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# Comparison of DNA methylation patterns of parentally imprinted genes in placenta derived from IVF conceptions in two different culture media

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**STUDY QUESTION:** Is there a difference in DNA methylation status of imprinted genes in placentas derived from IVF conceptions where embryo culture was performed in human tubal fluid (HTF) versus G5 culture medium?

**SUMMARY ANSWER:** We found no statistically significant differences in the mean DNA methylation status of differentially methylated regions (DMRs) associated with parentally imprinted genes in placentas derived from IVF conceptions cultured in HTF versus G5 culture medium.

**WHAT IS KNOWN ALREADY:** Animal studies indicate that the embryo culture environment affects the DNA methylation status of the embryo. In humans, birthweight is known to be affected by the type of embryo culture medium used. The effect of embryo culture media on pregnancy, birth and child development may thus be mediated by differential methylation of parentally imprinted genes in the placenta.

**STUDY DESIGN, SIZE, DURATION:** To identify differential DNA methylation of imprinted genes in human placenta derived from IVF conceptions exposed to HTF or G5 embryo culture medium, placenta samples ($n = 43$ for HTF, $n = 54$ for G5) were collected between 2010 and 2012 s as part of a multi-center randomized controlled trial in the Netherlands comparing these embryo culture media. Placenta samples from 69 naturally conceived (NC) live births were collected during 2008–2013 in the Netherlands as reference material.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** To identify differential DNA methylation of imprinted genes, we opted for an amplicon-based sequencing strategy on an Illumina MiSeq sequencing platform. DNA was isolated and 34 DMRs associated with well-defined parentally imprinted genes were amplified in a two-step PCR before sequencing using MiSeq technology. Sequencing data were analyzed in a multivariate fashion to eliminate possible confounding effects.

**MAIN RESULTS AND THE ROLE OF CHANCE:** We found no statistically significant differences in the mean DNA methylation status of any of the imprinted DMRs in placentas derived from IVF conceptions cultured in HTF or G5 culture medium. We also did not observe any differences in the mean methylation status per amplicon nor in the variance in methylation per amplicon between the two culture medium

groups. A separate surrogate variable analysis also demonstrated that the IVF culture medium was not associated with the DNA methylation status of these DMRs. The mean methylation level and variance per CpG was equal between HTF and G5 placenta. Additional comparison of DNA methylation status of NC placenta samples revealed no statistically significant differences in mean amplicon and CpG methylation between G5, HTF and NC placenta; however, the number of placenta samples exhibiting outlier methylation levels was higher in IVF placenta compared to NC ($P < 0.00001$). Also, we were able to identify 37 CpG sites that uniquely displayed outlier methylation in G5 placentas and 32 CpG sites that uniquely displayed outlier methylation in HTF. In 8/37 (G5) and 4/32 (HTF) unique outliers CpGs, a medium-specific unique outlier could be directly correlated to outlier methylation of the entire amplicon.

**LIMITATIONS, REASONS FOR CAUTION:** Due to practical reasons, not all placentas were collected during the trial, and we collected the placentas from natural conceptions from a different cohort, potentially creating bias. We limited ourselves to the DNA methylation status of 34 imprinted DMRs, and we studied only the placenta and no other embryo-derived tissues.

**WIDER IMPLICATIONS OF THE FINDINGS:** It has often been postulated, but has yet to be rigorously tested, that imprinting mediates the effects of embryo culture conditions on pregnancy, birth and child development in humans. Since we did not detect any statistically significant effects of embryo culture conditions on methylation status of imprinted genes in the placenta, this suggests that other unexplored mechanisms may underlie these effects. The biological and clinical relevance of detected outliers with respect to methylation levels of CpGs and DMR require additional analysis in a larger sample size as well. Given the importance and the growing number of children born through IVF, research into these molecular mechanisms is urgently needed.

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**TRIAL REGISTRATION NUMBER:** Placental biopsies were obtained under Netherlands Trial Registry number 1979 and 1298.

**Key words:** embryo culture medium / imprinting / placenta / epigenetics / human / DNA methylation

# Introduction

In the past two decades, over 1 million children have been conceived by medically assisted reproduction including IVF in Europe alone (Ferraretti *et al.*, 2017). While most of these children are born healthy after IVF, the risk for preterm delivery, pregnancy complications, perinatal mortality and low birthweight is higher in IVF pregnancies than in naturally conceived (NC) pregnancies (Helmerhorst *et al.*, 2004; Jackson *et al.*, 2004; Romundstad *et al.*, 2006; Chen *et al.*, 2009; Esh-Broder *et al.*, 2011; Molgaard-Hansen *et al.*, 2011; Davies *et al.*, 2012; Hansen *et al.*, 2013).

The mechanisms behind this observed susceptibility of IVF children to complications during pregnancy, birth and child development remain largely unclear. According to the Developmental Origins of Health and Disease (DOHaD) hypothesis, an altered environment during early development may lead to an altered health status later in life (Feuer and Rinaudo, 2012; Donjacour *et al.*, 2014). During IVF, gametes and embryos are kept in an artificial environment that cannot fully recapitulate the *in vivo* environment of the fallopian tubes and uterine cavity (Feuer and Rinaudo, 2012). Given that the exact composition of the optimal micro-environment for the embryo *in vivo* is largely unknown, we are currently still in the dark when it comes to the ideal embryo culture conditions in the laboratory. Pivotal developmental processes, including fertilization, early cell divisions, early lineage commitment and epigenetic reprogramming occur within the time frame that human embryos are *in vitro* during IVF. Hence, it is likely that the *in vitro* culture environment may affect these processes and therefore underlie the increased prevalence of adverse gestational, peri- and postnatal outcomes associated with IVF.

Direct evidence of an effect of the *in vitro* culture environment on the children born comes from clinical observations comparing different embryo culture media. In retrospective and prospective human studies, IVF culture media and protein supplementation have affected birthweight (Dumoulin *et al.*, 2010; Nelissen *et al.*, 2012; Eskild *et al.*, 2013; Wu *et al.*, 2015; Zandstra *et al.*, 2015), although such an effect was not seen in several other studies (Eaton *et al.*, 2012; Carrasco *et al.*, 2013; Lin *et al.*, 2013; Lemmen *et al.*, 2014; Zandstra *et al.*, 2015). In a recently published large randomized controlled trial (RCT) a mean difference of 158 g in birthweight was detected upon using two different media (Kleijkers *et al.*, 2016). Evidence emerging from RCTs is of great value and therefore strengthens the idea that IVF culture media indeed have an effect on the *in utero* development of the newborn and weight and adiposity at the age of 9 years (Zandstra *et al.*, 2018).

It is known that medium composition directly affects the embryo at multiple levels. For example, it has been shown in mouse, bovine and human embryos that medium or medium supplementation affects gene expression (Rinaudo and Schultz, 2004; Kleijkers *et al.*, 2015; Mantikou *et al.*, 2015), IVF effectiveness (Kleijkers *et al.*, 2015; Youssef *et al.*, 2015), histone modification and blastocyst development (Ikeda *et al.*, 2018), expression of imprinted genes (Khosla *et al.*, 2001; Mann, 2004; Market-Velker *et al.*, 2010) and post-natal glucose tolerance (Donjacour *et al.*, 2014).

