**Table SIV: Comparisons of cord blood and placental methylation between groups**

	Cord Blood				Placenta			
	Spontaneous (n = 48)	IVF/ICSI (n = 51)	Univariate P-value	Multivariate P-value	Spontaneous (n = 48)	IVF/ICSI (n = 51)	Univariate P-value	Multivariate P-value
<b>Repeats</b>								
LINE-1Hs	69.01 [67.03-71.30]	68.92 [66.40-70.60]	0.502	0.532	43.12 [41.87-45.01]	42.05 [40.84-43.46]	<b>0.020</b>	<b>0.025</b>
ERVFRD-1	59.73 [55.79-63.31]	59.49 [55.15-64.41]	0.912	0.942	26.40 [23.86-28.48]	24.38 [21.44-27.17]	<b>0.011</b>	<b>0.018</b>
AluYa5	60.07 [59.42-60.92]	59.89 [59.04-60.66]	0.165	0.118	46.85 [45.72-47.87]	46.52 [45.22-47.63]	0.176	0.311
ERVW-1	90.01 [88.47-91.05]	90.72 [88.79-92.28]	0.305	0.853	88.59 [87.12-89.86]	88.61 [85.94-89.99]	0.486	0.306
<b>Imprinted genes</b>								
<i>H19/IGF2</i> <i>CTCF3</i>	53.41 [48.20-57.44]	53.51 [50.02-57.14]	0.895	0.974	53.97 [51.11-59.86]	50.73 [47.34-55.30]	<b>0.002</b>	<b>0.005</b>
<i>KCNQ1OT1</i>	37.97 [35.63-40.48]	38.25 [34.49-41.98]	0.862	0.792	38.83 [35.52-40.84]	35.89 [33.15-38.84]	<b>0.002</b>	<b>0.018</b>
<i>SNURF</i>	38.99 [36.69-40.22]	38.99 [36.70-40.80]	0.812	0.342	40.75 [38.55-42.27]	39.39 [37.59-41.43]	<b>0.042</b>	0.140

Median [IQR] of methylation percentages, Multivariate analyses were adjusted for maternal age and parity, gestational age at delivery and sex of the new-born, Significant differences in bold

**Table SV: Comparisons of methylation levels in cord blood and placenta between boys and girls on the whole study population**

	Cord Blood				Placenta			
	Boys (n = 46)	Girls (n = 52)	Univariate P-value	Multivariate P-value	Boys (n = 46)	Girls (n = 52)	Univariate P-value	Multivariate P-value
<b>Repeats</b>								
LINE-1Hs	68.89 [66.75-70.89]	68.99 [66.34-70.08]	0.878	0.961	42.16 [40.89-44.61]	42.59 [41.26-44.10]	0.447	0.528
ERVFRD-1	60.98 [56.73-64.41]	57.62 [55.08-62.88]	0.112	0.155	24.38 [22.13-28.57]	26.34 [23.51-27.52]	0.366	0.680
AluYa5	60.22 [59.30-60.70]	59.84 [59.38-60.69]	0.890	0.683	47.01 [45.67-48.00]	46.41 [45.40-47.44]	0.099	0.190
ERVW-1	90.15 [88.83-91.17]	90.35 [88.50-91.80]	0.586	0.415	89.09 [86.83-90.91]	88.42 [86.53-89.83]	0.216	0.287
<b>Imprinted genes DMRs</b>								
<i>H19/IGF2</i> <i>CTCF3</i>	52.65 [48.70-56.56]	53.82 [49.15-58.11]	0.321	0.448	53.25 [49.39-58.25]	52.18 [48.85-57.61]	0.435	0.358
<i>KCNQ1OT1</i>	38.25 [36.04-40.42]	37.88 [34.34-41.44]	0.762	0.824	37.69 [34.31-39.89]	37.44 [34.07-39.52]	0.814	0.865
<i>SNURF</i>	38.20 [36.70-40.32]	39.27 [36.63-40.70]	0.281	0.153	40.41 [37.52-41.43]	39.97 [38.30-42.20]	0.517	0.686

Median [IQ range], Multivariate analyses were adjusted for maternal age and parity, gestational age at delivery and method of conception

**Table SVI. Comparison of cord blood and placental methylation between IVF and ICSI**

	Cord Blood				Placenta			
	ICSI (n = 36)	IVF (n = 15)	Univariate P-value	Multivariate P-value	ICSI (n = 36)	IVF (n = 15)	Univariate P-value	Multivariate P-value
<b>Repeats</b>								
LINE-1Hs	68.97 [66.44-71.22]	68.36 [65.93-69.72]	0.364	0.324	42.17 [40.87-43.26]	41.29 [40.81-44.13]	0.867	0.796
ERVFRD-1	59.78 [55.81-64.80]	59.31 [54.08-62.60]	0.333	0.285	25.06 [22.84-27.31]	23.20 [20.47-26.90]	0.169	0.158
AluYa5	59.89 [59.13-60.60]	59.79 [57.70-60.67]	0.536	0.487	46.56 [45.24-47.47]	45.88 [44.72-47.82]	0.866	0.770
ERVW-1	90.73 [88.81-92.25]	90.66 [86.82-92.40]	0.520	0.511	88.94 [87.22-90.22]	86.34 [83.58-89.53]	0.276	0.320
<b>Imprinted genes</b>								
<i>H19/IGF2</i> <i>CTCF3</i>	53.51 [50.41-57.41]	52.82 [36.39-55.99]	0.061	0.051	50.96 [48.94-55.68]	49.11 [42.83-52.04]	<b>0.041</b>	<b>0.026</b>
<i>KCNQ1OT1</i>	38.25 [35.72-42.06]	36.64 [30.67-40.48]	0.074	0.069	35.82 [33.86-39.04]	36.49 [32.69-38.01]	0.351	0.220
<i>SNURF</i>	39.41 [37.17-40.94]	37.22 [34.73-39.62]	0.109	0.068	39.40 [37.94-42.41]	38.18 [36.48-40.74]	0.216	0.173

Median [IQ range], Multivariate analyses were adjusted for maternal age and parity, gestational age at delivery and sex of the new-born, Significant differences in bold

**Table SVII: Comparisons between groups of relative expression in cord blood and placenta**

	Cord Blood				Placenta			
	Spontaneous (n = 48)	IVF/ICSI (n = 51)	Univariate P-value	Multivariate P-value	Spontaneous (n = 48)	IVF/ICSI (n = 51)	Univariate P-value	Multivariate P-value
<b>Repeats</b>								
LINE-1 ORF2	0.93 [0.73-1.21]	0.83 [0.56-1.01]	<b>0.036</b>	<b>0.020</b>	0.77 [0.55-1.05]	0.61 [0.44-0.90]	0.075	0.145
ERVFRD-1	1.18 [0.85-1.55]	0.93 [0.73-1.26]	<b>0.036</b>	<b>0.039</b>	28.37 [17.88-37.39]	17.96 [12.18-26.95]	<b>0.010</b>	<b>0.037</b>
AluYa5	0.94 [0.80-1.17]	1.01 [0.74-1.24]	0.954	0.568	0.79 [0.55-0.96]	0.66 [0.42-1.02]	0.284	0.553
ERVW-1	0.95 [0.78-1.07]	1.02 [0.82-1.27]	0.067	0.069	10.52 [5.13-19.76]	7.20 [3.52-17.77]	0.210	0.495
<b>Imprinted genes</b>								
<i>H19</i>	0.32 [0.10-1.29]	0.56 [0.17-1.00]	0.615	0.227	209.69 [160.44-323.96]	254.90 [132.84-375.00]	0.748	0.679
<i>KCNQ1</i>	1.80 [1.45-2.32]	1.68 [1.34-2.06]	0.244	0.436	0.07 [0.05-0.11]	0.08 [0.04-0.15]	0.712	0.660
<i>SNRPN</i>	1.27 [1.04-1.68]	1.35 [1.11-1.55]	0.615	0.787	0.19 [0.11-0.29]	0.18 [0.12-0.35]	0.678	0.839

Median [IQ range] of relative fold expression using *GAPDH*, *SDHA* and *TBP* as reference genes, Multivariate analyses were adjusted for maternal age and parity, gestational age at delivery and sex of the new-born, Significant differences in bold

## II. Post-translational histone modifications

### 1) Introduction: histones have a fundamental role in placental regulation

A wealth of data have underlined that histones could have a fundamental role in placental physiology (Kohan-Ghadr *et al.*, 2016). Notably, chronic ischemia in the rodent placenta was linked to decreased histone H3 acetylation levels (Eddy *et al.*, 2018). In human, abnormal histone methylation at some DMRs was linked with the development of placental disorders (Rahat *et al.*, 2017). Moreover, the interest of studying histone modifications in the context of ART is reinforced by the fact that histones are sensitive to environment and thus could mediate long-term effects of environment stressors (Barouki *et al.*, 2018).

From the precedent cohort, we selected the 16 patients from IVF/ICSI group who presented with below the 5<sup>th</sup> percentile of percentage placenta DNA methylation for at least one of the previously studied DMRs. These patients were compared with 16 controls matched for parity, new-born's sex, and gestational age at delivery. The controls were selected among the 48 women with natural pregnancy in the precedent study.

Thanks to a technique of Chromatin ImmunoPrecipitation associated with qPCR, both permissive (H3K4me2, H3K4me3 and H3K9ac) and repressive (H3K9 me3 and H3K9me2) histone marks were studied.

The aim of this original study was to profile permissive and repressive histone marks in placenta biopsies to reveal a better understanding of the epigenetic modifications in the context of ART.

### 2) Article 3: "The hypomethylation of imprinted genes in IVF/ICSI placenta samples is associated with concomitant changes in histone modifications"

This article will shortly be submitted in Clinical Epigenetics.

**Title: The hypomethylation of imprinted genes in IVF/ICSI placenta samples is associated with concomitant changes in histones modifications**

**Running title:** Methylation changes are associated with histones modifications in IVF-placentas

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## Abstract

### *Background*

Although more and more children are born by Assisted Reproductive Technologies (ART), the safety of these techniques has not fully been demonstrated. Notably, ART could disturb the delicate step of implantation, and trigger placenta-related adverse outcomes with potential long-term effects, through disrupted epigenetic regulation. We have previously demonstrated that DNA methylation in the placenta was significantly lower after IVF/ICSI than following natural conception at two differentially methylated regions (DMRs) associated with imprinted genes (IGs): *H19/IGF2* and *KCNQ1OT1*. As histone modifications are critical for trophoblast establishment and placental physiology, the aim of this study was to profile permissive and repressive histone marks in placenta biopsies to reveal a better understanding of the epigenetic changes in the context of ART.

### *Results*

Utilizing chromatin immunoprecipitation (ChIP) coupled with quantitative PCR, permissive (H3K4me3, H3K4me2 and H3K9ac) and repressive (H3K9me3 and H3K9me2) post-translational histone modifications were quantified. The analyses revealed significantly higher quantity of H3K4me2 precipitation in the IVF/ICSI group than in the natural conception group for *H19/IGF2* and *KCNQ1OT1* DMRs ( $P = 0.016$  and  $0.003$ , respectively). The quantity of both repressive marks H3K9me3 and H3K9me2 at *H19/IGF2* and *SNURF* DMRs was significantly lower in the IVF/ICSI group than in the natural conception group ( $P = 0.011$  and  $0.027$  for *H19/IGF2*, respectively; and  $P = 0.010$  and  $0.035$  for *SNURF*, respectively).

### *Conclusions*

These novel findings highlight that DNA hypomethylation at imprinted DMRs following ART is linked with increased permissive/decreased repressive histones marks, altogether promoting a more “active” chromatin conformation. This concomitant change in epigenetic state at IGs at birth might be an important developmental event as a consequence of ART manipulations.

Keywords: Assisted reproductive technologies, epigenetics, histone modifications, *in vitro* fertilization, DNA methylation, placenta



## Introduction

It is estimated that more than five million children have been born by Assisted Reproductive Technologies (ART) worldwide, representing ~4% of all births [1]. However, the safety of these techniques has not fully been demonstrated. ART has been associated with an increased risk of placenta-related adverse pregnancy, perinatal outcomes and imprinting disorders [2-5]. As ART take place during the epigenetic-sensitive period of preimplantation when genome-wide erasure and selective reprogramming occur, these techniques could affect the implantation step, when the dialogue between endometrium and embryo conditions the placental invasion into the uterine wall [6]. Together, these data raise the concern of the potential epigenetic vulnerability associated with ART.

Epigenetic mechanisms have been demonstrated to have a fundamental role in regulating placental function [6]. Notably, imprinted genes (IGs) are known to modulate foetal and placental growth, for example by regulating nutrients transfer, cell cycle and insulin metabolism [7-9]. Among imprinting mechanisms, DNA methylation in human placenta has been extensively studied, but literature about histones modifications after ART is relatively scarce. These modifications, called 'the histone code', lead to changes in regulation of DNA transcription, replication, recombination and repair. For example, acetylation of the lysine 9 of histone H3 (H3K9ac) neutralizes the positive charge of histone H3, decreasing the histone's affinity to bind DNA, resulting in a more "relaxed" chromatin state which is permissive to gene expression. More complex than acetylation, histone methylation can be either a permissive or a repressive mark, according to the residuals location on the histone tail. Though tri-methylation of lysine 4 on histone H3 (H3K4me3) is permissive, tri-methylation of lysine 9 on histone H3 is repressive when located in the promoters regions [10]. A wealth of data have underlined that histone modifications are critical for trophoblast establishment [11] and placental physiology [12]. Notably, chronic ischemia in the rodent placenta was linked to decreased histone H3 acetylation levels [13]. In human, abnormal histone methylation at

some imprinted DMRs was linked with the development of placental disorders such as preeclampsia and molar pregnancy [14]. Moreover, the interest of studying histone modifications in the context of ART is reinforced by the fact that histones marks could be disturbed by environmental stressors and thus could mediate long-term health effects of ART [15].

We previously demonstrated that DNA methylation in the placenta was significantly lower after IVF/ICSI than following natural conception at two imprinted DMRs: *H19/IGF2* and *KCNQ1OT1* [16]. The aim of this study was to determine whether DNA hypomethylation could be associated with particular histones profiles, to reveal a better understanding of the epigenetic modifications in the context of ART.

## **Materials and methods**

### **Study population**

Patients were prospectively included from January 1<sup>st</sup> 2013 to April 30<sup>th</sup> 2015 in the Department of Obstetrics, Gynaecology and Reproductive Biology at Dijon University Hospital, France. Controls were singleton pregnancies of women that had conceived spontaneously within 1 year after stopping contraception. IVF/ICSI patients were singleton pregnancies achieved following fresh embryo transfer after two days of *in vitro* culture. This cohort has previously been described [16] and used to compare the DNA methylation, by pyrosequencing, of 51 IVF/ICSI vs. 48 control placentas for three imprinted DMRs associated with the *H19/IGF2*:IG-DMR, *KCNQ1OT1*:TSS-DMR, and *SNURF*:TSS-DMR, named according to the recommendations for nomenclature [17]. For the present study, 16 patients from the IVF/ICSI group who presented with below 5<sup>th</sup> percentile for methylation for at least one of these DMRs were selected (Figure1). They were compared with 16 controls matched for parity, new-born's sex, and gestational age at delivery. The controls were selected among the 48 women with natural pregnancy from the previous study.

### **Sample preparation**

Placenta samples (1 cm<sup>3</sup>) were extracted from the foetal side near the umbilical cord insertion point within 15 min after delivery, washed twice in 0.9% NaCl before being snap frozen in liquid nitrogen and conserved at -80°C.

### **DNA methylation and expression**

Data for expression and DNA methylation experiments were obtained from our previous publication using real-time PCR and pyrosequencing, respectively [16], and analysed on this new cohort of 32 samples.

### ***Histones modifications analyses by Chromatin ImmunoPrecipitation (ChIP)***

For the three imprinted DMRs previously analysed three permissive histone marks (di/trimethylation of lysine 4 of histone H3, H3K4me<sub>2/3</sub>; acetylation of lysine 9 of histone H3, H3K9ac) [18] and two repressive histone marks associated with heterochromatic states (di/trimethylation of lysine 9 of histone H3, H3K9me<sub>2/3</sub>) [19,20] were studied.

#### *Preparation of chromatin from placenta samples*

Approximately 2 grams of frozen placenta was rinsed two times in cold PBS and placed in lysis tubes (Zymo Research BashingBeads Lysis Tubes - 0.5 mm) containing 1 mL buffer I (0.5 M Tris-HCl pH 7.5, 0.5 M KCl, 2.5 M NaCl, 0.5 M MgCl<sub>2</sub>, 25 mM EGTA, 0.3 M sucrose, 0.5 mM DTT, 0.1 mM PMSF, 3.6 ng/mL aprotinin, 5 mM sodium butyrate) and subject to three intervals of agitation (90 sec, 5000 rpm) using a Precellys24 homogenizer (Bertin technologies) with 5 minutes on ice between each agitation cycle. The cell suspension was then placed in 7 mL of buffer II (buffer I with NP40 at a final concentration of 0.2%) to purify nuclei by centrifugation at 8500 rpm/12720g for 20 minutes with low acceleration and low deceleration on a sucrose gradient (8 mL from the previous step carefully placed on 25 mL of buffer III (0.5 M Tris-HCl pH 7.5, 0.5 M KCl, 2.5 M NaCl, 0.5 M MgCl<sub>2</sub>, 25 mM EGTA, 1.2 M sucrose, 0.5 mM DTT, 0.1 mM PMSF, 3.6 ng/mL aprotinin, 5 mM sodium butyrate) in Sorvall™ RC 6 Plus Centrifuge (ThermoScientific™). The nuclear pellet was resuspended in digestion buffer (0.32 M sucrose, 50 mM Tris-HCl pH 7.5, 4 mM MgCl<sub>2</sub>, 1mM CaCl<sub>2</sub>, 0.1 mM PMSF, 5mM sodium butyrate) to 0.4 mg DNA/mL (Quantification by absorbance). Aliquots of 500 µL were distributed in 1.5 mL tubes. Micrococcal nuclease (Nuclease S7 15 IU/µL, Roche; final concentration 30 mIU/µL) was used to digest the chromatin to yield fragments one to five nucleosomes in length, which typically presented an incubation time of 3 minutes at 37°C. Digestion was stopped by adding 0.5 M EDTA at a final concentration of 20 mM and cooling on ice. After centrifugation (10 min, 15800g, 4°C), the supernatant was designated fraction S1. The pellet was resuspended in 500 µL lysis buffer (1 mM Tris-HCl pH 7.5, 0.2 mM EDTA, 0.2 mM PMSF, 5 mM sodium butyrate) and left 20-30 minutes on ice and subject

to a second centrifugation step (10 min, 15800g, 4°C), the supernatant of which was designated fraction S2. The size of the nucleosomes were determined following Nucleospin gel and PCR clean-up (Macherey-Nagel) of ~100 µL of each fraction, to ensure the S1 chromatin fraction mainly comprised of mono and dinucleosomes and the S2 poly-nucleosomes of 2 to 5 nucleosomes (Supplemental Figure 1).

#### *Immunoprecipitation of fresh chromatin*

For ChIP, we used antibodies directed against H3K4me3 (Diagenode C15410003-50), H3K4me2 (Millipore 07-030), H3K9ac (Cell Signaling 9649S), H3K9me3 (Abcam AB8898), H3K9me2 (Diagenode C15410060) and a negative control (mock precipitation with mouse IgG Millipore 12-371).

Chromatin was quantified by absorbance. For each condition, 4 µg of chromatin was used (constituted of 75% S1 and 25% S2) and suspended in incubation buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 20 mM sodium butyrate, 5 mM EDTA, 0.1 mM PMSF) in a total volume of 500 µL for each condition. We precleared chromatin by agitating overnight at 4°C with 4% Dynabeads® Protein G for immunoprecipitation (Invitrogen) washed three times in PBS-BSA 5%. In parallel, antibodies were combined to Dynabeads® Protein G for immunoprecipitation (Invitrogen), each antibody being agitated overnight in 250 µL of PBS-BSA 5% containing 16% of beads previously washed three times in PBS-BSA 5%.

The following day, beads were removed from precleared chromatin and antibodies-beads complexes were washed two times in PBS-BSA 5%. ChIP was then carried out for 4h at 4°C. The antibody-chromatin complexes were subsequently washed three times with each buffer A (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 5 mM sodium butyrate, 75 mM NaCl), B (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 5 mM sodium butyrate, 125 mM NaCl) and C (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 5 mM sodium butyrate, 175 mM NaCl) to ensure only the fraction of chromatin linked to the antibodies was retained. Elution was performed in 400 µL of elution buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM EDTA, 1% SDS). After a digestion with proteinase K (100 µg/mL) for 1 hour at 65°C, DNA was obtained from the input and bound

fractions with Nucleospin gel and PCR clean-up (Macherey-Nagel), according to the manufacturer's protocol (protocol for samples SDS rich for bound fractions) with a final elution with 40  $\mu$ L of water.

#### *Quantification of immunoprecipitated chromatin*

For an initial check of precipitated DNA quality, allelic specificity PCR assays were performed on all heterozygous samples. The PCR regions incorporated a Single Nucleotide Polymorphisms (SNPs) to allow both alleles to be discriminated. PCR and direct sequencing as used interrogate sequence traces, using Sequencher v4.6 (Gene Codes Corporation, MI). Primers, PCR mix and conditions are available in Supplemental Table S1.

Levels of immunoprecipitated chromatin at each region of interest were determined by quantitative real-time PCR amplification with the QuantStudio™ 5 Real-Time PCR system (Applied Biosystems™), using the SYBR™ Green PCR Master Mix (Applied) (see supplemental Table S2). Data were analysed with QuantStudio™ Design & Analysis Software (v1.3.1). Each PCR was run in triplicate and level of immunoprecipitation was quantified as a percentage of total input material as follows: % of input =  $2^{(-\Delta Ct)}$  where  $\Delta Ct$  is the difference in mean Ct triplicate between the DNA of interest and the DNA of the input.

To overcome the inherent variability of different immunoprecipitations, precipitation levels obtained at the region of interest were normalized to the level obtained for positive control intervals. Interrogation of placenta ChIP-seq datasets in the Genome Data viewer function in the GEO data repository revealed that the promoter of *KLF10* was enriched for the permissive histones marks H3K4me3, H3K4me2 and H3K9ac and was selected as a control region. For a control of repressed chromatin, we selected a heterochromatic satellite region on chromosome 4, which is ubiquitously associated with both H3K9me3 and H3K9me2.

### ***Methylation-sensitive genotyping***

Approximately 500 ng of heterozygous placenta DNA was digested with 10 units of *Hpa*II and BstU1 restriction endonuclease for 6 hours at 37°C. The digested DNA was subject to ethanol precipitation and resuspended in a final volume of 20 µl TE. Approximately 50 ng of digested DNA was used in each amplification reaction. The resulting amplicons were sequenced and the sequences traces were compared to those obtained for the corresponding undigested DNA template.

