Human Reproduction, Vol.00, No.0, pp. 1–15, 2021
doi:10.1093/humrep/deab078

human reproduction

ORIGINAL ARTICLE ***Reproductive genetics***

DNA methylation patterns within whole blood of adolescents born from assisted reproductive technology are not different from adolescents born from natural conception

B. Penova-Veselinovic [1], P.E. Melton[2,3,4], R.C. Huang[5,6], J.L. Yovich [3,7], P. Burton[8,9], L.A. Wijs [1], and R.J. Hart [1,10,*]

[1]Division of Obstetrics and Gynaecology, Faculty of Health and Medical Sciences, University of Western Australia, Perth, WA, Australia [2]School of Population and Global Health, University of Western Australia, Perth, WA, Australia [3]School of Pharmacy and Biomedical Science, Curtin University, Perth, WA, Australia [4]Menzies Institute for Medical Research, University of Tasmania, Hobart, TAS, Australia [5]Faculty of Health and Medical Sciences, Centre for Child Health Research, University of Western Australia, Perth, WA, Australia [6]Telethon Kids Institute, Nedlands, WA, Australia [7]PIVET Medical Centre, Perth, WA, Australia [8]Concept Fertility Centre, Subiaco, WA, Australia [9]School of Health and Medical Sciences, Faculty of Health Science, Edith Cowan University, Perth, WA, Australia [10]Fertility Specialists of Western Australia, Bethesda Hospital, Claremont, WA, Australia

*Correspondence address. Division of Obstetrics and Gynaecology, University of Western Australia, Faculty of Health and Medical Sciences, 374 Bagot Road, 2nd Floor, A Block, Perth 6008, WA, Australia. Tel: +61-8-6458 1328; Fax: +61-8-6458 1045; E-mail: roger.hart@uwa.edu.au https://orcid.org/0000-0002-6610-3040

Submitted on October 25, 2020; resubmitted on March 4, 2021; editorial decision on March 10, 2021

STUDY QUESTION: Do the epigenome-wide DNA methylation profiles of adolescents born from ART differ from the epigenome of naturally conceived counterparts?

SUMMARY ANSWER: No significant differences in the DNA methylation profiles of adolescents born from ART [IVF or ICSI] were observed when compared to their naturally conceived, similar aged counterparts.

WHAT IS KNOWN ALREADY: Short-term and longer-term studies have investigated the general health outcomes of children born from IVF treatment, albeit without common agreement as to the cause and underlying mechanisms of these adverse health findings. Growing evidence suggests that the reported adverse health outcomes in IVF-born offspring might have underlying epigenetic mechanisms.

STUDY DESIGN, SIZE, DURATION: The Growing Up Healthy Study (GUHS) is a prospective study that recruited 303 adolescents and young adults, conceived through ART, to compare various long-term health outcomes and DNA methylation profiles with similar aged counterparts from Generation 2 from the Raine Study. GUHS assessments were conducted between 2013 and 2017. The effect of ART on DNA methylation levels of 231 adolescents mean age 15.96 ± 1.59 years (52.8% male) was compared to 1188 naturally conceived counterparts, 17.25 ± 0.58 years (50.9% male) from the Raine Study.

PARTICIPANTS/MATERIALS, SETTING, METHODS: DNA methylation profiles from a subset of 231 adolescents (13–19.9 years) from the GUHS, generated using the Infinium Methylation Epic Bead Chip (EPIC) array were compared to 1188 profiles from the Raine Study previously measured using the Illumina 450K array. We conducted epigenome-wide association approach (EWAS) and tested for an association between the cohorts applying Firth's bias reduced logistic regression against the outcome of ART versus naturally conceived offspring. Additionally, within the GUHS cohort, we investigated differences in methylation status in fresh versus frozen embryo transfers, cause of infertility as well as IVF versus ICSI conceived offspring. Following the EWAS analysis we investigated nominally significant probes using Gene Set Enrichment Analysis (GSEA) to identify enriched biological pathways. Finally, within GUHS we compared four estimates (Horvath, Hanuum, PhenoAge [Levine], and skin Horvath) of epigenetic age and their correlation with chronological age.

MAIN RESULTS AND THE ROLE OF CHANCE: Between the two cohorts, we did not identify any DNA methylation probes that reached a Bonferroni corrected P-value $<$ 1.24E−0.7. When comparing IVF versus ICSI conceived adolescents within the GUHS cohort, after adjustment for participant age, sex, maternal smoking, multiple births, and batch effect, three methylation probes (cg15016734,

© The Author(s) 2021. Published by Oxford University Press on behalf of European Society of Human Reproduction and Embryology. All rights reserved.
For permissions, please email: journals.permissions@oup.com

Downloaded from https://academic.oup.com/humrep/advance-article/doi/10.1093/humrep/deab078/6247390 by John Yovich on 28 April 2021

cg26744878 and cg20233073) reached a Bonferroni correction of 6.31E−08. After correcting for cell count heterogeneity, two of the aforementioned probes remained significant and an additional two probes (cg 0331628 and cg 20235051) were identified. A general trend towards hypomethylation in the ICSI offspring was observed. All four measures of epigenetic age were highly correlated with chronological age and showed no evidence of accelerated epigenetic aging within their whole blood.

LIMITATIONS, REASONS FOR CAUTION: The small sample size coupled with the use of whole blood, where epigenetic differences may occur in other tissue. This was corrected by the utilized statistical method that accounts for imbalanced sample size between groups and adjusting for cell count heterogeneity. Only a small portion of the methylome was analysed and rare individual differences may be missed.

WIDER IMPLICATIONS OF THE FINDINGS: Our findings provide further reassurance that the effects of the ART manipulations occurring during early embryogenesis, existing in the neonatal period are indeed of a transient nature and do not persist into adolescence. However, we have not excluded that alternative epigenetic mechanisms may be at play.

STUDY FUNDING/COMPETING INTEREST(S): This project was supported by NHMRC project Grant no. 1042269 and R.J.H. received funding support from Ferring Pharmaceuticals Pty Ltd. R.J.H. is the Medical Director of Fertility Specialists of Western Australia and a shareholder in Western IVF. He has received educational sponsorship from Merck Sharp & Dohme Corp.- Australia, Merck-Serono Australia Pty Ltd and Ferring Pharmaceuticals Pty Ltd. P.B. is the Scientific Director of Concept Fertility Centre, Subiaco, Western Australia. J.L.Y. is the Medical Director of PIVET Medical Centre, Perth, Western Australia. The remaining authors have no conflicts of interest.