Epigenetic regulation is thought to be a key player in the molecular mechanisms that underlie the effects of embryo culture medium, especially the DNA methylation status of imprinted genes. Genomic imprinting refers to mono-allelic expression of genes in a parent-of-origin dependent manner, and genes that exhibit this type of expression often have a role in growth and development (Reik and Walter, 2001; Woodfine *et al.*, 2011). Differences in expression of imprinted genes were previously identified in mouse embryos cultured in different media (Khosla *et al.*, 2001; Mann, 2004; Market-Velker *et al.*, 2010). Due to the plasticity of DNA methylation, i.e. both gene-specific and global DNA methylation tend to change rapidly upon exposure to environmental stimuli, it is considered to be the perfect candidate to explain such DOHaD-related effects.

The placenta could be an important mediator in the effect of IVF culture media on the *in utero* development of the child, as insufficient placental functioning has been implicated in fetal growth restriction

and low birthweight (Nelissen et al., 2011). Interestingly, animal and human studies have suggested that the placenta is especially sensitive to preimplantation epigenetic disturbance in imprinted genes (Mann, 2004; Rivera et al., 2008; Choufani et al., 2019). Therefore, the aim of this study was to investigate the DNA methylation status of imprinted genes in placentas derived from IVF conceptions where embryo culture was performed in human tubal fluid (HTF) or G5 culture medium.

# Materials and Methods

## Ethical approval

Ethical approval was requested for this study but was waived as, in accordance with Dutch law, spare placenta tissues can be used for research after informed consent of the patient, without further permission of an ethical committee since no interventions were needed to obtain the samples.

## Study population and sample collection

Placental biopsies from IVF conceptions were obtained in five out of six IVF clinics in the Netherlands participating in a multi-center RCT (Amsterdam UMC, location AMC in Amsterdam, Catharina Hospital in Eindhoven, St. Elisabeth Hospital in Tilburg, Maastricht University Medical Center in Maastricht and University Medical Center Groningen in Groningen) and five IVF clinics affiliated to these hospitals (Netherlands Trial Registry (NTR) number 1979) (Kleijkers et al., 2016). In this RCT, embryo culture after IVF or ICSI was performed in either G5 series medium (a sequential medium from Vitrolife, Göteborg, Sweden) or HTF (a continuous medium from Lonza, Verviers, Belgium), and allocation occurred via an online computer program with a 1:1 allocation using a random block design. Embryo culture was performed at 5–6% $CO_2$ in either low $O_2$ or in 20% $O_2$ depending on the IVF center. All other procedures including ovarian hyperstimulation, ovum pick up and embryo transfer (all fresh Day 2 or Day 3 transfers) were similar between the two groups and performed according to local protocols (Kleijkers et al., 2016). As all embryo transfers were fresh, we assume that none of the women had developed ovarian hyperstimulation syndrome (OHSS) at the time of transfer. During the period between July 2010 and May 2012, 836 couples were randomly assigned for the culture medium G5 ($n = 417$) or HTF ($n = 419$) in their IVF cycles (Kleijkers et al., 2016). In the five clinics that participated in this study, 273 live births were achieved. In total, 115 placental biopsies were collected and available for this study.

All placental biopsies were obtained after vaginal delivery or cesarean section after at least 37 weeks of gestation, with exception of two biopsies obtained at 36.4 weeks (HTF) and 36.9 weeks (G5) of gestation. Biopsies that were collected more than 30 minutes after delivery of the placenta were deemed unsuitable ($n = 16$) and therefore excluded, and two samples had to be excluded because of a low DNA yield after nucleic acid isolation. In the end, 43 HTF-derived biopsies and 54 G5-derived biopsies were included.

For both groups, six placental biopsies (~5 $mm^2$ each) per patient were taken from the fetal side of the placenta within 30 minutes after delivery of the placenta. The biopsies were taken near the umbilical insertion point as this part of the placenta is considered to be derived from the embryonal trophectoderm and therefore consists of primarily fetal tissue with minimal maternal contamination. Because of the intraplacental variation in gene expression and probably also DNA methylation caused by different proportions of cell types within a region or clustering of clonally related cells, multiple samples from one placenta are needed to accurately represent the placenta at a molecular level. Therefore, we opted to pool all six biopsies of each placenta (Avila et al., 2010). Chorionic and amnionic membranes were removed before the villi were stored in RNAlater (Ambion, Thermo Fisher Scientific, USA) at −20°C according to the manufacturer's instructions.

Prior to proceeding with the experimental protocol, all samples were given a new random non-identifying number. Only one of the investigators (A.P.A.v.M) had the key to disclose the group to which each sample belonged to. All others involved in the experimental execution and data analysis were blinded. The grouping code was only released after multivariate analysis was performed.

## NC control population samples

To compare DNA methylation levels of genomically imprinted genes of placentas derived from IVF and natural conception, we collected placenta tissue from NC conceptions at term. Placenta biopsies were collected from 69 live births after informed consent between May 2008 and November 2013 (NTR 1298) in the obstetric outpatients' clinic in Maastricht University Medical Center and cooperating midwife practices (Nelissen et al., 2014). These biopsies originated from healthy pregnancies that were NC without the use of (hormonal) treatment or other types of medically assisted reproduction. These samples were collected in the same way as the G5 and HTF IVF samples and processed simultaneously with the IVF samples.

## DNA extraction from placental tissue

Genomic DNA was extracted from placental tissue using the AllPrep DNA/RNA Mini kit (QIAGEN, Germany) using the protocol for animal tissues, with some minor modifications. In brief, the placental biopsies from each individual were pooled and were minced using tweezers and a surgical blade. Then 20–30 mg of placental tissue was transferred into an Eppendorf tube and disrupted using a micro-pestle in 300 µl lysis buffer containing $\beta$-mercaptoethanol. After adding an additional 300 µl lysis buffer the total lysate was homogenized by expelling it through a 20-gauge needle fitted to a syringe. The lysate was centrifuged for 3 minutes at 21380*g* after which the supernatant was transferred to the AllPrep DNA spin column. To increase purity of the DNA sample, the column was washed twice using AW2 (QIAGEN) buffer and the DNA was eluted in a total volume of 100 µl of pre-warmed (65°C) elution buffer (QIAGEN). DNA samples were cleaned up by using the DNA Micro kit (QIAGEN) using the 'Clean-up of Genomic DNA' protocol. DNA concentrations were measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, USA).

## Amplicon library preparation for MiSeq sequencing

Genomic DNA (600 ng) was bisulfite converted using the EZ-96 DNA Methylation-Gold kit (Shallow well format, Zymo Research,

USA). All samples were distributed over two 96 well plates and were converted simultaneously in two identical Bio-Rad PTC-100 thermal cyclers (Bio-Rad Laboratories Inc., USA). In both plates a commercially available unmethylated DNA sample was included (QIAGEN). Bisulfite converted DNA samples were eluted in 30 µl (10 ng/µl, assuming a loss of 50% due to chemical degradation of the DNA during the conversion). To generate indexed paired-end MiSeq libraries, a custom PCR-based protocol was used to create an ampliconic library of the differentially methylated regions (DMRs) of interest. The 34 used primer sets have been previously described and validated to be specific to the DMRs associated with genomic imprinted genes or loci with no preference for amplification of either methylated or unmethylated sequences (Woodfine *et al.*, 2011; Nickkholgh *et al.*, 2014) (Supplementary Table SI). We opted for two rounds of PCR: a first round with these DMR-specific primer sets extended with an Illumina-specific adapter sequence and a second round where patient-specific dual TruSeq indices (N500 and N700 series, Illumina, USA) were attached to the amplicons.

