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ARTICLE

Does underlying infertility in natural conception modify the epigenetic control of imprinted genes and transposable elements in newborns?

BIOGRAPHY
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KEY MESSAGE
With increasing time to conception, DNA methylation and transcriptional changes in imprinted genes and transposable elements were observed in both placenta tissue and cord blood. This study provides evidence that infertility itself, and not only assisted reproductive technology, could contribute to potential epigenetic risks for children.

ABSTRACT

Research question: Does the epigenetic control of imprinted genes and transposable elements at birth differ according to time to conception in natural conception and after intrauterine insemination (IUI)?

Design: A total of 144 singletons were included in four groups: 50 natural pregnancies obtained within 6 months after stopping contraception (group 1); 34 natural pregnancies with infertility period between 6 and 12 months (group 2); 36 pregnancies with an infertility period of more than 12 months (group 3) and 24 pregnancies obtained after IUI (group 4).

Results: The placental DNA methylation levels of *H19/IGF2* and *KCNQ1OT1* were lower in groups 2, 3 and 4 than in group 1 ($P = 0.025$ in the overall comparison). The DNA methylation rate for LINE-1 was higher in placentas from group 2 than in group 1 ($P = 0.022$). In cord blood, DNA methylation levels were not significantly different between groups except for *H19/IGF2* for which the DNA methylation levels were higher in group 2 than in group 1 (*H19/IGF2*-seq1 and seq2: $P = 0.023$ and $P = 0.002$, respectively). In placenta tissue, compared with group 1, relative expression for *SNRPN* and for LINE-1 was significantly higher in group 2 ($P = 0.002$ and $P < 0.001$, respectively). The relative expression of *KCNQ1* in placenta was lower in group 4 than in group 1 ($P = 0.013$). In cord blood, compared with group 1, the relative expression for *H19* was significantly higher in group 3 ($P = 0.026$), and the relative expression of LINE-1 was higher in groups 2 and 3 and in group 4 ($P < 0.001$).

Conclusions: Infertility itself, and not only ART techniques, could contribute to potential epigenetic risks for children.

KEYWORDS
DNA methylation
Imprinted genes
Infertility
Singletons
Transposable elements

INTRODUCTION

Millions of children have been born through assisted reproductive technology (ART) worldwide, and about 4% of all births are now conceived through ART (*Adamson et al., 2018*). Although most of these children are deemed healthy, awareness about the complications that could potentially be linked to epigenetic deregulation is increasing (*Argyraki et al., 2019*). These include some adverse perinatal outcomes, e.g. abnormal placental function (*Choux et al., 2015a*; *Qin et al., 2016*) and especially rare imprinting disorders, e.g. Beckwith–Wiedemann and Russell–Silver syndromes (*DeBaun et al., 2003*; *Gicquel et al., 2003*; *Maher et al., 2003*; *Sutcliffe et al., 2006*; *Doornbos et al., 2007*; *Hiura et al., 2012*).

The increased rate of imprinting disorders after ART raises the issue of a potential epigenetic risk in situations in which ART is used. The many manipulations and processes of ART, e.g. hormonal stimulation and gamete manipulation, are concurrent with epigenetic reprogramming and imprinting, leading to concerns that ART could negatively affect epigenetics and genomic imprints (*Choux et al., 2015a*). Studies analysing epigenetic regulation in placenta or cord blood from ART-conceived newborns compared with naturally conceived infants (*Katari et al., 2009*; *Turan et al., 2010*; *Wong et al., 2011*; *Rancourt et al., 2012*; *Lou et al., 2014*; *Sakian et al., 2015*; *Song et al., 2015*) have reported differences in the methylation, expression of imprinted genes in cord blood or placenta, or both, but most of these studies have heterogeneous designs (*Choux et al., 2015b*). In addition, the infertility and subfertility status of the parents may also play a role in the increased incidence of epigenetic imprinting-related disorders (*Fauque et al., 2020*). In humans, however, it is challenging to distinguish the responsibility of ART procedures from the effects inherent to infertility problems *per se*. A Dutch study conducted in families with a child with imprinting disorders underlined the likely association between parental infertility and epigenetic abnormalities in offspring (*Doornbos et al., 2007*). Another more recent study (*Litzky et al., 2017a*) has also demonstrated that infertility could modify placental gene expression. It is still unclear, however, whether it is the ART procedures or the subfertility itself that leads to some of the epigenetic changes found in ART-conceived infants.

In the present study, groups of in-vivo singleton pregnancies, stratified by the time to conception or obtained through intrauterine insemination (IUI), were prospectively included. The aim of the study was to better understand whether the underlying infertility itself could influence the risk of epigenetic changes in newborns.

DNA methylation and transcriptional levels of specific imprinted genes and transposable elements in cord blood and placenta samples were analysed, and a robust methodology that took into account potential confounding factors, was applied. The focus was on three differentially methylated regions (DMR) of imprinted genes (*H19/IGF2*:IG-DMR, *KCNQ1OT1*:TSS-DMR, and *SNURF*:TSS-DMR, named according to the recommendations for nomenclature (*Monk et al., 2018*), because they have been reported to be deregulated or particularly sensitive to assisted reproductive procedures in animals and humans (*Oliver et al., 2012*; *Whitelaw et al., 2014*; *Choux et al., 2015b*). Two types of transposable elements have also been identified as indicators of global DNA methylation (HERV-FRD and LINE-1 part of respectively, long terminal repeat and non-long terminal repeat families).