Key words: IVF / ICSI / DNA methylation / long-term / epigenetic age

Introduction

It is estimated that approximately 1 in 25 children born in Australia (Newman et al., 2019), and over 8 million children and adults worldwide (Fauser, 2019) have been born following ART. For the purposes of this paper, ART includes IVF and ICSI, both from fresh and frozen transfers. It is well established that pregnancies resulting from ART are at an increased risk of major birth anomalies (Hansen et al., 2013), intrauterine growth restriction (Brezinka and Khanjani, 2018) and neurological problems (Hansen et al., 2018) for the newborn. Potential differences in respiratory (Kallen et al., 2013), cardiovascular (Weinrauch et al., 2018) and cardiometabolic health (Ceelen et al., 2008), and possibly altered thyroid function (Sakka et al., 2009) are amongst those adverse health concerns reported in children born from ART treatment (Hart and Norman, 2013a). However, it is important to note that children born to couples with a degree of subfertility have an increased risk of congenital abnormalities and longer-term health concerns (Bellver and Donnez, 2019). This may relate to the cause of the difficulty conceiving such as the health of the couple (Bellver and Mariani, 2019), the age of male and female partners (Bergh et al., 2019) and the cause of infertility, such as the presence of polycystic ovary syndrome (Doherty et al., 2015).

Several short-term, and a limited number of longer-term studies have investigated the general health outcomes of children born from ART treatment, although, without common agreement as to the cause and underlying mechanisms of these adverse health findings (Davies, 2013; Hart and Norman, 2013a, b; Kallen et al., 2013; Lu et al., 2013; Shankaran, 2014; Song et al., 2015; Catford et al., 2018; Hann et al., 2018). Consequently, the long-term health of children conceived using IVF and ICSI is of substantial public health interest.

A growing body of evidence is emerging, that the observed adverse health outcomes in ART-born offspring may have underlying epigenetic mechanisms (Maher et al., 2003; Lucas, 2013; Jiang et al., 2017; Huntriss et al., 2018). Epigenetics is defined as the study of mechanisms that control gene expression in a mitotically heritable manner, which are influenced by genetic, environmental and developmental factors (Cavalli and Heard, 2019). Epigenetic modification adds an additional level of regulation over the message within the genotype through four major mechanisms: DNA methylation, histone modification, chromatin restructure and non-coding RNA regulation. Of the four epigenetic mechanisms, DNA methylation is the most common and involves the addition of a methyl group (CH_3) onto the C5 position of a cytosine, preceding a guanine (CpG) forming the ‘fifth base’ in the DNA alphabet. The major role of DNA methylation is regulation of gene expression (Waddington, 2012; Moore et al., 2013; Zhang and Pradhan, 2014). A comprehensive method for examining epigenetics is to conduct an epigenome-wide DNA Methylation association study (EWAS), which is a high-throughput analysis scanning the whole-genome associating differential DNA methylation with a disease or trait (Rakyan et al., 2011). EWAS studies are commonly conducted using whole-blood, as collection of this tissue is non-invasive and previous research has shown moderate to strong correlations between blood tissue DNA methylation and other tissue types such as subcutaneous fat (Wahl et al., 2017) when properly accounting for cell count heterogeneity.

The very early stages of embryo development, preimplantation advancement and germ cell development are characterized by extensive developmental epigenetic reprogramming (Reik et al., 2001; Cantone and Fisher, 2013). It is, therefore, probable that ART manipulations occurring during this dynamic developmental period may disrupt epigenetic processes in the gametes and in the developing embryo, potentially indirectly altering normal development and long-term health outcomes (El Hajj and Haaf, 2013).

The majority of studies have investigated differential DNA methylation between ART and naturally conceived offspring in placental tissue (Katari et al., 2009; Choux et al., 2018; Choufani et al., 2019), cord blood (Melamed et al., 2015; Castillo-Fernandez et al., 2017; El Hajj et al., 2017) or buccal cells (Whitelaw et al., 2014) focusing on the neonatal period using small sample sizes. Changes in the overall DNA methylation levels, altered methylation profiles of imprinted genes as

Downloaded from https://academic.oup.com/humrep/advance-article/doi/10.1093/humrep/deab078/6247390 by John Yovich on 28 April 2021

well as the possible impact on gene expression have been observed in ART-born offspring when compared to naturally conceived children (Katari et al., 2009; Lazaraviciute et al., 2014; Choufani et al., 2019). In contrast, other studies have reported that ART-born offspring are at no increased risk of epigenetic alterations (Gentilini et al., 2018), imprinting disorders (Tierling et al., 2010; Oliver et al., 2012), and exhibit an overall stable DNA methylation profile in the imprinted genes (Feng et al., 2011). Few studies have investigated the potential long-term stability of ART-induced DNA methylation changes in puberty and adolescence, which are critically important and informative periods in human development (Han et al., 2019).

The available evidence suggests that some of the ART-associated adverse health outcomes reported in childhood and adolescence are short-lived and may be mitigated by adulthood (Halliday et al., 2019). A study by Novakovic et al. suggested that the observed differential DNA methylation patterns in ART-born offspring do not persist into adulthood (Novakovic et al., 2019).

Variations in the DNA methylation levels observed with aging have been perceived as possible mechanism underlying human senescence (Horvath, 2013). This has led to the concept of 'DNA methylation age' (DNAmAge), epigenetic marker that calculates an estimate of a person's biological age based on the DNA methylation status of informative DNA probes across different tissues and at various stages through the lifespan. Using these markers, one can determine 'accelerated epigenetic aging', a term describing the difference between the DNAmAge and the chronological age and has been associated with several chronic diseases related to aging and mortality (Fransquet et al., 2019).

In one of the first studies of its kind, we established a cohort of ART-conceived adolescents and young adults born in Western Australia: the Growing Up Healthy Study (GUHS). This cohort was established to determine the long-term consequences of ART upon the development of the offspring, and uniquely compared their adolescent health parameters to a well-established representative cohort of naturally conceived children from the Raine Study (Straker et al., 2017). To our knowledge, there have been no studies that investigated the DNA methylation status of ART-born adolescents. Hence, we have a unique opportunity to investigate the differences in the DNA methylation levels in whole blood between the ART-conceived adolescents from the GUHS cohort and their naturally conceived, similar aged counterparts from the Raine Study. To investigate this potential epigenetic difference, we compared DNA methylation profiles between the GUHS and Raine cohorts using EWAS. In addition, we investigated the risk of 'accelerated aging' in ART-born offspring.