For the first round PCR, amplification was performed using the EpiTect MSP kit (QIAGEN) using 5 ng of bisulfite converted DNA and 4 µM primerset as follows: 10 minutes at 95°C, followed by 37 cycles of 1 minute at 95°C, 1 minute at 56°C and 30 seconds at 72°C, followed by a final extension step of 10 minutes at 72°C. A commercially available human methylated DNA sample was included as a control (QIAGEN). To confirm the presence of the correct PCR products and the efficacy of the PCR, a gel electrophoresis was performed after the first round PCR on a randomly selected placenta-sample (data not shown).

Prior to the second round PCR, all DMR PCR products from each patient were pooled after the first round and purified using Agencourt Ampure beads (BD, USA). Subsequently, dual barcodes indices identifying individual placenta samples were annealed to the pooled first round PCR product and products were amplified further using the Invitrogen Platinum PCR SuperMix (High Fidelity, Invitrogen, USA) as follows: 1 minute at 95°C, 5 minutes at 72°C, followed by 6 cycles of 30 seconds at 95°C, 2 minutes at 62°C and 2 minutes at 72°C, followed by a final extension step of 3 minutes at 72°C. PCR products were stored cool at 4°C until visualization using agarose gel electrophoresis. After the second round PCR, all placenta samples showed a sufficient DNA smear indicating that the majority of amplicons were amplified sufficiently (data not shown).

PCR products were cleaned using Agencourt Ampure beads and mixed in an equimolar fashion in order to create two independent 10 nM libraries for MiSeq sequencing. In order to do so, DNA concentration of all individual second round PCR products was measured using a Qubit Fluorometer (Invitrogen) according to the manufacturer's protocol. We had to resort to two libraries because a maximum of 96 unique combinations can be made from the barcode indices. Library quality was evaluated using Bioanalyzer equipped with an Agilent High Sensitivity DNA chip (Agilent Technologies, USA). In both libraries methylated and unmethylated controls were included.

The two libraries were sequenced paired-end, each on a separate flow cells using Illumina's MiSeq system and the v2 500 cycle kit (Illumina). Libraries were loaded in a 9 pM concentration and mixed with 15% phiX control (Illumina).

## Statistical and bioinformatical analysis

All statistical and bioinformatical analyses were performed blindly. For the baseline characteristics, statistical analyses were performed using IBM SPSS Statistics 24. The normality of the variables was tested with the Shapiro–Wilk test (when >0.05, the data are considered to be normally distributed). If the variables were normally distributed, the differences in outcomes between the two groups were compared and evaluated using Student's *t*-test. If the variables were not normally distributed, a Mann–Whitney test was performed. To test categorical variables a Pearson's $\chi^2$ test was used. VassarStats was used for the calculation of proportions of pregnancy complications and their respective confidence intervals (Newcombe, 1998).

R statistical software (version 3.4.1) was used for the statistical analysis of the MiSeq data (R Core Team, 2012). Reads (2 × 250 bp) were trimmed using trimGalore after which reads were aligned to hg18 utilizing Bismark v0.19.0 (Krueger and Andrews, 2011). Methylation values and the genomic coordinates were extracted using Bismark for use with the R-package BiSeq 1.16.0 (Hebestreit *et al.*, 2013). Reads were specifically mapped to the defined amplicon sequences, and the coverage at identified CpG sites was extracted. We predicted the coverage of 483 CpG sites *in silico,* covering multiple CpG sites for each of the 34 amplicons in the hg18 build (Supplementary Fig. S1). However, for 33 CpG sites within H19 and the ICR of IGF2/H19 region we were unable to acquire data. In addition, for amplicons representing GRB10 (germline DMR), one out of two amplicons representing MEG3 and the amplicon representing the somatic DMR of DIRAS3, we were unable to acquire sufficient data for the two groups. Box plots were created to visualize the distribution of the mean methylation values per amplicon.

We used the variancePartition R-package 1.6.0 (Hoffman and Schadt, 2016) to determine the biological and technical variation and performed a multivariate analysis in a linear model with known variables using limma 3.32.10 (Ritchie *et al.*, 2015). In addition, we assessed differential methylation per CpG and used a general omnibus test and the p.adjust function in R to calculate FDRs via the Benjamini–Hochberg procedure. In all analyses of the Miseq data, one sample (number 5051, group G5) had to be excluded because of a high number of missing values. To identify variables that contribute to the variety in the data, a surrogate variable analysis (SVA; 3.24.4) was performed (Leek and Storey, 2007). In brief, an SVA attempts to identify patterns within the data of overall DNA methylation of the amplicons. These patterns are assigned to surrogate variables (SVs) which in theory are subsequently be correlated to known variables. For the calculation of proportions of outliers per group, VassarStats was used for the calculation of proportions and their respective CIs (Newcombe, 1998) followed by a Pearson's $\chi^2$ test or Fisher's exact using IBM SPSS Statistics 24. The magnitude of an outlier was defined as above or below the 1.5 interquartile range (IQR) calculated for each group and amplicon or CpG separately.

# Results

Sufficient DNA could be harvested from 43 HTF-derived placental biopsies and 54 G5-derived placental biopsies. Baseline characteristics of these groups are described in Tables I and II and in Supplementary Table SII. All parental characteristics and neonatal outcomes were similar.

**Table I Baseline characteristics.**

| Characteristic | Culture medium | | *P*-value |
|---|---|---|---|
| | HTF (*n* = 43) | G5 (*n* = 54) | |
| **Maternal characteristics** | | | |
| Age (years) | 33.1 ± 3.8 | 33.3 ± 3.7 | 0.821 |
| Height (cm) | 170.6 ± 6.4 | 169.4 ± 7.8 | 0.410 |
| Weight (kg) | 70.4 ± 14.1 | 67.2 ± 11.2 | 0.459 |
| BMI | 24.2 ± 4.6 | 23.4 ± 3.7 | 0.656 |
| Smoking before pregnancy, yes | 7 (18) | 5 (10) | 0.266 |
| Smoking during pregnancy, yes | 4 (10) | 2 (4) | 0.249 |
| Nulliparous | 31 (74) | 39 (72) | 0.932 |
| **Paternal characteristics** | | | |
| Age (years) | 36.7 ± 6.2 | 37.9 ± 5.7 | 0.199 |
| Height (cm) | 182.3 ± 7.2 | 181.7 ± 7.4 | 0.733 |
| Weight (kg) | 87.0 ± 13.4 | 86.1 ± 12.1 | 0.741 |
| BMI | 26.1 ± 3.1 | 26.1 ± 3.2 | 0.970 |
| Smoking, yes | 8 (21) | 9 (18) | 0.719 |
| **IVF/ICSI characteristics** | | | |
| IVF | 16 (37) | 17 (32) | 0.554 |
| ICSI | 27 (63) | 37 (69) | |
| Duration of subfertility (years) | 3.7 ± 2.3 | 2.9 ± 2.0 | 0.058 |
| Delivery mode | | | |
| Section | 10 (23) | 8 (15) | 0.288 |
| Vaginal | 33 (77) | 46 (85) | |
| Live birth after first cycle | 27 (64) | 36 (67) | 0.520 |
| Cycle rank | | | |
| 1 | 27 (64) | 36 (67) | |
| 2 | 10 (24) | 15 (28) | |
| 3 | 5 (12) | 3 (6) | |
| No of embryos transferred | | | |
| 1 | 25 (60) | 37 (69) | 0.361 |
| 2 | 17 (41) | 17 (32) | |
| Day of embryo transfer | | | |
| Day 2 | 12 (29) | 16 (30) | 0.910 |
| Day 3 | 30 (71) | 38 (70) | |
| Primary indication for fertility treatment | | | |
| Unexplained | 6 (14) | 9 (17) | 0.754 |
| Male factor subfertility | 28 (67) | 32 (59) | |
| Female factor subfertility | 8 (19) | 13 (24) | |