MATERIALS AND METHODS

Study population
Patients were prospectively included from 1 January 2013 to 31 December 2019 from the Departments of Obstetrics, Gynaecology and Reproductive Biology at the Dijon-Bourgogne University Hospital, France. The first group, defined as the control group, included singleton pregnancies obtained through natural conception within 6 months after stopping contraception (group 1).

Three groups of patients were then included: a group in whom singleton pregnancies were achieved between 6 months and 1 year (group 2), a group with more than 1 year of infertility (group 3) and pregnancies resulting from intrauterine insemination (IUI) (group 4). All IUI cycles were managed by gonadotrophins, as previously described (*Fauque et al., 2014*). Briefly, ovarian stimulation was carried out with FSH. The treatment was started on the second day of the cycle, and HCG was administered when the leading follicle diameter measured over 15 mm. Intrauterine insemination was carried out the day after ovulation triggering.

Exclusion criteria were fetuses with an abnormal karyotype, maternal neurological, cardiac or pulmonary disorders, diabetes, hypertension, HIV and hepatitis B or C infections. Pregnancies resulting from ovulation induction (clomiphene citrate treatment) were not included in the study. All couples declared no sexual issues and regular intercourse.

Clinical data collection
The medical history of the mother and father, and treatments undertaken, were collected prospectively during the IVF and intracytoplasmic sperm injection procedures, throughout the pregnancy and after birth. In addition, the characteristics of the placenta and the newborn at birth (weight, birth defects and neonatal data) were exhaustively recorded.

Ethics approval and consent to participate
All women provided written informed consent in accordance with the Declaration of Helsinki. The study was approved by the Institutional Review Board and the Ethics Committee of the Dijon-Bourgogne University Hospital (Comité de Protection des Personnes [CPP] Est I, number 2012-A01010-43; 20 September 2012).

Sample preparation
Biological samples were collected within 15 min after delivery. Placenta samples (1 cm^3) were extracted from the fetal side near the umbilical cord insertion point. Protocols for RNA or DNA extraction were carried out as previously described (*Choux et al., 2018*). Briefly, RNA was extracted from approximately 100 mg of placenta using TRI Reagent® (Sigma-Aldrich, St Louis, MO, USA), according to the manufacturer's protocol with an additional DNase digestion step (Ambion® TURBO DNA-free™) (Invitrogen, Waltham, MA, USA). Blood RNA was extracted using the PAXgene Blood RNA kit® (Qiagen, Hilden, Germany) from cord blood collected in PAXgene blood RNA Tubes® (PreAnalytiX, Hombrechtikon, 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.

Quantitative DNA methylation analyses

The imprinted genes and transposable elements were studied by pyrosequencing after sodium bisulfite DNA treatment. The DNA methylation assays investigated several CpG sites depending on the sequences and genes studied (note that two *H19/IGF2*:IG-DMR sequences were distinguished because they display two different DNA methylation levels: sequences 1 and 2, and included a conversion bisulfite treatment control. Bisulfite conversion of genomic DNA and pyrosequencing analysis were carried out as previously described (*Bruno et al., 2015*; *2018*; *Choux et al., 2018*; *Barberet et al., 2021*). Briefly, genomic DNA (500 ng) was modified using the Epitect Bisulfite Kit® (Qiagen, Hilden, Germany). Bisulfite-treated DNA (10 ng) was used as the template for polymerase chain reaction amplification. Pyrosequencing reactions were carried out in a PyroMark Q24 MDx® system (Pyrosequencing AB, Stockholm, Sweden) with the PyroGold Reagents kit® (Qiagen, Hilden, Germany). The DNA methylation level was calculated as the ratio of the C to T peaks at a given CpG site using PyroMark® Q24 Software v.2.0.6 (Qiagen, Hilden, Germany). Primers and conditions (*Choux et al., 2018*) have previously been described. To overcome potential between-plate variability, a common control was placed in each pyrosequencing plate, and the other DNA methylation values were normalized in accordance with this control. Moreover, a DNA methylation scale (0–50–100%), obtained with EpiTect methylated human Control DNA, was placed in each pyrosequencing plate. Samples were processed, analysed and interpreted in a blinded manner. The linkage was only established for the interpretation of DNA methylation data.

Expression analysis

Real time polymerase chain reaction was used to study the expression of genes associated with the three differentially methylated regions (DMR) (*H19* for *H19/IGF2* DMR, *KCNQ1* for *KCNQ1OT1* DMR and the common *SNRPN* for *SNURF* DMR) and two transposable elements (the LINE-1 protein, ORF2; the envelope proteins of the retrovirus ERVFRD-1, syncytin-2). Expression was normalized on three reference genes (*GAPDH*, *SDHA* and *TBP*). cDNA was synthesized using Maxima Reverse Transcriptase (Thermo Scientific, Waltham, MA, USA). The level of expression of each imprinted gene or transposable element (called X in the following formula) was normalized to the geometric mean of expression levels of the three reference genes, using the following formula: X/geometric mean (R1, R2, R3) = 2(Ct[X]-arithmetic mean [Ct(R1), Ct(R2), Ct(R3)]), where Ct is the threshold cycle, and R1, R2, R3 are the reference genes. Bio-Rad CFX Manager (Version 3.0.1224.1015) was used to analyse the data. Primers and conditions were previously reported (*Choux et al., 2018*).