Materials and methods

Study populations

The Growing Up Healthy Study (*GUHS*) is a prospective and observational study to the long-term follow-up of adolescents and young adults (aged 13–22 years), conceived through ART between 1991 and 2001. Four hundred and four families were recruited from the only two fertility clinics operating in Western Australia at the time: PIVET Medical Centre and Concept Fertility Centre in Perth, Western Australia. In total, 303 adolescents and young adults consented to undertake in the assessments defined by the study protocols at ages 14, 17 and 20 years.

Blood and urine samples were collected at each follow-up, for age-specific biochemical analyses. Blood for DNA extraction was collected at one point in time, commonly at their first assessment. Their long-term health parameters, such as cardiovascular, metabolic, endocrine, respiratory and mental health outcomes were investigated at age-specific follow-up assessments (including questionnaires) and compared to their naturally conceived counterparts from the Raine Study Generation 2 (Gen2) by replicating the Raine Study assessments.

Ethical approval

The following committees approved the assessments and subsequent multiple analyses conducted within the scope of the Growing Up Healthy Study project: The University of Western Australia Human Research Ethics Office (RA/4/1/5860). The Department of Health Western Australia, Human Research Ethics Committee with project number 2013/25. Informed and written consents were obtained from the participating families at each follow up including genetic assessment consent.

The Raine Study was formed from a pregnancy cohort study (https://www.rainestudy.org.au). The Raine Study Gen2 is a cross-section of the larger longitudinal and multigenerational study that recruited pregnant mothers between 1989 and 1991 to investigate the safety and effects of ultrasound on the foetus (Newnham et al., 1991; Straker et al., 2017). A total of 2900 women were enrolled by the 18th week of gestation from antenatal booking clinics. The resulting 2868 children born to 2804 mothers were retained to form the Raine Study cohort, to investigate the role of perinatal events on subsequent childhood and adult health (Straker et al., 2017). The cohort is unique, as detailed antenatal and childhood measurements have been recorded throughout life. The current cohort includes 1800 men and women aged 30 years. There is close and frequent contact with study participants and the cohort has a current retention rate of over 70%. The Raine Study is recognized to be representative of the population of Western Australian children (Dontje et al., 2019). Gen2 has been comprehensively phenotyped through questionnaires, anthropometric, clinical and biochemical data, as well as collected and stored biological samples (cord blood, blood, urine, milk saliva, DNA). Assessments occurred annually until age 3, and then at ages 5, 8, 10, 14, 17, 18, 20, 22, 27 and 28. The approval for conducting the epigenetics analysis at the Gen2-17 year follow-up was given by the Human Ethics Committee of the University Western Australia.

Clinical data collection (GUHS)

Clinical data regarding the cause of a couple's subfertility, the previous obstetric and medical history were recorded, and additional data relating to their IVF treatment cycles, embryological data and pregnancy outcomes were obtained from medical records and presented in Table I. As was universally standard at the time, the embryo cryopreservation techniques used was the traditional 'slow freezing' approach with cryoprotectants and embryo cultured in desiccators. The embryo culture media used was made 'in-house', with few exceptions, in which cases a commercial media was utilized (Medicult Medium Denmark (n = 1); and Quinn's Media (n = 4) from PIVET Medical Centre). Outcome data from the index ART conceived pregnancy were

Downloaded from https://academic.oup.com/humrep/advance-article/doi/10.1093/humrep/deab078/6247390 by John Yovich on 28 April 2021

Table 1 Clinical information regarding ART cycles of the mothers of 'Growing Up Healthy Study' (GUHS) offspring aged 13–19.9 years for the index pregnancy.

Clinical information for GUHS parental generation	
Cause of infertility	
Tubal	74 [32.03]
Endometriosis	26 [11.26]
Male factor	75 [32.47]
Unexplained	42 [18.18]
Other *	37 [16.02]
Data N/A	13 [5.63]
Type of procedure	
Fresh IVF	96 [41.56]
Fresh ICSI	33 [14.29]
FET (IVF)	64 [27.71]
FET (ICSI)	24 [10.39]
Unknown	14 [6.06]
Hormone replacement therapy (HRT)	
HRT for endometrial preparation	13 [5.63]
Natural cycle	187 [80.95]
Data N/A	31 [13.42]
Day of embryo transfer	
Day 1	34 [14.72]
Day 2	151 [63.37]
Day 3	28 [12.12]
Data N/A	17 [7.36]
Donor used	
Egg	4 [1.73]
Sperm	2 [0.87]
Embryo	1 [0.43]

Data presented as n [%]; n = 231. *Endocrine; hostile mucous; ovarian; uterine; ovulation disorder; anti-sperm antibodies; PCOS, polycystic ovary syndrome; female sterile; FET (IVF), Frozen embryo transfer from an IVF cycle; FET (ICSI), frozen embryo transfer from an ICSI cycle; HRT, hormone replacement therapy for the cycle of index pregnancy; Data N/A, data not available.

collected using core health data sets (www.datalinkage-wa.org/data-linkage/data-collections), within the linked data from the Western Australia Data Linkage System, validated previously and used extensively for health research (Holman et al., 1999). The Western Australia Data Linkage System facilitates systematic record linkage from population-based administrative health data sets within Western Australia encompassing all pregnancies beyond 20 weeks of gestation, recorded in the Midwives' Notifications System.

Clinical data collection (the Raine Study, Gen1)

Relevant clinical data for The Raine Study Generation1 (Gen1), the parents of Gen 2 (the comparator group), was obtained from the Raine data repository. Six of the seventeen children born from infertility treatment within the Raine Study had epigenome profiles available for comparison. Three were conceived from either IVF or Gamete Intrafallopian Transfer (GIFT) cycles, and, therefore, excluded from the analysis, allowing for a clean non-ART/GIFT phenotype group, and a total of 1188 participants for comparison.

Sample preparation—DNA extraction (GUHS)

Whole blood for DNA extraction was collected from the participants at a single time point, commonly at their first assessment. The whole blood was kept in −80°C freezers until genomic DNA was extracted using the Promega Reliaprep Large volume HT g DNA Isolation System and quantitated on the Qubit 4.0 System in the Western Australian DNA Bank at the Centre for Genetic Origins of Health and Disease, Australia. Thirty participants refused to have their bloods drawn due to needle phobia or declined consent for DNA extraction (Fig. 1).