Continuous variables are mean ± SD, and categorical variables are n (%).

## Analysis of overall DNA methylation in imprinted genes

We first performed a comparison of overall DNA methylation levels of all CpGs measured in DMRs associated with imprinted genes in placenta-derived conceptions in HTF or in G5. One G5 sample had to be excluded from the analysis due to a high number of missing values. The origin of the placentas (HTF or G5) could not be correlated to overall DNA methylation patterns in an unsupervised clustering analysis (Fig. 1). Supervised clustering analysis also revealed no apparent difference in the pattern of DNA methylation level between the two medium groups based on the mean methylation status per CpG in these placental biopsies (Fig. 2).

Next, we assessed differential methylation using an empirical Bayes moderated t-test within Limma's linear model framework, considering the possible effects of the known variables gestational age, parental age, parental BMI, parental smoking, parity, gender and birthweight of the child, mode of delivery and method of conception (IVF or ICSI). Also, after this correction, we were unable to find any statistically

**Table II Neonatal outcomes of live born singletons.**

| Outcome | Culture medium | | *P*-value |
|---|---|---|---|
| | HTF (*n* = 43) | G5 (*n* = 54) | |
| **Gender, female** | 22 (51.4) | 26 (48.1) | 0.681 |
| **Birthweight (g)** | 3471.1 ± 417.0 | 3374.8 ± 476.8 | 0.302 |
| **Low birthweight (<2500 g)** | 0 (0) | 2 (4) | 0.208 |
| **High birthweight (>4500 g)** | 0 (0) | 0 (0) | N.A. |
| **GA at birth (weeks)** | 39.3 ± 1.2 | 39.5 ± 1.3 | 0.436 |
| **Preterm birth (<37 weeks)** | 1 (2) | 1 (2) | 0.857 |
| **Pregnancy complications** | 9 (21) | 17 (32) | 0.244 |

Pregnancy complications are described in Supplementary Table II. N.A. = not applicable.

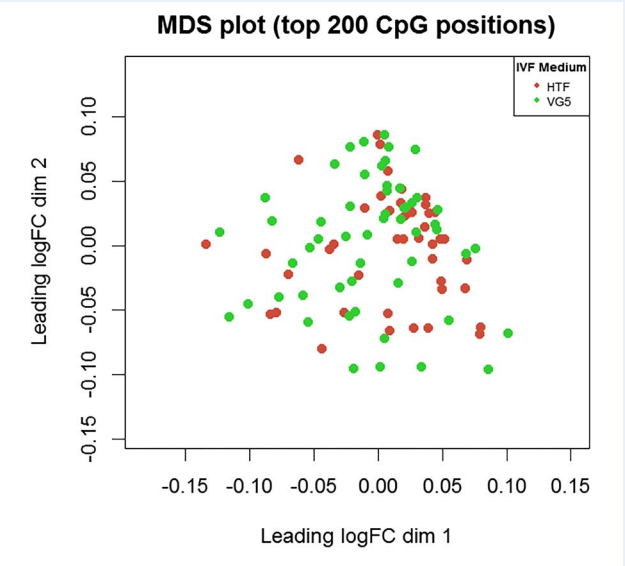


**Figure 1 Multi-dimensional scaling (MDS) plot of unsupervised clustering based on DNA methylation levels of the top 200 differentially methylated CpG positions in placentas derived from embryos cultured in HTF or G5 medium.** Green dots represent G5 samples, red dots HTF samples.

significant (adjusted *P*-value < 0.05) differences between the two medium groups in mean CpG methylation (Supplementary Table SIII).

To identify variables that contribute to the variety in the data, an SVA was performed. We were unable to couple SVs obtained from an SVA to known variables indicating that the slight variation observed between all these samples cannot be attributed to one of the known experimental variables, including IVF medium. Even though for example maternal smoking status, birthweight and gestational age could explain some of the variance found in DNA methylation levels (Fig. 3), most variance could not be explained by known factors.

## Amplicon-specific differences in DNA methylation

Aside from overall DNA methylation changes per CpG in parentally imprinted genes, the DNA methylation status of each DMR was studied per amplicon. Based on mean methylation per amplicon, we did not identify statistically significant differences between the two medium groups for 31 out of 34 targeted regions (Fig. 4). For amplicons representing GRB10 (g), MEG3 (2) DIRAS3 (3), we were unable to acquire methylation data for the two groups. Of note, the amplicons representing ZAC, L3MBTL, KCNQ1, UBE3A, GRB10, DLK, MKRN3 and GNASXL displayed low methylation (median below 25%) in both groups, and PEG3/ZIM2, ZIM3 and IGF2R-2 displayed high methylation in both groups (median above 75%).

## Variance in DNA methylation in G5- and HTF-derived placenta

It has been suggested that a high variance (often defined as a higher number of outliers) in DNA methylation status of imprinted genes might be important (Choux *et al.*, 2017). In our cohort, both groups displayed outliers (above or below the 1.5 IQR based on the median methylation level per amplicon) in the majority of amplicons that we studied (Fig. 4). We were, however, unable to identify an unambiguous pattern based on the outliers detected in the different amplicons. On the amplicon level, we identified 78 outliers from the median methylation per amplicon in DNA methylation in all samples (HTF 37 outliers in 25 samples, G5 41 outliers in 28 samples). The direction of methylation was equally distributed as well; 20 out of 37 (54.1%) outliers displayed hypomethylation and 17 out of 37 (45.9%) showed hypermethylation in the HTF group. For the G5 placentas, 20 out of 41 (48.8%) outliers displayed hypomethylation and 21 out of 41 (51.2%) outliers displayed hypermethylation as compared to the median.

In most placentas a single outlier was found, while some placentas exhibited outlier values in multiple amplicons (Supplementary Tables SIV and SV). For HTF, one or more outliers were seen in 25 out of 43 placentas (58%, 95% CI: 42–73) for all amplicons tested, of which 11 had outliers in multiple amplicons (26% of all HTF cases, 95% CI: 14–41). In the G5 group, in 28 out of 53 placentas (53%, 95% CI: 39–66) one or more outliers were seen, of which 8 had outliers in multiple amplicons (17% of all G5 cases, 95% CI: 7–28). The difference in proportion of placentas that exhibited at least one outlier or outliers in multiple amplicons was not statistically significant different between groups ($P = 0.603$ and 0.200, respectively).