Statistical analyses

Categorical variables were expressed as numbers (percentages) and compared using chi-squared or Fisher's exact tests, as appropriate. Continuous variables were expressed as means ± SD or median and interquartile range, and compared using analysis of variance or Kruskall–Wallis according to their distribution. Pairwise comparison was carried out when the $P < 0.200$ using the Student's t-test or Mann–Whitney test with post-hoc correction by false discovery rate (FDR) method (q-value) (*Simes 1986*; *Benjamini 1995*; *Benjamini, 2001*).

Before fitting the multivariable regression linear models, standard regression assumptions were checked, including the homoscedasticity assumption. DNA expression levels constituting our dependent variables were log-transformed for the dependent variable in case of departure from the normal distribution. In this case, the results were expressed in terms of relative difference, which corresponds to the exponential of the coefficient (exp[β]) associated with each explanatory variable of the model with its 95% confidence interval. This estimate can be assimilated to a ratio of the means of the expressional levels in the compared groups. To detect outliers, univariate linear least squares regressions were carried out for each DNA methylation and expression in type of conception (groups 1, 2 and 3). The following indicators were used to measure observation influence: Cook's distance, leverage and Dfits. In case the percentage of outliers was above 10%, as the standard variance estimator for ordinary least squares regression is highly sensitive to outliers, multiple robust regression linear models were fitted.

Linear regression models were adjusted for the delay and type of conception (natural or after IUI) with group 1 (natural conception within 6 months after stopping contraception) as the reference, and then for delay and type of conception, maternal age, sex of the newborn (girl versus boy), term (weeks of gestation), and parity (≥1 versus nulliparous). All *P*-values were adjusted for multiple comparisons using the FDR method (q-value) (*Simes, 1986*; *Benjamini, 1995*; *2001*). Statistical significance was set at a two-tailed $P < 0.05$ or $q < 0.05$. Stata Statistical Software: Release 15 (College Station, TX: StataCorp LLC) was used for all statistical analyses.


RESULTS

Epigenetic analyses were carried out on a total of 144 singletons, including 50 natural pregnancies obtained within 6 months after stopping contraception (group 1), 34 natural pregnancies with an infertility period between 6 and 12 months (group 2), 36 spontaneous pregnancies with an infertility period of more than 12 months (group 3), and 24 pregnancies obtained with IUI (group 4) (**FIGURE 1**). The demographic and obstetric characteristics of the pregnancies according to conception delay and type of conception are presented in **TABLE 1**.

Infertility was associated with changes in DNA methylation profiles of imprinted genes and transposable elements in placenta and cord blood

To evaluate whether infertility in in-vivo conception changed the DNA methylation patterns of selected imprinted genes and transposable elements in placenta and cord blood, the results according to the time to natural conception were compared, and IUI data were compared with the results obtained in group 1.

Concerning imprinted genes in placenta tissue, the mean DNA methylation level of *H19/IGF2* DMR was significantly different in the four groups (*H19/IGF2*-seq1: $P = 0.025$ in the overall comparison)
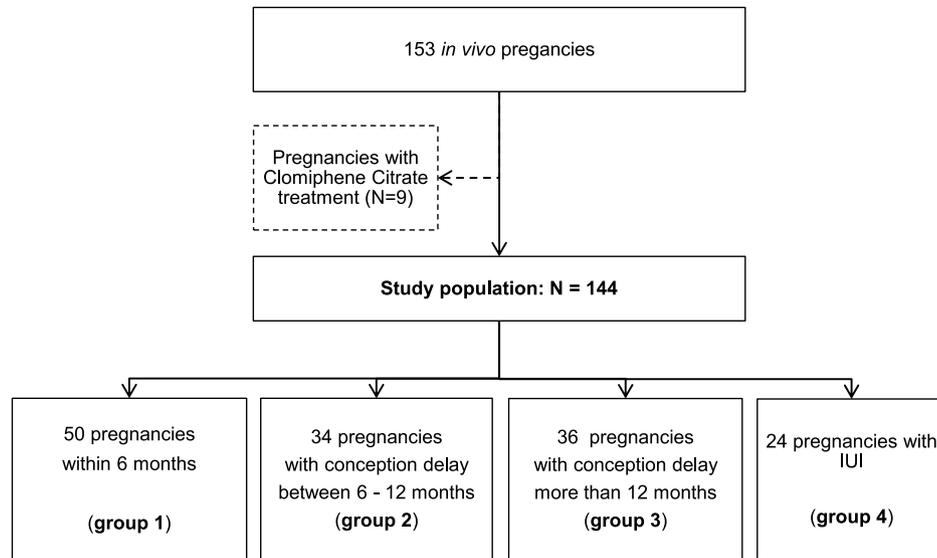
FIGURE 1 Study population. IUI, intrauterine insemination.

(**FIGURE 2**). After adjustment for maternal age, parity, sex of newborn and term of pregnancy, DNA methylation of *H19/IGF2*-seq1 remained significantly lower in groups 2 (–3.03 points, *P* = 0.041), 3 (–4.39 points, *P* = 0.007) and 4 (–4.71 points, *P* = 0.008) compared with group 1 (**TABLE 2**). Similarly, the DNA methylation level of *KCNQ1OT1* was also lower in groups 2, 3 and 4 by almost two points compared with group 1 (groups 2 [–2.19

points, *P* = 0.032], 3 [–1.76 points, *P* = 0.024) and 4 [–2.10 points, *P* = 0.015]) (**TABLE 2**).

