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# At age 9, the methylome of assisted reproductive technology children that underwent embryo culture in different media is not significantly different on a genome-wide scale

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**STUDY QUESTION:** Can we detect DNA methylation differences between ART children that underwent embryo culture in different media?

**SUMMARY ANSWER:** We identified no significant differences in site-specific or regional DNA methylation between the different culture medium groups.

**WHAT IS KNOWN ALREADY:** Embryo culture in G3 or K-SICM medium leads to differences in embryonic, neonatal and childhood outcomes, including growth and weight. The methylome may mediate this association as the period of *in vitro* culture of ART treatments coincides with epigenetic reprogramming.

**STUDY DESIGN, SIZE, DURATION:** This study was conducted as a follow-up to a previous culture medium comparison study in which couples were pseudo-randomized to embryo culture in G3 or K-SICM medium. Of the resultant singletons, 120 (n = 65 G3, n = 55 K-SICM), were recruited at age 9.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** The ART children provided a saliva sample from which the methylome was analysed using the Infinium MethylationEPIC array. After quality and context filtering, 106 (n = 57 G3, n = 49 K-SICM) samples and 659 708 sites were retained for the analyses. Differential methylation analyses were conducted using mixed effects linear models corrected for age, sex, sample plate and cell composition. These were applied to all cytosine-guanine dinucleotide (CpG) sites, various genomic regions (genes, promoters, CpG Islands (CGIs)) and as a targeted analysis of imprinted genes and birth weight-associated CpG sites. Differential variance was assessed using the improved epigenetic variable outliers for risk prediction analysis (iEVORA) algorithm and methylation outliers were identified using a previously defined threshold (upper or lower quartile plus or minus three times the interquartile range, respectively).

<sup>†</sup>These authors jointly supervised this study.

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**MAIN RESULTS AND THE ROLE OF CHANCE:** After correcting for multiple testing, we did not identify any significantly differentially methylated CpG sites, genes, promoters or CGIs between G3 and K-SICM children despite a lenient corrected *P*-value threshold of 0.1. Targeted analyses of (sites within) imprinted genes and birth weight-associated sites also did not identify any significant differences. The number of DNA methylation outliers per sample was comparable between the culture medium groups. iEVORA identified 101 differentially variable CpG sites of which 94 were more variable in the G3 group.

**LARGE SCALE DATA:** Gene Expression Omnibus (GEO) GSE196432

**LIMITATIONS, REASONS FOR CAUTION:** To detect significant methylation differences with a magnitude of <10% between the groups many more participants would be necessary; however, the clinical relevance of such small differences is unclear.

**WIDER IMPLICATIONS OF THE FINDINGS:** The results of this study are reassuring, suggesting that if there is an effect of the culture medium on DNA methylation (and methylation-mediated diseases risk), it does not differ between the two media investigated here. The findings concur with other methylome studies of ART neonates and children that underwent embryo culture in different media, which also found no significant methylome differences.

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**TRIAL REGISTRATION NUMBER:** Dutch Trial register—NL4083

**Key words:** ART / culture medium / follow-up / ART children / methylome

## Introduction

Year-on-year a worldwide increase in the number of ART procedures has led to the birth of more than 8 million babies (Adamson et al., 2019) and currently, ~3% of births in European countries are conceived through ART (Wyns et al., 2020). Even though most ART children appear healthy at birth, follow-up studies have reliably shown that, compared to their naturally conceived counterparts, ART offspring are at increased risk of adverse perinatal (Berntsen et al., 2019), childhood and later life outcomes (Ceelen et al., 2009; Hann et al., 2018). The perinatal risks include premature birth, low birth weight, being small for gestational age and perinatal mortality (Berntsen et al., 2019), while the later life outcomes relate mainly to cardiometabolic health, including weight (Ceelen et al., 2009; Guo et al., 2017; Hann et al., 2018; Bay et al., 2019). Specifically, the affected cardiometabolic parameters include an increase in both systolic and diastolic blood pressure (Guo et al., 2017) and features of cardiovascular dysfunction, such as suboptimal cardiac diastolic function and increased blood vessel thickness (Guo et al., 2017). Additionally, ART children have been shown to have significantly lower weights than their naturally conceived counterparts from birth until the age of 4 (Bay et al., 2019). These observations are in line with the developmental origins of health and disease (DOHaD) paradigm, which states that early life adversity predisposes an individual to disease in later life (Vadhwa et al., 2009), therefore raising concerns about the impact of ART procedures on the resultant children.

During ART treatments, embryos undergo *in vitro* culture for 2–5 days prior to intrauterine transfer to establish a pregnancy. Throughout this time, embryos are exposed to an artificial *in vitro* environment consisting of the culture medium, the atmospheric conditions (oxygen levels) and laboratory-specific factors, such as laboratory plastics. Over time, a number of compositionally different culture media have been used for ART procedures (Mantikou et al., 2013; Morbeck et al., 2014; Sunde et al., 2016; Morbeck et al., 2017) and these have been associated with differences in short- and long-term outcomes of the resultant offspring both in animal and human studies (Fernández-Gonzalez et al., 2004;

Donjacour et al., 2014; Zandstra et al., 2015; Bouillon et al., 2016; Velazquez et al., 2018). We previously conducted a culture medium trial, in which couples undergoing ART treatments in Maastricht were pseudo-randomized, by strict alternation, to embryo culture in G3 (Vitrolife) or Sydney IVF cleavage medium (K-SICM, Cook). Although several components of these culture media are known, their precise concentrations and composition are not fully disclosed by the manufacturers. However, a possible difference between G3 and K-SICM is that the version of K-SICM used for the treatments described in these studies, contained an unstable form of the amino acid glutamine (according to product inserts from the media in this period), while G3 contained a more stable dipeptide form of the same amino acid. Several studies have shown that ammonium accumulation during storage of media containing the unstable form of glutamine is significantly higher (Kleijkers et al., 2016) and that an increased ammonium concentration in culture medium has an adverse effect on embryonic development (Virant-Klun et al., 2006; Hashimoto et al., 2008). Embryos cultured in G3 were found to have a greater number of cells, while their morphological grade was lower than K-SICM embryos (Dumoulin et al., 2010). Thereafter, implantation and pregnancy rates were higher in the G3 group (Dumoulin et al., 2010). Interestingly, growth differences, namely increased growth in the G3 group, could already be detected by ultrasound in the second trimester (Nelissen et al., 2013), were evident at birth (Dumoulin et al., 2010; Nelissen et al., 2012) and persisted at age 2 (weight) (Kleijkers et al., 2014) and age 9 (Zandstra et al., 2018a). At age 9, children from the G3 group remained heavier with higher waist circumferences and truncal adiposity than children from the K-SICM group (Zandstra et al., 2018a). Other markers of cardiovascular health, including blood pressure, lipid profile and endothelial function, were comparable between the culture medium groups (Zandstra et al., 2018a). Similarly, cognitive development was comparable between the culture medium groups (Zandstra et al., 2018b).