On the CpG level, a grand total of 1897 outliers were identified that displayed a methylation level above or below the 1.5 IQR based

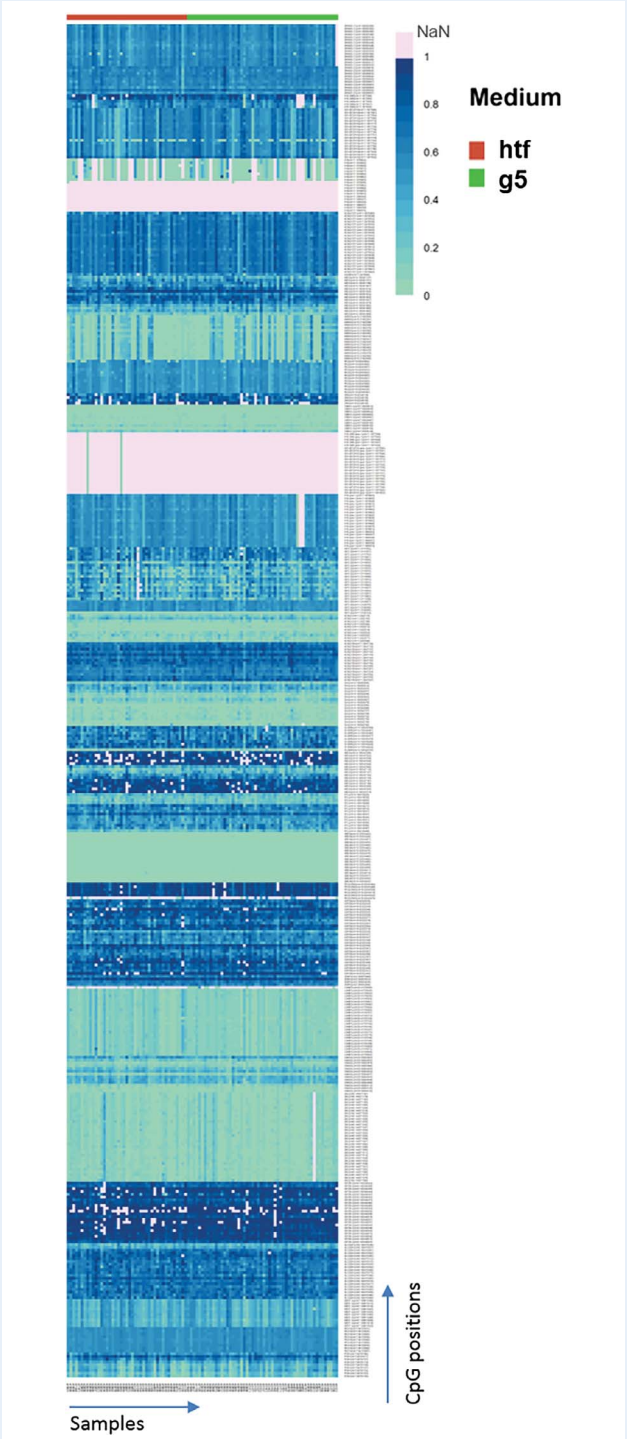


**Figure 2 DNA methylation levels per CpG in placentas derived from embryos cultured in HTF or G5 medium.** DNA methylation levels per CpGs range between no methylation (0, light green) and fully methylated (1, dark blue). CpGs are in light pink when no data could be acquired of this specific CpG site and sample (NaN). For amplicons GRB10 (g), MEG3 (2) DIRAS3 (3), we were unable to acquire methylation data for all CpGs in these amplicons in all samples and are therefore not represented in this figure. Placental samples are on the horizontal axis, and CpGs are on the vertical axis. Clustering is based on group of origin.

on mean methylation level per CpG (Supplementary Tables SVI and SVII). All medium placenta samples exhibited at least one CpG outlier. The majority of CpG outliers were directed to hypermethylation (HTF 660 outlier CpGs, G5 726 outlier CpGs); the rest directed to hypomethylation (HTF 231 outlier CpGs, G5 280 outlier CpGs).

### Comparison DNA methylation levels of G5 and HTF conceptions to NC conceptions

Knowing that no differences are found between the medium groups based on our results, we explored whether the DNA methylation levels of genomically imprinted genes in the placenta of G5 and HTF conceptions is different from NC placenta. We opted to analyze the data with NC placenta samples as a reference (Nelissen *et al.*, 2014) of which baseline characteristics are compared to that of the G5 and HTF IVF conceptions in Supplementary Table SVIII. Parental age, maternal smoking and pregnancy complications were statistically significant different between the NC compared to HTF and G5 IVF conceptions, but birthweight was not different between groups.

DNA isolation, bisulphite conversion, library preparation and sequencing of these NC samples occurred simultaneously with the HTF and G5 samples. In an unsupervised clustering analysis based on the top 200 differentially methylated CpGs, the origin of the placenta (HTF, G5 or natural conception) could not be correlated to overall DNA methylation patterns (Fig. 5). The mean methylation level per amplicon was not statistically significant different between G5, HTF and NC placenta samples (Supplementary Fig. S2). Also on the CpG level we were unable to find any statistically significant (adjusted *P*-value $< 0.05$) differences between the three groups of conceptions (Supplementary Table SIX).

We also reviewed the number of outliers per amplicon and per CpG. Compared to the median methylation level per amplicon, one or more outliers were found in 29 out of 69 NC placentas (42%, 95% CI:30–55), of which 14 placentas had outliers in multiple amplicons (20% of all NC cases, 95% CI: 12–32). The difference in proportion of placentas that exhibited at least one outlier or outliers in multiple amplicons was not statistically significant between groups ($P = 0.230$ and 0.836, respectively). On the CpG level we were able to identify 430 CpGs that displayed outlier hypermethylation and 256 CpGs that displayed hypomethylation in NC placentas (Supplementary Table SX). While in the IVF cases all placentas displayed at least one CpG outlier, 16 out of 69 NC control placentas displayed no CpG outlier (23% of NC controls, 95% CI: 14–35 versus 0% of IVF cases, $P < 0.00001$). While taking a closer look to the CpG-specific outliers in HTF and G5, we were able to identify 37 CpG sites that uniquely displayed outlier methylation in G5 placentas and 32 CpG sites that uniquely displayed outlier methylation in HTF, albeit with a low frequency (frequency, 1–4) (Table III, Supplementary Tables SVI, SVII and SX). In 8/37 (G5) and 4/32 (HTF) unique outliers CpGs, a medium-specific unique outlier could be directly correlated to outlier methylation of the entire amplicon (Table III, Supplementary Tables SIV and SV).