In cord blood, the mean DNA methylation levels of *H19/IGF2* were significantly different between the four groups (*H19/IGF2*-seq2, *P* = 0.002) (**FIGURE 2**). After adjustment, the time to conception remained associated with DNA methylation changes in *H19/IGF2*, but the difference

was only significant between group 2 and group 1. More precisely, the DNA methylation levels of *H19/IGF2*-seq1 and *H19/IGF2*-seq2 were found to be significantly higher by 3.4 points (*P* = 0.023) and 1.98 points (*P* = 0.002), respectively, in group 2 compared with group 1 (**TABLE 2**).

Concerning transposable elements in placenta tissue, the mean DNA methylation levels of HERV-FRD were

TABLE 1 MATERNAL AND BIRTH CHARACTERISTICS

	Overall (*n* = 144)	Group 1 (*n* = 50)	Group 2 (*n* = 34)	Group 3 (*n* = 36)	Group 4 (*n* = 24)	*P*-value
Maternal characteristics						
Age, years	30.5 ± 4.3	28.8 ± 3.7	29.4 ± 3.5	32.8 ± 4.3	32.1 ± 4.3	<0.001[c]
Pre-pregnancy BMI, kg/m²	23.9 ± 4.7	23.4 ± 4.7	24.5 ± 4.8	24.4 ± 4.8	23.6 ± 4.3	0.666
Pre-pregnancy parity						
Nulliparous	91 (63.2)	24 (48.0)	25 (73.5)	24 (66.7)	18 (75.0)	0.043[b,c]
≥1	53 (36.8)	26 (52.0)	9 (26.5)	12 (33.3)	6 (25.0)	
Birth characteristics						
Term, weeks	39.9 ± 1.5	40.0 ± 1.3	39.7 ± 1.7	39.7 ± 1.6	39.9 ± 1.4	0.771
Birth weight, g	3335.4 ± 476.1	3397.8 ± 507.3	3290.3 ± 467.3	3303.1 ± 479.9	3317.9 ± 428.4	0.718
Z-score of birth weight[a]	0.1 ± 1.0	0.2 ± 1.1	0.1 ± 1.0	0.1 ± 1.0	0.0 ± 0.9	0.902
Placenta weight, g[a]	534.6 ± 105.9	537.0 ± 120.3	536.6 ± 107.6	537.0 ± 102.4	523.0 ± 77.6	0.956
Z-score of placenta weight[a]	–1.0 ± 0.8	–1.0 ± 0.9	–1.0 ± 0.8	–1.0 ± 0.8	–1.1 ± 0.6	0.901
Sex of newborn[a]						
Male	72 (50.0)	24 (48.0)	16 (47.1)	18 (50.0)	14 (58.3)	0.834
Female	72 (50.0)	26 (52.0)	18 (52.9)	18 (50.0)	10 (41.7)	

Group 1: <6 months; group 2: 6–12 months; group 3: >12 months; group 4: intrauterine insemination.

[a] z-score of birth weight unknown for two cases; placenta weight unknown for five cases; z-score of placenta weight unknown for seven cases; and sex unknown for one case. Pairwise comparison with adjusted *P*-value using false discovery rate method.

[b] (1) – (2): q-value = 0.207, (1) – (3): q-value = 0.419, (1) – (4): q-value = 0.207, (2) – (3): q-value = 1.000, (2) – (4): q-value = 1.000, (3) – (4): q-value = 1.000.

Data are presented as mean ± SD or *n* (%) unless specifically designated as z-score.

[c] Statistically significant.

BMI, body mass index.
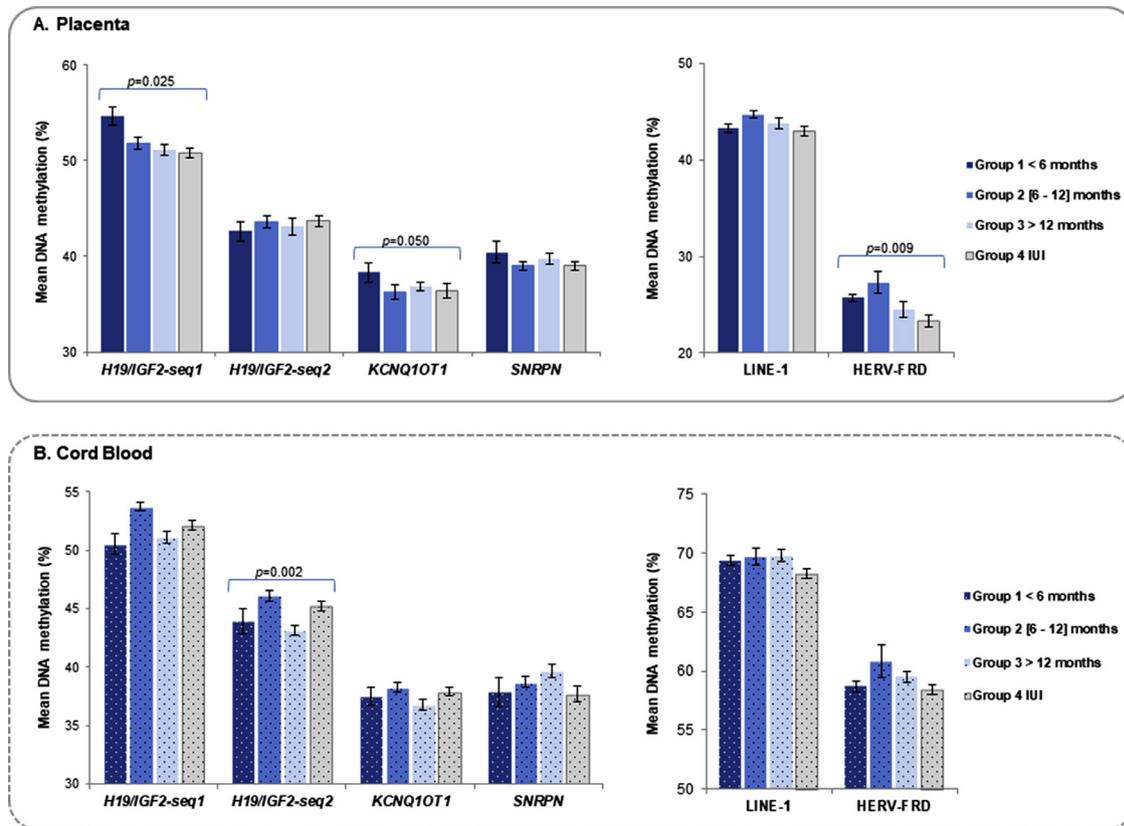
FIGURE 2 Mean DNA methylation levels of the specific imprinted gene differentially methylated regions and transposable elements using pyrosequencing in (A) the placenta and (B) in cord blood. *P*-values from analysis of variance. Bars represent SEM. IUI, intrauterine insemination.