### **Statistical analyses**

Categorical variables were expressed as numbers (percentages) and compared using the Chi-2 test or Fisher exact test when appropriate. Continuous variables were expressed as means ± standard deviations (SD) or medians and interquartile ranges [IQR], and compared using the Student or Mann-Whitney test, as appropriate. Birth weights were normalized by conversion to z-scores calculated using normal birthweight curves of our population accounting for gestational age and new-born's sex [21]. Placental weights were also converted into z-scores according to gestational age and new-born's sex [22]. All statistical analyses were performed with SAS software, version 9.4 (SAS Institute Inc, USA). A two-tailed  $P < 0.05$  was considered significant.

### **Ethical approval**

All women had given written informed consent in accordance with the Declaration of Helsinki. The study was approved by the Institutional Review Board and the Ethics Committee of Dijon University Hospital (*Comité de Protection des Personnes [CPP] Est I, n°2012-A01010-43*).

## Results

### Confirmation of *in silico* histone modifications profiles in term placentas

To ensure the PCR amplified intervals mapping to the imprinted DMRs were enriched for the histone modification of interest, we performed an *in silico* analysis to ensure primer design coincided with the largest peaks in placenta-derived ChIP-seq datasets (GEO accession numbers GSM1160199 for H3K4me3; GSM753439 for H3K4me2, GSM818049 for H3K9ac and GSM1160204 for H3K9me3). Following primer optimization, amplicons of ~120-200bp, which would allow for amplification of dinucleosome fragments and larger, were used to quantify the precipitation levels for the three imprinted loci of interest: *H19/IGF2* (Figure 2A), *KCNQ1OT1* (Figure 2B) and *SNURF* (Figure 2C) DMRs.

Subsequently we analysed the allelic precipitation of the ChIP material in naturally conceived control samples, since we anticipated that permissive and repressive histone marks should be on opposite parental alleles at these imprinted DMRs. PCR were performed using primers that flanked highly informative SNPs and the resulting amplicons sequenced. In total 9 samples were heterozygous for *H19/IGF2* (*rs2107425*), 9 for *KCNQ1OT1* (*rs11023840*) and 9 for *rs4906939* within the *SNURF* DMR. The allelic precipitation levels were compared to methylation-sensitive genotyping, which revealed that the permissive marks were solely on the unmethylated allele and the repressive marks preferentially on the opposite chromosome (Figure 3).

### Comparison between IVF and natural conception

The two groups were comparable in terms of parental and new-born characteristics (Table 1). The mean group DNA methylation of *H19/IGF2*, *KCNQ1OT1* and *SNURF* DMRs was significantly lower in the IVF/ICSI group (45.1% [43.2-48.9]; 32.8% [31.7-35.7] and 38.3% [35.5-40.5], respectively) compared to those conceived naturally (53.5% [49.6-59.3],  $P =$



0.004; 39.4% [34.8-41.9],  $P = 0.001$  and 41.2% [38.4-42.1],  $P = 0.036$ , respectively; Table 2, Figure 4A). Relative expression was not different between groups (Table 2).

Quantitative PCR targeting *H19/IGF2* and *KCNQ1OT1* DMRs in the H3K4me2 precipitated material revealed significantly higher quantity of H3K4me2 in the IVF/ICSI group than in the natural conception group ( $P = 0.016$  and  $0.003$ , respectively; Figure 4B). There was no significant difference for H3K4me2 for *SNURF*, or for the other two permissive marks (H3K4me3, H3K9ac; Figure 4B).

The quantity of both repressive H3K9me3 and H3K9me2 modifications at *H19/IGF2* and *SNURF* DMRs was significantly lower in the IVF/ICSI group than in the natural conception group ( $P = 0.011$  and  $0.027$  for *H19/IGF2*, respectively; and  $P = 0.010$  and  $0.035$  for *SNURF*, respectively; Figure 4C) but there was no significant difference for either repressive mark at *KCNQ1OT1* DMR (Figure 4C).

One hypothesis that could explain the increased of permissive histones modifications in some samples was the presence of these marks in the repressed allele. To address this, we focused on the allelic precipitation profiles in IVF/ICSI samples with highest precipitation levels of permissive marks at the *H19/IGF2* DMR. Sequencing of samples heterozygous for SNPs revealed that the normally methylated allele was decorated with H3K4me2 and H3K9ac (Supplemental Figure 2). However similar experiments targeting the *KCNQ1OT1* and *SNURF* regions revealed maintained monoallelic precipitation patterns, consistent with mutually exclusivity of these histone modifications comparable to spontaneously conceived controls (Supplemental Figures 3 and 4, respectively).

## Discussion

These data demonstrate that DNA hypomethylation at imprinted DMRs could be associated with an increase in permissive histone marks and/or with a decrease in repressive histone modifications. This is consistent with a more “active” chromatin conformation on the normally repressed allele. However, by focusing on outlier samples with highest precipitation levels of permissive marks and heterozygous for SNPs, we observed the enrichment of H3K4 methylation and H3K9 acetylation on the normally repressed and DNA methylated allele at the *H19/IGF2* region. Since DNA methylation and H3K4 methylation are assumed to be mutually exclusive, this suggests that some cells within the samples could lose their allelic methylation and subsequently gained the permissive histone modifications. Single cell studies would be required to clarify this observation. In the *KCNQ1OT1* and *SNURF* regions, *ie* maternally imprinted genes, the monoallelic imprint seemed to be preserved.

Several studies have addressed the stability of DNA methylation in placenta after IVF. The first reported lower DNA methylation levels in placentas after IVF than after natural pregnancy [23], whilst other observed hypomethylation at the *MEST* and *H19* loci [23-25]. Our previous work evidenced lower DNA methylation levels of two imprinted loci (*H19/IGF2* and *KCNQ1OT1*) and two retroviruses (LINE-1 and ERVFRD-1) in IVF placentas while there was not any statistical difference between IVF and controls for *SNURF* DNA methylation [16]. However not all studies have shown such clear-cut differences [26]. Higher levels of expression of some IGs such as *MEST* and *H19* have been demonstrated after IVF [23,24], but other studies found lower levels for *IGF2* and *H19* [27].

To our knowledge, this study is the first reporting altered post-translational histone modification abundance in the human placenta after ART. Indeed, most studies focusing on histone regulation have been conducted in mouse models and mainly in pre-implantation embryos. For example, a study profiling epigenetic modifications at the *Mest* and *H19* loci in mouse blastocysts cultured *in vitro* found an increased abundance of permissive histone marks and a decrease in repressive histone modifications [19]. The same team confirmed

these trends at the *H19/Igf2* region on two cohorts of 2-cells embryos cultured *in vitro* until the blastocyst stage or vitrified/thawed and then cultured *in vitro* until the blastocyst stage [28]. Similarly, altered methylation of histones and DNA at the *H19/Igf2* region has also been shown in embryonic stem cells derived from mice pre-implantation embryos [29]. Overall, our results are consistent with those reported in these models.

The increased in permissive and decrease of repressive histones marks observed in our study in hypomethylated samples after ART, together with the lower methylation levels [16], support the hypothesis that chromatin could be more “open” and permissive to transcription. However, increased expression was evidenced neither in this study nor in the previous one [16]. However, as we worked on term placentas, plasticity and adaptability of placenta to environment [6] suggest that the altered expression could occur throughout pregnancy and no longer be visible at birth. This is well demonstrated by increased *Igf2* after ART in mice during gestation but no longer visible at birth [30,31] and by the observation that a positive correlation between placental *IGF2* expression and birth weight is only present during the first trimester and not at term [32]. Indeed, as the placenta undergoes rapid epigenomic changes, a placenta collected at birth may not reflect the changes occurring throughout pregnancy [15]. However, these epigenetic changes occurring during prenatal period, probably participating in compensation mechanisms [6,16], raise question about the potential long-term effects of such modifications on children conceived by ART.

A limitation of this study could be the restricted number of IGs and histone marks studied. It would be interesting to extend analyses to other imprinted DMRs as well as imprinted genes with unmethylated promoters, regulated by neighbouring DMRs *in cis*, such as *CDKN1C* and *PHLDA2* [20]. Furthermore, studying non-imprinted loci associated with early and late placental development could be revealing. Moreover, as ART are not limited to IVF, it would also be useful to include groups of patients having undergone frozen-thawed embryo transfer or intra-uterine insemination. Indeed, some studies have demonstrated the negative impact of frozen-thawed embryo transfer on obstetrical and neonatal outcomes [33,34].

## **Conclusion**

These novel findings highlight that DNA hypomethylation at imprinted DMRs after ART is linked with increased permissive/decreased repressive histones marks, altogether promoting an “active” conformation of the chromatin. This concomitant change in epigenetic state at IGs at birth might be an important developmental event as a consequence of ART. To date, exact causes and consequences of these changes are not known. Better knowledge of the mechanisms at stake could enable to adapt our daily practice in order to reduce the impact of these changes.

## **Authors’ roles**

PF, DM and CC were the principal investigators and take primary responsibility for the paper. PF, DM, PP and CC were responsible for the study design. CC and PF recruited the patients. CC, DM, PP, MS, JH and AM were involved in experiments. PF, DM and CC coordinated the research. CC performed the statistical analyses. DM, PF and CC drafted the manuscript.

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## **Conflict of interest**

None declared.

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## Tables

**Table 1. Population characteristics**

	Natural Conception (n = 16)	IVF/ICSI (n = 16)	P
<b>Maternal characteristics</b>			
Age (years)	28.5 +/- 4.2	31.3 +/- 6.3	0.137
Pre-pregnancy parity	0 [0 - 0.5]	0 [0 - 0.5]	0.980
Tobacco Smoking	2 (1.3%)	1 (6.3%)	1
Pre-pregnancy BMI (kg/m <sup>2</sup> )	21.7 +/- 2.8	24.6 +/- 4.8	0.050
<b>Paternal characteristics</b>			
Age (years)	30.7 +/- 5.8	32.8 +/- 5.5	0.306
Tobacco smoking	3 (1.9%)	5 (3.1%)	0.685
<b>New-born characteristics</b>			
Term (weeks of gestation)	39.7 +/- 1.1	38.9 +/- 2.1	0.194
Birth weight (grams)	3310.9 +/- 461.6	3184.1 +/- 593.7	0.505
z-score of birth weight	0.06 +/- 1.13	0.10 +/- 1.23	0.930
Placenta weight (grams)	483.4 +/- 109.1	517.8 +/- 135.1	0.435
z-score of placenta weight	-1.39 +/- 0.82	-1.02 +/- 0.93	0.235
Sex ratio M/F [95 % CI]	0.60 [0.43 - 0.77]	0.60 [0.43 - 0.77]	1

Results are displayed as: n (%), mean ± standard deviation, or median [interquartile range] and compared with Student's test or Mann-Whitney according to the distribution, significant results in bold, BMI: body mass index

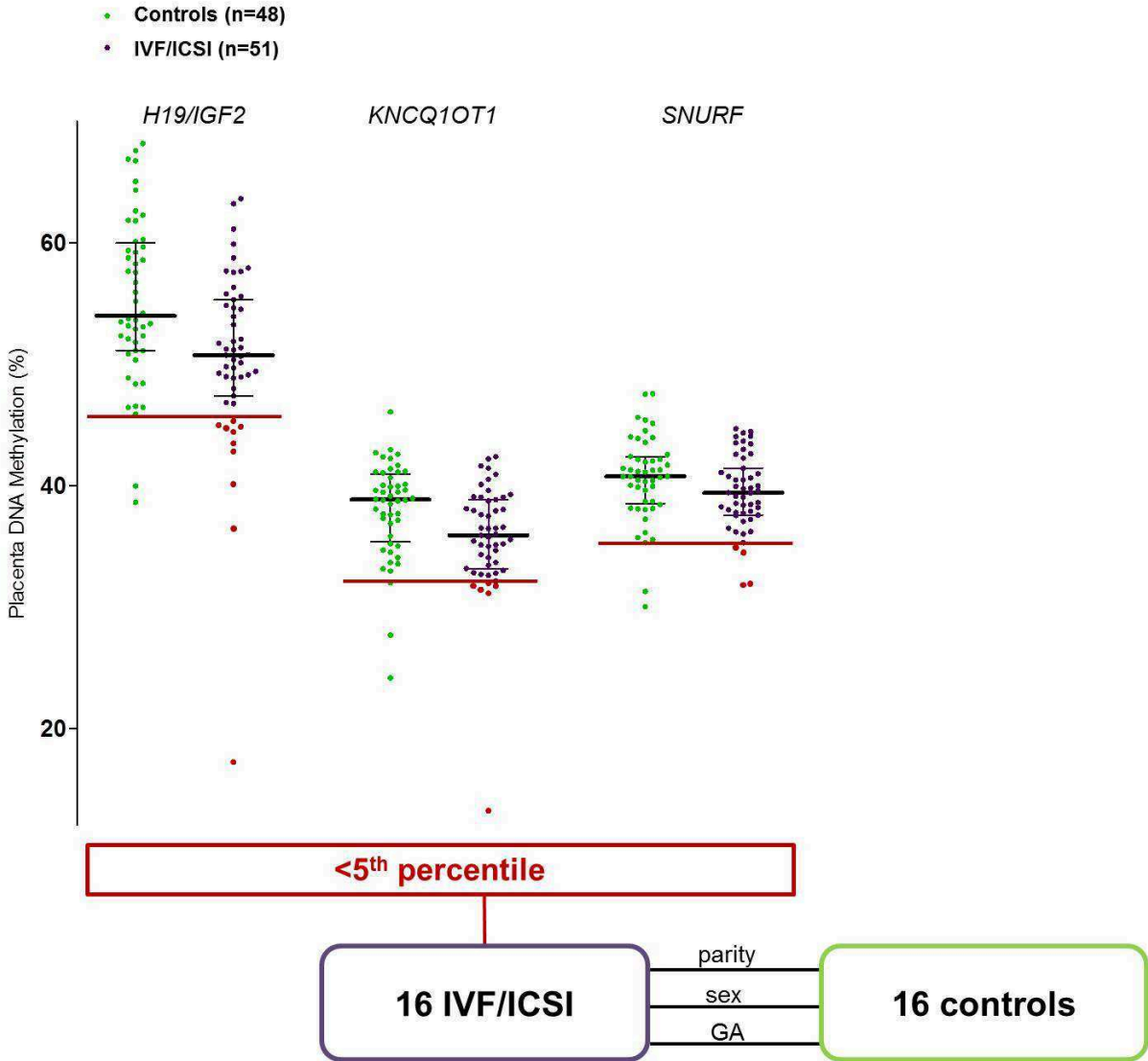
**Table 2. DNA methylation and expression according to the mode of conception**

	Methylation			Expression		
	Natural conception	IVF/ICSI	P	Natural conception	IVF/ICSI	P
<b>Imprinted genes</b>						
<i>H19/IGF2</i>	53.54 [49.59-59.29]	45.09 [43.16-48.94]	<b>0.004</b>	228.34 [186.14-350.50]	301.83 [173.01-506.40]	0.395
<i>KCNQ1OT1</i>	39.38 [34.76-41.94]	32.79 [31.70-35.73]	<b>0.001</b>	0.06 [0.05-0.11]	0.06 [0.04-0.23]	0.594
<i>SNURF</i>	41.20 [38.41-42.09]	38.30 [35.54-40.47]	<b>0.036</b>	0.23 [0.09-0.31]	0.29 [0.11-0.54]	0.244

Results are displayed as median [interquartile range]. P-values are the result of Student or Mann-Whitney test, as recommended according to the distribution

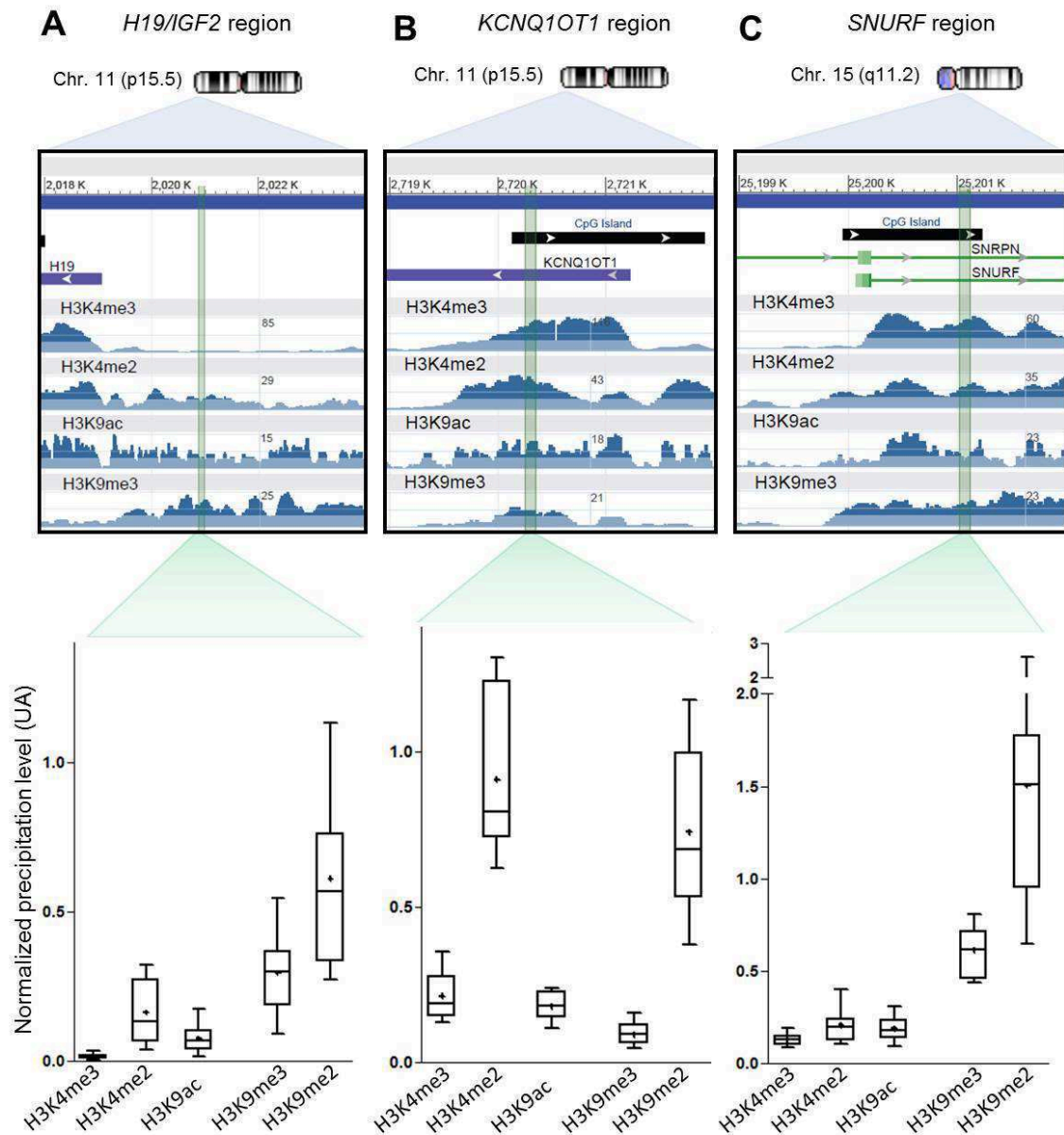


# Figures



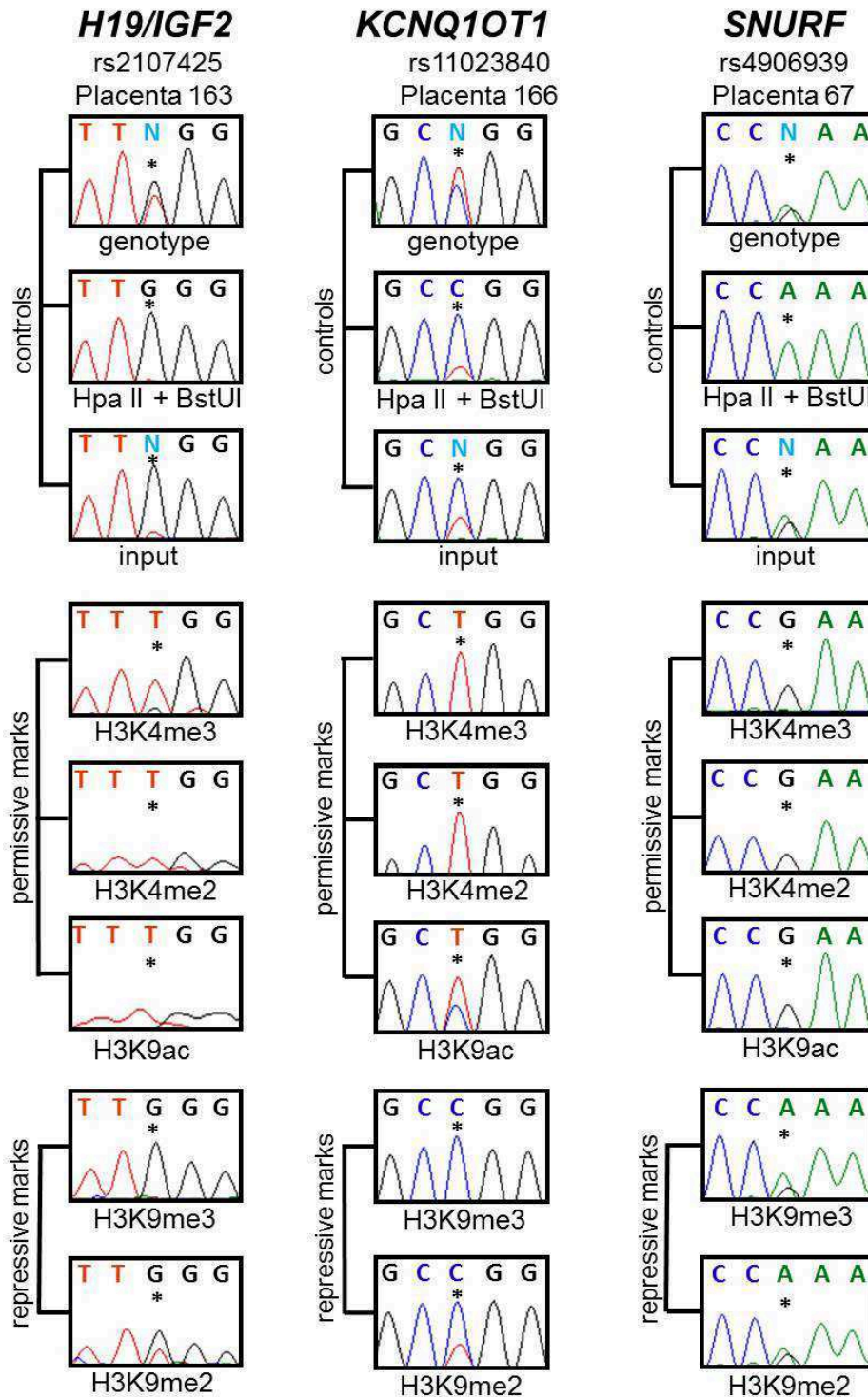
**Figure 1: Flowchart.**

From our precedent study (Choux *et al.*, 2018) we selected the patients from IVF/ICSI group who presented with below the 5<sup>th</sup> percentile of percentage methylation for at least one of the studied DMRs (*H19* DMR, *KNCQ1OT1* DMR and *SNURF*). The 16 selected patients were then matched for 16 controls from the same cohort for parity, new-born’s sex, and gestational age at delivery.