## Discussion

In this study, we found no differences in the overall DNA methylation status, the mean amplicon and mean CpG-specific DNA methylation status nor in the variance of methylation of imprinted DMRs in

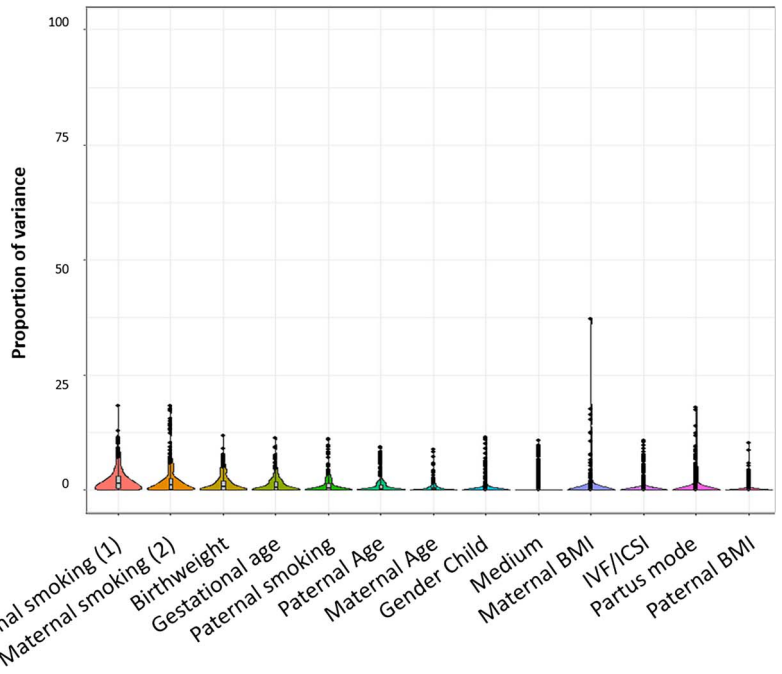


**Figure 3 Violin plots of SVA of overall DNA methylation.** Violin plot for the variance seen in the DNA methylation data in placenta samples derived from IVF embryos cultured in HTF or G5 medium. The y-axis represents the proportion of variance that can be explained by the variables on the x-axis. Maternal smoking (1) represents the variable maternal smoking before pregnancy, and maternal smoking (2) represents the variable maternal smoking during pregnancy.

placentas derived from conceptions after IVF with HTF culture medium or conceptions after IVF with G5 culture medium. Comparison of NC placenta samples with IVF conceived samples revealed minor differences in the variation of CpG-specific methylation levels, resulting in a statistically significant difference in the number of placenta exhibiting CpG outliers between IVF samples and NC samples and a number of unique embryo culture-specific outlier CpG sites between HTF and G5.

This is to our knowledge the first report where the DNA methylation status of parentally imprinted genes is being studied in the placenta derived from human IVF conceptions in two different culture media. While others have identified statistically significant epigenetic changes in imprinted genes in embryos after exposure to different culture media in IVF in animals (Khosla *et al.*, 2001; Mann, 2004; Market-Velker *et al.*, 2010), a culture medium effect on the DNA methylation status of imprinted genes had not been studied in human placentas before. Our study is unique because it covered a wide range of parentally imprinted genes and allowed distinction between IVF conceptions in two different culture media and subsequent comparison to natural conception. A major strength of this study is the fact that placenta samples were derived from an RCT on the effects of embryo medium, which is reflected in the lack of statistically significant differences in baseline characteristics. Furthermore, a multivariate approach allowed us to build a robust statistical model to study the precise effect of the embryo culture medium by considering known possible confounding variables, strengthening our methodology even further. As a reference, DNA from NC placenta samples from a pregnancy cohort was extracted and sequenced simultaneously, reducing technical bias. Moreover, we used an amplicon-based sequencing approach that allowed for an in-depth analysis of the status of imprinted genes. With this approach, the methylation status of 483 CpG sites per sample was covered, including multiple CpG sites for each of the 34 amplicons.

While most DMRs showed the expected methylation status of ~50% for somatic cells, some amplicons displayed extremely low or high DNA methylation. Low placental DNA methylation levels of the amplicons representing the DMRs of KCNQ1 (16.4%), UBE3A (2.4%), MKRN3 (19.4%) and GBR10 (11.8%) were reported previously by the researchers that designed the primer sets used in this study (Woodfine *et al.*, 2011). This is also the case for the high methylation level of the IGF2R-2 DMR (80.9%) and ZIM3 (83.5%). On the contrary, 50% methylation levels were reported in the placenta for ZAC, L3MBTL, DLK and GNASXL, while we measured hypomethylation levels. For PEG3/ZIM2 no placenta-specific methylation level was previously reported (Woodfine *et al.*, 2011). In that study, tissue from only three individual placentas was used, which may explain the discrepancies between their results and our results. For L3MBTL, our results are consistent with the report of a low DNA methylation level observed in one ICSI case in a previous study (Feng *et al.*, 2011).

The IVF placenta samples used in this study were collected during a study where patients were randomly allocated to either IVF with embryo culture in G5 or IVF with embryo culture in HTF (Kleijkers *et al.*, 2016). In this RCT, a statistically significant difference in birthweight was found between these two groups. We used all the placenta samples available to us from this study, irrespective of the occurrence of (mild) pregnancy complications in some of the pregnancies; however, not all placentas were collected during this trial