significantly different between the four groups (*P* = 0.009 in the overall comparison) (FIGURE 2). After adjustment, the association between groups and HERV-FRD methylation levels were no longer significant. In contrast, the LINE-1 methylation rate was found to be significantly higher in group 2 compared with group 1 (1.40 points, *P* = 0.022) (TABLE 2). In cord blood, no association was observed between the in-vivo conception delay or type of conception and the DNA methylation of transposable elements.

Using the FDR method to account for multiple testing, no significant association was found between DNA methylation of imprinted genes and transposable elements, except for *H19/IGF2* methylation levels, which remained significantly higher in cord blood from group 2 compared with group 1 (*H19/IGF2*-seq1: *P* = 0.023, *H19/IGF2*-seq2: 0.002) (TABLE 2).

Infertility was associated with relative modifications in the expression of imprinted genes and transposable elements in placenta and cord blood
In placenta, the comparison of relative fold expression revealed no significant changes in imprinted gene expression

between the four groups. After adjustment for maternal age, parity, sex of newborn and term, however, the expression of *SNRPN* and *KCNQ1* was found to be significantly different according to in-vivo conception delay or type of conception. Compared with group 1, the relative mean *SNRPN* expression in placenta was 72% higher in placentas from group 2 (exp[β] = 1.72, *P* = 0.002), and the expression of *KCNQ1* was 76% lower in placentas from singletons conceived after IUI (group 4) (exp[β] = 0.24, *P* = 0.013) (FIGURE 3 and Supplementary Table 1).

In cord blood, no significant change took place in imprinted gene expression between groups in univariate analysis (using linear regression models); however, in multivariable analyses, *H19* expression was increased (112% [exp[β] = 2.12], *P* = 0.026) in singletons from group 3 compared with those from group 1 (FIGURE 3 and Supplementary Table 1).

For the transposable elements, the comparison of relative expression in placenta revealed significant changes in relative LINE-1 ORF2 expression between the four groups (*P* < 0.001). After

adjustment, LINE-1 ORF2 expression was increased in placenta from group 2 (89% [exp[β] = 1.89], *P* < 0.001) and group 3 (94% [exp[β] = 1.94], *P* < 0.001) compared with placenta from group 1 (FIGURE 3 and Supplementary Table 1). In cord blood, the time to conception was also associated with LINE-1 ORF2 expression changes (*P* < 0.001). After adjustment, LINE-1 ORF2 expression was 103% (exp[β] = 2.03, *P* < 0.001) and 130% (exp[β] = 2.30, *P* < 0.001) higher in singletons from groups 2 and 3, respectively, compared with those from group 1 (FIGURE 3 and Supplementary Table 1). In addition, the LINE-1 ORF2 expression was increased (75% [exp[β] = 1.75], *P* < 0.001) in cord blood from singletons conceived after IUI (group 4) compared with those from group 1 (FIGURE 3 and Supplementary Table 1).

Using the FDR method to account for multiple testing, most of these expressional differences were still observed (Supplementary Table 1).