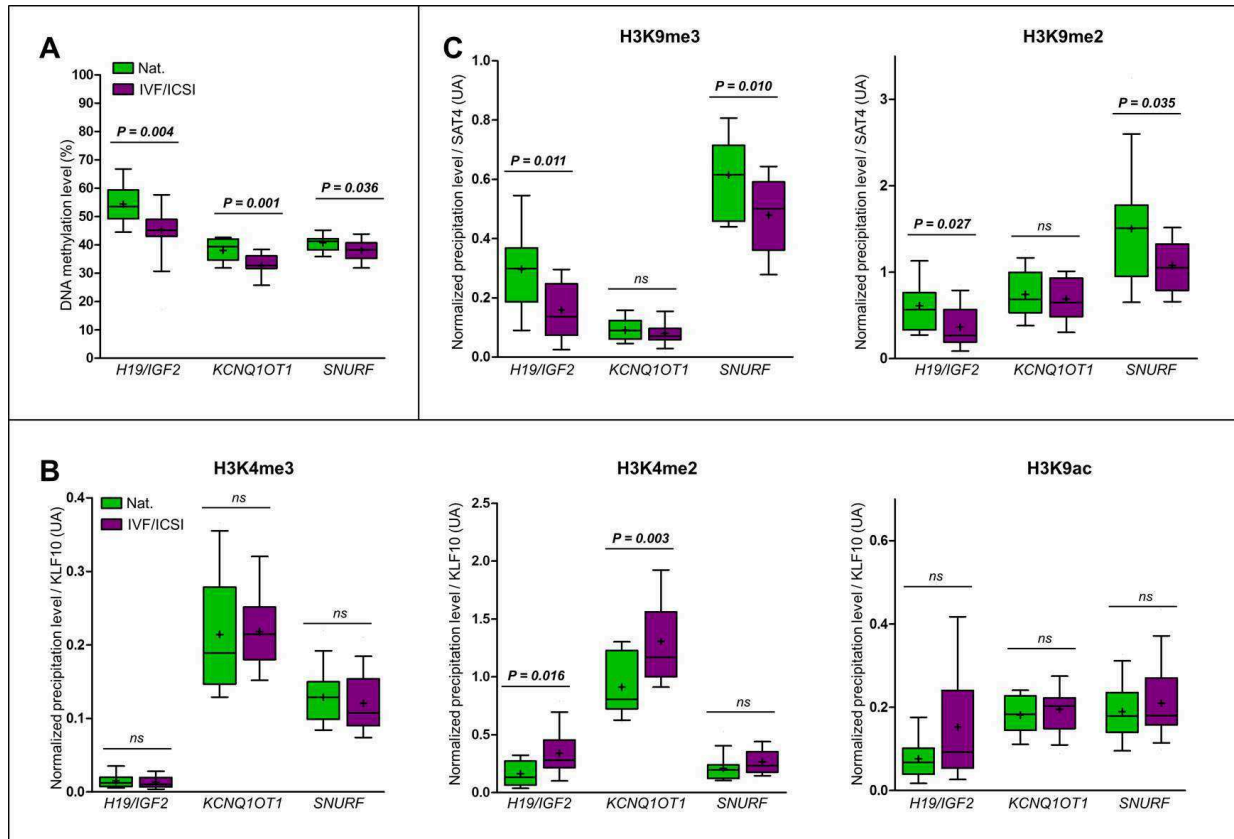
**Figure 2: Mapping of histone marks in placenta for each region of interest**

For each region of interest (*H19* DMR (A), *KCNQ1OT1* DMR (B) and *SNURF* DMR (C)), we marked in green the sequence amplified by the qRT-PCR ChIP primers. To ensure the PCR amplified intervals mapping to the imprinted DMRs were enriched for the histone modification of interest, we performed an *in silico* analysis to ensure primer design coincided with the largest peaks in placenta-derived ChIP-seq datasets. We used the Gene Expression Omnibus (GEO) application, available at <https://www.ncbi.nlm.nih.gov/geo/>. The GEO accession numbers for H3K4me3, H3K4me2, H3K9ac, and H3K9me3 were GSM1160199 (Histone H3K4me3 ChIP-Seq of Fetal Placenta), GSM753439 (ChIP-Seq Analysis of H3K4me2 in BMP4 Trophoblast Cells), GSM818049 (ChIP-Seq Analysis of H3K9ac in BMP4 Trophoblast Cells) and GSM1160204 (Histone H3K9me3 ChIP-Seq of Fetal Placenta), respectively. In parallel, normalized precipitation levels obtained in the 16 control samples of our cohort are displayed for each studied region. Precipitation levels of permissive marks H3K4me3, H3K4me2 and H3K9ac were normalized on precipitation levels of the *KLF10* gene whereas repressive marks H3K9me3 and H3K9me2 were normalized on the satellite region SAT4.



**Figure 3: Histone post-translational modifications are imprinted in the placenta**

For each region of interest, an informative SNP was selected, the control DNA was genotyped, and heterozygous samples were studied. DNA was digested by HpaII and BstUI before sequencing to evidence the methylated allele. Then the ChIP products were also sequenced to assess which allele was the most represented in either permissive or repressive marks. It appears that the unmethylated allele is mostly represented in the permissive marks H3K4me3, H3K4me2 and H3K9ac. On the contrary, the methylated allele is mostly represented in the repressive marks H3K9me3 and H3K9me2. Thus methylated regions are associated with repressive histone marks whereas unmethylated regions are associated with permissive histone marks.



**Figure 4: Comparisons between IVF/ICSI group and controls, DNA methylation levels were lower in the IVF/ICSI group than in the natural conception group (A), some permissive and repressive marks normalized precipitation levels were higher and lower, respectively, in the IVF/ICSI group compared to the natural conception group (B and C, respectively).**

Each box represents the interquartile range (IQR). Lines inside the boxes are the median. Whiskers represent the 10<sup>th</sup> and 90<sup>th</sup> percentiles. Crosses represent the mean. For the histone marks profiling, the figures present the ratio between the % of input obtained at the region of interest and the % of input obtained at the control region. IVF: *In Vitro* Fertilization, ICSI: IVF with Intra Cytoplasmic Sperm Injection, ns: non-significant. Nat.: natural conception group

## Supplementary material

**Table S1: Primers for sequencing**

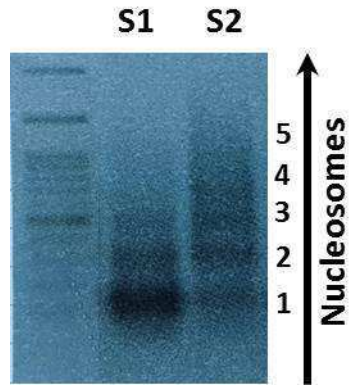
Region	Data base Reference	Sequence Number Nucleotide position	Primers (sequencing primer in bold)	Product Reaction temperature
<b>Imprinted genes</b>				
<i>H19/IGF2: IG-DMR</i>	UCSC	hg38: chr11:1,997,582-2,003,510	<b>F: GGGCTGTCCTTAGACGGAGTC</b> R: GTATTTCTGGAGGCTTCTCC	409 pb 56°C
<i>KCNQ1OT1: TSS-DMR</i>	UCSC	hg38 : chr11:2,698,718-2,701,029	<b>F: GATGCCACCCGGGCTCAGATTGG</b> R: ACCCCGGGGTGGTGAACACATCA	216 pb 56°C
<i>SNURF: TSS-DMR</i>	UCSC	hg38 : chr15:24,954,857-24,956,829	<b>F: ACTGCGCCACAACCGGAAAGGAA</b> R: GTAGAGCCGCCAGTGGGGAGGG	320 pb 56°C

Bioline products were used for the PCR mix as follows: water 11.55  $\mu$ L, 5M betaine 7.5  $\mu$ L, 10xNH4 Reaction Buffer 2.5  $\mu$ L, 50 mM MgCl<sub>2</sub> 0.75  $\mu$ L, 2 mM dNTP 0.5  $\mu$ L, BIOTAQ DNA polymerase 5U/ $\mu$ L 0.2  $\mu$ L, with 1  $\mu$ L DNA and 2 ng/ $\mu$ L of each primer, for a final volume of 25  $\mu$ L. Amplification was performed with the following conditions: 5 min denaturation phase at 96°C, followed by 40 cycles of three steps: 30 s denaturation at 96°C, 30 s annealing at 56°C and 30 s extension at 72°C with final extension 7 min at 72°C.

**Table S2: Primers for qRT-PCR CHIP**

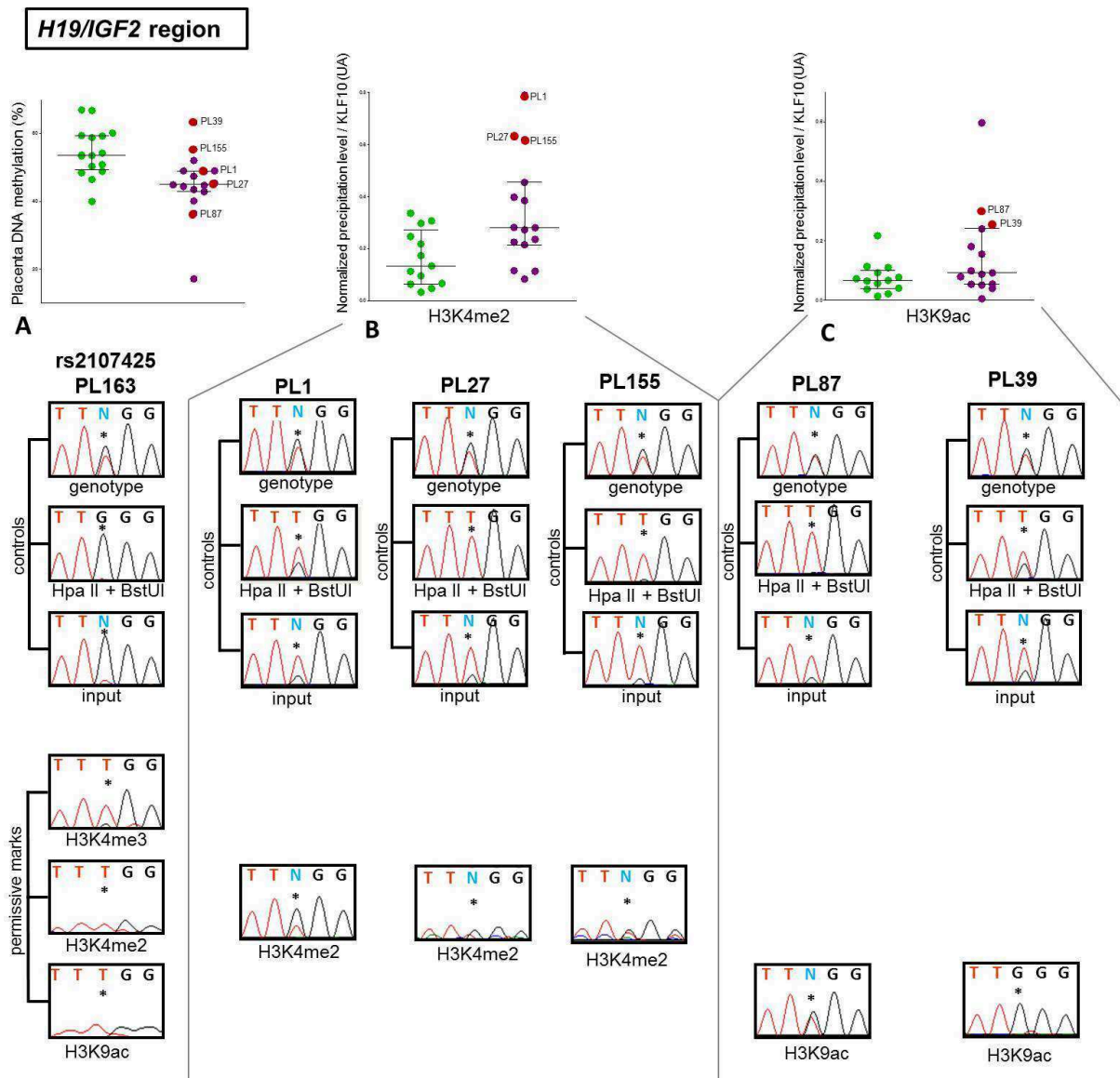
Region	Data base Reference	Sequence Number Nucleotide position	Primers	Product Reaction temperature
<b>Housekeeping genes</b>				
<i>KLF10</i>	Ensembl	Ensembl ENSG00000155090 hg 38: chr 8:102,648,779-102,655,902 reverse strand	F: GACAAGACCAGGCGAGGAAG R: GCCAACCATGCTCAACTTCG	89 pb 60°C
<i>SATα chr4</i>	NCBI Alexiadis <i>et al.</i> , 2017	M38467	F: CTGCACTACCTGAAGAGGAC R: GATGGTTCAACACTCTTACA	139 pb 60°C
<b>Imprinted genes</b>				
<i>H19/IGF2: IG-DMR</i>	UCSC	hg38: chr11:1,997,582-2,003,510	F: AGCTGTGCTCTGGGATAGATG R: ATGATCACAGTGTGTTCCACC	60 pb 60°C
<i>KCNQ1OT1: TSS- DMR</i>	UCSC	hg38 : chr11:2,698,718-2,701,029	F: ATTTCCGACTCCGGTCCCAA R: CATCGTGGTTCTGAGTCCGC	94 pb 60°C
<i>SNURF: TSS-DMR</i>	UCSC	hg38 : chr15:24,954,857-24,956,829	F: CTGTGCTACTGCCCTTCTG R: GGAGTACTAAGGGACGCTGAATG	68 pb 60°C

4.5 µL 2X SYBR™ Green PCR Master Mix (Applied) was used with 0.1 µL primers (0.1 µg/µL), 0.4 µL water and 5 µL DNA (diluted 1/40), for a final volume of 10 µL. Amplification was performed in triplicate using QuantStudio™ 5 Real-Time PCR system (Applied Biosystems™) with the following conditions: 10 min denaturation phase at 95°C, followed by 40 cycles of two steps: 15 s denaturation at 95°C and 1 min annealing/extension at 60°C.



**Supplemental Figure 1: Nucleosome ladder**

100  $\mu$ L of each fraction of chromatin S1 and S2 were cleaned and migrated on an agarose gel. Then the gel was immersed in a midori green bath during 2 hours. S1 chromatin fraction was mostly composed of mono and di nucleosomes whereas S2 fraction contained mostly fragments of 2 to 5 nucleosomes.



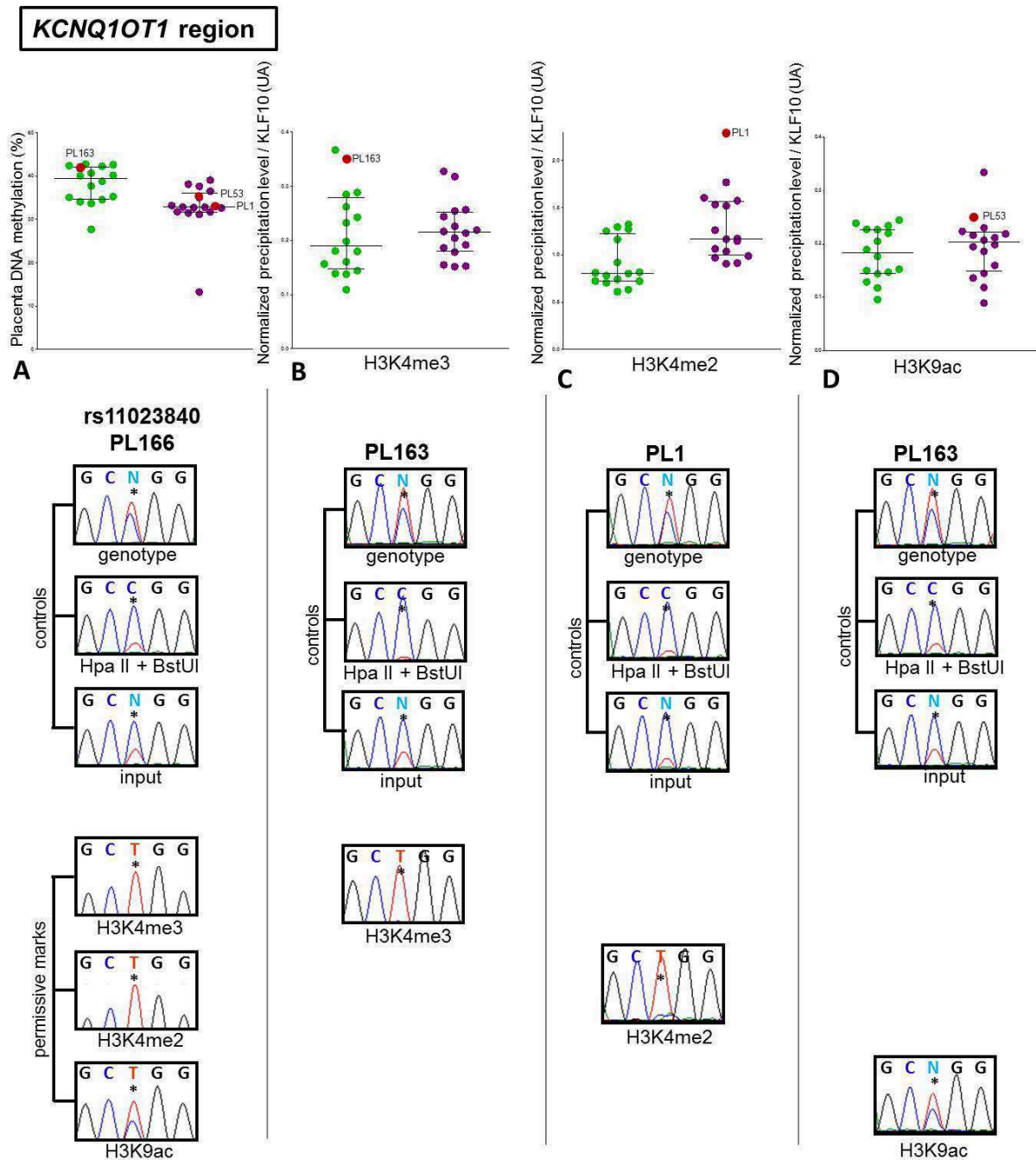
**Supplemental Figure 2: Analysis of the outliers in the *H19/IGF2* region**

A: Placental DNA methylation of IVF/ICSI group (in purple) vs. controls (in green) with the outliers marked with red dots. PL163 represents the control at the SNP rs2107425 of this region. Sequencing of cDNA (genotyping), DNA digested with Hpa II and input chromatin for ChIP and chromatin precipitated with permissive histone marks H3K4me3, H3K4me2 and H3K9ac are displayed.

B: Precipitation levels of H3K4me2 normalized on KLF10, with the 3 outliers studied marked with red dots (PL1, 27 and 155). Sequencing of the 3 outliers for controls (genotype, HpaII and input) and for H3K4me2

C: Precipitation levels of H3K9ac normalized on KLF10, with the 2 outliers studied marked with red dots (PL87 and 39). Sequencing of the 2 outliers for controls (genotype, HpaII and input) and for H3K9ac





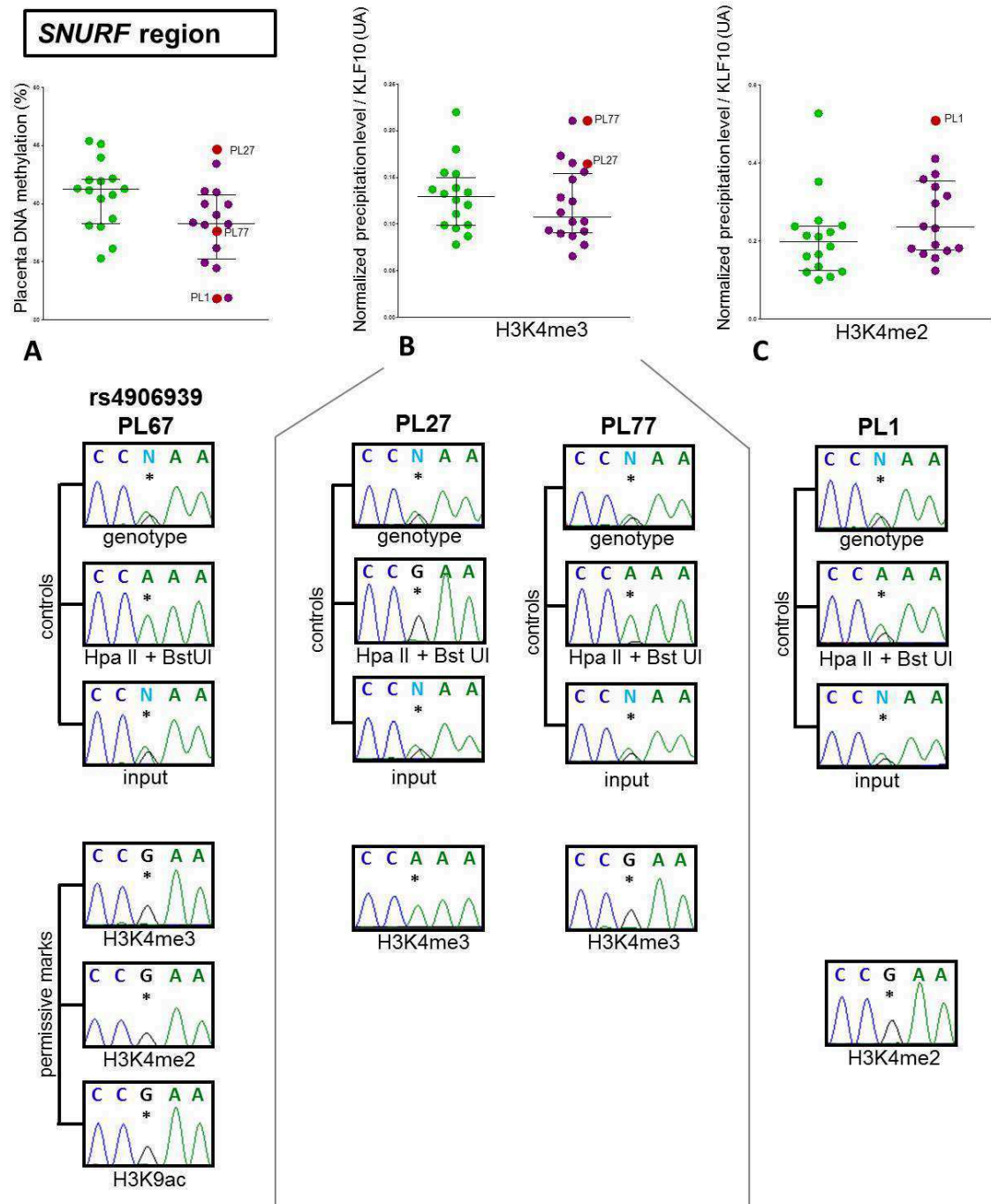
**Supplemental Figure 3: Analysis of the outliers in the *KCNQ1OT1* region**

A: Placental DNA methylation of IVF/ICSI group (in purple) vs. controls (in green) with the outliers marked with red dots. PL166 represents the control at the SNP rs11023840 of this region. Sequencing of cDNA (genotyping), DNA digested with Hpa II and BstUI and input chromatin for ChIP and chromatin precipitated with permissive histone marks H3K4me3, H3K4me2 and H3K9ac are displayed.