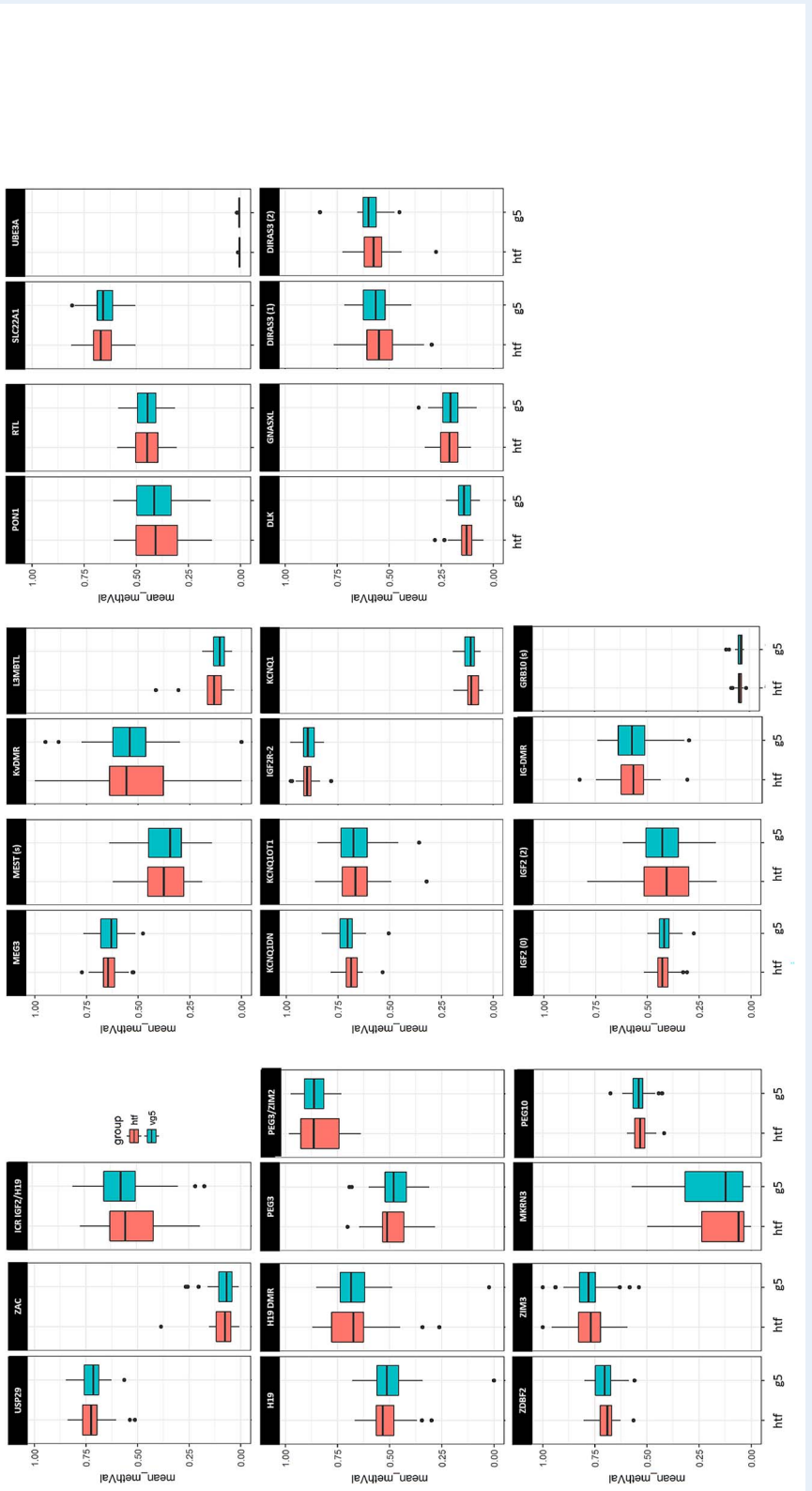


**Figure 4 Distribution of mean methylation values per amplicon.** The distribution of mean methylation of all CpGs in each amplicon is depicted in standard box plots, where the median is represented as the horizontal line in the box. Outliers with mean methylation value per amplicon above or below 1.5× quartiles from the median are depicted as black dots (see Supplementary Tables SIV and SV for details).

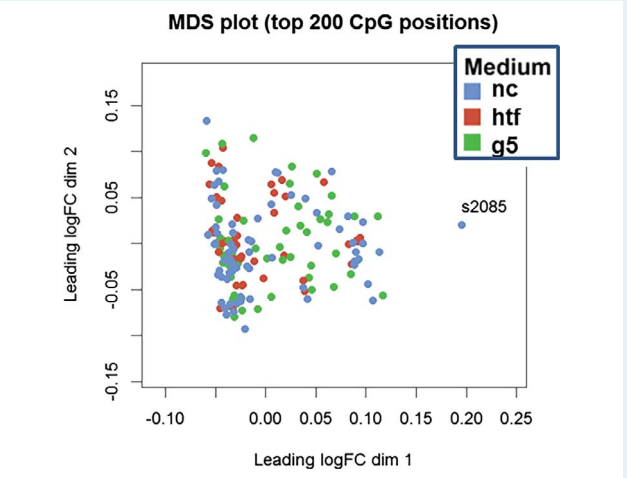


**Figure 5 Multi-dimensional scaling (MDS) plot of unsupervised clustering based on DNA methylation levels of the top 200 differentially methylated CpG positions in placentas derived from embryos cultured in HTF or G5 medium and placentas derived from** NC **pregnancies.** Green dots represent G5 samples, red dots HTF samples and blue dots natural conception.

due to practical reasons (e.g. lack of time at delivery room, emergency delivery and unexpected pre-term birth). For the NC reference group, placentas from another study were used, which were collected in a similar fashion (Nelissen *et al.*, 2014). Since in the reference group the inclusion of mothers with a pregnancy complication was avoided, the number of pregnancy complication is considerably lower than in the G5 and HTF group. A potential selection bias in collecting the placentas and the decreased number of available samples caused that the statistically significant difference in birthweight found in the RCT could not be confirmed in the study population used in this study (Kleijkers *et al.*, 2016). In addition, birthweights from G5 and HTF conceptions were not different from NC. Despite this, we expected that a medium known to cause a difference in birthweight would induce an epigenetic effect in the placenta due to a gradation effect, even if the birthweight was normal. Since we did not find differences in mean DNA methylation between the two culture media we need to conclude that we were unable to identify this gradation effect. Also, one must keep in mind that this RCT had not been powered to identify methylation-induced differences in placenta, which is a limitation of our study.

Aside from the DOHaD-related concept that the embryo may adapt to the *in vitro* environment through directed DNA methylation changes, IVF-associated techniques and processes including embryo medium composition might more or less randomly perturb epigenetic reprogramming of these embryos at a crucial stage in development, leading to stochastic errors in the epigenetic landscape of the developing embryo. This may be reflected in differences in the variability of DNA methylation. A high frequency of DNA perturbations in imprinted genes in human preimplantation embryos in IVF has been reported (White *et al.*, 2015). Interestingly, it was shown that newborns with low birthweight display a deviant CpG methylation pattern in cord blood samples (Ghosh *et al.*, 2016). These 'outlier individuals' were more often children conceived through assisted reproduction (Ghosh *et al.*, 2016), suggesting an association between deviant or variable DNA methylation, low birthweight and assisted reproduction. It has been suggested that the *in vitro* environment during IVF may lead to a higher variation in genome-wide DNA methylation patterns compared to natural conception, leading to a larger variation in health problems in these IVF children (Melamed *et al.*, 2015). The higher variation in methylation levels in imprinted DMRs that others have identified in IVF-conceived newborns compared to NC newborns provides suggestive evidence for this theorem (Turan *et al.*, 2010; Choux *et al.*, 2017). We were unable to find an effect in imprinted genes between embryo culture medium, as samples with outliers occurred at a similar frequency in IVF conceived in HTF and IVF conceived in and G5. However, in this study, the number of placentas exhibiting CpG outlier methylation appeared to be higher in IVF-conceived placenta than NC, thereby adding support to the theory that IVF might induce a higher variation in DNA methylation. Also, a number of outlier CpG sites unique for G5 or HTF were identified that in some cases correlated to outlier methylation of the entire DMR. As the frequency of these unique outlier CpG was low in our cohort, we cannot decipher whether there is a causal relationship between the embryo culture conditions and outlier methylation or that they simple arose due to chance. It is unclear as well if these unique outlier CpGs influence molecular pathways that lead to a difference in biological and clinical outcome. Therefore, the biological and clinical significance of these CpG outliers remains elusive and requires more research.

Our results suggest that the mechanism behind the effect of IVF culture medium on the susceptibility for complications during pregnancy, birth and child development is not likely to be mediated through the mean DNA methylation status of DMRs and CpGs associated with our studied panel of parentally imprinted genes. Certainly, DNA methylation differences in other genes may be identified using whole genome bisulfite sequencing. However, it is important to note that when studying the epigenetic status of the placenta, the effect of IVF culture medium is studied 38 weeks after the exposure took place, making the identification of a possible gradation effect challenging. Therefore, the effect may be diluted or even reversed to normal through known and unknown epigenetic resetting mechanisms during pregnancy. Biologically, it would be interesting to study the effect of IVF culture medium in human embryos and tissue derived from the three different germ layers (Deglincerti *et al.*, 2016; Shahbazi *et al.*, 2016). Even though these types of experiments are practically and ethically challenging to set up, it would give tremendous amounts of data that can be used to further optimize the efficiency and safety of fertility treatments.