DNA methylation profiling for GUHS and quality control

Genomic DNA from a total of 273 GUHS participants was used for epigenomic profiling, on the Infinium MethylationEpic BeadChip (EPIC Illumina Inc., San Diego, CA) platform through PathWest Laboratory Medicine (Perth, Western Australia). EPIC profiles over 850 000 CpG sites, roughly 3% of the human epigenome (Heiss et al., 2020), at a single nucleotide resolution allowing for EWA to be done in a population of adolescents born through ART. Additionally, independent repeated measurements of the quantified epigenetic marks from 15 participants were used as technical replicates for a total of 288 DNA methylation profiles.

Quality control

The pre-processing of the raw EPIC array files was conducted using the *RnBeads* package in the R (Assenov et al., 2014). Single nucleotide probes (SNPs)—enriched sites, probes with a high likelihood of cross-hybridization and probes with the highest fraction of unreliable measurement were removed. In addition, one DNA methylation profile was removed due to a high number of probes with unreliable measurements. A total of 287 profiles (including 15 replicates where one replicate was used to replace the profile with unreliable measurement) and 793 224 probes were then normalized using the Beta-Mixture Quantile dilation (BMIQ) model for comparative purposes (Teschendorff et al., 2013). Following the BMIQ normalization, 1120 probes were removed due to missing values resulting in 287 profiles (corresponding to n = 273 participants and 14 technical replicates) and a total of 792 104 probes for downstream analysis. To account for potential cell count heterogeneity, six cell types were estimated (CD8T, CD4T, NK, B cell, monocytes and granulocytes) using the *minfi* package in R (Aryee et al., 2014).

To be consistent with the overlap with adolescent age in participants from the Raine Study Gen2, a further 35 participants, aged 20 years or over, were excluded. Additional seven participants were removed due to being conceived using GIFT giving a final population of 231 ART-born participants for comparative analysis (a schematic diagram is shown in Fig. 1).

Downloaded from https://academic.oup.com/humrep/advance-article/doi/10.1093/humrep/deab078/6247390 by John Yovich on 28 April 2021

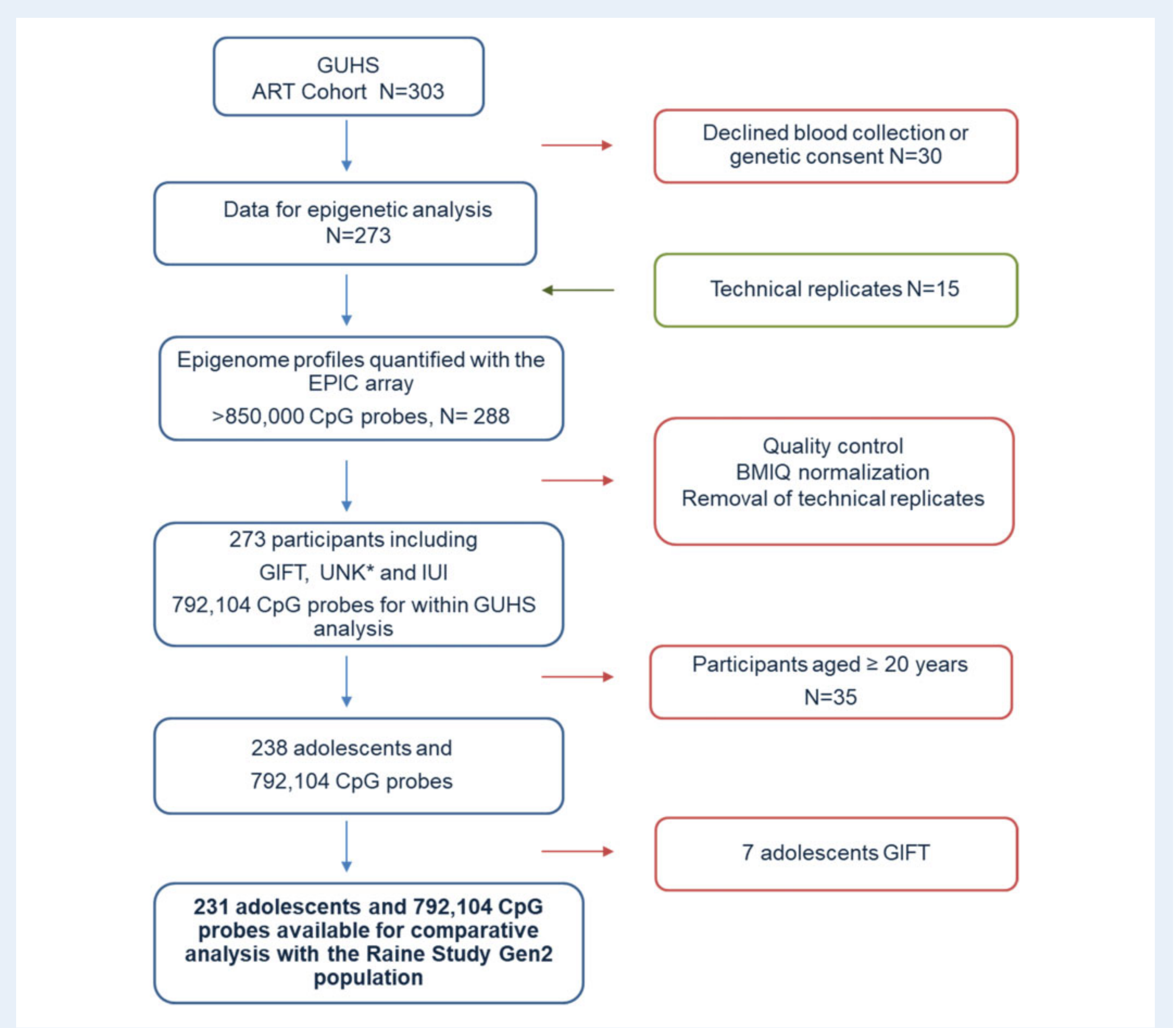

Figure 1. Flow chart of the Growing Up Healthy Study (GUHS) population included in the epigenetic analysis and quality control of the raw EPIC files of the quantified epigenomes. EPIC, Infinium Methylation EpicBead Chip Array; BMIQ, Beta Mixture Quantile dilation normalization; GIFT, Gamete intrafallopian transfer; UNK*, Unknown type (ART procedure confirmed, IVF/ICSI not specified).

To investigate the methylation differences within the GUHS cohort, from the 273 participants with epigenome profiles, 15 conceived through GIFT, one using IUI and another 18 participants with unknown, unspecified IVF/ICSI status, were omitted resulting in a total of 239 ART-born participants with clean phenotype. Demographic and clinical characteristics of the entire GUHS cohort (n = 273) are provided in the Supplementary Tables SI and SII.