B: Precipitation levels of H3K4me3 normalized on KLF10, with the outlier studied marked with red dot (PL163). Sequencing of the outlier for controls (genotype, HpaII + BstUI and input) and for H3K4me3

C: Precipitation levels of H3K4me2 normalized on KLF10, with the outlier studied marked with red dot (PL1). Sequencing of the outlier for controls (genotype, HpaII + BstUI and input) and for H3K4me2

D: Precipitation levels of H3K9ac normalized on KLF10, with the outlier studied marked with red dot (PL163). Sequencing of the outlier for controls (genotype, HpaII and input) and for H3K9ac



**Supplemental Figure 4: Analysis of the outliers in the *SNURF* region**

A: Placental DNA methylation of IVF/ICSI group (in purple) vs. controls (in green) with the outliers marked with red dots. PL67 represents the control at the SNP rs4906939 of this region. Sequencing of cDNA (genotyping), DNA digested with Hpa II and BstUI and input chromatin for ChIP and chromatin precipitated with permissive histone marks H3K4me3, H3K4me2 and H3K9ac are displayed.

B: Precipitation levels of H3K4me3 normalized on KLF10, with the outliers studied marked with red dots (PL27 and 77). Sequencing of the outliers for controls (genotype, HpaII + BstUI and input) and for H3K4me3

C: Precipitation levels of H3K4me2 normalized on KLF10, with the outlier studied marked with red dot (PL1). Sequencing of the outlier for controls (genotype, HpaII + BstUI and input) and for H3K4me2

***DISCUSSION  
AND  
PERSPECTIVES***

## DISCUSSION AND PERSPECTIVES

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### I. Discussion

This thesis aimed to comprehensively describe the epigenetic modifications of some IGs and TEs induced by ART in human placenta and cord blood at birth and to link these findings to obstetrical and perinatal outcomes. The final objective was to better understand the causes and consequences of ART on epigenetic regulation and to minimize these variations in the interest of full epigenetic safety after ART.

The inclusion of an extensive cohort of patients helped us to answer a certain number of questions. These new findings complete the data in the literature and open new perspectives.

#### **Methylation and expression of IGs and TEs in human placenta and cord blood at birth after IVF**

In the first study (Choux *et al.*, 2018), we analysed a very homogeneous cohort of 51 patients pregnant after IVF with fresh embryo transfer after 2 days of culture and compared them with 48 controls pregnant within 1 year of stopping contraception. In cord blood and placenta collected at birth, we studied the DNA methylation of 3 imprinted DMRs and 4 TEs and the expression of genes associated with these DMRs or sequences. We found changes in the epigenetic regulation of IGs and TEs after IVF in placenta. Indeed, DNA methylation levels for H19/IGF2 and KCNQ1OT1 DMRs, LINE-1 and ERVFRD-1 in placenta were lower in the IVF/ICSI group than in the control group. Expression level of ERVFRD-1 in placenta was also lower in the IVF/ICSI group than in the control group. To our knowledge, this is the first report of the impact of ART on the regulation of syncytins and the first study to address both the methylation and expression of TEs and IGs in human cord blood and placenta at birth. **Table 5** summarizes existing studies on methylation and/or expression of IGs and/or TEs in human placenta and/or cord blood after ART.

The modifications evidenced at birth witness some epigenetic changes after ART, but it is unclear if they demonstrate dysregulation at birth or if it reveals some mechanisms settled to compensate prior dysfunctions at the beginning of the pregnancy. From our results combined with data in the literature, the second option seems more persuasive.

Indeed, the hypomethylation of 2 IGs which are part of the IGN and the increased variations in DNA methylation levels after IVF clearly illustrates the plasticity of the placenta. It appears that some imprinted genes could participate in gene networks with reciprocal functions, resulting in highly coordinated regulation of foetal and placental growth during pregnancy, and even after birth (Azzi *et al.*, 2014). This IGN was first described in mice (Varrault *et al.*, 2006), and more recently in the human placenta (Iglesias-Platas *et al.*, 2014), in which the paternally-expressed zinc-finger transcription factor PLAGL1 regulates multiple imprinted and non-imprinted genes including *IGF2*, *CDNK1*, *SLC2A4* and *PPAR $\gamma$ 1*. It has been hypothesized that this set of genes could be upregulated in a coordinated fashion after ART, the final goal being to correct primary dysfunctions of the placenta (Fauque *et al.*, 2010). Given the redundant functions of the members of this network, the failure of one gene would be compensated by the upregulation of others. In addition, we did not find any difference in expression at birth, showing that the levels may have returned to normal. The negative correlation between H19 expression and gestational age at delivery could also be evidence of the plasticity of the placenta throughout pregnancy. The unavailability of placenta during pregnancy in humans prevented us from confirming this hypothesis but, given animal studies that evidenced dynamic changes throughout pregnancy but normal levels of expression at birth (Fortier *et al.*, 2008; Fortier *et al.*, 2014), we can suppose that, depending on the severity of the primary injury and the efficacy of the compensation mechanisms involved, modifications in levels of expression may return to normal at birth or persist.

Moreover, data about TEs also support the idea of the placenta being a smart organ that is able to adapt during pregnancy to meet foetal demands. Indeed, syncytin-1 and -2 are fusion proteins encoded by retroviruses ERVW-1 and ERVFRD-1, respectively (Bolze *et al.*, 2017). They are known to be involved in the fusion of

cytotrophoblasts, a necessary step in the formation of the syncytiotrophoblast, the placental exchange area between mother and foetus. Their dysregulation has been demonstrated in cases of preeclampsia and IUGR (Vargas *et al.*, 2014; Bolze *et al.*, 2017). A recent study even suggested that decreased placental DNA methylation of these retroviruses could be predictive of IUGR and that syncytins could be used as biomarkers of IUGR (Makaroun *et al.*, 2018). In our study, DNA methylation of ERVFRD-1 in the placenta was lower in the IVF/ICSI group than in the control group, arguing for a dysregulation of syncytins after IVF/ICSI, which might promote an increase in cell fusion to maintain foetal growth during pregnancy. The expression level of ERVFRD-1 in placenta correlated negatively with the gestational age at delivery in the IVF/ICSI group, suggesting that the compensatory mechanism developed throughout pregnancy could be exhausted by the end of the pregnancy following ART. Moreover, expression of ERVFRD-1 in placenta correlated positively with birth weight and placental weight, but only in the control group, showing the importance of syncytin-2 for placental physiology, and the potential dysregulation in cases of ART.

Thus, this first study suggests that ART modifies placental epigenetic regulation and, given that TEs and IGs are known to be involved in foetal and placental development, this could explain in part the increased rate of placenta-related adverse pregnancy outcomes after ART.

### **Post-translational histone modifications in the placenta after IVF**

Epigenetic regulation not only is represented by DNA methylation but also encompasses histone modifications. A wealth of data underlines the fact that histones could have a fundamental role in placental physiology (Kohan-Ghadr *et al.*, 2016). Notably, chronic ischemia in the rodent placenta was linked to decreased histone H3 acetylation levels (Eddy *et al.*, 2018). In human, abnormal histone methylation at some DMRs was linked with the development of placental disorders (Rahat *et al.*, 2017). Moreover, the interest of studying histone modifications in the context of ART is reinforced by the fact that histones are sensitive to environment

and thus could mediate the long-term effects of environment stressors (Barouki *et al.*, 2018).

Thus we decided to complete the study of the epigenetic control of IGs in the placenta by studying histone modifications in the context of ART.

The results revealed a significantly higher quantity of H3K4me2 in the IVF/ICSI group than in the natural conception group for *H19/IGF2* and *KCNQ1OT1* DMRs.

The quantity of both repressive marks H3K9me3 and H3K9me2 at *H19/IGF2* and *SNURF* DMRs was significantly lower in the IVF/ICSI group than in the natural conception group.

These data demonstrate that DNA hypomethylation at imprinted DMRs could be associated with an increase in permissive and a decrease in repressive histone marks. This is consistent with a more “active” chromatin conformation on the normally repressed allele.

To our knowledge, this is the first study reporting post-translational histone modifications in the human placenta after ART. Indeed, most studies focusing on histone regulation have been conducted in mouse models and mainly in pre-implantation embryos. For example, a study profiling epigenetic modification at the *Mest* and *H19* loci in mouse blastocysts found an increase in permissive and a decrease in repressive histone modifications (Jahangiri *et al.*, 2014). Similarly, altered methylation of histones and DNA at the *H19/Igf2* region has also been shown in embryonic stem cells from mice pre-implantation embryos (Li *et al.*, 2005). Overall, our results are consistent with those reported for mouse models.

Our findings, together with the literature data, reinforce the hypothesis of some mechanisms settled in placenta after ART, probably useful to mediate placental plasticity to compensate primary disorders, and written through epigenetic changes such as DNA methylation but also histone modifications.

Though some questions remain to be answered from the original project objectives, this thesis paves the way for further original studies.

## II. Perspectives

During this thesis, an extensive cohort of almost 250 patients was prospectively included, resulting in 4 groups of ART techniques (IUI, IVF/ICSI, frozen/thawed embryo transfer, prolonged culture) and 4 groups of controls (natural pregnancy within 6 months after stopping contraception, within 6 months to 1 year or within more than 1 year of infertility, and pregnancies after ovulation induction), opening the door to multiple new studies.

### 1) Epigenetic regulation after frozen-thawed embryo transfers

The recent meta analyses on the potential detrimental effects of FET on pregnancy such as increased rates of gestational hypertension, preeclampsia and large for gestational age new-borns (Sazonova *et al.*, 2012; Wennerholm *et al.*, 2013; Ishihara *et al.*, 2014; Korosec *et al.*, 2014; Litzky *et al.*, 2018; Roque *et al.*, 2018), the discovery of differential foetal growth kinetics according to the mode of conception (**Annex 2**) (Ginod *et al.*, 2018) and our recently accepted article demonstrating the modification of placental volume and other first-trimester parameters with opposite trends for fresh embryo transfer and FET (**Annex 3**), raise questions about epigenetic regulation in cases of FET.

Indeed, it is now largely recognized that endometrium-embryo synchrony is essential in IVF. Indeed, the duration of the window of implantation, defined by the perfect balance of oestradiol and progesterone, may only last 2 to 5 days (Ozgur *et al.*, 2018). Progesterone in particular has to be tightly regulated to ensure the highest rates of implantation. In addition, there is now converging evidence suggesting that hormonal therapy could affect endometrial receptivity, thus trophoblastic invasion and, finally, pregnancy outcomes. A high dose of total FSH negatively impacted live birth rates in fresh ET compared to subsequent FET (Munch *et al.*, 2017).



First-trimester maternal serum PAPP-A is currently recognized to be predictive of placenta-related diseases such as preeclampsia and small for gestational age (Tan *et al.*, 2018). Increased oestrogen levels at ovulation triggering before fresh ET were associated with lower first-trimester PAPP-A levels, preeclampsia, low birth-weight and preterm birth (Imudia *et al.*, 2012; Giorgetti *et al.*, 2013; Hu *et al.*, 2014; Liu *et al.*, 2017). PAPP-A values were found to be lower after fresh ET than after FET (Amor *et al.*, 2009; Gjerris *et al.*, 2009). FET in artificial cycles was associated with increased PPH risk compared with FET in natural cycles (Healy *et al.*, 2010; Nakamura *et al.*, 2015). The use of hCG triggering or progesterone supplementation for FET has been associated with lower clinical pregnancy rates than FET after natural cycles (Montagut *et al.*, 2016). Particularly, micronized progesterone has been shown to increase placental volume if given in women with first trimester threatened abortion (Turgal *et al.*, 2017). In our centre, the different conditioning between fresh ET and FET patients result in a cumulated dose of progesterone nearly four times lower for fresh ET, but in this case, the proper secretion of hormones by the multiple *corpus lutea* also has to be taken into account (Conrad *et al.*, 2013).

As a potential explanation of these consequences of hyperstimulation, a recent study investigated the endometrial gene expression between hyperstimulated and non-hyperstimulated women and demonstrated that superovulation alters the expression of genes implicated in endometrial remodelling during implantation, potentially leading to impaired trophoblastic invasion (Senapati *et al.*, 2018). Another study evidenced significant differences in endometrial epigenetic markers (DNA methylation and histone modifications) between women with high or normal progesterone levels on the day of hCG administration in IVF cycles (Xiong *et al.*, 2017).

Consequently, studying the variations in epigenetic regulation in placenta between different techniques of ART is fundamental because it would explore the potential dysregulation of the dialogue between endometrium and embryo.

One recent study has investigated the differences between FET and fresh ET concerning LINE-1 DNA methylation in placenta: it found hypomethylation in the fresh ET group compared to controls but did not find any difference between FET and

controls (Ghosh *et al.*, 2017). However, this study did not take into account potential confounding factors such as gestational age or maternal age. Thanks to our comprehensive collection, we would be able to design a study to address this issue by taking into account the potential confounding factors.

2) Epigenetic regulation according to the cause of infertility: endometriosis, PCOS, male infertility

Concerning endometrium quality, our cohort could also be useful to study the epigenetic regulation in the placenta in the particular context of specific diseases such as PCOS and endometriosis, because these diseases are known to affect endometrium quality.

It is now largely demonstrated that epigenetics plays a role in the pathogenesis of endometriosis through epigenetic modifications (global DNA methylation decrease, decreased levels of histones acetylation, dysregulation of microRNAs) in ectopic lesions (Hsiao *et al.*, 2017), but also in endometrium. Indeed, a study found altered DNA methylation pattern in endometrial biopsies in women with endometriosis, associated with altered expression of genes involved in endometrial function, cell proliferation, immune response, angiogenesis and steroid hormone response, thus potentially disrupting the embryo-endometrium dialogue necessary to proper implantation and placenta formation (Houshdaran *et al.*, 2016).

It would thus be interesting to assess the potential consequences of this disease on epigenetic regulation in the placenta and cord blood of new-borns.

Concerning PCOS, a recent study in cord blood identified differential picture of methylation in new-borns from PCOS women compared to non-PCOS women (Lambertini *et al.*, 2017). It would be valuable to confirm these data on our cohort and study epigenetic regulation in the placenta in the context of PCOS.

### 3) The effects of infertility per se

In our work, we compared IVF with natural pregnancies within 1 year of stopping contraception. As infertility *per se* might also trigger epigenetic changes (Litzky *et al.*, 2017), it would be interesting to compare IVF patients and other ART groups with a cohort of infertile patients and, in addition, to compare the different groups of natural pregnancy patients stratified on the time to pregnancy in order to decipher the consequences of infertility on epigenetic regulation.

### 4) Follow-up of the cohort

The DOHaD stated that modified maternal-foetal interactions *in utero* might have consequences for outcomes in infancy and even in adulthood (Gillman, 2005; Barker, 2007). A recent review confirmed the increased risk of cardiometabolic diseases in individuals conceived by ART (Vrooman *et al.*, 2017). To date, among studies that assessed epigenetic regulation in ART children, the effect of ART was not obvious (Kanber *et al.*, 2009; Oliver *et al.*, 2012; Puumala *et al.*, 2012) and only one study evidenced small differences in *SNRPN* methylation in ICSI children versus standard IVF and spontaneous conception (Whitelaw *et al.*, 2014). However, the cohorts were small and rather heterogeneous.

The follow-up of our cohort may make it possible to assess the potential long-term consequences of ART on epigenetic regulation.

### 5) Other analyses

Because we carefully conditioned cord blood and placenta samples with reagents to protect RNA and DNA, a tissue, blood, DNA and RNA bank is now available to launch other analyses such as proteomics, transcriptomics, exomes, and histological analyses.

# ***CONCLUSION***

## CONCLUSION

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### (Figure 15)

This thesis, together with the existing data in the literature, provides significant evidence regarding modified the epigenetic regulation in the context of ART, not only on methylation, but also on histone modifications.

ART may be responsible for abnormal implantation due to an alteration of the dialogue between the endometrium and embryo. This altered trophoblastic invasion is responsible for a primary imbalance. Although alterations of the embryo after ART have been largely explored in the literature, both in animal and human studies, the potential modifications of endometrium receptivity in superovulation and other hormonal treatments remain largely unknown.

If the unbalance is too severe, it would lead to implantation failures or miscarriage. However, if the pregnancy is able to continue, the various mechanisms of placental plasticity will correct the primary unbalance in most cases, resulting in a healthy new-born. The IGs members of the IGN, together with other factors such as the syncytins, may contribute to this placental plasticity.

If the primary unbalance is too severe and/or the correction mechanisms are not sufficient, placenta-related pregnancy diseases such as SGA, gestational hypertension or preeclampsia may result.

However, even in the case of a healthy new-born, because gene expression has been tuned to suit the current environment, and as the environment at birth is changing, it might not correspond to the phenotype developed throughout pregnancy. Thus a further unbalance could appear and be responsible for diseases later in life.

Our data reinforces the hypothesis that the aforementioned unbalance and corrections could be written through epigenetic changes of the placenta and foetus, with the epigenetic marks serving as the link between the environment, the individual and the potential long-term consequences.

New studies based on our cohort of patients, divided into different ART treatments groups, will be useful to unravel the consequences of each technique. The final goal is to define the 'best option', namely the technique whose epigenetic regulation is as close as possible to what occurs in the context of a natural pregnancy.

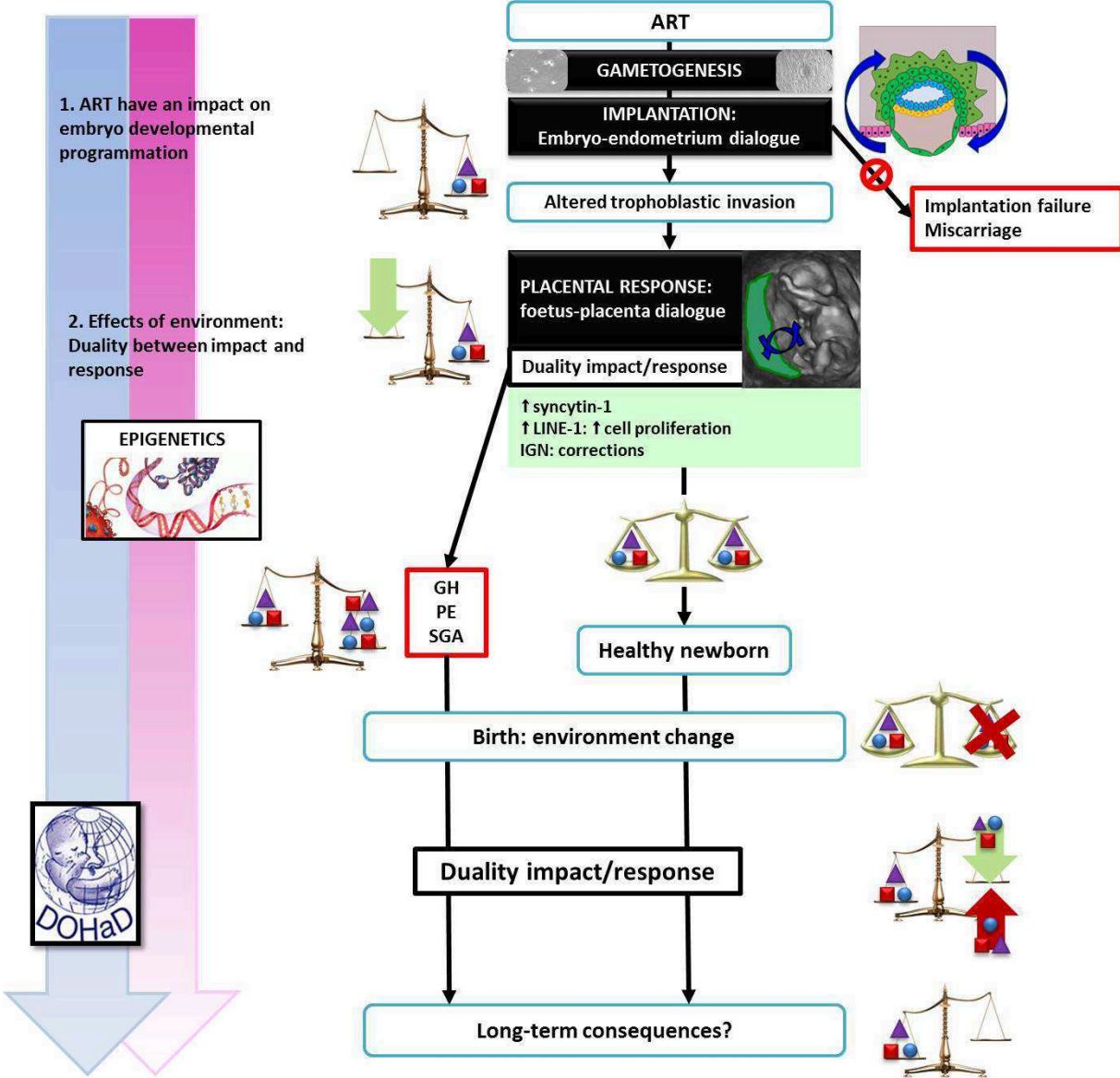


Figure 15: Conclusion

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## REFERENCES

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# ***ANNEXES***

## ANNEXES

### **Annex 1. Characteristics of the Conçue Cohort**

	<b>Natural Conception (n = 102)</b>	<b>Use of CC (n = 8)</b>	<b>IUI (n = 22)</b>	<b>IVF/ICSI (n = 69)</b>	<b>FET (n = 47)</b>
<b>Maternal characteristics</b>					
Age (years)	30.2 ± 4.2	29.3 ± 3.3	32.5 ± 4.4	31.4 ± 5.1	32.5 ± 4.1
Pre-pregnancy parity					
0	62 (60.8%)	5 (62.5%)	16 (72.7%)	49 (71%)	27 (57.5%)
1	36 (35.3%)	2 (25.0%)	5 (22.7%)	16 (23.2%)	18 (38.3%)
2 or more	4 (3.92%)	1 (12.5%)	1 (4.6%)	4 (5.8%)	2 (4.3%)
Tobacco Smoking	13 (12.75%)	2 (25.0%)	1 (4.6%)	6 (8.7%)	2 (4.3%)
Pre-pregnancy BMI (kg/m <sup>2</sup> )	23.7 ± 4.6	26.4 ± 6.8	23.9 ± 4.7	23.7 ± 4.4	23.5 ± 4.4
<b>Paternal characteristics</b>					
Age (years)	32.6 ± 5.2	31.4 ± 4.0	34.7 ± 6.3	34.1 ± 5.6	34.1 ± 4.5
Tobacco smoking	39 (38.2%)	2 (25.0%)	5 (22.7%)	23 (33.3%)	16 (34.0%)
<b>New-born characteristics</b>					
Term (weeks of gestation)	39.9 ± 1.5	40.0 ± 1.1	40.1 ± 1.1	39.6 ± 1.6	40.2 ± 1.5
Birth weight (grams)	3326 ± 476	3308 ± 439	3328 ± 427	3258 ± 482	3467 ± 511
z-score of birth weight	0.09 ± 1.0	-0.10 ± 1.10	-0.02 ± 0.98	0.05 ± 1.03	0.32 ± 1.08
Placenta weight (grams)	530 ± 106	496 ± 96	526 ± 75	528 ± 119	577 ± 117
z-score of placenta weight	-1.06 ± 0.78	-1.36 ± 0.73	-1.12 ± 0.58	-1.03 ± 0.85	-0.76 ± 0.84
Sex ratio M/F [95 % CI]	1.04	1.67	1.2	1.09	0.88
C-section	3 (2.94%)	0	2 (9.1%)	12 (17.39%)	7 (14.9%)

Results are displayed as: n (%) or mean ± standard deviation, BMI: body mass index, CC: clomiphene citrate, FET: frozen-thawed embryo transfer, ICSI: intra-cytoplasmic sperm injection, IUI: intra-uterine insemination, IVF: *in vitro* fertilization

### **Annex 2: Article “Singleton fetal growth kinetics depend on the mode of conception”**

By Ginod P, Choux C, Barberet J, Rousseau T, Bruno C, Khallouk B, Sagot P, Astruc K and Fauque P.