Effect of maternal age, parity, sex of newborn and term of pregnancy
In this cohort of singletons conceived *in vivo*, further analysis was carried out to explore the effect of maternal

TABLE 2 DIFFERENCES IN DNA METHYLATION OF DIFFERENTIALLY METHYLATED REGIONS OF IMPRINTED GENES AND TRANSPOSABLE ELEMENTS IN PLACENTA AND CORD BLOOD ACCORDING TO THE TIME OF CONCEPTION

	Group 2 versus group 1	Group 3 versus group 1	Group 4 versus group 1	Group 2 versus group 1		Group 3 versus group 1		Group 4 versus group 1	
				P–value	q–value	*P*–value	q–value	*P*–value	q–value
Placenta Imprinted genes									
H19/IGF2–seq1	–3.03[a] (–5.92; –0.13)	–4.39[a] (–7.58; –1.20)	–4.71[a] (–8.16; –1.26)	0.041[a]	0.112	0.007[a]	0.054	0.008[a]	0.082
H19/IGF2–seq2	1.31 (–0.61; 3.22)	0.50 (–1.67; 2.67)	1.18 (–0.78; 3.13)	0.180	0.275	0.650	0.841	0.236	0.371
KCNQ1OT1	–2.19[a] (–4.19; –0.20)	–1.76[a] (–3.28; –0.23)	–2.10[a] (–3.78; –0.42)	0.032[a]	0.099	0.024[a]	0.112	0.015[a]	0.082
SNRPN	–1.42 (–3.00; 0.16)	–0.61 (–2.63; 1.40)	–1.34 (–2.83; 0.14)	0.077	0.170	0.548	0.841	0.075	0.236
Transposable elements									
LINE–1	1.40[a] (0.20; 2.59)	0.42 (–1.06; 1.91)	–0.34 (–1.83; 1.14)	0.022[a]	0.085	0.575	0.841	0.649	0.719
HERV–FRD	1.91 (–0.45; 4.26)	–0.91 (–2.67; 0.85)	–2.07 (–4.28; 0.13)	0.111	0.204	0.309	0.566	0.066	0.236
Cord blood Imprinted genes									
H19/IGF2–seq1	3.40[a] (0.47; 6.32)	1.13 (–1.64; 3.89)	1.59 (–1.76; 4.94)	0.023[a]	0.085	0.422	0.715	0.351	0.482
H19/IGF2–seq2	1.98[a] (0.75; 3.20)	1.00 (–0.27; 2.27)	0.90 (–0.43; 2.23)	0.002[a]	0.013[a]	0.121	0.381	0.183	0.334
KCNQ1OT1	0.80 (–0.60; 2.20)	0.24 (–1.36; 1.84)	0.36 (–1.38; 2.09)	0.261	0.359	0.767	0.854	0.686	0.719
SNRPN	1.09 (–0.06; 2.25)	1.16 (0.02; 2.31)	0.33 (–1.17; 1.82)	0.064	0.156	0.047	0.172	0.664	0.719
Transposable elements									
LINE–1	0.45 (–0.79; 1.69)	0.31 (–0.93; 1.56)	–1.07 (–2.58; 0.44)	0.472	0.546	0.619	0.841	0.163	0.334
HERV–FRD	2.60 (–0.37; 5.58)	1.93 (–0.64; 4.51)	0.79 (–1.90; 3.49)	0.086	0.172	0.140	0.385	0.562	0.686

Group 1: <6 months; group 2: 6–12 months; group 3: >12 months; group 4: intrauterine insemination.

Multivariate linear regression models used to adjust for time of conception, maternal age, parity, sex of newborn and term (robust 95% confidence Interval).

Q–value is the adjusted *p*–value using the false discovery rate method.

[a] Statistically significant.

IUI, intrauterine insemination.

age, parity, sex of newborn and term of pregnancy on imprinted genes and transposable elements DNA methylation and expression in placenta and cord blood (Supplementary Tables 2–5). Overall, imprinted genes and transposable elements DNA methylation profiles were unchanged except for LINE-1 methylation, which increased by 1.04 points ($P = 0.024$) in placentas from multiparous pregnancies (versus nulliparous) and decreased by 0.40 points ($P = 0.032$) in cord blood with each 1-week increase in the term of gestation. Concerning imprinted genes and transposable elements expression, only the sex of the newborn was associated with significant transcriptional modifications in placenta. The expression of *H19* and *SNRPN* was higher in the placenta of girls compared with boys ($P = 0.013$ and $P = 0.030$, respectively) (Supplementary Table 4).

DISCUSSION

One of our objectives was to determine whether underlying infertility itself influences epigenetic changes. To the best of our knowledge, this is the first time that epigenetic outcomes have been studied directly in naturally conceived children according to the time to conception.

During this unique study, after several potential biases were limited, we observed DNA methylation and transcriptional changes in imprinted genes and transposable elements in newborns according to the time to conception. Interestingly, IUI conceptions seemed to follow trends, i.e. on *H19/IGF2* and *KCNQ1OT1* DMRs, similar to those observed after long delays in natural conception. The *H19* gene is an imprinted genes transcribed from the maternally inherited allele (and so normally methylated only on the paternally inherited allele) and known to play a role in both limiting placental growth and controlling fetal growth (*Fowden et al., 2006*). More particularly, even though no associated significant transcriptional changes for