DNA methylation profiling (the Raine Study Gen2)

Epigenome-wide DNA methylation profiling and quality control for the Raine Study Gen2 have been previously described (Rauschert *et al.*, 2019). Briefly, whole-blood samples collected at age 17 years, epigenome-wide DNA methylation profiles for 1260 (58 technical replicates) individuals were generated at the Centre for Molecular Medicine and Therapeutics, University of British Columbia using the Illumina Infinium HumanMethylation450 BeadChip array (Illumina, San Diego, CA). After quality control, removal of technical replicates and those undergoing ART as described above a total of 1188 participants remained available for analysis. These DNA methylation values were adjusted using BMIQ normalization (Teschendorff *et al.*, 2013).

Statistical analysis

Univariate analysis of differences between cohorts

The demographic data, maternal characteristics at time of conception and neonatal details for both studies, as well as the clinical information

Downloaded from https://academic.oup.com/humrep/advance-article/doi/10.1093/humrep/deab078/6247390 by John Yovich on 28 April 2021

Table II **Pregnancy details for the 'Growing Up Healthy Study' (GUHS) and Raine Study Gen2 participants and maternal demographics at the time of conception.**

Pregnancy details	GUHS participants	Gen2 participants	P-value
Sample size (n)	231	1188	
Age (years) mean ± SD	16 ± 1.6	17 ± 0.6	< 0.001
Sex male, n [%]	122 [52.8]	605 [50.9]	< 0.001
Sex female, n [%]	109 [47.2]	583 [49.1]	
Gestational age (weeks) n = 217, median (Q1—Q3)	38.57 (37.0–39.71)	39.27 (38.43–40.57)	< 0.001
Birth weight (g) n = 230, median (Q1—Q3)	3210 (2803.8–3582.5)	3312 (3010–3682.5)	< 0.001
Plurality n [%]			
Singleton	178 [77.1]	1165 [98.1]	< 0.001
Twin*	49 [21.2]	22 [1.9]	
Triplet	3 [1.3]	1 [0.1]	
Data N/A	1 [0.4]		
Maternal demographics at the time of conception	**Mothers of GUHS participants**	**Mothers of Gen2 participants (Raine Study)**	**P-value**
Age (years) n = 230, mean ± SD	33.9 ± 3.9	28.5 ± 5.8	< 0.001
Smoking n [%]			
Yes	15 [6.5]	339 [28.5]	< 0.001
Unknown	33 [14.3]	119 [10.0]	

Independent t-test and Chi-squared test were used to weigh differences between the cohorts for continual and categorical traits respectively. Data N/A, data not available.
*In one set of the twins only one of the twins participated.

regarding the IVF cycles of the mothers of GUHS offspring, are presented as mean ± SD, Median (Q1–Q3) and n (%) in Tables I and II. Independent *t*-tests and Pearson's Chi-squared tests were used to evaluate the differences between the cohorts for continual (quantitative) and categorical variables respectively.

Epigenome-wide DNA methylation association analysis between GUHS and the Raine Study Gen2

For association between 231 ART-born (GUHS) and 1188 naturally conceived (the Raine Study Gen2) adolescents, Firth's bias reduced logistic regression (Firth, 1993) was used with group as outcome and adjusting the model for 401 022 overlapping normalized BMIQ DNA methylation probes and technical variation due to the different EWAS arrays. Firth's method logistic regression allows for finite estimates in cases of separation using maximum likelihood (Fijorek and Sokoowski, 2012) and corrects for any potential sample size imbalance differences between the cohorts. Briefly, a total of 401 022 independent regression analyses were performed using the *logistf* package in R (Puhr et al., 2017) accounting for the previously mentioned six cell count estimates, age, sex and multiple births. To correct for multiple testing, a Bonferroni correction was applied to all EWAS analyses, resulting in a critical *P*-value threshold of 1.24E−07 (0.05/401 022).

In an additional EWAS, the methylation profiles from the multiple births in the GUHS cohort were compared to the whole Raine Study Gen2 using the same method as described above.

Epigenome-wide DNA methylation analysis within the GUHS cohort

To explore the potential differences within the GUHS cohort, we used linear regression to investigate differential DNA methylation with 792 104 DNA methylation probes in fresh versus frozen embryo transfer, cause of infertility (male, female, unexplained), IVF versus ICSI offspring and IVF versus ICSI offspring corrected for the type of embryo transfer using the *shinyGEM* (https://github.com/Hobbeist/shinyGEM) package in R. This package accounts for batch effects using the ComBat adjustment (Johnson et al., 2007). We used two models, the first adjusted for age, sex, maternal smoking, multiple births and the second model added these covariates along with estimated cell count heterogeneity. To correct for multiple testing, we used a Bonferroni corrected *P*-value threshold of 6.31E−08.

Differentially methylated regions (DMRs) GUHS Versus Raine Gen2

To identify differently methylated regions (DMRs) we used the *dmrff* package (https://github.com/perishky/dmrff), which identifies differentially methylated regions by combining EWAS summary statistics from nearby differentially methylated probes (Suderman et al., 2018). Significant differentially methylated regions were defined as regions spanning a set of DNA methylation sites with at most 500 bp between consecutive sites with nominal EWAS *P* values < 0.05 and effect estimates with the same direction between the GUHS and Raine Study participants. Resulting DMRs were annotated to the UCSC Refgene panel from the Illumina annotation file.

Downloaded from https://academic.oup.com/humrep/advance-article/doi/10.1093/humrep/deab078/6247390 by John Yovich on 28 April 2021

Gene set enrichment analysis

Gene set enrichment analysis was performed using the R-package *methylGSA* using DNA methylation sites with *P*-value < 0.001 and minimum size of 100 and maximum of 1000 (Ren and Kuan, 2019). This approach takes the varying CpG density per gene of the 450K array into account. We set the threshold for an enriched pathway to the false discovery rate (FDR) corrected *P*-value of 0.05 and report significant Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. We investigated these both between GUHS and the Raine Study Gen2 as well as between fresh versus frozen embryo transfer and IVF versus ICSI.

Calculation of epigenetic age within the GUHS cohort

We investigated the correlation between chronological age and four estimates of epigenetic age: Horvath's (Horvath, 2013) DNA age predictor based on the methylation levels of 353 informative DNA methylation probes and skin Horvath (Horvath *et al.*, 2018); Hannum's (Hannum *et al.*, 2013) 71 methylation markers for epigenetic age prediction; PhenoAge (Levine *et al.*, 2018), a composite biomarker of aging with 513 methylation probes within whole blood of the GUHS participants using the R-package *methylClock* (https://github.com/isglobal-brge/methylclock). This package accounts for both normalized data and cell count heterogeneity while calculating epigenetic age and allows for rapid correlation with chronological age.