To date, the molecular mechanisms mediating the relationship between culture medium composition and the observed outcomes are not fully understood. It has been suggested that the epigenome, and

specifically DNA methylation in which a methyl ( $-CH_3$ ) group is added to the cytosine base of cytosine-guanine dinucleotides (CpG), is sensitive to environmental perturbations and subsequently 'programs' an individual's disease susceptibility (Felix and Cecil, 2019). Furthermore, pre-implantation human embryos are undergoing epigenetic reprogramming, consisting of virtually complete erasure and re-establishment of DNA methylation marks (Li *et al.*, 2018; Hanna *et al.*, 2018), during which they may be especially sensitive to environmental regulation of the epigenome. DNA methylation-associated imprinting disorders, although still rare, are also more common amongst IVF children (DeAngelis *et al.*, 2018). Consequently, methylome profiling of ART offspring born after culture medium trials has been carried out. For instance, our group examined DNA methylation in tissues collected at birth, namely the placenta (Mulder *et al.*, 2020) and umbilical cord blood (Koeck *et al.*, 2022), from neonates born after a multicentre randomized controlled trial comparing G5 (Vitrolife) and human tubal fluid (HTF, Lonza) media. Neither study identified any significant DNA methylation differences between the culture medium groups and the group mean differences at the sites analysed were small (largely <10%) (Mulder *et al.*, 2020; Koeck *et al.*, 2022). Interestingly, we identified several CpG sites with differential variability between the culture medium groups (Koeck *et al.*, 2022). These could relate to prenatal factors, such as the pregnancy complications, only experienced by some individuals in each group, alternatively, they could represent epigenetic marks indicative of disease that only some individuals will develop later in life. To investigate this further, later life or longitudinal methylome studies of ART offspring are required. Thus far, the methylome of ART children (age 7 or 8) has only been characterized in one cohort, in the context of a culture medium trial (global medium—LifeGlobal vs single-step medium—Irvine Scientific), finding no significant DNA methylation differences, and small intragroup differences (mostly <10%) (Barberet *et al.*, 2021; Ducreux *et al.*, 2021).

Not in the context of culture media trials, the methylome has also been compared between ART and naturally conceived offspring and such studies were the focus of a recent meta-analysis (Barberet *et al.*, 2022). In neonates, the meta-analysis only identified consistent differential methylation at the paternally expressed gene 1/mesoderm specific transcript (*PEG1/MEST*) imprinting gene locus when including all targeted methylome studies of placenta and cord blood samples (Barberet *et al.*, 2022). The findings from genome-wide analyses conducted on samples collected during the neonatal period have yielded contradictory result, with some studies identifying differentially methylated sites, while other studies found no differences (El Hajj and Haaf, 2013; Melamed *et al.*, 2015; Barberet *et al.*, 2022; Håberg *et al.*, 2022). In children, on the other hand, the meta-analysis of targeted methylation studies on blood and saliva samples, identified no significant differences between ART and naturally conceived individuals (Barberet *et al.*, 2022). Similarly, genome-wide studies comparing ART and naturally conceived children (Ducreux *et al.*, 2021; Yeung *et al.*, 2021), adolescents (Penova-Veselinovic *et al.*, 2021) or adults (Novakovic *et al.*, 2019), found no or few significant differences. Although this suggests that any ART-associated methylation differences present at birth do not persist into adulthood, these results come from a small number of studies that have not been validated by other groups. Additionally, the studies lack a detailed description of the ART culture conditions that these individuals were exposed to, meaning that their effect on the methylome cannot be established. As already described, clinical

differences are observed between ART offspring that were exposed to different culture environments, highlighting the need to specifically examine the methylomes of these ART sub-groups.

Here, we describe the saliva methylome of ART children (aged 9 or 10) that underwent embryo culture either in G3 (Vitrolife) or K-SICM (Cook) media. For this, we profiled DNA methylation on a genome-wide scale, using the EPIC array, in the largest cohort of its kind to date. Comparison to naturally conceived children was attempted using data from the Flemish Environment Health Study (FLEHS) (Fig. 1A).

## Materials and methods

### Ethical approval

This study was registered in the Dutch Trial register (trial number NL4083) and was approved by the ethical review board of the Maastricht University Medical Centre (MUMC+). Both parents of the children provided written, informed consent.