# Singleton fetal growth kinetics depend on the mode of conception

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**Objective:** To study the impact of in vitro fertilization, with or without intracytoplasmic sperm injection (IVF/ICSI), frozen-embryo transfer (FET), and intrauterine insemination (IUI) on fetal growth kinetics throughout pregnancy and to compare the different modes of conception.

**Design:** Retrospective cohort study.

**Setting:** University.

**Patient(s):** A total of 560 singleton pregnancies were included (96 IVF, 210 ICSI, 121 FET, and 133 IUI).

**Intervention(s):** None.

**Main Outcome Measure(s):** We compared crown-rump length (CRL) at the first trimester (T1: 11–13 weeks of gestation [WG] + 6 days), estimated fetal weight (EFW) at the second (T2: 21–23 WG + 6 days) and third (T3: 31–33 WG + 6 days) trimesters, and birth weight (BW) z-scores with those in the reference curves (Papageorgiou for T1, and Ego M2 for T2, T3, and birth). Multivariate analyses were performed.

**Result(s):** For T1, the CRL was longer than the reference curve whatever the assisted reproductive technique (ART). For T2, EFW was significantly greater for all groups compared with the reference curve, and for T3 only FET singletons had a greater EFW. ICSI, IVF, and IUI singletons had a significantly lower BW compared with reference curves. For all ART fetuses, growth kinetics differed from T2. Only FET fetuses maintained their significantly above-reference growth values. The proportion of fetuses for which at least one period of growth loss was observed from T2 to birth was higher after IVF, ICSI, and IUI than after FET.

**Conclusion(s):** For the first time, we have highlighted that fetal growth kinetics differed from T2 depending on the ART protocols used. They could have an impact on trophoblastic invasiveness and might lead to long-term health effects. (Fertil Steril® 2018; ■:■-■. ©2018 by American Society for Reproductive Medicine.)

**Key Words:** Assisted reproductive technologies, birth weight, fetal growth kinetics, singletons, small for gestational age

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In Europe and the USA, ~2% of children are born after assisted reproductive technology (ART) every year (1, 2). Although these techniques are considered to be safe, they seem to be associated with an excess risk of worse obstetrical and perinatal outcomes (3, 4). In particular, several

studies have found increased rates of preeclampsia (5, 6), prematurity (7–10), and babies small for gestational age (SGA) (7, 8, 11–13).

Some authors have suggested that the ART procedures themselves may cause these complications (14, 15). Indeed, birth weights could depend on

the technique of ART used: Singletons born after fresh and frozen embryo transfers have increased risks of being SGA or large for gestational age (LGA), respectively (16, 17).

Moreover, during the past decade, it has been reported that in infants born to fertile parents, abnormal birth weight (BW) and fetal growth could affect the child's health and the development of chronic diseases in adulthood (18–21). In particular, a gestational age (GA) calculated according to crown-rump length (CRL) of 2–6 days lower than that expected (calculated from the day of fertilization) is associated with a higher risk of severe prematurity or low BW (22–27) and accelerated growth in the first years of life (28). In addition, a

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P.G. and C.C. should be considered similar in author order.

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CRL-GA of 3.5 days greater than the expected value is predictive of LGA (29, 30).

Given the above, and because fetal growth during ART and intrauterine insemination (IUI) pregnancy has never been investigated extensively, the present study aimed to evaluate fetal growth kinetics throughout ART pregnancies and to compare the different modes of conception.

The first objective was to compare fetal growth parameters from ART and IUI singletons with those in reference curves: CRL in the first trimester (T1), estimated fetal weight (EFW) in the second and third trimesters (T2 and T3), and BW. The second aim was to compare fetal growth kinetics and trends up to birth according to the reproductive technique used.

## MATERIALS AND METHODS

### Study Population and Ethical Approval

In this single-center retrospective cohort study women were included at their first-trimester ultrasound scan. The inclusion criteria were: singleton pregnancies (i.e., only one gestational sac observed at the first ultrasound scan at ~6 weeks of gestation [WG]) after classic in vitro fertilization (IVF) or with intracytoplasmic sperm injection (ICSI), or transfer of frozen-thawed embryos (FET), or IUI performed in Dijon University Hospital (the only ART center within the Burgundy perinatal network) from January 1, 2010, to March 31, 2015.

Conventional IVF and ICSI were performed as previously described (31). The FET group included patients who were conditioned in artificial cycles with the use of a sequential protocol with a first phase started on day 2 of the menstrual cycle and including estrogens. The uterus was assessed by means of transvaginal ultrasound on day 18; if the endometrial thickness was >7 mm, the second phase with estrogens and intravaginal progesterone was started and continued until 12 WG. Embryo cryopreservation and FET were performed as previously described (31). Exclusion criteria were: uterine malformation, history of adverse obstetrical outcomes (SGA, LGA, preterm birth <37 WG), birth before 22 WG, fetal malformation detected by ultrasound, fetal aneuploidy, and pregnancy after oocyte or sperm donation (Supplemental Fig. 1, available online at [www.fertstert.org](http://www.fertstert.org)). For IUI, IVF, and ICSI groups, we used the day of fertilization as the start of the pregnancy in accordance with the recommendations of the French National College of Gynecologists and Obstetricians (CNGOF) (32). Therefore, the date of the beginning of the pregnancy was the date of insemination for IUI or the date of oocyte retrieval for IVF/ICSI. For FET, the date of pregnancy was calculated as the date of the transfer minus the number of days of embryo culture.

Maternal and paternal characteristics before the attempt were collected (age, weight, height, body mass index) as well as parity, cesarean history, active smoking, type 1 or 2 diabetes, and chronic hypertension for the mother.

Infertility data were gathered, such as the etiology, the stimulation parameters (type of protocol, total dosage of FSH, duration of stimulation), the number of transferred embryos, and the history of intrauterine surgery (polyp, intrauterine adhesion, submucosal fibroids).

Data about obstetrical complications were collected. Preterm labor was defined as contractions of the uterus resulting in changes in the cervix that start before 37 WG (33). Gestational hypertension (GH) was defined as systolic blood pressure (BP)  $\geq 140$  mm Hg and/or diastolic BP  $\geq 90$  mm Hg after 20 WG. Preeclampsia (PE) was the association of GH and proteinuria >0.3 g/24 h. Gestational diabetes mellitus (GDM) was diagnosed according to World Health Organization criteria (34). SGA and LGA were defined as weights below the 10th percentile and above the 90th percentile for the GA, respectively (35). Intrauterine growth retardation (IUGR) was diagnosed according to criteria as established by the French College of Gynecologists and Obstetricians as SGA associated with abnormal uterine and/or umbilical Doppler or evidence indicating abnormal growth (stopping or shifting of the growth longitudinally in at least two measurements 3 weeks apart) (36, 37).

The characteristics of the newborns were also collected: term, BW, sex, and APGAR score.

This was a noninterventional study, in which subjects were not assigned to a treatment. Epidemiologic methods were used to analyze the data, and information used in the study was collected for clinical care. According to the French Public Health Law, approval from Institutional Review Board and written consent are not required for human noninterventional studies. Nevertheless, formal confirmation that ethical approval was not required for this observational (noninterventional) study was obtained.

### Ultrasound Data

All ultrasound scans were performed transabdominally by staff skilled in antenatal diagnosis (and who are required to undergo quality control annually) with the use of the same devices (Voluson E8; GE Medical Systems).

In the first trimester (T1; 11–13 WG + 6 days) measurements of CRL and nuchal translucency in mm were collected. Only images with a reasonable (4–7 points) or excellent (8–9 points) Herman score were used in this study (38).

At the second (T2; 21–23 WG + 6 days) and third (T3; 31–33 WG + 6 days) trimesters, biometric measurements in mm were collected: biparietal diameter, head circumference (HC), abdominal circumference (AC), femur length (FL), and estimated fetal weight (EFW) in grams, according to the Hadlock et al. formula with three parameters (HC, AC, FL) (39):

$$\text{Log}_{10} \text{ EFW} = 1,326 - 0.00326(\text{AC})(\text{FL}) + 0.0107(\text{HC}) \\ + 0.0438(\text{AC}) + 0.158(\text{FL})$$

### Reference Ultrasound Curves

The reference curves used in this study are presented in Supplemental Table 1 (Supplemental Tables 1–5 are available online at [www.fertstert.org](http://www.fertstert.org)). We decided to use validated curves because they were established from large cohorts of non-ART singletons (from 4,000 to 1.5 million), thus forming a robust control group.

For T1, we used the curve from the Intergrowth 21st project (40), based on 4,265 women. That project included

singleton fetuses conceived spontaneously in eight countries with no exposure to toxic substances or tobacco smoke and a very low risk of SGA or IUGR. The curve is recognized as an international standard for early fetal growth.

For T2 and T3, we chose to use a customized national French curve, which was, among existing curves, the most appropriate for our study population (Supplemental Table 1). The M2 model of Ego et al. was obtained with live births without birth defects from 37 to 42 WG (41, 42). From the BW, the construction of an in utero growth model was calculated with the Hadlock model (43) using the method developed by Gardosi et al. (44). The M2 model is a customized curve based on maternal height, weight, and parity and fetal sex, and it is used at T2, T3 and at birth.

### Statistical Analyses

Categorical variables were expressed as n (%), and compared by means of  $\chi^2$  or Fisher exact test as appropriate. Continuous variables were expressed as mean  $\pm$  SD and compared with the use of the Student *t* test.

CRL, EFW, and BW were expressed as z-scores and percentiles. The z-scores were calculated with the use of the following formula:  $z\text{-score} = (\text{value observed} - \text{mean for GA})/\text{SD for GA}$ , where GA is the gestational age. For each trimester and at birth, we compared the z-scores of each group with those in the reference curve (by comparing them with 0) with the use of a Student *t* test. A positive z-score meant that the growth was higher than that of the reference curve and vice versa. Intergroup comparisons were then made at T1, T2, T3, and birth with the use of an analysis of variance (ANOVA) global test. In addition, multivariate analyses were performed with the use of multiple linear regression models from T1 to birth. These analyses included our variable of interest, the ART technique (IVF/ICSI/FET) or IUI, and other factors, such as parity, the mother's age, the mother's and father's weight and height, active tobacco smoking in the mother, sex of the newborn, and other possible confounders that may affect fetal growth: GH or PE and GDM (except those already taken into account in the reference curve model: parity, the mother's weight and height, sex of the newborn [Supplemental Table 1]).

The significance level for this study was set at  $P < .05$ . Stata 10 software was used for all statistical analyses.

### RESULTS

A total of 560 women were included: 96 women who underwent IVF, 210 ICSI, 121 FET, and 133 IUI (Supplemental Fig. 1). The groups were comparable (Supplemental Table 2). A detailed description of the infertility cohort is provided in Supplemental Table 3. No significant differences were found between groups for obstetrical complications such as GDM, premature labor, cholestasis, chorioamnionitis, hemorrhagic placenta previa, PE, and IUGR (Supplemental Table 4).

### Ultrasound Parameters

The CRL in each group was significantly greater than the reference curve, as shown by the positive z-scores at T1 (Table 1). At this time, the global univariate test (ANOVA) revealed no significant differences between groups.

At T2, the fetuses of the four groups were significantly larger than those represented by the Ego M2 curves (Table 1). Again, at this stage, the T2 univariate intergroup analysis found no significant difference between groups (Table 1).

At T3, only fetuses of the FET group were significantly larger (Table 1). The same trends throughout the pregnancy were observed in multivariate analyses (Supplemental Table 5).

### Birth Data

Term, prematurity, and severe prematurity were similar in all of the groups (Supplemental Table 4).

The BW distribution was significantly different between groups (Supplemental Fig. 2 [available online at [www.fertstert.org](http://www.fertstert.org)]; Supplemental Table 4). BWs in the IVF, ICSI, and IUI groups were below the FET BWs (Supplemental Table 4). There was no significant difference between the groups for SGA rates compared with the Ego M2 curve (Supplemental Table 4) or for the proportion of SGA infants in the IVF, ICSI, and IUI groups.

TABLE 1

Mean z-score calculations according to reference curves, with comparisons to 0 and between groups.

Term	Reference curve	IVF		ICSI		FET		IUI		Intergroup comparison Global test P value <sup>b</sup>
		Mean (SD)	P value <sup>a</sup>	Mean (SD)	P value <sup>a</sup>	Mean (SD)	P value <sup>a</sup>	Mean (SD)	P value <sup>a</sup>	
T1: CRL	Papageorghiou	0.30 (0.59)	<.01*	0.27 (0.62)	<.01*	0.42 (0.78)	<.01*	0.29 (0.61)	<.01*	0.23
T2: EFW	Ego M2	0.52 (0.69)	<.01*	0.56 (0.85)	<.01*	0.75 (0.94)	<.01*	0.59 (0.84)	<.01*	.23
T3: EFW	Ego M2	0.02 (0.79)	.82	0.04 (0.97)	.56	0.24 (0.82)	<.01*	0.04 (0.93)	.62	.29
Birth: BW	Ego M2	-0.26 (1.07)	.02*	-0.30 (1.11)	<.01*	0.18 (1.04)	.07	-0.27 (1.08)	<.01*	<.01*

Note: Values are expressed as mean of z-score = (value observed - mean for GA)/SD for GA, where GA is the gestational age. BW = birth weight; CRL = crown-rump length; EFW = estimated fetal weight.

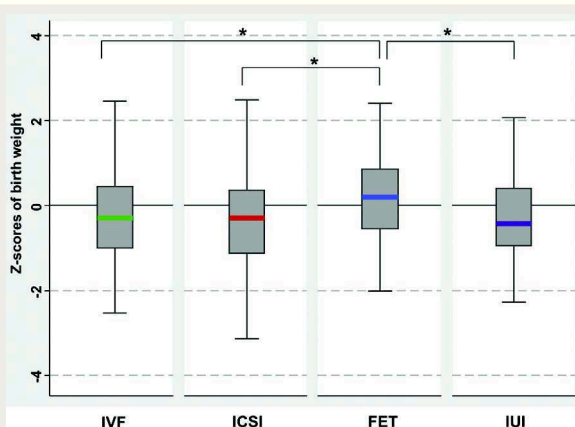
<sup>a</sup> P value for mean z-score in comparison to 0.

<sup>b</sup> P value for intergroup comparison with the use of analysis of variance global test.

\* Significant difference.

Ginod. Fetal growth and mode of conception. Fertil Steril 2018.

FIGURE 1



Distributions of birth weight z-scores according to the reproductive technology used (compared with the Ego M2 curve). FET = frozen-embryo transfer; ICSI = intracytoplasmic sperm injection; IUI = intrauterine insemination; IVF = in vitro fertilization. \* $P < .05$ .

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Analysis of z-scores for BW in the different groups showed a significantly smaller BW in the ICSI, IVF, and IUI groups and a nonsignificant trend toward a greater BW with the use of FET (Table 1). The global comparison showed significant intergroup differences in BW (Table 1). Significant differences were found between FET and the other groups in the univariate intergroup analyses (Fig. 1).

The multivariate analysis, which took into account significant prognostic factors, such as maternal and paternal

height and maternal weight, confirmed significant differences between groups (Supplemental Table 5). The BW in the FET group was confirmed to be significantly greater than that in the IVF, ICSI, and IUI groups (Supplemental Table 5).

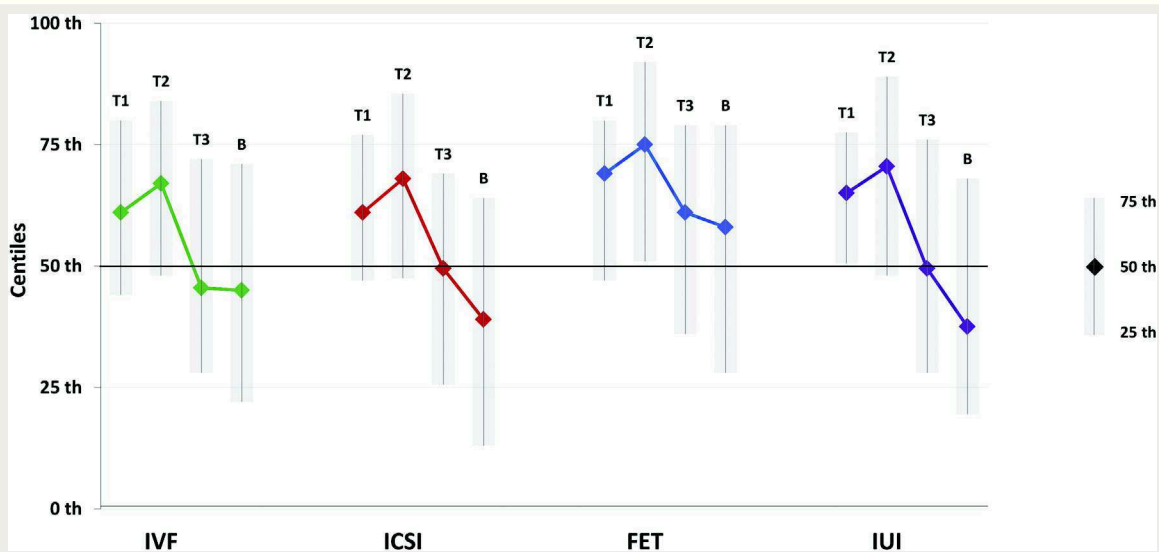
### Growth Kinetics

The mean percentiles for CRL and EFW at the T1 and T2 ultrasounds, respectively, were higher in all studied groups (Fig. 2). Then, from T2 (between 24 and 32 WG), the growth curves for IVF, ICSI, and IUI fetuses leveled off before dropping to below the 50th percentile until delivery, while those for FET fetuses continued to be higher than the standard values (Fig. 1). In addition, the proportions of IVF, ICSI, and IUI fetuses with a loss of centile class from T2 to birth were 14.7%, 16.2%, 15.1%, respectively, whereas only 6.6% of FET fetuses lost a centile class (Fig. 3). The proportion of fetuses that maintained their growth above the 90th percentile until birth was higher in the FET group (12.4%) than in the other groups (4.2%, 7.0%, and 8.3% in the IVF, ICSI, and IUI groups, respectively; Fig. 3).