Results

Descriptive statistics

Pregnancy details for the subset of 231 GUHS adolescents aged 13–19.9 years and the 1188 Raine Study Gen2 participants, as well as maternal demographics at the time of conception are presented in Table II. The mothers in the GUHS cohort were on average older at conception (33.9 vs. 28.5 years), less likely to be smokers (6.5% vs. 28.5%), with higher percentage of pregnancies resulting in multiple births (22.5% vs. 1.9%), delivering at an earlier gestation (38.6 vs. 39.3 weeks), and there were more male offspring than the Raine cohort (52.8% vs. 50.9%) ($P < 0.001$ for all variables). Relevant clinical information regarding the ART cycles and characteristics of subfertility of the parental generation in the GUHS cohort for the index pregnancy are summarized in Table I.

Epigenome-wide DNA methylation analysis

Comparative EWAS between the GUHS and the Raine Study Gen2 cohorts

The association of ART on DNA methylation levels of 231 ART-born adolescents was compared to the DNA methylation profiles of 1188 naturally conceived participants from the Raine Study *Gen2*. After adjustment for batch effects, as well as technical variation due to utilizing different methylation platforms between the cohorts, no DNA methylation probe reached a Bonferroni correction *P*-value threshold of < 1.24E−07 (0.05/401 022; Supplementary Fig. S1). Overall, 1437 DNA methylation profiles between the two groups showed nominal difference with a *P*-value <0.05 accounting for participant age, sex and multiple births. After adjustment for cell count heterogeneity, 38 of the compared DNA methylation probes were nominally significant.

Further, no significant difference was observed when we compared 52 DNA methylation profiles from multiple births (49 from 25 twin births, where in one set of twins, only one twin participated; and 1 set of triplets) in the GUHS cohort with the whole Raine Study Gen2. One thousand five hundred one methylation probes showed nominal significance ($P < 0.05$) after correcting for participant age, sex and multiple births. In the model where the correction for cell count was added to the previous mentioned covariates, only 18 methylation probes showed nominal significance.

EWAS analysis within the GUHS cohort

A total of 792 104 DNA methylation probes were investigated for difference in methylation marks comparing 146 fresh versus 93 frozen embryo transfers. A separate analysis, examined the methylation profiles of the 215 ART participants, adjusting for cause of infertility (male, female and unexplained). Twenty-six participants, where a single cause of infertility was not established (couples with both male and female cause of infertility) were omitted from the analysis. In both analyses, no DNA methylation probes reached a Bonferroni correction of 6.31E−08 for statistical significance for a positive correlation (data not presented).

When comparing the DNA methylation profiles between 181 IVF and 58 ICSI offspring, and after adjusting for age, sex, maternal smoking, multiple births and batch effect, three DNA methylation probes [cg 26744878 ($P = 2.86E{-}09$), cg 15016734 ($P = 2.59E{-}08$;) cg20233073 ($P = 4.09E{-}08$)] reached a Bonferroni correction of 6.31E−08 (Fig. 2A and data included in Table III). After correcting for cell count, two of these DNA methylation probes [cg 15016734 ($P = 5.87E{-}09$), cg 26744878 ($P = 1.61E{-}08$)] remained significant and further two more methylation probes have been identified: cg 20235051 ($P = 6.18E{-}08$) and cg 0331628 ($P = 3.92E{-}08$; Fig. 2B and data included in Table IV).

Additional CpG probes were identified between the IVF and ICSI offspring at an FDR of 5% after adjusting for age, sex, maternal smoking, multiple births, and batch effect (Table III) and cell count (Table IV). The observed difference in methylation marks, although significant, exerted a small effect size with a trend towards hypomethylation in the ICSI offspring.

The detected significant difference in methylation marks between IVF and ICSI offspring was lost when we further corrected for the type of embryo transfer, fresh IVF versus fresh ICSI ($n = 112$ versus $n = 34$) and frozen IVF versus frozen ICSI ($n = 69$ versus $n = 24$), potentially due to small sample size and loss of power.

In our cohort, ICSI was used predominantly for male factor infertility, with only small number of cases of ICSI ($n = 3$) performed for previous poor or failed fertilization with IVF.

Differentially methylated regions (DMRs) GUHS versus Raine Study Gen2

Between the GUHS and Raine Study cohorts, a total of 1499 DMR's were identified for the age and sex EWAS model. When adjusting for cell count heterogeneity in the model, the number of DMRs was reduced to 19. No DMRs reached statistical significance after correcting for multiple testing.

Downloaded from https://academic.oup.com/humrep/advance-article/doi/10.1093/humrep/deab078/6247390 by John Yovich on 28 April 2021