### Study population and sample collection

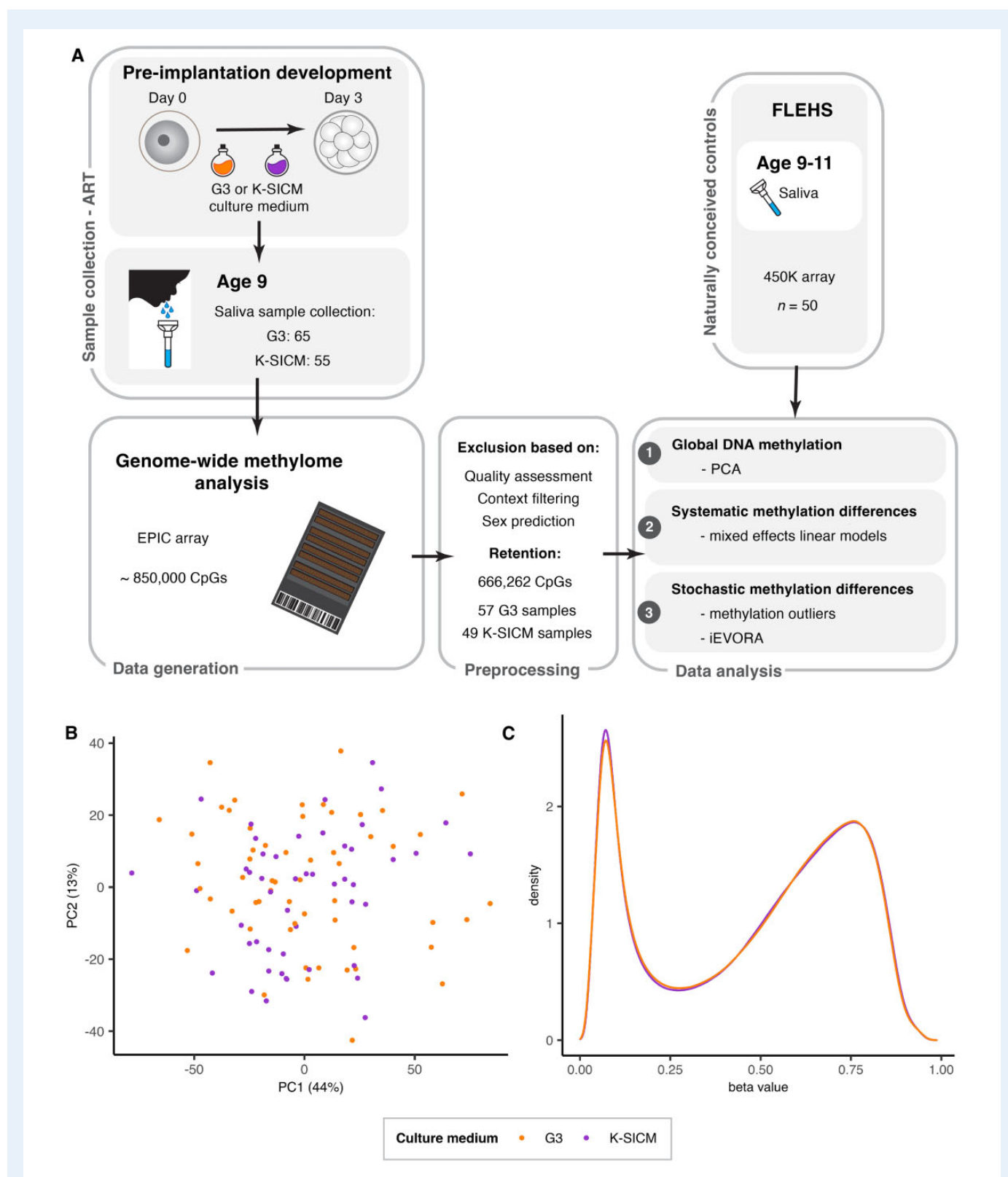
Samples for methylome analysis were collected during medical follow-up of children born after a previously conducted culture medium comparison study (Zandstra *et al.*, 2018a). Between July 2003 and December 2006, Vitrolife G1™ Version 3 (G3) (Göteborg, Sweden) and K-SICM from Cook (Brisbane, Australia) were used at MUMC+. Consecutive IVF treatments (with or without ICSI) were strictly alternated between the two media types, while all other ART procedures remained consistent (Dumoulin *et al.*, 2010; Nelissen *et al.*, 2012). Parents of all liveborn singletons from this study were approached for a follow-up investigation after the 9th birthday of the child. In addition to growth and cardiometabolic measurements, 2 ml of saliva were collected using the Saliva DNA Collection, Preservation and Isolation Kit (Norgen Biotek, Thorold, Canada). The saliva samples were collected after an overnight fast (necessary for blood glucose measurements) and after the children had rinsed their mouths with water. Subsequently, the preservation liquid was added to the tube and the samples were stored at room temperature, according to the manufacturer's instructions.

### DNA extraction

DNA was extracted using the Saliva DNA Collection, Preservation and Isolation Kit (Norgen Biotek, Thorold, Canada) according to the manufacturer's instructions. Briefly, 2 ml of preserved saliva was mixed with 80 µl of proteinase K, 800 µl of Binding buffer B and 2.88 ml of isopropanol. After centrifugation, the resulting DNA pellet was washed in 70% ethanol and air-dried. The pellet was rehydrated in 300 µl of Tris-EDTA buffer. DNA quantity and quality were determined using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA).

### Bisulfite conversion and DNA methylation profiling by EPIC array

Prior to DNA quantification, DNA samples containing precipitated material were heated to 65°C and centrifuged. Thereafter, 1 µg of



**Figure 1. Genome-wide DNA methylation analysis of ART children that underwent embryo culture in different media revealed no significant differences.** (A) Schematic overview showing sample collection/inclusion of ART-conceived and naturally conceived children, methylome data generation alongside data processing and analyses included in this study. (B) Principal component analysis (PCA) of all cytosine-guanine dinucleotide (CpG) sites passing our quality control criteria in data from saliva samples of ART children that underwent embryo culture in G3 (orange) or Sydney IVF cleavage medium (K-SICM, purple) medium. (C) Density plot showing the distribution of beta values from all sites and samples within each group (G3 = orange, K-SICM = purple).

DNA was bisulfite-treated using the EpiTect<sup>®</sup> Fast 96 DNA Bisulfite Kit (Qiagen Hilden, Germany) and analysed using the Infinium Human MethylationEPIC BeadChip Kit (Illumina, CA, USA) according to the manufacturer's protocol.

## Data analysis

All data analysis was conducted using R (version 3.6.3) (R Core Team, 2021) and visualized using the ggplot2 and ComplexHeatmap packages (Gu *et al.*, 2016). Custom R code used for the processing and analysis of the data described in this article are available at [https://github.com/CellularGenomicMedicine/saliva\\_methylome](https://github.com/CellularGenomicMedicine/saliva_methylome).

### Participant characteristics

The participant characteristics were compared for differences between the culture medium groups using Student's *t*-tests for continuous variables and Pearson's chi-squared tests for categorical variables.