In conclusion, our data show no apparent differences in DNA methylation status of parentally imprinted genes in placenta derived from IVF conceptions cultured in HTF and placenta derived from IVF conceptions cultured in G5 culture medium. We also found no significant differences between the IVF conception compared to natural conception other than an increased number of outlier CpG methylation in IVF placenta compared to NC placentas. We propose to study the effects of *in vitro* culture and choice of culture media beyond the epigenetic state of imprinted genes. After all, the epigenetic state of imprinted genes is only a part of the regulation of embryogenesis, and therefore there are more fields to explore.

**Table III Frequency and direction of CpG-specific outliers unique for placenta derived from embryos cultured in G5 or HTF.**

| G5 | *Frequency and direction of outlier* | | HTF | *Frequency and direction of outlier* | |
|---|---|---|---|---|---|
| **CpG** | **Hypermethylated** | **Hypomethylated** | **CpG** | **Hypermethylated** | **Hypomethylated** |
| DLK(chr14:100262614) | 1 | 0 | IGF2 (2)(chr11:2110838) | 1 | 0 |
| DLK(chr14:100262727) | 3 | 0 | IGF2 (2)(chr11:2110870) | 1 | 0 |
| GNASXL(chr20:56863903) | 1* | 0 | IGF2 (2)(chr11:2110893) | 1 | 0 |
| H19(chr11:1979962) | 0 | 1* | IGF2 (2)(chr11:2110916) | 2 | 0 |
| ICR_IGF2/H19(chr11:1977779) | 0 | 3* | IGF2 (2)(chr11:2110963) | 1 | 0 |
| ICR_IGF2/H19(chr11:1977784) | 0 | 2 | IGF2R-2(chr6:160346573) | 0 | 1 |
| ICR_IGF2/H19(chr11:1977796) | 0 | 3 | KCNQ1(chr11:2422183) | 1 | 0 |
| IG-DMR(chr14:100345459) | 1 | 1 | KCNQ1(chr11:2422198) | 2 | 0 |
| IG-DMR(chr14:100345461) | 1 | 2 | MEG3(chr14:100347092) | 1 | 0 |
| IG-DMR(chr14:100345477) | 0 | 1 | MEG3(chr14:100347164) | 0 | 1 |
| IGF2 (2)(chr11:2110764) | 0 | 2 | MEG3(chr14:100347178) | 0 | 1 |
| IGF2 (2)(chr11:2110801) | 0 | 1 | MEG3(chr14:100361807) | 1 | 1 |
| IGF2 (2)(chr11:2110832) | 2 | 0 | MKRN3(chr15:21362280) | 3 | 0 |
| IGF2 (2)(chr11:2110898) | 1 | 2 | MKRN3(chr15:21362299) | 3 | 0 |
| IGF2 (2)(chr11:2110957) | 0 | 1 | MKRN3(chr15:21362393) | 3 | 0 |
| KCNQ1DN(chr11:2847128) | 1 | 1* | MKRN3(chr15:21362416) | 6 | 0 |
| KCNQ1DN(chr11:2847194) | 2 | 1 | MKRN3(chr15:21362421) | 3 | 0 |
| KCNQ1DN(chr11:2847206) | 1 | 1 | MKRN3(chr15:21362424) | 3 | 0 |
| KvDMR(chr11:2678696) | 2* | 0 | PEG3(chr19:62044040) | 1* | 2 |
| MEG3(chr14:100347020) | 0 | 2 | PON1(chr7:94791590) | 0 | 2 |
| MEG3(chr14:100361798) | 1 | 0 | PON1(chr7:94791617) | 0 | 1 |
| MEG3(chr14:100361852) | 0 | 1 | RTL(chr14:100419220) | 3 | 4 |
| MEG3(chr14:100361857) | 0 | 1 | SLC22A1(chr6:160475291) | 0 | 1 |
| MEST (s)(chr7:129913425) | 0 | 1 | SLC22A1(chr6:160475305) | 0 | 3 |
| PEG10(chr7:94123826) | 0 | 3* | SLC22A1(chr6:160475438) | 0 | 1 |
| PON1(chr7:94791707) | 0 | 1 | SLC22A1(chr6:160475452) | 0 | 1 |
| PON1(chr7:94791719) | 1 | 1 | USP29(chr19:62322230) | 1 | 3 |
| RTL(chr14:100419273) | 0 | 1 | USP29(chr19:62322337) | 0 | 2* |
| RTL(chr14:100419315) | 1 | 2 | USP29(chr19:62322349) | 1 | 1 |
| RTL(chr14:100419409) | 2 | 0 | USP29(chr19:62322358) | 0 | 1* |
| SLC22A1(chr6:160475257) | 2 | 0 | USP29(chr19:62322412) | 0 | 2* |
| SLC22A1(chr6:160475312) | 0 | 1 | ZDBF2(chr2:206834090) | 1 | 0 |
| SLC22A1(chr6:160475459) | 0 | 1 | **TOTAL (32 sites)** | **46** | **88** |
| USP29(chr19:62322259) | 0 | 2 | | | |
| USP29(chr19:62322318) | 0 | 2* | | | |
| USP29(chr19:62322381) | 0 | 1* | | | |
| ZIM3(chr19:62348180) | 0 | 2 | | | |
| **TOTAL (37 sites)** | **23** | **44** | | | |

*Association with medium amplicon outlier methylation (see Supplementary Tables SIV and SV). **G5.** ICR_IGF2/H19(chr11:1977779): placenta samples 3117, 5077 and 4023. PEG10(chr7:94123826): placenta samples 1046, 4023, 5106. USP29(chr19:62322318): placenta sample 3163. USP29(chr19:62322381): placenta sample 3163. H19 (chr11:1979962): placenta sample 4067. KvDMR(chr11:2678696): placenta sample 5053 and 5106. GNASXL(chr20:56863903): placenta sample 3059. KCNQ1DN(chr11:2847128): placenta sample 3095. **HTF.** USP29(chr19:62322337): placenta sample 2008. PEG3(chr19:62044040): placenta samples 3126. USP29(chr19:62322358): placenta sample 3168. USP29(chr19:62322412): placenta sample 3168.

## Supplementary data

Supplementary data are available at *Human Reproduction* online.

## Authors' roles

The study was performed under NTR number 1979 and 1298 was designed and performed by A.P.A.v.M, J.C.M.D, S.M, J.v.E-A., D.C and Y.W. This specific study was designed by A.P.A.v.M., A.M.M.P., S.M., S.R. and C.L.M. Nucleic acid isolation was performed by A.P.A.v.M and C.L.M. The library preparation protocol was designed by R.B.S., and library preparation was done by T.M.W., C.M.W.K, S.K.M.D., S.C.M. and C.L.M. Bioinformatical and statistical analyses were performed by A.J., assisted by R.B.S., C.L.M. and T.M.W. The original manuscript was drafted by C.L.M. All authors critically reviewed and revised the manuscript and approved the final version.

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## Conflict of interest

The authors report no financial or other conflict of interest relevant to the subject of this article.

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