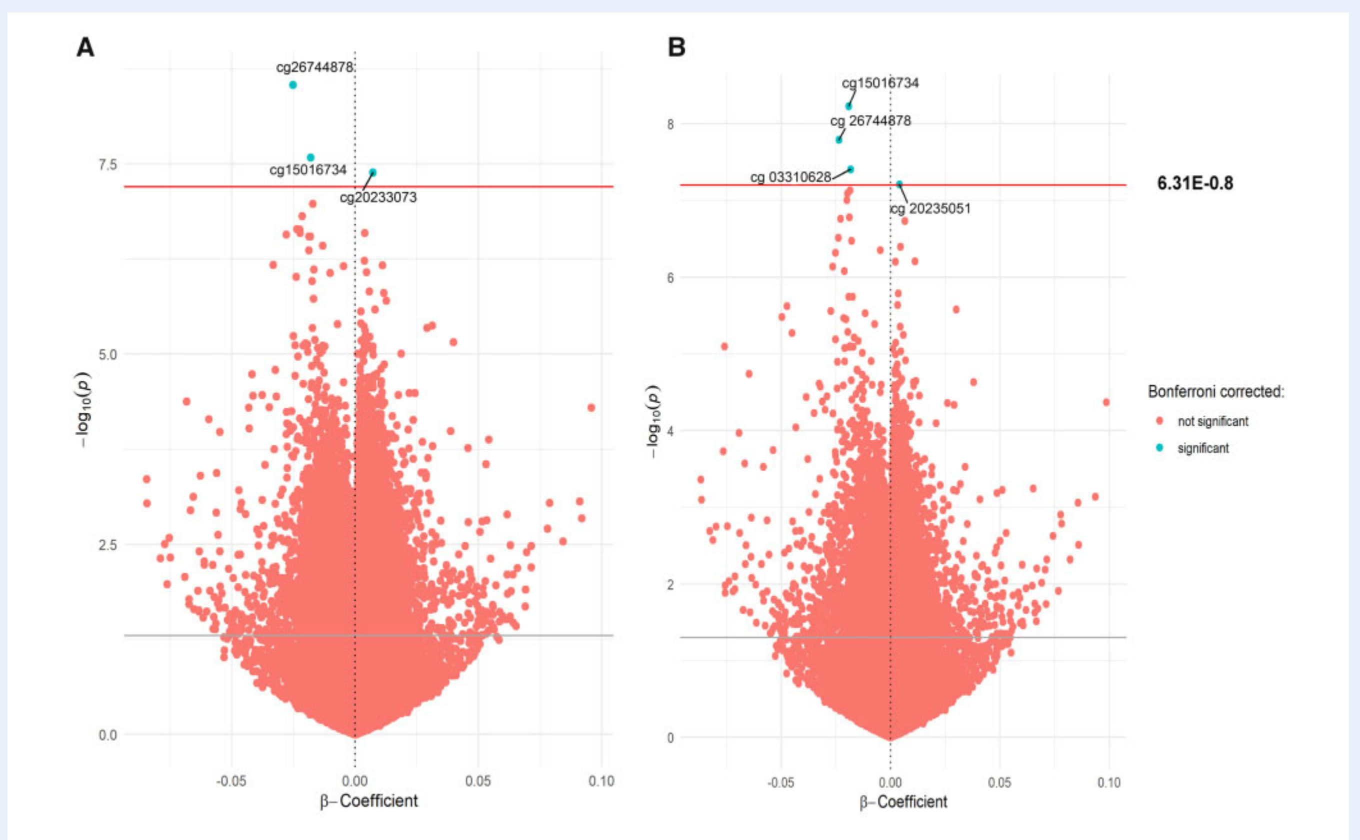

Figure 2. Volcano plots showing the effect size for each of the 792 104 DNA methylation probes plotted against the *P*-value for the comparative EWAS analysis between IVF and ICSI offspring within the GUHS cohort. The red lines represent the threshold barrier set to 6.31E−08 (0.05/792 104) for Bonferroni correction, taking into account the number of probes used in the analysis. **(A)** After adjusting for age, sex, maternal smoking, multiple births and batch effect the following methylation probes reached a Bonferroni correction significance: cg 26744878 ($P = 2.86E{-}09$), cg 15016734 ($P = 2.59E{-}08$) and cg 20233073 ($P = 4.09E{-}08$). **(B)** After adjusting for cell count, the DNA methylation probes: cg 15016734 ($P = 5.87E{-}09$), cg 26744878 ($P = 1.61E{-}08$), cg 03310628 ($P = 3.92E{-}08$) and cg 20235051 ($P = 6.18E{-}08$) reached the pre-set Bonferroni correction.

Gene set enrichment analysis (GSEA)

To enhance the understanding of potential functional significance of differential methylation, we applied GSEA. Results from the EWAS analyses were inputted to identify KEGG pathways enriched amongst the most significantly altered CpGs.

Comparative GSEA between GUHS and the Raine Study Gen2 cohorts

For the comparison between the Raine Study Gen2 and GUHS participants, no significant biological pathways were identified.

GSEA analysis within GUHS cohort

The GSEA identified the neuroactive ligand–receptor interaction pathway, which remained significant ($P = 0.00048$) after adjusting for age and sex when comparing the IVF and ICSI offspring (Table V). The neuroactive ligand-receptor pathway incorporates some 302 genes that have been implicated in addictive disorders.

There were no enriched biological pathways identified when comparing fresh versus frozen embryo transfers.

DNA methylation age (DNAmAge) within GUHS cohort analysis

In the analysis of epigenetic aging, within whole blood of 239 IVF/ICSI born participants, all four measures of DNAmAge (Hannum, skinHorvath, PhenoAge, Horvath) were significantly correlated with chronological age and did not demonstrate an elevated epigenetic aging. The PhenoAge age estimate provided the weakest correlation for accelerated aging ($r^2 = 0.23$) and skin Horvath had the best fit with ($r^2 = 0.61$), followed by Horvath ($r^2 = 0.35$) and Hannum ($r^2 = 0.28$; Fig. 3).

Discussion

Our comparative analysis of the DNA methylation signatures of ART and naturally conceived children demonstrated no significant differences in their overall DNA-methylation signatures, utilizing the 401 022 overlapping DNA methylation probes. The DNA methylation profiles were not quantified contemporaneously, and, hence, were performed with the most available DNA methylation platform at the time.

Downloaded from https://academic.oup.com/humrep/advance-article/doi/10.1093/humrep/deab078/6247390 by John Yovich on 28 April 2021

Table III **List of DNA methylation probes, significantly different between IVF and ICSI offspring, which reached 5% false discovery rate (FDR) significance after adjusting for age, sex, maternal smoking, multiple births and batch effect.**