### Quality control and pre-processing

Data were pre-processed using pre-processing functions contained within the RnBeads package (Müller *et al.*, 2019). Data were normalized using the subset-quantile within array normalization (SWAN) method (Maksimovic *et al.*, 2012). Poor quality probes and samples were removed using the greedyCut algorithm with a detection *P*-value threshold of 0.05. Sites were further filtered out based on the following criteria: (i) if they were located on the sex chromosomes, (ii) if they were in close proximity to single-nucleotide polymorphisms, (iii) if more than 5% of the samples contained missing values and (iv) if they were not in a CpG context. Sites containing any missing values (between 0% and 5% missing values) were only used to aggregate sites into regions, they were excluded for all single site-based analyses. Unless indicated otherwise, we used methylation beta values which represent the methylated signal divided by the sum of the methylated and unmethylated signal at each CpG site. Sample sex prediction was carried out as described by Jung *et al.* (2018) and implemented in the sEst package. Briefly, the X and Y chromosome beta value distributions of the test (ART) samples were combined with those of the reference male and female samples (Jung *et al.*, 2018). Subsequently, a principal component analysis (PCA) was conducted separately for each sex chromosome (referred to as PCA.X and PCA.Y). Then, *k*-means clustering (number of groups = 2) was conducted on the results from PCA.X and PCA.Y to determine if the test (ART) samples cluster with the female or male samples of the reference samples, respectively. When the *k*-means clustering for both sex chromosomes assigned the same sex, samples were labelled as male or female, all other samples were labelled 'not specified'. The X principal component (PC)1 threshold, to distinguishing between the presence of one or two X chromosomes, was set at 0.05. Principal component PC1 of the PCA.X and PCA.Y are shown in Fig. 2A and Supplementary Fig. S1A, without showing the reference samples that were analysed alongside the test (ART) samples.

### Cellular deconvolution of saliva samples

Cell composition of the samples was estimated using the reference-based Houseman algorithm (Houseman *et al.*, 2014) implemented using the ewastools package (Heiss and Just, 2018). Probes for cell-type deconvolution were identified using DNA methylation signatures from sorted saliva samples collected from

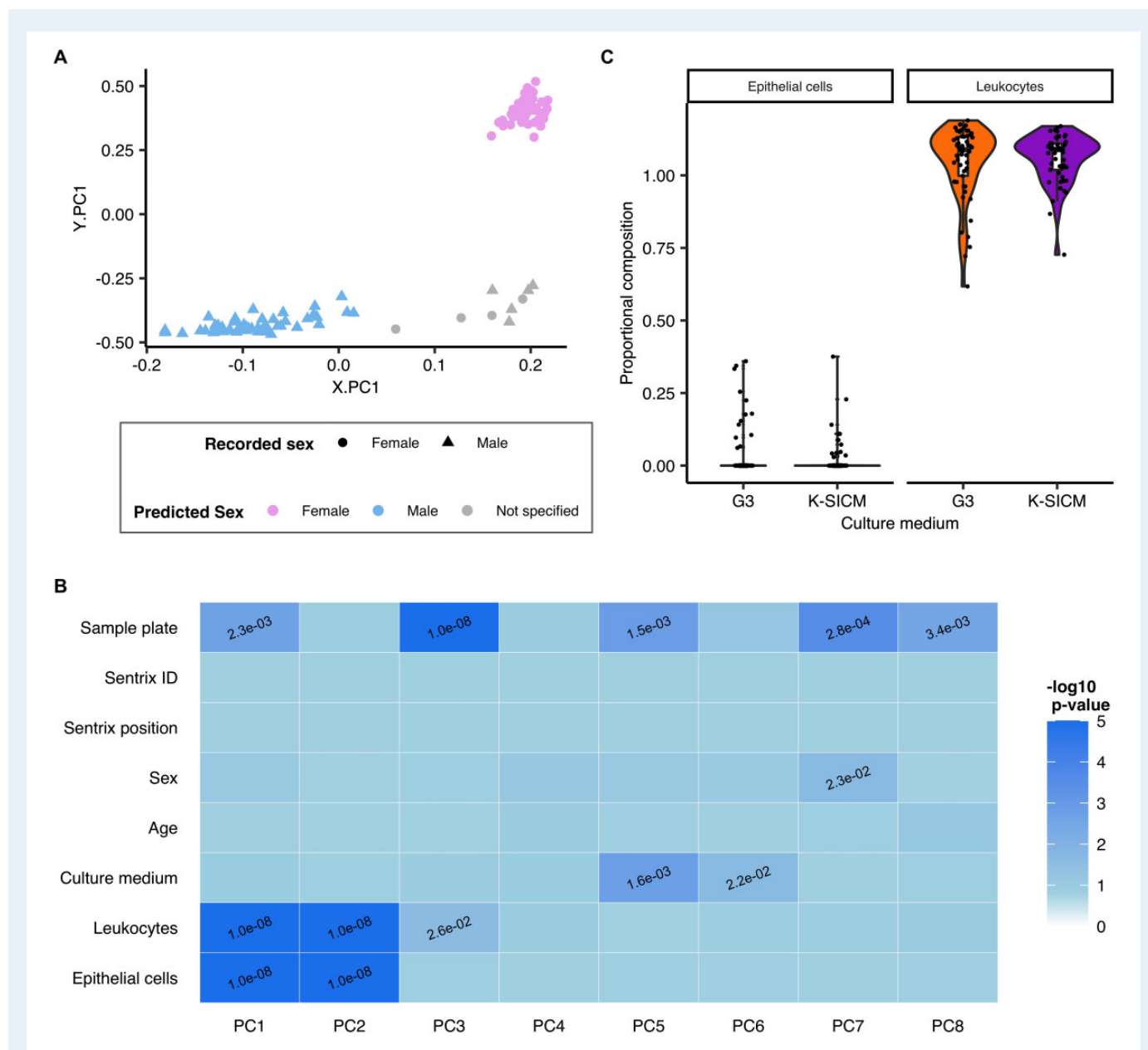
children (Middleton *et al.*, 2022) (available at Gene Expression Omnibus GSE147318) yielding an estimate of the proportion of leucocytes and epithelial cells.

### Comparison of G3 and K-SICM children

We applied PCA on all high-quality CpG sites. The beta values were centred but not scaled for the PCA. Associations between the PCs and technical or demographic features of our samples were tested either (i) using permutation tests with 10 000 permutations to determine the significance of correlations (age, leucocytes, epithelial cells), (ii) using a two-sided Wilcoxon rank test for categorical variables creating two groups (sex, culture medium, sample plate) or (iii) using a Kruskal–Wallis one-way ANOVA for categorical variables generating three or more groups (Sentrix ID, Sentrix position—chip number and sample position, respectively).