H19 were observed here, the DNA methylation of *H19/IGF2* was decreased along with increasing time to naturally obtained pregnancy or IUI compared with controls. Interestingly, it has been reported by several groups that *H19* DNA methylation levels were also lower in placentas from newborns conceived after IVF compared with those from natural conceptions (*Wong et al., 2011*; *Rancourt et al., 2012*; *Nelissen et al., 2013*; *Choux et al., 2018*). Therefore, the placental *H19* DNA methylation changes observed in this infertility study could contribute in part to the results observed in children born from in-vitro conception. In contrast, in cord blood, the *H19* DNA methylation levels were high after a delay in conception lasting more than 6 months. Infertility, however, does not seem to increase the potential effects on the *H19* methylation in cord blood. The *H19* transcription in cord blood was increased in the group with a long time to conception and tended to be high in the IUI group, suggesting that the *H19* regulation could nevertheless
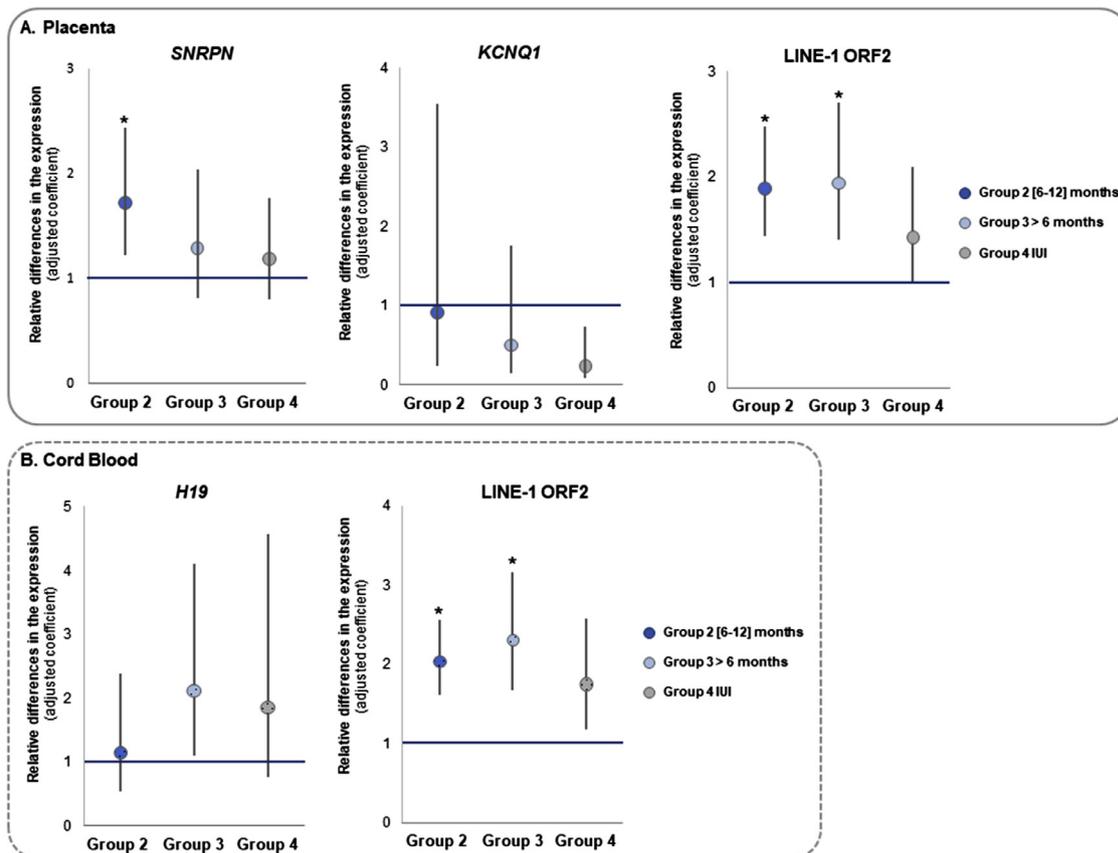
FIGURE 3 Relative differences in the expression of imprinted genes and transposable elements in (A) placenta and (B) cord blood according to the time and type of in-vivo conception. Group 1 is used as the reference group. Only significant imprinted genes and transposable elements using multivariate linear regression models are shown. Marks represent variable log-transformed, thus exp(coefficient β). Bars represent 95% confidence interval. *, significant q-value, which is the adjusted *P*-value using the false discovery rate method (exact *P*- and q-values are available in Supplementary Table 1). IUI, intrauterine insemination.

be affected in newborns conceived from infertile parents.

The finely tuned regulation of *KCNQ1OT1* is also required for normal placentation during development (*Oh-McGinnis et al., 2010*). Again, after adjusting for confounding factors, we found that *KCNQ1OT1* was less methylated after longer infertility periods and after IUI. Previous reports on *KCNQ1OT1* DMR found similar results in IVF compared with spontaneous conceptions (*Gomes et al., 2009*; *Choux et al., 2018*), reinforcing the possible role of underlying infertility on a potential *KCNQ1OT1* dysregulation. *KCNQ1OT1* is a paternally expressed non-coding RNA that is transcribed from a maternally methylated promoter (KvDMR) of the *KCNQ1* gene in the antisense orientation (*Horike et al., 2000*; *Mancini-DiNardo et al., 2003*; *Horike et al., 2009*). *KCNQ1OT1* transcripts recruit polycomb-group complexes, mediating repressive histone marks on the paternal allele, and repressing the surrounding

genes *in cis*. Therefore, transcription of the *KCNQ1OT1* gene silences several maternally expressed genes on the paternal chromosome (*Monk et al., 2006*). Therefore, the hypomethylation at *KCNQ1OT1* DMR associated with infertility could potentially enhance the silent chromatin structure and negatively regulate imprinted genes that are important for placental function, e.g. *KCNQ1, CDKN1C, SLC22A18* and *PHLDA2* (*Horike et al., 2000*; *Fitzpatrick et al., 2002*; *Thakur et al., 2003*). The present study reinforces this idea considering that *KCNQ1* expression was found to be downregulated after a long infertility period (even though not statistically significant) or after IUI.