### DISCUSSION

Previous studies have reported that BW can be affected by the reproductive technique used. The present in-depth analysis based on a cohort of 560 IVF, ICSI, FET, and IUI singletons included from the first trimester and followed until birth has allowed us, for the first time, to highlight that the mode of conception also influences fetal growth kinetics. Indeed, CRL and EFW at T1 and T2 ultrasounds, respectively, were significantly higher than those in reference curves. Then, throughout the second half of the pregnancy and until

FIGURE 2

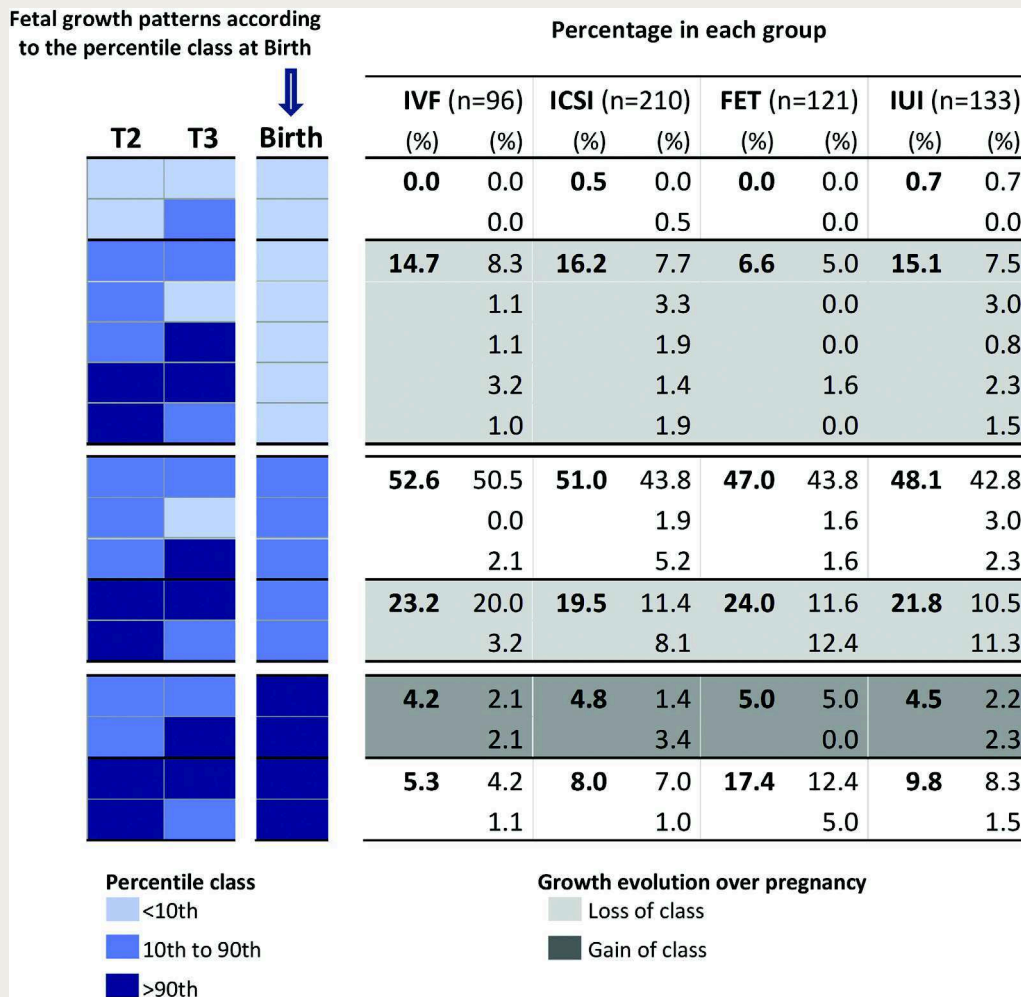


Fetal growth kinetics according to the assisted reproductive technology (ART) used (abbreviations as in Fig. 1). Median and interquartile ranges of centiles for (left to right for each ART) crown-rump length at the first trimester (T1), estimated fetal weight at the second (T2) and third (T3) trimesters, and birth (B) weight, compared with the Papageorghiou curve at T1 and the Ego M2 curve at T2, T3, and B.

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FIGURE 3



Patterns of fetal growth evolution over pregnancy according to the percentile class at birth (<10th, 10–90th, and >90th) in each ART group, according to the Ego M2 curve. Abbreviations as in Figures 1 and 2.

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delivery, the growth curve for ICSI, IVF, and IUI fetuses dropped below the reference curves although that for FET fetuses remained significantly above. In addition, the proportion of singletons with decelerated growth from the second trimester onward was higher in the IVF, ICSI, and IUI groups than in the FET group. Thus, we demonstrate that fetal growth can be modified from the earliest stages of development until birth depending on the mode of conception.

Although several studies have focused on embryonic growth in the first trimester after ART and its correlation with BW (22–25, 27, 45, 46) or with the occurrence of complications, such as PE (5, 6) or SGA (7, 8, 11–13, 47), no study has analyzed fetal growth by including data for the second and third trimesters. In addition, our study aimed to compare growth parameters with regard to the different reproductive techniques used (after IVF and ICSI, as well as FET and IUI). To our knowledge, no previous studies have explored EFW throughout pregnancy (including T3) for these different reproductive techniques.

One limitation could be the inaccuracy of T3 EFW, due to the growing fetus and decreasing amniotic fluid. However, performing this exam between 31 and 33 WG + 6 days and using the criteria issued by the French College of Fetal Sonography (37, 48) ensures good reproducibility of the data.

In addition, we took into account many factors well known to influence fetal weight, such as fetal sex (45–47), maternal age, height, and weight (27, 47, 49, 50), GH or PE (51), GDM (52–54), parity, and maternal smoking (49, 50, 55). Moreover, in contrast to most studies, we also adjusted for paternal height and weight, because they could represent significant risk factors (49, 56).

In this series, even though we can not exclude margins of error at the early term of pregnancy (57), the CRL in each ART group was greater than that in the reference curve. In the literature, CRL has been reported to be a predictor of BW (22–27, 29, 30), which is discordant with our results, probably because the potential impact of the reproductive technique used was not previously considered. At the T2 ultrasound, the EFW

for each group was greater than the Ego M2 adjusted curve. In line with these results, two studies that assessed EFW up to 20 WG in IVF and ICSI populations reported a correlation between CRL and EFW in T2 (57, 58). At the T3 ultrasound, fetal growth assessed in comparison with the same reference curve (Ego M2 curve) differed from those at the T1 and T2 ultrasound recordings, as we found that fetal growth slowed down for the IVF, ICSI, and IUI fetuses, but not for FET fetuses, which remained significantly larger. In addition, we highlighted that even though the EFW was in the normal or upper range in the second half of ART pregnancies, fetal growth kinetics decreased in about one-third of cases in our series (including in some FET fetuses), leading to IUGR. This could potentially be a source of adverse perinatal outcomes, such as newborn encephalopathy and cerebral palsy due to antepartum hypoxia (36, 37, 59). Altogether these data suggest that an additional ultrasound growth screening at 36–37 WG for all ART/IUI pregnancies might reduce these complications.

In line with data in the literature, we found that BWs were lower in the ICSI group and greater in the FET group (16, 17) than in the general population. In addition, our comparisons between various reproductive techniques showed that BWs of FET infants were significantly higher than those of IVF, ICSI, and IUI infants. Very little data are available on BW in IUI children, but like us, one study reported a higher incidence of low BW in children after the use of IUI (60). Therefore, each technique influences fetal development in different ways.

One explanation is that reproductive techniques could impair trophoblastic invasion by altering the dialog between the endometrium and the embryo (61), resulting in higher rates of vascular complications such as PE and IUGR after IVF (62, 63). Notably, the endometrium could be disturbed by the hormones received during IVF and ICSI (63). Indeed, the hormonal treatments can cause an excessive elevation of progesterone and earlier luteinization of the endometrium, leading to a disturbed implantation window (64–67). This has been demonstrated in mice, in which treatments with gonadotropins led to delayed implantation or altered embryonic and fetal development (68, 69). Thus, the lower BW observed after IVF or ICSI cycles could be related to the adverse effects of controlled ovarian hyperstimulation on placentation leading to the depletion of placental functions during the last in utero stages of development. In contrast, we can hypothesize that the trend toward a higher BW in children born after FET than after fresh-embryo transfers could be linked to increased trophoblastic invasiveness induced by hormone replacement therapy used for FET cycles (61).

According to the developmental origin of health and disease hypothesis, an adverse perinatal environment may be associated with negative effects on long-term health, particularly regarding chronic metabolic or cardiovascular diseases (18, 20, 21, 61, 70). Even though the question remains as to whether these mechanisms might affect the long-term health of children born after ART procedures, we have to strive to minimize these effects in the interest of safety for ART children.

This growth shifting observed after IVF, ICSI, or IUI raises the question of the benefit of introducing low doses of aspirin from embryo transfer onward. Although controversial for IUGR or PE prevention, this could be beneficial for fetoplacental development in IVF, ICSI, or IUI (71–73).

Because infertility itself could have an effect on BW (10, 11, 74), including a group of infertile patients with a long time to natural pregnancy would be valuable for deciphering the potential impact of infertility itself on fetal growth.

## CONCLUSION

Although BW differences had already been reported in newborns after ART, this is the first report of fetal growth kinetics throughout the pregnancy. The differences in CRL and EFW observed for the different ART techniques suggest that the type of hormones and protocols used could have different effects on trophoblastic invasiveness, effects that are clinically visible from the second trimester. Because epigenetics plays a major role in placental and fetal growth, our results highlight the need for further studies to assess changes in placental function and epigenetic modifications induced by each of the ART techniques (61). These novel findings also highlight the importance of taking the mode of conception into account in fetal growth follow-up, and they may lead to the development of research protocols (preventive or therapeutic) to minimize the adverse fetal outcomes potentially linked to some ART procedures. In addition, these results should encourage us to analyze the consequences of these fetal growth changes on the infants' growth and health in later life.

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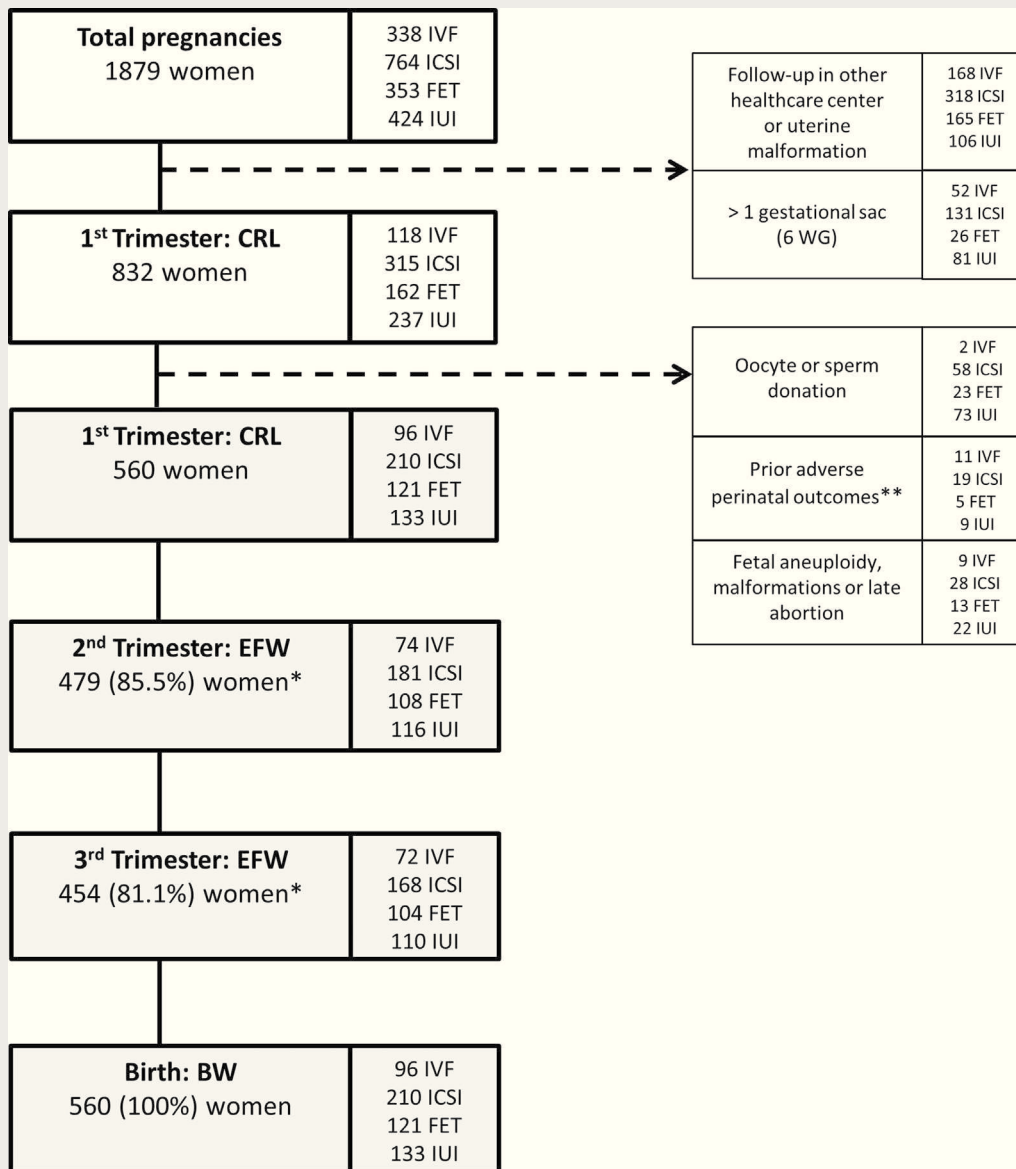
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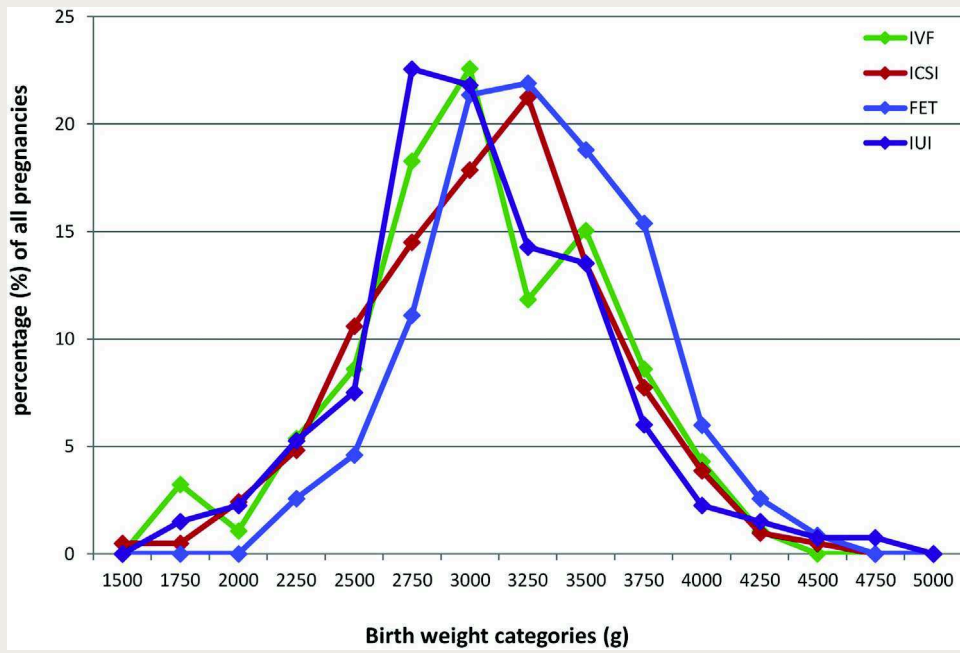
SUPPLEMENTAL FIGURE 1



Study population flow chart. Four groups: in vitro fertilization (IVF), intracytoplasmic sperm microinjection (ICSI), frozen-embryo transfer (FET), and intrauterine insemination (IUI). BW = birth weight; CRL = crown-rump length; EFW = estimated fetal weight. \*EFW was not available owing to missing data; \*\*previous adverse perinatal outcomes defined as preterm delivery, small for gestational age, or large for gestational age.

Ginod. Fetal growth and mode of conception. Fertil Steril 2018.

SUPPLEMENTAL FIGURE 2



Distribution of live-born singleton birth weights according to the mode of conception. This graph shows the percentage of newborns by birth weight category. Abbreviations as in Supplemental Figure 1.

Ginod. Fetal growth and mode of conception. Fertil Steril 2018.

UNCORRECTED

**Annex 3:** Article “Placental volume and other first-trimester outcomes: are there differences between fresh embryo transfer, frozen-thawed embryo transfer and natural conception?”

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1  
2 **Placental volume and other first-trimester outcomes: are there differences between**  
3 **fresh embryo transfer, frozen-thawed embryo transfer and natural conception?**  
4

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47 **Declarations of interest: None**  
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## ABSTRACT

**Research question:** Does the mode of conception influence placental volume and other first-trimester outcomes?

**Design:** This retrospective single-centre case-control study led in Dijon University Hospital included 252 singleton pregnancies (84 *in vitro* fertilization [IVF] with either fresh embryo transfer [fresh ET] or frozen-thawed embryo transfer [FET] and 168 natural conceptions). First-trimester placental volume, uterine artery pulsatility index and maternal serum PAPP-A and  $\beta$ -hCG were measured. Statistical analyses were adjusted for gestational age, the newborn's gender, maternal age, parity, body mass index and smoking status.

**Results:** Placental volume was significantly greater in the FET group than in control ( $P = 0.043$ ) and fresh ET ( $P = 0.023$ ) groups. At birth, fresh ET new-borns were significantly smaller than controls ( $P = 0.01$ ) and FET new-borns ( $P = 0.008$ ). Post-partum haemorrhage was far more frequent in FET than in controls and fresh ET group (38.1%, 2.6% and 1.9%, respectively;  $P < 0.0001$ ). Placental volume positively correlated with PAPP-A,  $\beta$ -hCG and the new-born's birth weight, and negatively correlated with uterine artery pulsatility index.

**Conclusions:** Placental volume and other first-trimester parameters are modified by IVF with fresh ET and FET as compared with natural conception, but with opposite trends. Given the different protocols used for these techniques, hormonal treatment *per se* may have a major impact on pregnancy outcomes through the modification of placental invasiveness.

### Keywords

fresh embryo transfer, frozen-thawed embryo transfer, *in vitro* fertilization, placenta, placental volume, first-trimester pregnancy

## INTRODUCTION

1  
2 It is estimated that more than five million children have been born by Assisted  
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4 Reproductive Technologies (ART) worldwide, representing up to 4% of all births (Messerlian  
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6 and Gaskins, 2017), and there is an increasing number of initiated cycles from year to year  
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8 (Dyer et al., 2016). Between 8 and 12% of reproductive-aged couples are currently infertile  
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10 (Vander Borgh and Wyns, 2018), and providing them with safe techniques to achieve  
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12 parenthood is a public health issue. However, the safety of these techniques has not been  
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14 fully demonstrated. Notably, the rate of placenta-related adverse pregnancy outcomes such  
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16 as miscarriages, preeclampsia, placenta *praevia*, placenta *accreta* and placental abruption  
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18 could be increased after ART (Thomopoulos et al., 2013). Adverse perinatal outcomes such  
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20 as small for gestational age (SGA) or large for gestational age (LGA) have also been  
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22 reported after fresh and frozen-thawed embryo transfer, respectively, even in singleton  
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24 pregnancies (Pinborg et al., 2014; Weinerman and Mainigi, 2014). Our team and others have  
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26 suggested that these conditions could be linked to inadequate trophoblastic invasion of  
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28 uterine decidua and spiral arteries during early pregnancy (Prefumo et al., 2007; Choux et  
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30 al., 2015).  
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35 In mammals, the placenta is a pregnancy-specific temporary organ that creates  
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37 intimate contact between mother and foetus to ensure the maintenance of gestation and  
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39 foetal well-being by the exchange of gases, nutrients and waste products (Zhang et al.,  
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41 2008). Its formation involves a molecular crosstalk between the maternal endometrium and  
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43 the peripheral multipotent cells of the blastocyst called trophoblasts (Chelbi and Vaiman,  
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45 2008). This finely-tuned temporal and spatial regulation of trophoblastic invasion, essential  
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47 for proper future functions of the placenta and foetal development, may be disrupted by ART  
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49 (Chelbi and Vaiman, 2008; Denomme and Mann, 2012; Choux et al., 2015). Given the  
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51 statements of the Developmental Origin of Health and Diseases (DOHaD) concept (Barker et  
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53 al., 1990), disturbed maternal-foetal interactions could not only have consequences for  
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55 outcomes in childhood but also in adulthood (Gillman, 2005; Isles and Holland, 2005;  
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1 Nathanielsz, 2006; Barker, 2007; Gluckman et al., 2008; Reynolds and Caton, 2012;  
2 Vrooman and Bartolomei, 2017).

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4 Thus, as placental and foetal growth are closely linked and given the increased  
5 incidence of placenta-related adverse outcomes after ART and their potential long-term  
6 consequences, studying the placenta in the context of ART is of particular interest. A  
7 previous large cohort study reported lower birth weight and higher placental weight after IVF,  
8 ICSI or other methods of ART (Haavaldsen et al., 2012). Additionally, in animal models,  
9 many placental modifications have been reported after ART throughout pregnancy (Miles et  
10 al., 2004; Miles et al., 2005; Giritharan et al., 2007; Delle Piane et al., 2010; Fauque et al.,  
11 2010; Fauque et al., 2010; Bloise et al., 2012; Ptak et al., 2013).

12  
13 Because placental weight cannot be assessed during pregnancy in humans, 3D-  
14 ultrasound has brought dramatic progress in the last decade by making it easy to measure  
15 placental volume (PV) (Wegrzyn et al., 2005; Cheong et al., 2010; Rizzo et al., 2015).  
16 Several authors have investigated the relationships between first-trimester PV and adverse  
17 obstetric outcomes. PV has been shown to be predictive of preeclampsia (Rizzo et al., 2008;  
18 Arakaki et al., 2015; Yucel et al., 2016), associated with birth weight below the 10<sup>th</sup> percentile  
19 (Effendi et al., 2014; Schwartz et al., 2014; Gonzalez-Gonzalez et al., 2017), strongly  
20 correlated with birth weight and placental weight at birth (Effendi et al., 2014) and even  
21 predictive of placental insufficiency-related complications (Papastefanou et al., 2018).  
22 Despite the data, few studies have investigated PV after *in vitro* fertilization (IVF), and their  
23 results were conflicting (Rifouna et al., 2014; Rizzo et al., 2015; Churchill et al., 2017;  
24 Sundheimer et al., 2018). Indeed, some studies found no significant difference in PV  
25 between IVF and spontaneous pregnancies at 10 WG (Rifouna et al., 2014) or at 12 WG  
26 (Churchill et al., 2017; Sundheimer et al., 2018), while Rizzo et al. (Rizzo et al., 2015)  
27 evidenced significantly reduced PV in IVF pregnancies. Only one other study distinguished  
28 IVF after fresh embryo transfer (fresh ET) from those after frozen-thawed embryo transfer  
29 (FET) (Rizzo et al., 2016), but without providing a full vision of first-trimester markers and  
30 their relationship with perinatal and obstetrical outcomes. Yet, some first-trimester

1 parameters have been associated with adverse pregnancy outcomes. For example, low  
2 levels of PAPP-A and beta-hCG in first-trimester maternal serum, high uterine PI and/or  
3  
4 notching in the waveform of uterine artery Doppler could be associated with low birth weight  
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6 (Shwarzman et al., 2013; Zhu et al., 2015; Gundu et al., 2016; Sirikunalai et al., 2016;  
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8 Stampalija et al., 2017) and first-trimester CRL could correlate positively with birthweight  
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10 (Hackmon et al., 2017). Currently, PAPP-A and uterine artery PI are even recognized to be  
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12 predictive of placenta-related diseases such as preeclampsia (Tan et al., 2018) and small for  
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14 gestational age (Tan et al., 2018).  
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20 The primary endpoint of this single-centre cohort study was to compare PV between  
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22 11 weeks of gestation (WG) and 13 WG + 6 days according to different modes of conception:  
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24 natural, IVF with fresh ET and IVF with FET. The secondary endpoints were comparisons  
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26 between groups for crown-rump length (CRL), uterine artery pulsatility index (PI), incidence  
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28 of notching, and maternal serum markers values (PAPP-A and  $\beta$ -hCG).  
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## MATERIALS AND METHODS

### Study population

This retrospective single-centre study included all singleton pregnancies after IVF with fresh ET or FET, with dates of pregnancy between 01/10/2013 and 31/01/2015, followed at the Dijon University Hospital for the first trimester ultrasound (11 WG – 13 WG + 6 days), and with available 3D placental acquisition. Exclusion criteria were: presence of pre-existing maternal diseases (diabetes, chronic hypertension), oocyte donation, foetal malformation or abnormal karyotype. For each patient, two controls fulfilling the same criteria were randomly selected during this period of time from our computerised database.