Methylation *M*-values, representing the log<sub>2</sub> ratio of the methylated probe intensity compared to the unmethylated probe intensity (Du *et al.*, 2010), were used to test for associations between DNA methylation and culture medium using mixed effects linear models implemented with the variancePartition package (Hoffman and Schadt, 2016). The models were adjusted for *a priori* chosen potential confounders: age at sample collection, sex and cell composition as fixed effects alongside batch correction (sample plate) as a random effect. They were applied to individual CpG sites or aggregate values of multiple CpG sites to identify differentially methylated positions (DMPs) and differentially methylated regions (DMRs), respectively. To aggregate sites into regions, we calculated the mean of all the beta values from probes attributed to the same gene, promoter or CpG island (CGI). For the targeted analyses, the aforementioned models were applied to (sites within) imprinted genes (Ginjala, 2001) and sites associated with birth weight (Küpers *et al.*, 2019). The Benjamini–Hochberg (Benjamini and Hochberg, 1995) method was used to correct all analyses for multiple testing, and an adjusted *P*-value of <0.1 was considered significant.

DNA methylation outliers were defined as previously described by Gentilini *et al.* (2015). Briefly, hypomethylation outliers were defined as beta values more than three interquartile ranges (IQRs) below the 25th percentile, while hypermethylation outliers were defined as beta values more than three IQRs above the 75th percentile. The IQR and percentile thresholds were calculated across all saliva samples. We tested for associations between the log<sub>10</sub> transformed number of outliers and culture medium using the mixed effects linear models described above. The significance of associations between the number of outliers per sample and various clinical and technical features were assessed using permutation tests with 10 000 permutations for continuous variables (age and weight at sample collection, birth weight, leucocyte proportion, epithelial cell proportion) and with two-sided Wilcoxon rank tests for categorical variables (sample plate). To identify differentially variable sites, the improved epigenetic variable outliers for risk prediction analysis (iEVORA) algorithm (Teschendorff *et al.*, 2016) was applied using the matrixTests package (Koncvičius, 2020). In brief, iEVORA applies Bartlett's test, a parametric test for differential variance, and a Student's *t*-test to each CpG site. Subsequently, sites reaching significance after multiple testing correction (false discovery rate (FDR) corrected *P*-value < 0.05) of the Bartlett's test and reaching nominal significance (*P*-value < 0.05 without multiple



**Figure 2. Preprocessing of saliva methylome data.** (A) Scatter plot showing the projection of high-quality saliva samples ( $n = 106$ ) into the principal component (PC) space generated using reference data for sex prediction. The shape of the dots represents the recorded sex of the participants (circles = female, triangle = male), while the colour shows the predicted sex based on results from sEst (blue = male, pink = female, grey = not specified). (B) Heatmap showing associations between the PCs and biological/technical aspects of the samples. The colour gradient represents the  $-\log_{10}$  of the  $P$ -values.  $P$ -values that are  $<0.05$  are shown. (C) Violin plot showing the predicted cellular composition of the saliva samples, split by cell type (leukocytes and epithelial cells) and culture medium group (orange and purple represent G3 and Sydney IVF cleavage medium (K-SICM) respectively). The violin plots are overlaid with boxplots where the horizontal lines represent the 25th percentile, median and 75th percentile, respectively, while the whiskers extend to the farthest data points that are no more than 1.5 times the interquartile range (IQR) from the upper or lower quartile.

testing correction) of the  $t$ -test are considered significant. This approach regularizes the result of the Bartlett's test which is usually overly sensitive to single outliers. The resulting differentially variable sites were used for Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses using functionality from the missMethyl package (Phipson et al., 2016).

### Comparison with naturally conceived children

Saliva methylome data were obtained from children enrolled in the longitudinal birth cohort FLEHS; the cohort has previously been described in detail (Langie et al., 2018; Van Den Heuvel et al., 2020).

From these children, saliva samples were collected at the age of 10–11 and the methylome was profiled using the 450K array (Illumina, CA, USA). Data preprocessing and the analysis procedures were largely the same as those described above, for more details see the [Supplementary materials and methods](#).

## Results

Of the 294 ART singletons from the culture medium study, 136 (48%) parent couples agreed for their child to participate. Three of the children failed or refused to provide 2 ml of saliva, a further 7 samples yielded insufficient DNA for processing and 6 samples did not meet quality criteria after bisulfite conversion. Therefore, methylome analysis by EPIC array was carried out on 120 saliva samples ( $n = 65$  for G3,  $n = 55$  for K-SICM). Quality control procedures on the generated data led to the exclusion of 5 poor quality samples ( $n = 3$  for G3 and  $n = 2$  for K-SICM) and 9 samples ( $n = 5$  for G3 and  $n = 4$  for K-SICM) with an undefined sex prediction (Fig. 2A). The characteristics of both groups were comparable (Table I). Although the weight of G3 offspring at birth (Dumoulin *et al.*, 2010) and age 9 (Zandstra *et al.*, 2018a) was reported to be higher than that of K-SICM offspring in the culture medium trial, these parameters were not significantly different in this subgroup of the original study. Nonetheless, the trend is the same.

Of ~850 000 profiled CpG sites, 666 262 were retained for analysis after the filtering procedures, of which 659 708 sites contained no missing values and were used for downstream analysis.

### Global analysis of DNA methylation

Global DNA methylation was first assessed by PCA (Fig. 1B), which did not show clear separation of the G3 and K-SICM groups within the first 4 PCs, which explain 49% of the variance in our data. Only PCs 5 and 6, representing 5.2% and 4.8% of the variance in our data, respectively, were significantly associated with the culture medium (Fig. 2B), thus suggesting that the culture medium is not the main source of variance within our data. Other significant associations were found between some of the first 8 PCs and certain technical or demographic factors, namely sample plate (PC1, PC3, PC5, PC7, PC8), sex (PC7), leucocytes (PC1–3) and epithelial cells (PC1, 2) (Fig. 2B and C). These factors are therefore corrected for in the subsequent analyses. Similarly, the distribution of beta values was very similar in both culture medium groups (Fig. 1C).