In accordance with published research on IVF infants (*Camprubi et al., 2013*; *Nelissen et al., 2013*), our findings show the absence of any effect of fertility problems on the methylation status of *SNRPN* in newborns. The placental *SNRPN* transcription was found to be increased when the couple had difficulty

conceiving (only significant in the group with 6–12 months of infertility). *SNRPN* (Small nuclear ribonucleoprotein-associated protein N), a paternally expressed gene, by participating in pre-mRNA processing events and coding (*Ozcelik et al., 1992*), could be involved in pathological gestation (*Rahat et al., 2017*).

Concerning transposable elements control, in multivariable analyses accounting for potential confounders, the methylation levels of LINE-1 in the placenta, which were previously found to be modified after ART (*Ghosh et al., 2017*; *Choux et al., 2018*), seemed to be modified by infertility.

The only statistically significant DNA methylation changes in placental transposable elements, however, were observed in the group with 6–12 months of infertility. After IVF, the DNA methylation of LINE-1 elements might have a directional difference. For instance, even though they found an

increased methylation overall (through the LUMA assay), *Ghosh et al. (2017)* observed hypomethylation of LINE-1 elements in the placental genome conceived by IVF compared with those from natural conceptions by pyrosequencing. In the present study, placental LINE-1 ORF2 expression was, in contrast, almost twice as high with underlying infertility. The ORF2 detected in vascular endothelial cells and syncytiotrophoblasts of the placenta is believed to be implicated in placental cell fusion processes (*Ergun et al., 2004*). Indeed, an increased expression has been observed in third-trimester placentas compared with first-trimester placentas, indicating that LINE-1 could play a role in placental function (*He et al., 2014*). Therefore, we can speculate that the increased expression in LINE-1 ORF2 in delayed-conception groups may be a biomarker of placental upregulation to sustain fetal development. Therefore, it would be interesting to extend the analyses on epigenetic regulation according to the time needed for in-vivo conception on a larger scale (both methylome and transcriptome). *Litzky et al., (2017b)* who examined epigenetic regulation in placentas from infant cohorts mostly selected for small and large for gestational age, found considerable epigenetic changes in conception obtained in the group of subfertile parents, i.e. parents who tried to conceive for more than a year, whereas, in contrast, they reported no differences between IVF placentas and normally conceived placentas (*Litzky et al., 2017b*).

One of the strengths of our study is the prospective and monocentric, i.e. one IUI laboratory, and design that ensured accurate data collection and standardized samples. In addition, multivariate analysis was carried out in which several adjustments were made, including gestational age at delivery, which is well known to highly influence DNA methylation levels at birth (*El Hajj et al., 2017*), also proved here to influence the placental LINE-1 regulation. Interestingly, different epidemiological studies have reported that the risk of congenital malformations was increased along with increasing time to naturally obtained pregnancy (*Zhu et al., 2006*; *Rimm et al., 2011*) or with the underlying infertility in parents, pertaining to male (*Davies et al., 2012*) or female infertility factors (such as endometriosis or

polycystic ovary syndrome) (*Fauque et al., 2021*). In the present study, the delayed-conception groups had no clinical subfertility diagnoses. We are, therefore, unable to determine the specific causes of subfertility related to the observed epigenetic changes, which makes it difficult to elaborate mechanistic hypotheses. We suspect, however, that infertility in parents can alter the epigenetic landscape of their gametes, as reported by several investigators who analysed spermatozoa from subfertile men (*Asenius et al., 2020*). Noticeably, from mid-gestation in mouse models with infertility problems, epigenetic modifications reported after IVF were mainly restricted to placental tissues, whereas the embryo seemed to be mostly unaffected (*Mann et al., 2004*). In humans, however, it seems that epigenetic changes could be observed in cells from both placentas and cord blood (*Choux et al., 2015b*; Fauque 2018). It is likely that the altered epigenetic processes associated with infertility are both complex and different from those linked to reproductive techniques. Furthermore, the interpretation of methylation and expression data is difficult owing to the lack of strong correlation between methylation and expression changes, especially in the placenta (*Wong et al., 2011*; *Rancourt et al., 2012*; *Nelissen et al., 2013*; *Sakian et al., 2015*; *Vincent et al., 2016*; *Choux et al., 2018*). Indeed, DNA methylation is one of the mechanisms controlling gene expression, with many other combined factors, i.e. histone modifications, non-coding RNAs, and transcription factors. Therefore, for future research, to broaden the vision of epigenetic regulation in newborns, further studies are needed to assess the role of other actors. Concerning the IUI group results, none of the observed epigenetic changes were stronger than in the in-vivo conception groups, suggesting that the sperm manipulation or mild ovarian stimulation are not major detrimental factors.

In conclusion, the results of the present study highlight the epigenetic effects of parental fertility problems in singletons conceived *in vivo*. These effects are also likely to contribute to the epigenetic changes reported after ART. Indeed, to date, epigenetic studies were not designed to be able to decipher the relative role of underlying infertility from the effect of the reproductive techniques.

Herein, even though confirmation is needed, this unique study provides new findings in this field of research, and the implications are particularly important considering that infertility itself, and not only the ART techniques, could contribute to potential epigenetic risks for children. The epigenetic modifications, however, are modest and their clinical significance is not well understood. In addition, it remains to be seen if environmental factors, such as exposure to toxins, could contribute to the epigenetic changes related to parental infertility.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2022.01.004.

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