Women from the fresh ET group underwent ovarian stimulation, where ovulation was triggered by recombinant hCG. Fertilization was achieved either by conventional IVF or by Intra Cytoplasmic Sperm Injection (ICSI). Depending on the woman's age, the number of previous cycles and the number and quality of available embryos, one to three fresh embryos were transferred at day 2, 3 or 5 after oocyte retrieval, 86.7% being transferred at day 2 or 3. Patients were given 400 mg of progesterone per day vaginally from the day after oocyte retrieval until 6 WG and thereafter 200 mg per day until 8 WG. In accordance with the French National College of Obstetrics and Gynaecology, the date of pregnancy was the date of oocyte retrieval (Salomon, 2011).

The FET group included patients who were conditioned by a sequential two phase protocol. The first phase starts on day 2 of the menstrual cycle and uses oestrogens alone (2 mg estradiol per day for three days followed by 4 mg per day for three days and then 6 mg per day until the embryo transfer). The second phase includes oestrogens (6 mg per day and then 8 mg per day if the pregnancy test is positive) and intravaginal progesterone (400 mg per day from 2, 3 or 5 days before the transfer of day-2, -3 or -5 frozen embryos, respectively, then 600 mg per day after the transfer and 800 mg per day if the pregnancy test is positive) until 12 WG. Embryo cryopreservation is done on day 2, 3 or 5, and frozen-thawed embryo transfers were performed as previously described (Desch et al., 2017), 83.3% of embryos being transferred at day 2 or 3. In accordance with fresh ET group, the

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date of pregnancy for FET group was calculated as follows: date of the transfer minus the number of days of embryo culture.

### Ultrasound data

All ultrasound scans were performed transabdominally between 11 WG and 13 WG + 6 days, with the same apparatus (Voluson E8, GE Medical Systems, Milwaukee, WI, USA) in the prenatal diagnosis department (Dijon University Hospital, France) by staff skilled in antenatal diagnosis. Natural pregnancies were dated as recommended by measuring CRL (Salomon, 2011). In accordance with Papageorghiou *et al.* (Papageorghiou *et al.*, 2014), the formulae were:

$$\text{CRL} = -50.6562 + (0.815118 \times \text{GA}) + (0.00535302 \times \text{GA}^2) \quad (\text{GA is the gestational age [days]})$$

$$\text{SD CRL} = -2.21626 + (0.0984894 \times \text{GA}) \quad (\text{SD is the standard deviation})$$

$$\text{GA} = 40.9041 + (3.21585 \times \text{CRL}^{0.5}) + (0.348956 \times \text{CRL})$$

$$\text{SD GA} = 2.39102 + (0.0193474 \times \text{CRL})$$

To measure placental volume, Virtual Organ Computer-aided AnaLysis (VOCAL II GE Medical Systems, Milwaukee, WI, USA) with a rotation angle of 15° is the most precise and reproducible technique (Wegrzyn *et al.*, 2005; Cheong *et al.*, 2010; Rizzo *et al.*, 2015; Papastefanou *et al.*, 2018). In our centre, 3D placental volume was acquired routinely according to standardized protocol with a sweep angle set at 85° and a volume box set so as to include the whole placenta. Subsequent volume analyses were performed later by the same observer, blinded to the method of conception and pregnancy outcomes. Placental shape was drawn manually in 12 sections, using a rotation angle of 15°. The volume was then calculated by computer. Each volume was measured twice and the mean was used for analyses. If the variation was greater than 5 cm<sup>3</sup>, measurements were repeated. The uterine artery PI was measured on the right and left arteries. Maximal PI was collected for analysis. PI is defined as  $\text{PI} = (\text{S}-\text{D})/\text{m}$ , where S is maximal velocity during systole, D is minimal velocity during diastole and m is mean velocity. Maternal serum PAPP-A and β-hCG values

1 were measured the same day as the first-trimester ultrasound in the Dijon University hospital  
2 laboratory and expressed in multiple of the median (MoM).  
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## 6 **Clinical data and definitions**

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8 Maternal and ART data were extracted from our prospective database and clinical  
9 records were consulted in case of missing or inconsistent data. CRL was expressed as z-  
10 scores, calculated as follows:  $z\text{-score} = (\text{CRL observed} - \text{mean CRL for GA}) / (\text{SD CRL})$ , in  
11 accordance with Papageorghiou *et al.* (Papageorghiou *et al.*, 2014). Concerning birth weight,  
12 the z-score was calculated according to our 2008 Burgundy population growth curves further  
13 adjusted in 2011 for new-born gender (Rousseau *et al.*, 2008). Using these curves, SGA and  
14 LGA were defined as birth weight < 10<sup>th</sup> and > 90<sup>th</sup> percentile, respectively (Rousseau *et al.*,  
15 2008). Gestational hypertension (GH) was defined as systolic blood pressure (BP)  $\geq$  140  
16 mmHg and/or diastolic BP  $\geq$  90 mmHg. Preeclampsia was the association of GH and  
17 proteinuria > 0.3 g / 24 hours after 20 WG. Gestational diabetes mellitus was diagnosed  
18 according to guidelines of National College of Obstetrics and Gynecology, either if first-  
19 trimester fasting glucose  $\geq$  0.92 g/L or if any of the following cut-offs for the 75 g oral glucose  
20 tolerance test (performed between 24 and 28 WG) was met: fasting glucose  $\geq$  0.92, 1-hour  $\geq$   
21 1.80, 2-hour  $\geq$  1.53 g/L (CNGOF, 2010). Post-partum haemorrhage (PPH) was defined as  
22 blood loss > 500 mL within 24 hours after delivery, regardless of the route of delivery,  
23 according to recent guidelines (Sentilhes *et al.*, 2016).  
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## 46 **Statistical analyses**

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48 Categorical variables were expressed as numbers (percentages) and compared  
49 using the Chi-2 test or Fisher exact test when appropriate. Continuous variables were  
50 expressed as mean  $\pm$  SD and compared using ANOVA when more than two groups or with  
51 Student's or the Mann-Whitney test, as appropriate. To compare the three groups, global  
52 tests were first used and if they resulted in  $P < 0.05$ , then each ART group was compared to  
53 the controls. Multivariate analyses were performed using multiple regression linear models.  
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1 Maternal age, height, weight, body mass index (BMI), parity, smoking habits, gestational age  
2 and sex of the new born, when associated with the dependent variable resulting in a P-value  
3 below 0.2 in bivariate analyses, were included in the multiple regression analysis. Indeed, as  
4 placental weight at birth can be modified by these factors (Ouyang et al., 2016), we  
5 considered that first-trimester PV could also be affected. A Shapiro-Wilk test was performed  
6 for the normality of quantitative variables. When rejected, log-transformation of the variable  
7 distribution was tested. Because the distribution of PV was not normal, logarithmic  
8 transformation was done. Seeing as placental volume is dependent on GA, all analyses were  
9 adjusted for GA. The linear form of continuous variables was checked by means of fractional  
10 polynomials.

11 All statistical analyses were performed with Stata software, version 10 (Stata  
12 corporation, College Station TX, USA). The tests were two-tailed, and a P-value below 0.05  
13 was considered statistically significant.

## 14 **Ethics Statement**

15 Our study is a human non-interventional study where subjects were not assigned to  
16 treatment. Epidemiological methods were used to analyze the data, and information used in  
17 the study was collected for clinical care. According to the French Public Health Law, approval  
18 from the Institutional Review Board and written consent are not required for non-  
19 interventional human studies. However, formal confirmation that ethical approval was not  
20 required for this observational study was obtained.



## RESULTS

### Population characteristics

A total of 84 singleton ART pregnancies (60 fresh ET [13 conventional IVF and 47 IVF with ICSI], 24 FET [9 conventional IVF and 15 IVF with ICSI]) and 168 singleton controls were included in this study.

Demographic and clinical characteristics of the pregnancies studied are summarized in Table 1. Notably, ART mothers were different from controls in terms of age, smoking habits and parity. ART mothers were older than controls. Tobacco smoking was more frequent in controls than fresh ET. Parity was lower in the fresh ET group than in controls and FET but was the same in controls and the FET group.

### First-trimester Data

As recommended, natural pregnancies were dated by measuring the CRL and using the curve of Papageorghiou *et al.* (Papageorghiou et al., 2014), so their z-score of CRL is 0. No difference was observed when comparing the FET and fresh ET groups to this curve or by comparing one group to the other (Figure 1A; Table 2).

After adjustments for gestational age and maternal height, log PV was significantly greater in the FET group ( $4.52 \pm 0.39 \text{ cm}^3$ ) than in the control group ( $4.40 \pm 0.28 \text{ cm}^3$ ;  $P = 0.043$ ) and in the fresh ET group ( $4.38 \pm 0.37 \text{ cm}^3$ ;  $P = 0.024$ , Figure 1B).

PI was significantly higher in the fresh ET group ( $1.86 \pm 0.64$ ) than in controls ( $1.52 \pm 0.59$ ;  $P = 0.001$ ; Table 2), even after adjustments for gestational age ( $P = 0.001$ ; Figure 1C). Conversely, PI was lower in the FET group ( $1.26 \pm 0.44$ ) than in the fresh ET group ( $P = 0.001$ ; Table 2), even after adjustment on gestational age ( $P=0.001$ , Figure 1C). PI was also lower in the FET group than in controls, but the difference was not significant.

Consistently, diastolic notching was more frequent in fresh ET patients (55.6%) than controls (32.5%,  $P = 0.003$ ; Table 2), even after adjusting for the sex of the new-born,

1 maternal age and parity ( $P = 0.016$ ), whereas the incidence of diastolic notching was similar  
2 between FET and controls (Table 2).

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4 Maternal serum markers were significantly lower in the fresh ET group than in  
5 controls:  $0.91 \pm 0.48$  vs.  $1.12 \pm 0.60$  ( $P = 0.016$ ) for PAPP-A and  $0.92 \pm 0.47$  vs.  $1.21 \pm 0.67$   
6 for  $\beta$ -hCG ( $P = 0.002$ ; Table 2).  
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### 10 **Obstetrical and neonatal outcomes**

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12 When comparing the obstetrical and neonatal outcomes between groups, ART  
13 patients were not different from controls except for PPH, instrumental delivery and birth  
14 weight (Table 3). In fact, the incidence of instrumental delivery was no different between the  
15 studied groups after adjusting for parity ( $P = 0.325$ ). Even after adjusting for birth weight,  
16 maternal age, parity and the occurrence of C-section, PPH remained significantly more  
17 frequent in the FET group than in the control group (38.1% vs. 2.6%,  $P = 0.003$ ) and in the  
18 IVF group (1.9%,  $P = 0.021$ ), whereas the incidence of PPH was similar in the fresh ET  
19 group and controls ( $P = 0.861$ ). In the FET group, PPH was due to retained placenta or  
20 retained placental fragments requiring manual removal ( $n = 4$ , including one placenta  
21 *accreta*), and during C-section ( $n = 4$ ). Of note, there was no correlation between the risks of  
22 PPH and the new-born's birth weight or the incidence of C-section.  
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40 After adjusting for BMI, tobacco smoking and parity, the z-score for birth weight  
41 remained significantly lower in the fresh ET group ( $-0.60 \pm 0.96$ ) than in the control group ( $-$   
42  $0.07 \pm 0.92$ ;  $P = 0.010$ ) and in the FET group ( $0.15 \pm 1.27$ ;  $P = 0.008$ ; Figure 1D).  
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### 48 **PV correlations**

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50 After adjusting for gestational age, log PV was positively associated with maternal  
51 serum  $\beta$ -hCG (r-squared for regression model:  $r^2 = 0.15$ , linear regression coefficient:  $\beta =$   
52  $0.09$ ,  $P = 0.006$ ) and maternal serum PAPP-A ( $r^2 = 0.18$ ,  $\beta = 0.13$ ,  $P < 0.0001$ ), and  
53 negatively associated with uterine artery PI ( $r^2 = 0.16$ ,  $\beta = -0.09$ ,  $P = 0.004$ ; Supplemental  
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Figure 1 A, B and C, respectively). Notably, the correlation between PAPP-A and log PV was stronger for the FET group ( $r^2 = 0.62$ ,  $\beta = 0.37$ ,  $P < 0.0001$ ; Supplemental Figure 1D).

The z-score for birth weight correlated positively with first-trimester maternal serum PAPP-A ( $r^2 = 0.12$ ,  $\beta = 0.57$ ,  $P < 0.0001$ ; Supplemental Figure 2A), with log PV ( $r^2 = 0.03$ ,  $\beta = 0.51$ ,  $P = 0.022$ ; Supplemental Figure 2B), inversely with the presence of unilateral or bilateral notching of uterine artery ( $r^2 = 0.03$ ,  $\beta = -0.37$ ,  $P = 0.012$ ) and did not correlated with  $\beta$ -hCG and PI ( $P > 0.05$ ).

## DISCUSSION

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4 With an original design providing a global vision of first-trimester data and robust  
5 methodology, our study gives novel insights about placental function and its link with  
6 pregnancy outcomes. We found increased first-trimester placental volume after FET  
7 compared to controls and fresh ET, suggesting that ART has an impact on placental  
8 development.  
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11 Data in the literature about placental growth throughout pregnancy following ART are  
12 conflicting. A study comparing IVF with natural pregnancies found a significantly smaller first-  
13 trimester PV in the IVF group, and even smaller PV in the oocyte-donor subgroup (Rizzo et  
14 al., 2015). On the contrary, Rifouna *et al.* (Rifouna et al., 2014) and two other studies  
15 (Churchill et al., 2017; Sundheimer et al., 2018) compared trophoblastic volumes at 10 WG  
16 and around 12 WG, respectively, and did not find any significant difference. In the three  
17 studies, the IVF group included patients who indistinctly received fresh or frozen-thawed  
18 embryos, but because of the major differences between the protocols, especially the  
19 additional freezing-thawing step and different hormonal stimulation used for FET, it is hardly  
20 conceivable to group them together. Like ours, another study found larger PV in pregnancies  
21 after FET compared with pregnancies after fresh ET (Rizzo et al., 2016). As PV correlated  
22 with birth weight, this is consistent with the findings of a higher incidence of LGA new-borns  
23 after FET (Pinborg et al., 2014; Spijkers et al., 2017).  
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44 Interestingly, in addition to the study of PV, our work also provides a full vision of first-  
45 trimester data and the associated obstetrical significance, and helps to decipher the  
46 differences in trophoblastic invasion according to the mode of conception. PAPP-A  
47 contributes to maternal tolerance towards the foetus (Zhabin et al., 2003) and is considered  
48 as an early marker of trophoblastic invasion (Fournier et al., 2008). The level of maternal  
49 serum first-trimester PAPP-A, as in our study, was found to positively correlate with birth  
50 weight (David and Jauniaux, 2016) and be associated with low birth weight and preeclampsia  
51 if low (Zhu et al., 2015; Gundu et al., 2016), and LGA if high (David and Jauniaux, 2016).  
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1 Produced by trophoblastic cells, hCG promotes the invasion of cytotrophoblasts and  
2 supports foetal and placental growth (Cole, 2010), making consistent our finding of its  
3 correlation with PV. Increased uterine artery PI is the expression of increased vascular  
4 resistances, which corrupt endovascular trophoblastic invasion (Prefumo et al., 2004;  
5 Prefumo et al., 2007), consistently with the negative correlation between PV and PI observed  
6 in the present study and in others (Hashish et al., 2015). Overall, PV correlated with these  
7 three markers, which supports the hypothesis that PV could be a surrogate marker of  
8 trophoblastic invasiveness.  
9

10 Altogether, by analysing the comprehensive “first-trimester overview” given by these  
11 markers, we observed interesting opposing trends between fresh ET and FET (Figure 2),  
12 suggesting insufficient or excessive placental invasion, respectively. Insufficient placental  
13 invasion after fresh ET is witnessed in our study and/or others by lower maternal hCG (Hui et  
14 al., 2005) and PAPP-A levels (Hui et al., 2005; Tul and Novak-Antolic, 2006; Anckaert et al.,  
15 2008; Gjerris et al., 2009; Cavoretto et al., 2017; Hunt et al., 2017), lower PV (Rizzo et al.,  
16 2016), higher PI and lower birth weight (Jackson et al., 2004) than natural pregnancies and  
17 increased frequency of *abruptio placentae* (Healy et al., 2010) and preeclampsia (Imudia et  
18 al., 2013). On the contrary, excessive placental invasion in FET is visible in our study and/or  
19 others through increased PV, increased maternal hCG (Hui et al., 2003), higher risk of  
20 manual placental extraction (Aziz et al., 2016), placenta *accreta* (Kaser et al., 2015; Ata and  
21 Seli, 2017), and PPH (Healy et al., 2010) due to the absence of spontaneous delivery of the  
22 placenta and higher frequency of LGA (Pinborg et al., 2014; Spijkers et al., 2017).  
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24 It suggests that a first-trimester evaluation for trophoblastic invasion combining all these  
25 markers would be highly valuable in ART pregnancies. Patients presenting with either an  
26 increase or a decrease in placental invasion could thus benefit from closer follow-up,  
27 particularly for foetal growth and for the risk of PPH during delivery.  
28

29 In addition to the measures for prevention to set up, these data raise the question of  
30 the origin of the differences between FET and fresh ET. Two points stand out: 1-the  
31 additional freezing-thawing step in FET; and 2- the different hormonal stimulation used  
32

1 between the two techniques. The freezing-thawing step could modify the developmental  
2 potential of the embryos and thus the obstetrical outcomes (Spijkers et al., 2017). However, it  
3 is now largely recognized that endometrium-embryo synchrony is essential in IVF. Indeed,  
4 the duration of the window of implantation, defined by the perfect balance of oestradiol and  
5 progesterone, may only last 2 to 5 days (Ozgun et al., 2018). Progesterone in particular has  
6 to be tightly regulated to ensure the highest rates of implantation. For example, the optimal  
7 mid-luteal progesterone concentration is thought to be 80-100 nmol/L for stimulated cycles  
8 (Yding Andersen and Vilbour Andersen, 2014) and 70-99 nmol/L in FET cycles (Yovich et al.,  
9 2015). In addition, there is now converging evidence suggesting that hormonal therapy could  
10 affect endometrial receptivity, thus trophoblastic invasion and finally pregnancy outcomes. A  
11 high dose of total FSH negatively impacted live birth rates in fresh ET compared to  
12 subsequent FET (Munch et al., 2017). Increased oestrogen levels at ovulation triggering  
13 before fresh ET were associated with lower first-trimester PAPP-A levels, preeclampsia, low  
14 birth-weight and preterm birth (Imudia et al., 2012; Giorgetti et al., 2013; Hu et al., 2014; Liu  
15 et al., 2017). PAPP-A values were found to be lower after fresh ET than after FET (Amor et  
16 al., 2009; Gjerris et al., 2009). In the same line as our results, FET in artificial cycles was  
17 associated with increased PPH risk compared with FET in natural cycles (Healy et al., 2010;  
18 Nakamura et al., 2015). The use of hCG triggering or progesterone supplementation for FET  
19 has been associated with lower clinical pregnancy rates than FET after natural cycles  
20 (Montagut et al., 2016). Particularly, micronized progesterone has been shown to increase  
21 placental volume if given in first trimester threatened abortion (Turgal et al., 2017). In our  
22 centre, the different conditioning between fresh ET and FET patients result in a cumulated  
23 dose of progesterone nearly 4 times lower for fresh ET, but in this case, the proper secretion  
24 of hormones by the multiple *corpus lutea* also has to be taken into account (Conrad and  
25 Baker, 2013).

26 The main strengths of our study, which was conducted in a single-centre, lie in its  
27 homogeneous population, well-standardized protocols and the good quality ultrasound  
28 measurements ensured by our highly skilled staff. In addition, all results were adjusted for  
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1 many potential confounding factors such as maternal age or parity. One weakness is the  
2 small number of patients, but the accuracy of our conclusions is ensured by the robust  
3 design and the comprehensive data collection, making our results consistent with most data  
4 in the literature. In order to ensure the homogeneity of the cohort and treatments used, we  
5 chose to lead a monocentric study, but it would be worthwhile for future studies to validate  
6 the results found here in other ART centres. Another limitation is the absence of data about  
7 the kinetics of placental and foetal growth. Indeed, crucial events for placental invasion are  
8 already completed before 12 WG (Moser and Huppertz, 2017), so it would be valuable to  
9 study the embryo from the very beginning of pregnancy, although the small size before 11  
10 WG would limit the accuracy of measurements. Otherwise, by the end of pregnancy, the  
11 plasticity of placenta can still change the whole picture of it (Choux et al., 2015), so late  
12 measures would be useful too, although measuring a larger placenta would also be  
13 technically difficult.  
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31 To conclude, ART can modify the delicate implantation step. Considering the  
32 potential effects of hormonal therapy on foeto-placental growth and even adverse outcomes  
33 such as PPH, we have to decipher the origin of these variations and strive to minimize them  
34 in order to avoid maternal and foetal morbidity, for example by reconsidering treatments for  
35 conditioning women before FET, with the aim of obtaining implantation similar to natural  
36 pregnancy.  
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## FIGURE LEGENDS

**Figure 1. Comparison of crown-rump length (A), placental volume (B), uterine artery pulsatility index (C) and birth weight (D) between groups.** Each box represents the interquartile range (IQR). Lines inside the boxes are the median. Whiskers represent the 10<sup>th</sup> and 90<sup>th</sup> percentiles. Crosses represent the mean. P is the result from multivariate analyses. All variables associated with the dependent variable with a P-value below 0.2 in bivariate analyses were included in the multiple regression analysis among maternal age, height, weight, body mass index (BMI), parity, smoking habits, gestational age and new-born's sex. ns: non-significant. Fresh ET: Fresh Embryo Transfer, FET: Frozen-thawed Embryo Transfer.

**Figure 2. ART is responsible for altered trophoblastic invasion, either decreased after *in vitro* fertilization (IVF) with fresh embryo transfer (Fresh ET) or exacerbated after frozen-thawed embryo transfer (FET).**

In most cases, the placenta is able to compensate for original injury throughout pregnancy, which results in a healthy new-born. However, if compensation is overwhelmed, the consequences could be adverse obstetrical and neonatal outcomes. The Developmental Origin of Health and Diseases (DOHaD) concept states that intra-uterine events can affect future life.