### Analysis of DNA methylation at individual CpG sites

We assessed the association of DNA methylation with culture medium at all individual CpG sites using mixed effects linear models corrected for potential confounders. We found no significant DMPs between the two culture medium groups after adjusting for multiple testing (adjusted  $P$ -value  $< 0.1$ ) (Fig. 3A, Supplementary Figs S2A and S3). Additionally, the group mean differences at all sites were small, with  $< 1\%$  of sites (17 in total) having a group mean difference of more than 10% and the maximum group mean difference being 13.5% (Supplementary Table S1).

To reduce the number of comparisons in our analysis, we focused on genomic regions of potential relevance to our cohort, i.e. sites

within imprinted genes (Ginjala, 2001) and sites associated with birth weight (Küpers *et al.*, 2019). After our quality control and filtering procedure, 8940 sites within 207 imprinted genes were retained for the analysis. Among these sites, no significant DMPs were identified, and the maximum group mean was 7.4% (Fig. 3A, Supplementary Fig. S2A). Of the 914 previously identified birth weight-associated CpG sites (Küpers *et al.*, 2019) 726 passed our quality control (QC) criteria and none were significantly differentially methylated with a maximal group mean difference of 3.0% (Fig. 3B, Supplementary Fig. S2B).

### Regional analysis of DNA methylation

We then analysed DNA methylation across larger regions of the genome, namely whole genes, promoters and CGIs. In total, 32 564 genes were included in the analysis. Of these, the maximal group difference was found to be 9.4% and no significantly differentially methylated genes were identified between the culture medium groups. A targeted analysis of only the imprinted genes showed that these had even lower group mean differences (maximum group mean difference 1.2%) than the individual sites within these genes (Fig. 3C, Supplementary Fig. S2C). Furthermore, no imprinted genes were found to be significantly differentially methylated between the G3 and K-SICM groups. A total of 41 519 promoters and 25 224 CGIs were included in our analysis. The maximal group mean differences were 9.4% and 8.4% for promoters and CGIs, respectively, and no promoters or CGIs were found to be significantly differentially methylated between the culture medium groups (Fig. 3D and E, Supplementary Fig. S2D and E).

### DNA-methylation variance in ART samples

To assess whether stochastic DNA methylation alterations contribute to the phenotypes observed in the culture medium trial, DNA methylation outliers were identified using previously defined thresholds (Gentilini *et al.*, 2015) and differential variance was assessed using the iEVORA method (Teschendorff *et al.*, 2016). Overall, we found a predominance of hypomethylation outliers compared to hypermethylation outliers (92 238 hypomethylation outliers vs 33 009 hypermethylation outliers). On average, we identified a total of  $254 \pm 485$  (median  $\pm$  IQR) outliers per G3 sample and  $186 \pm 153$  (median  $\pm$  IQR) outliers per K-SICM sample, which was not found to be significantly different ( $p = 0.368$ ) (Fig. 4). Additionally, there was no significant difference between the culture medium groups when the numbers of hypomethylation outliers ( $P = 0.86$ ) and hypermethylation outliers ( $P = 0.238$ ) were analysed separately (Fig. 4). Outlier burden, i.e. the total number of outliers per sample, was not significantly associated with age at sample collection or weight at birth or follow-up. On the other hand, technical features, specifically leucocyte proportion and sample plate, were significantly associated with outlier burden (Supplementary Table SII). An association between the number of samples and the presence of common diseases (atopy, autism, urological problems) was not tested statistically, but one-third of the children with a diagnosis (8 out of 24) had a very high number of methylation outliers (more than the upper quartile).

Using iEVORA we identified 101 differentially variable CpG sites between the two groups, of which 94 sites were more variable in the G3 group than the K-SICM group. While 20 of the sites were unannotated, the remainder belonged to 80 unique genes. Two genes,



**Table 1** Characteristics of the child, pregnancy and fertility treatment.

Characteristic	Culture medium		P-value
	G3 (n = 57)	K-SICM (n = 49)	
<b>Characteristics of the child</b>			
Sex (female)	29 (51)	26 (53)	0.977
Age at sample collection (years)	9.5 ± 0.3	9.5 ± 0.3	0.802
Weight (kg)	34.1 ± 6.7	31.8 ± 5.5	0.054
Height (cm)	139.0 ± 5.9	138.5 ± 7.5	0.706
Medical diagnoses			
Urological	3 (5)	2 (4)	1.000
Asthma/allergy	9 (16)	4 (8)	0.370
Autism	4 (7)	2 (4)	0.818
<b>Pregnancy characteristics</b>			
Maternal age (years)	32.8 ± 3.4	33.0 ± 3.4	0.772
Paternal age (years)	35.8 ± 4.5	35.6 ± 4.7	0.827
Gestational age	39.7 ± 1.5	39.5 ± 2.2	0.583
Birth weight (g)	3425.4 ± 486.4	3308.6 ± 537.0	0.247
<b>Fertility treatment</b>			
Fertilization method			0.985
IVF	18 (32)	17 (35)	
ICSI	39 (68)	32 (65)	
Treatment indication			0.985
Unknown	12 (21)	11 (22)	
Male factor	38 (67)	32 (65)	
Female factor	7 (12)	6 (12)	

Continuous variables shown as mean ± SD, categorical variables shown as n (%). Maternal and paternal age at time of ovum pick-up is shown.

*DOCK1* and *PDZRN3*, contained two differentially variable sites. Additionally, two of the sites were within imprinted genes, namely *KCNQ1* and *APBA1* (Supplementary Table SIII, sheet 1). The differentially methylated sites identified by iEVORA were not found to be significantly enriched (FDR < 0.05) in any pathways or ontologies according to the KEGG and GO analyses (Supplementary Table SIII, sheets 2–3).

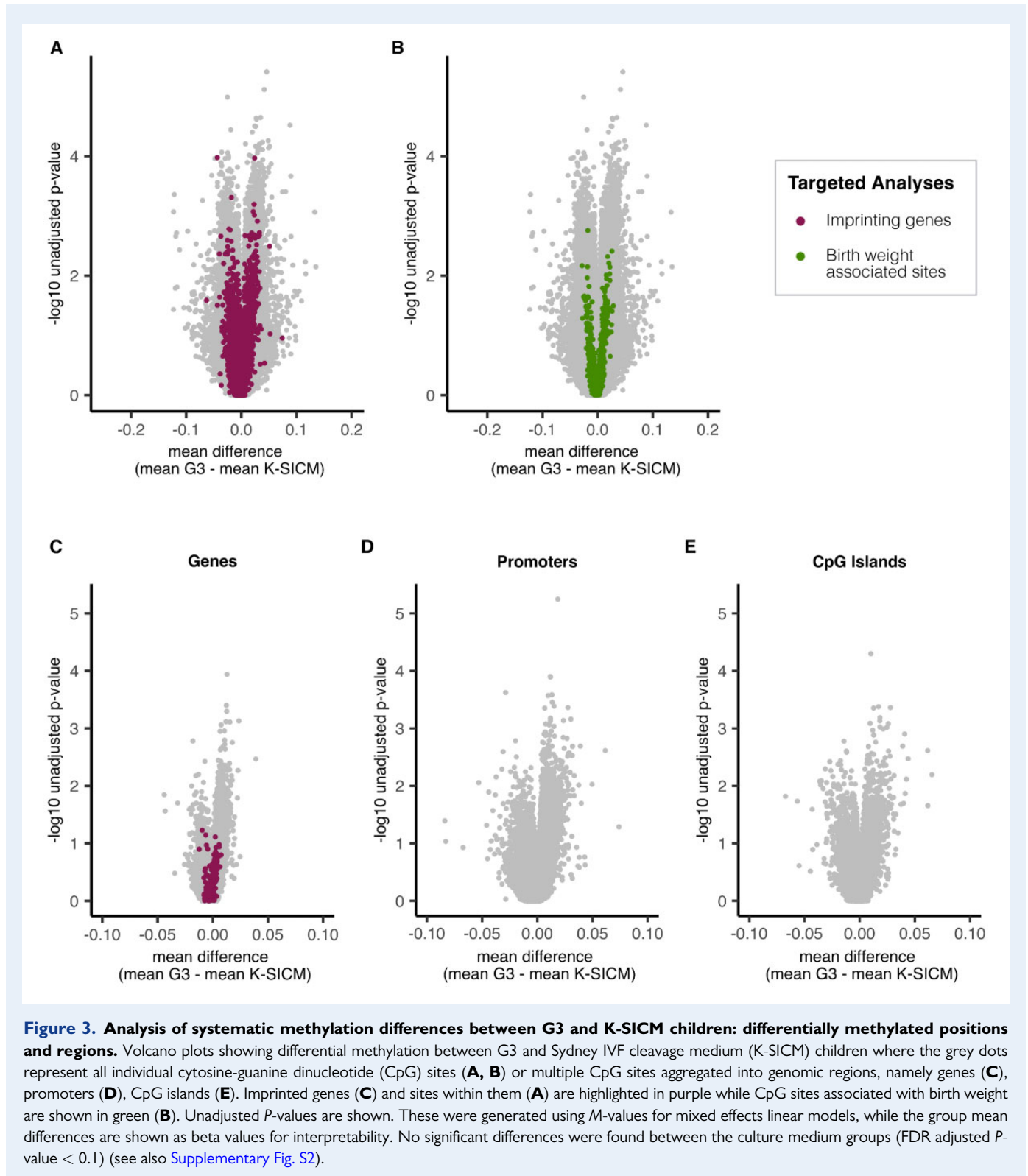
### Comparison of DNA methylation levels of ART children compared to naturally conceived individuals

All the saliva methylomes (n = 50) available from the FLEHS cohort passed our QC criteria (Supplementary Fig. S1) and the characteristics of these individuals are summarized in Supplementary Table SIV. Of note is that saliva samples were collected from significantly older naturally conceived children than ART children (ART age: 9.5 ± 0.3, naturally conceived age: 10.6 ± 0.3, mean ± SD,  $P < 0.001$ , Welch's two-sample *t*-test) with a higher incidence of asthma (12% vs 64% in the ART and naturally conceived cohorts, respectively). PCA showed significant separation of the ART and naturally conceived samples on PCs 1, 3, 4 and 5 (Supplementary Fig. S4A and B). Seventy-two percent of CpG sites were found to be significantly differentially methylated (adjusted  $P$ -value < 0.05) with a strong predominance of sites that were

hypomethylated in the ART samples compared to the naturally conceived samples (Supplementary Fig. S4C and D). Such marked methylation differences in phenotypically similar individuals suggest systematic differences in the data that could be due to technical or cohort differences, such as the array type (EPIC or 450K) used, differences induced by the array scanner, laboratory environmental factors or minor pipetting or laboratory protocol deviations. When the sample groups of interest are processed separately, these features align perfectly with the groups of interest and therefore cannot be differentiated from meaningful biological variation. Further analyses were therefore not carried out.

### Discussion

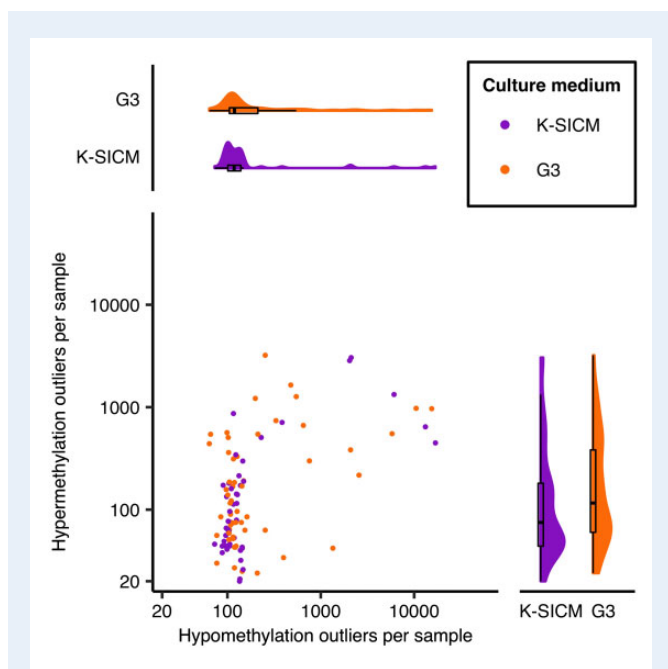
Here, we present the largest study to date of the saliva methylome of ART children that underwent embryo culture in different media, namely G3 or K-SICM. At age 9, we have found no significant DNA methylation differences between the culture medium groups when considering individual CpG sites, multiple CpG sites aggregated into genomic regions or when conducting a targeted analysis of (sites within) imprinted genes or birth weight-associated CpG sites. The number of methylation outliers per sample was found to be comparable between the culture medium groups and was only significantly associated with



technical features of the samples. Interestingly, iEVORA identified that the majority of differentially variable sites between the G3 and K-SICM groups were more variable in the G3 group.