LGA: large for gestational age, PI: uterine artery pulsatility index, PPH: post-partum haemorrhage, PV: placental volume, SGA: small for gestational age

**Supplemental Figure 1. Correlations between log PV and  $\beta$ -hCG (A) PAPP-A (B), uterine artery PI (C) in the whole population. Correlation between log PV and PAPP-A in the FET group (D).**

PI: pulsatility index, PV: placental volume at the first-trimester ultrasound.

**Supplemental Figure 2. Correlation between birth weight and first trimester PAPP-A**

**(A) and log PV** adjusted for gestational age **(B)** in the whole population.

PV: placental volume at the first-trimester ultrasound.

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**Table 1** Population Characteristics.

	<b>Controls</b> <b>(n = 168)</b>	<b>Fresh ET</b> <b>(n = 60)</b>	<b>FET</b> <b>(n = 24)</b>	<b>P</b> <b>(global test)</b>	<b>P</b> <b>(Fresh ET vs. C)</b>	<b>P</b> <b>(FET vs. C)</b>	<b>P</b> <b>(Fresh ET vs. FET)</b>
<b>Maternal Age</b>	28.9 ± 5.6	32.1 ± 4.3	33.3 ± 3.4	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>0.0003</b>	0.237
Age > 35 years	24 (14.3)	12 (20.0)	6 (25.0)	0.306			
<b>Maternal Height (cm)</b>	165.6 ± 10.2	165.5 ± 6.2	165.3 ± 6.2	0.987			
<b>Maternal Weight (kg)</b>	65.1 ± 13.4	64.5 ± 13.2	58.8 ± 9.3	0.089			
<b>Maternal BMI (kg/m<sup>2</sup>)</b>	23.9 ± 5.1	23.5 ± 4.6	21.5 ± 3.2	0.081			
BMI > 30 kg/m <sup>2</sup>	18/154 (11.7)	8/59 (13.6)	1 (4.2)	0.466			
BMI > 25 kg/m <sup>2</sup>	48/154 (31.2)	15/59 (25.4)	2 (8.3)	0.061			
<b>Tobacco smoking during pregnancy</b>	37/167 (22.2)	5 (8.3)	1 (4.2)	<b>0.011</b>	<b>0.018</b>	0.052	0.669
<b>Parity before ongoing pregnancy</b>	0.92 ± 1.17	0.20 ± 0.41	0.58 ± 0.83	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	0.167	<b>0.006</b>
Nulliparous	66/155 (42.6)	47/59 (79.7)	14 (58.3)	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	0.149	0.046
<b>Indication for ART</b>				*	*	*	<b>0.032</b>
Male infertility	*	37 (61.7)	9 (37.5)				
PCOS	*	3 (5.0)	5 (20.8)				
Endometriosis	*	4 (6.7)	5 (20.8)				
Tubal	*	9 (15.0)	2 (8.3)				
Anovulation	*	3 (5.0)	0 (0)				
Other	*	4 (6.7)	3 (12.5)				

Quantitative data: mean ± SD, qualitative data: n (%)

ART: Assisted reproductive technologies, BMI: Body Mass Index, C: Controls, FET: Frozen-thawed Embryo Transfer, Fresh ET: Fresh Embryo Transfer, PCOS: PolyCystic Ovary Syndrome

P is the result from univariate analyses. P<0.05 in bold

**Table 2** First-trimester data.

	<b>Controls</b> (n = 168)	<b>Fresh ET</b> (n = 60)	<b>FET</b> (n = 24)	<b>P</b> (global test)	<b>P</b> (Fresh ET vs. C)	<b>P</b> (FET vs. C)	<b>P</b> (Fresh ET vs. FET)
<b>First-trimester ultrasound</b>							
maximal uterine artery PI	1.52 ± 0.59	1.86 ± 0.64	1.26 ± 0.44	<b>&lt;0.001</b>	<b>0.001</b>	0.112	<b>0.001</b>
presence of diastolic notching	53/163 (32.5)	30/54 (55.6)	5/17 (29.4)	<b>0.008</b>	<b>0.003</b>	0.794	0.060
placental volume (log cm <sup>3</sup> )	4.40 ± 0.283	4.38 ± 0.37	4.52 ± 0.39	0.068			
z-score of CRL	0	0.07 ± 0.53	0.09 ± 0.48	0.186			
<b>PAPP-A (MoM)</b>	1.12 ± 0.60	0.91 ± 0.48	1.02 ± 0.64	<b>0.049</b>	<b>0.016</b>	0.457	0.407
<b>β-hCG (MoM)</b>	1.21 ± 0.67	0.92 ± 0.47	1.19 ± 0.53	<b>0.007</b>	<b>0.002</b>	0.902	0.067

Quantitative data: mean ± SD, qualitative data: n (%)

C: Controls, FET: Frozen-thawed Embryo Transfer, Fresh ET: Fresh Embryo Transfer, PI: placental index, P is the result from univariate analyses, P<0.05 in bold

**Table 3** Obstetrical and neonatal outcomes.

	<b>Controls</b> <b>(n = 168)</b>	<b>Fresh ET</b> <b>(n = 60)</b>	<b>FET</b> <b>(n = 24)</b>	<b>P</b> <b>(global test)</b>
<b>Obstetric outcomes</b>				
severe preeclampsia	4/156 (2.6)	2/54 (3.7)	0/21 (0)	0.803
preterm labour	15/156 (9.6)	6/54 (11.1)	2/21 (9.5)	0.939
gestational hypertension	0/156 (0)	0/54 (0)	1/21 (4.8)	0.091
gestational diabetes	22/156 (14.1)	6/54 (11.1)	1/21 (4.8)	0.542
premature preterm rupture of membranes	0/156 (0)	1/54 (1.9)	0/21 (0)	0.325
chorioamniotitis	4/156 (2.6)	2/54 (3.7)	0/21 (0)	0.803
<i>abruptio placentae</i>	1/156 (0.6)	1/54 (1.9)	0/21 (0)	0.545
<b>Delivery</b>				
gestational age (WG)	39.5 ± 2.5	39.0 ± 2.7	40.0 ± 1.4	0.060
premature birth < 37 WG	8/156 (5.1)	3 (5.0)	1 (4.2)	1
premature birth < 34 WG	3/156 (1.9)	1 (1.7)	0 (0)	1
labour induction	42/156 (26.9)	18/54 (33.3)	8/21 (38.1)	0.443
post-partum haemorrhage	4/156 (2.6)	1/54 (1.9)	8/21 (38.1)	<b>&lt;0.0001</b>
instrumental delivery	22/156 (14.1)	20/57 (35.1)	6 (26.1)	<b>0.003</b>
caesarean section	30/156 (19.2)	7/59 (11.9)	6 (25.0)	0.270
<b>Birth weight (grams)</b>	<b>3281.8 ± 549.7</b>	<b>3040.8 ± 598.1</b>	<b>3526.5 ± 540.8</b>	<b>&lt;0.001</b>
birth weight < 10 <sup>th</sup> centile (SGA)	11/142 (7.8)	10/52 (19.2)	1/18 (5.6)	0.076
birth weight > 90 <sup>th</sup> centile (LGA)	12/142 (8.5)	2/52 (3.9)	2/18 (11.1)	0.368
<b>Birth weight (z-score)</b>	<b>-0.074 ± 0.92</b>	<b>-0.60 ± 0.96</b>	<b>0.15 ± 1.27</b>	<b>0.002</b>

<b>APGAR score at 5'</b>	9.73 ± 1.19	9.73 ± 1.43	9.68 ± 1.43	0.982
<b>Respiratory distress</b>	12/156 (7.7)	3/54 (5.6)	0/21 (0)	0.477

Quantitative data: mean ± SD, qualitative data: n (%)

C: Controls, FET: Frozen-thawed Embryo Transfer, Fresh ET: Fresh Embryo Transfer, LGA: Large for Gestational Age, SGA: Small for Gestational Age, P is the result from univariate analyses, P<0.05 in bold

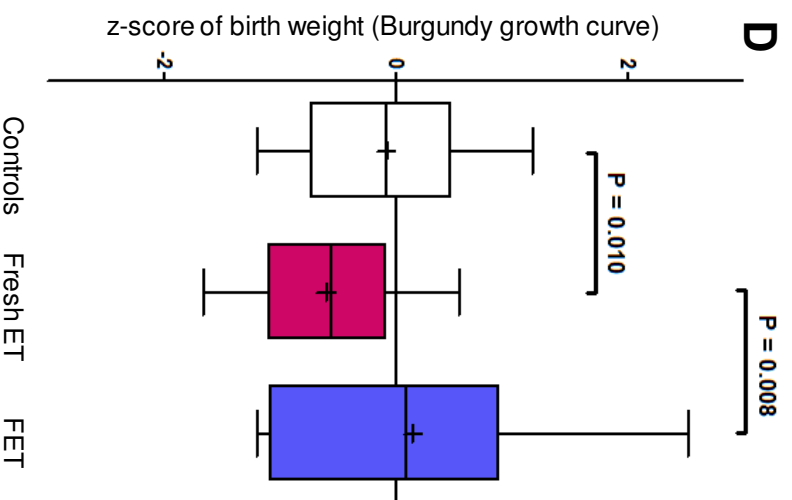
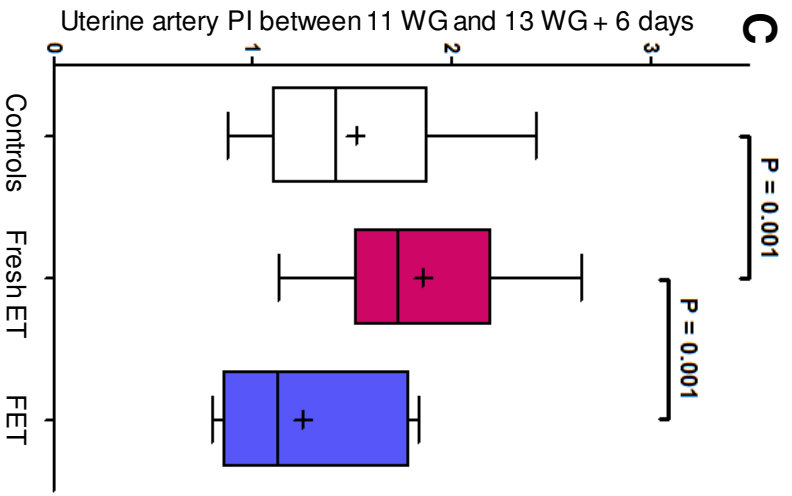
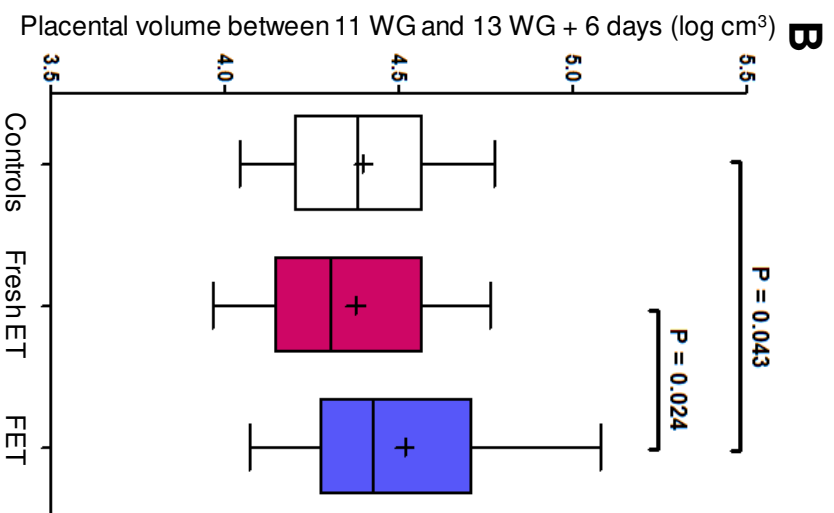
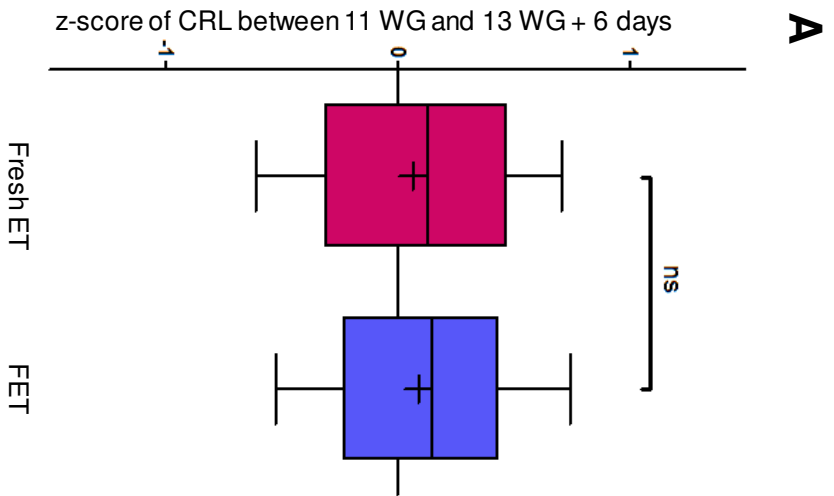


Figure 1

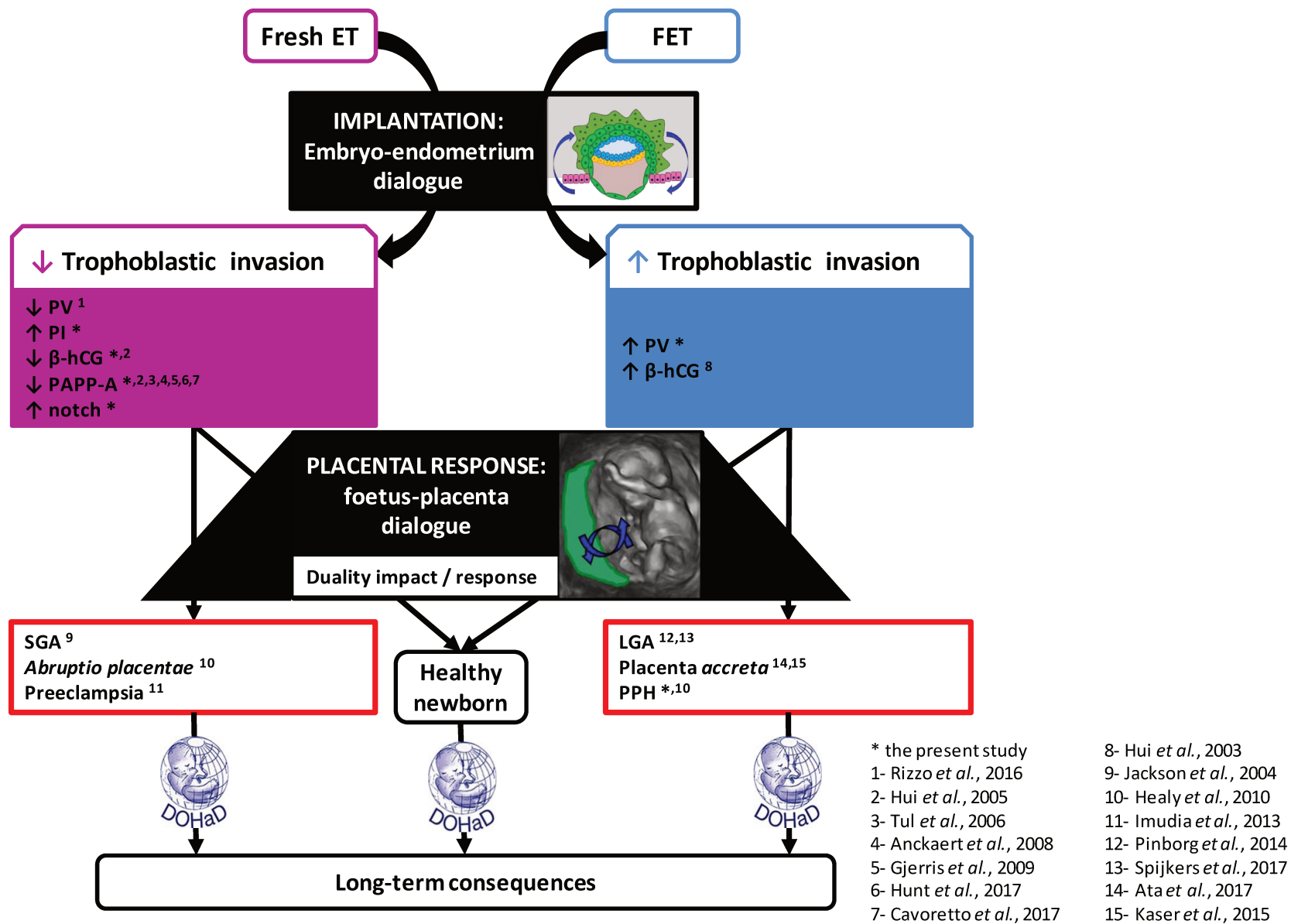
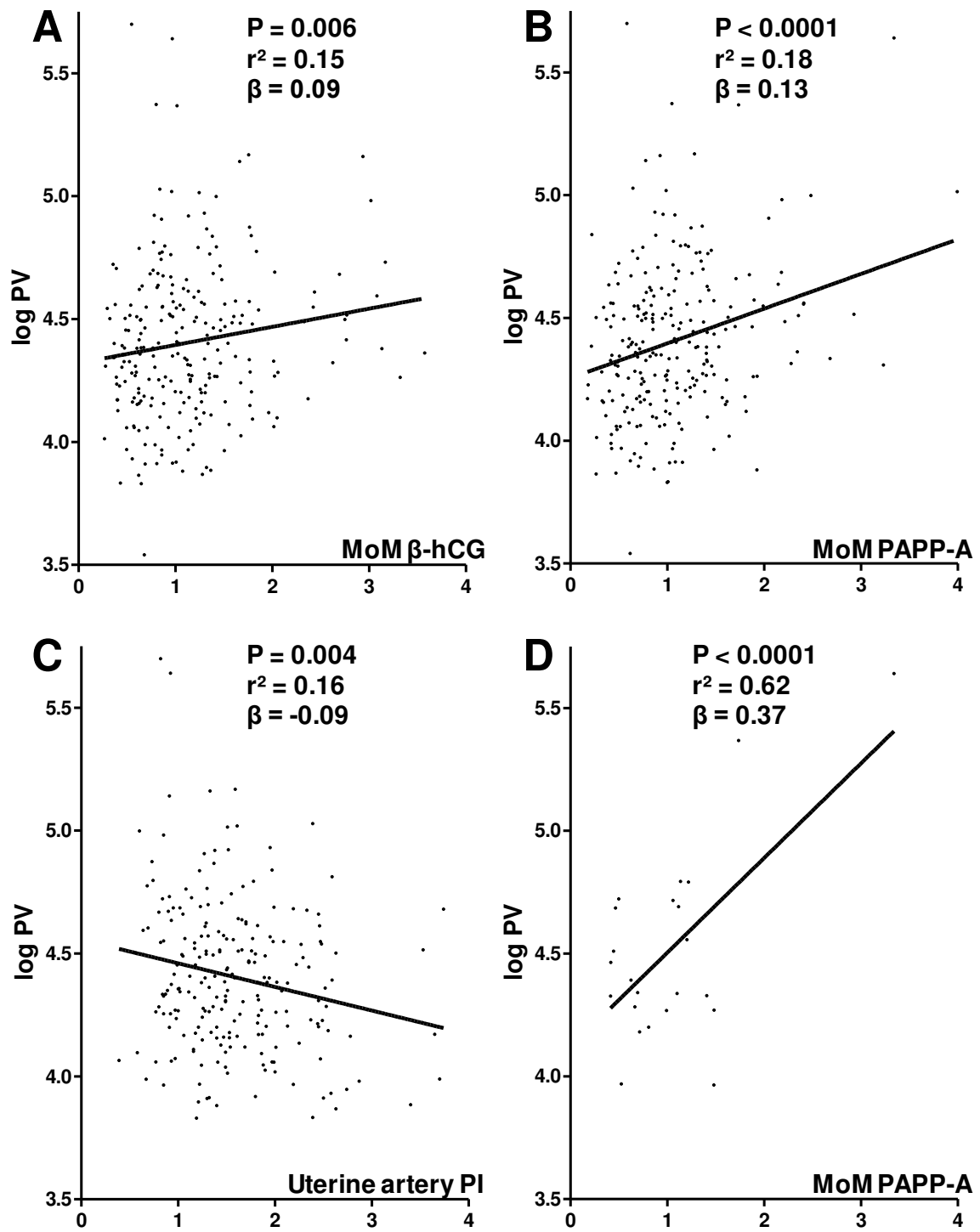
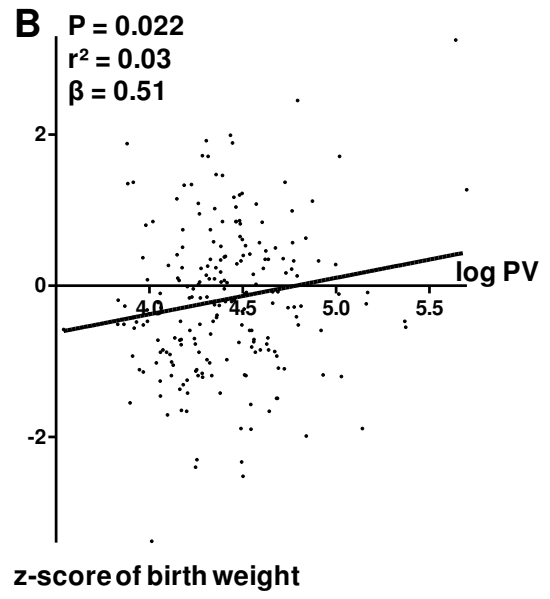
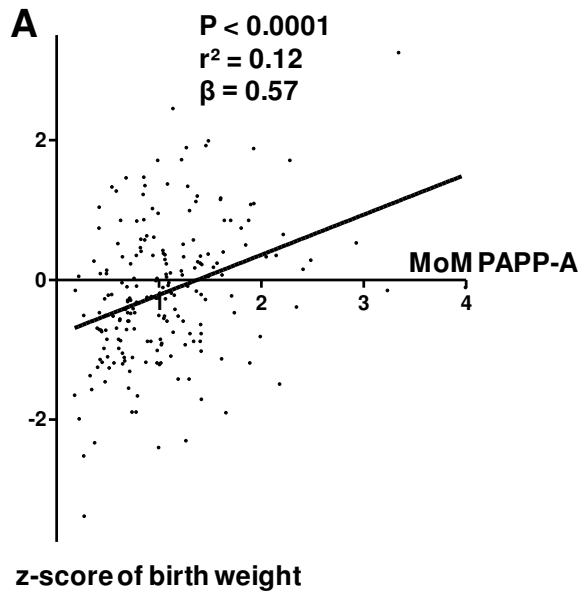


Figure 2



Supplemental figure 1



Supplemental figure 2