Our findings are consistent with those from the only other ART culture medium cohort in which the saliva methylome was investigated

during childhood (ages 7–8) (Barberet *et al.*, 2021; Ducreux *et al.*, 2021). Although the ART children included in that study underwent embryo culture in other media than those compared in the present work, no significant DNA methylation differences were found at any of the sites or regions (Barberet *et al.*, 2021; Ducreux *et al.*, 2021).



**Figure 4. Methylation outliers.** The main panel shows the number of hypomethylation (*x*-axis) and hypermethylation (*y*-axis) outliers per saliva sample (G3 = orange, Sydney IVF cleavage medium (K-SICM) = purple). Distribution summaries, in the form of a density plot and boxplot, are shown for hypomethylation outliers and hypermethylation outliers in the top and right side-panels, respectively. Lines of the boxplot represent the 25th percentile, median and 75th percentile, respectively, while the whiskers extend to the farthest data point that is no more than 1.5 times the interquartile range (IQR) from the upper or lower quartile. The axes are shown on a log<sub>10</sub> scale. The groups were not found to be significantly different (*P*-value > 0.1).

The group mean differences that they observed between their culture medium groups were also similar to those observed in our cohort (Barberet et al., 2021). Due to the notably larger sample size of our study, we anticipate that we would have sufficient power (0.8) to detect smaller methylation differences compared to what was possible in the previously described cohort. The results from the current study were also comparable to those of the methylome studies conducted on placenta or umbilical cord blood samples collected from ART neonates cultured in G5 or HTF media (Mulder et al., 2020; Koeck et al., 2022). In addition to finding no significant DMPs or DMRs, these studies also reported that there was no difference in the number of outliers per sample between the culture medium groups (Mulder et al., 2020; Koeck et al., 2022).

Interestingly, in the recent umbilical cord blood methylome study (Koeck et al., 2022), 90% of the differentially variable sites identified using iEVORA were more variable in the G5 group than in the HTF culture medium group. Although the effect was less striking once data from neonates that had experienced pregnancy complications had been excluded, 56% of the identified sites were still more variable in the G5 group. In both studies, the culture medium from Vitrolife was associated with a higher number of more variable CpG sites amongst

the differentially variable sites (Koeck et al., 2022). Several explanations have been proposed for the relevance of differentially variable sites. For instance, low methylation variance could result from (environmental) selection pressures that only facilitate the survival of individuals of certain methylation signatures (Tobi et al., 2018). Alternatively, in oncological samples, sites with differential variability in pre-cancerous tissues are commonly found to be differentially methylated in tumours, when compared with healthy tissues, thus suggesting that these epimutations play a role in disease pathogenesis (Teschendorff et al., 2016). Therefore, in our study, less variability in DNA methylation, as seen in the K-SICM group, could indicate a greater environmental selection pressure which would be in concordance with the observed lower implantation and pregnancy rates in this group (Dumoulin et al., 2010). However, these sites with differentially variable methylation levels could also be indicative of (cardiometabolic) diseases that certain individuals may develop in later life, which would need to be investigated with further longitudinal follow-up studies of these individuals.

The main aim of the study was to compare the methylomes of ART children who had undergone embryo culture in different media, therefore saliva samples from naturally conceived children were not collected and processed alongside the ART samples. Although a comparison with children from the FLEHS cohort was attempted, the results indicate a large contribution of technical or cohort differences that cannot be corrected in samples that are not processed simultaneously. As such, we recommend that future studies analysing the methylome of ART offspring also collect samples from naturally conceived individuals for simultaneous processing.

The strengths of our study lie in the use of a methylome analysis method that captures methylation status across the whole genome and the sample size which is the largest of any methylome study of ART children in the context of a culture medium study. Nonetheless, to reach sufficient power (0.8) to detect methylation differences of <10% between the culture medium groups a bigger sample size is likely required (Tsai and Bell, 2015; Saffari et al., 2018). On the other hand, it is unclear whether such small differences would represent clinically meaningful differences. Since methylation is a binary state (methylated or unmethylated) a 5% methylation increase in any given individual signifies that 5% more of their cells are methylated at the given position. Whether this contributes to pathology or phenotypic variance remains to be determined.

Overall, the findings of this study are reassuring. Although phenotypic differences are observed, even at the age of 9, between ART offspring that had undergone embryo culture in G3 or K-SICM medium, there is no evidence that the epigenome of these individuals differs greatly. As the epigenome retains its plasticity throughout life it is possible that epigenetic dysregulation experienced during the pre-implantation period is no longer evident in ART offspring at the age of 9. Further research, profiling the epigenome of ART embryos, is required to understand how the ART culture environment modulates the epigenome during the period of *in vitro* culture and the significance of sites with differentially variable methylation for later life (disease) outcomes should be evaluated with longitudinal studies.

## Supplementary data

Supplementary data are available at *Human Reproduction* online.

## Data availability

The raw and processed array data from this study have been uploaded to the Gene Expression Omnibus (GEO) database and are available under the accession number GSE196432 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE196432>). The raw array data from the FLEHS study are available within the GEO database under the accession number GSE110128.

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## Authors' roles

R.M.K., A.P.A.v.M. and M.Z.E. study design and conception. H.Z., R.v.G. and J.C.M.D. were involved in sample collection for the ART cohort. A.P.A.v.M. and F.B. carried out the lab work for the ART samples. S.R. and S.L. were involved in sample collection and processing for the naturally conceived cohorts. R.M.K., F.B., J.T., A.P.A.v.M. and M.Z.E. were involved in the data analysis and interpretation. R.M.K. wrote the first draft of the manuscript. M.G., A.P.A.v.M. and M.Z.E. contributed to the writing of the manuscript. All authors provided textual comments and approved the manuscript. H.B., A.P.A.v.M. and M.Z.E. supervised the study.

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## Conflict of interest

The authors do not report any conflicts of interest relevant to this study.

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