DNA methylation probe	β	Stats	*P*-value	FDR	UCSC Reference Gene Name	Position
cg26744878	−0.025219768	−6.148237621	2.86E−09	0.0023	*NA*	Chr2: 27348040
cg15016734	−0.018003349	−5.739120863	2.59E−08	0.0102	*ADD2*	Chr2: 70742832
cg20233073	0.007104223	5.651451635	4.09E−08	0.0108	*NA*	Chr4: 54654983
cg00693157	−0.023647994	−5.315129417	2.26E−07	0.0186	*CES7*	Chr16: 54467995
cg01303685	−0.02803103	−5.282927597	2.65E−07	0.0186	*NA*	Chr20: 24037848
cg03310628	−0.017241501	−5.468988528	1.04E−07	0.0186	*WDR33*	Chr2: 128179975
cg09935822	−0.018809712	−5.271398511	2.80E−07	0.0186	*DLAT*	Chr11: 111405510
cg16046769	−0.01821944	−5.270141861	2.82E−07	0.0186	*NA*	Chr10: 50021217
cg16903016	−0.021569131	−5.39329178	1.53E−07	0.0186	*NA*	Chr1: 55548240
cg20235051	0.003845792	5.292324287	2.53E−07	0.0186	*C17orf82*	Chr17: 56844634
cg22994586	−0.02233529	−5.291989617	2.53E−07	0.0186	*MLLT1*	Chr19: 6214524
cg23157501	−0.022446932	−5.312272546	2.29E−07	0.0186	*NA*	Chr1: 209446585
cg05916456	−0.013150599	−5.21277594	3.74E−07	0.0228	*NA*	Chr10: 65468928
cg08861930	−0.018792251	−5.183614891	4.31E−07	0.0244	*NA*	Chr8: 56928808
cg06694040	0.011000582	5.090915405	6.75E−07	0.0302	*GALNTL4*	Chr11: 11258275
cg07396904	−0.004727153	−5.087132278	6.87E−07	0.0302	*OR10G2*	Chr14: 21173344
cg08004620	−0.033233051	−5.09319965	6.68E−07	0.0302	*MYOM2*	Chr8: 2066776
cg21034023	0.003752798	5.118741709	5.90E−07	0.0302	*UGGT2*	Chr13: 95503930
cg10897045	−0.01684985	−5.064721872	7.65E−07	0.0319	*MLL3*	Chr7: 151507397
cg10953604	−0.010256689	−5.041176693	8.56E−07	0.0323	*ERBB4*	Chr2: 212340065
cg23531640	0.004555139	5.046562047	8.35E−07	0.0323	*ETS1*	Chr11: 127882462
cg01410279	−0.023902056	−5.016683705	9.62E−07	0.0347	*MYOC*	Chr1: 169888564
cg16764236	−0.017474797	−4.991242501	1.09E−06	0.0374	*CCL11*	Chr17: 29635403
cg09866569	0.005749833	4.924556289	1.49E−06	0.0491	*KCTD5*	Chr16: 2677341
cg12584702	0.011646186	4.914444158	1.56E−06	0.0494	*ETV7*	Chr6: 36462411

β—represents the effect size, with ‘−’ and ‘+’ values indicating the direction of the effect to be hypo-methylated and hyper-methylated respectively; USCS, University of California Santa Cruz Genome Browser.

The lack of differences between the ART offspring and their naturally conceived counterparts was observed despite significant difference in maternal age (Adkins *et al.*, 2011) and smoking (Joubert *et al.*, 2016; Rauschert *et al.*, 2019), as well as gestational age at birth (Merid *et al.*, 2020), all factors that have previously been shown to affect the DNA methylation in their offspring.

The observed significant differences in the demographic characteristics between the two studies were expected and may in part be explained by the imbalanced sample size, as well as by the expected features of an ART cohort, such as older mothers and who are less likely to smoke cigarettes due to them embarking on ART treatment.

The ‘Clinical Review of the Health of adults conceived following Assisted Reproductive Technologies’ (CHART) study (Lewis *et al.*, 2017; Halliday *et al.*, 2019), in Melbourne, found no difference in the growth, respiratory health, cardiovascular and cardiometabolic risk in 193 adults aged 22–35 conceived by IVF, when compared to 86 naturally conceived adults. Novakovic *et al*, additionally used longitudinal EWAS approach to investigate the DNA methylation signature in the neonatal period and adulthood and demonstrated that the potentially early ART-related epigenetic variation detected at birth was not persistent (Novakovic *et al.*, 2019) and mitigated by adulthood. They were able to replicate and confirm their findings of altered DNA methylation profiles in neonates in an independent cohort (Estill *et al.*, 2016).

Other, albeit significantly smaller studies (Katari *et al.*, 2009; Melamed *et al.*, 2015; Estill *et al.*, 2016; El Hajj *et al.*, 2017) have also demonstrated findings in favour of correlation between ART and/or couples’ infertility and changes in the DNA methylation signature in the offspring which contradicts the notion that the epigenomes of the ART born offspring are stable (Feng *et al.*, 2011), and do not have an increased risk of DNA methylation defects (Manning *et al.*, 2000; Oliver *et al.*, 2012).

Embryological laboratory procedures undertaken during IVF procedures, such as the differing oxygen tensions (5% vs. 20% O_2), the type of embryo used (fresh vs. frozen) have also been associated with differences in DNA methylation levels when comparing the placentas of subgroups of IVF and naturally conceived pregnancies (Ghosh *et al.*, 2017). We found no difference in the methylation profiles when comparing offspring developed from fresh or frozen embryo transfer, and

Downloaded from https://academic.oup.com/humrep/advance-article/doi/10.1093/humrep/deab078/6247390 by John Yovich on 28 April 2021

Table IV List of DNA methylation probes, significantly different between the IVF and ICSI offspring, which reached 5% false discovery rate (FDR) significance after adjusting for cell count.

DNA methylation probes	β	Stats	*P*-value	FDR	UCSC reference gene name	Position
cg15016734	−0.019042303	−6.021322166	5.87E−09	0.0047	*ADD2*	Chr2:70742832
cg26744878	−0.023572265	−5.83330929	1.61E−08	0.0064	*NA*	Chr2:27348040
cg03310628	−0.018168669	−5.663364851	3.92E−08	0.0104	*WDR33*	Chr2:128179975
cg20235051	0.004112412	5.575422926	6.18E−08	0.0107	*C17orf82*	Chr17:56844634
cg10897045	−0.018461505	−5.542158822	7.32E−08	0.0107	*MLL3*	Chr7:151507397
cg09935822	−0.019724953	−5.523175613	8.07E−08	0.0107	*DLAT*	Chr11:111405510
cg08861930	−0.019962081	−5.483185571	9.89E−08	0.0112	*NA*	Chr8:56928808
cg16046769	−0.018862608	−5.381101969	1.65E−07	0.0146	*NA*	Chr10:50021217
cg22994586	−0.022915999	−5.37298285	1.72E−07	0.0146	*MLLT1*	Chr19:6214524
cg20233073	0.006504555	5.358701441	1.85E−07	0.0146	*NA*	Chr4:54654983
cg00693157	−0.023824988	−5.255879357	3.07E−07	0.0221	*CES7*	Chr16:54467995
cg10397223	−0.017796342	−5.238149075	3.35E−07	0.0221	*ZBTB16*	Chr11:113576772
cg23531640	0.004472103	5.201787797	4.00E−07	0.0244	*ETS1*	Chr11:127882462
cg07396904	−0.004691177	−5.180591471	4.44E−07	0.0251	*OR10G2*	Chr14:21173344
cg01410279	−0.025134712	−5.164827249	4.79E−07	0.0253	*MYOC*	Chr1:169888564
cg06694040	0.011158974	5.111575616	6.20E−07	0.0292	*GALNTL4*	Chr11:11258275
cg10785051	0.00223237	5.108968897	6.28E−07	0.0292	*TCF7*	Chr5:133477826
cg01303685	−0.026422902	−5.079302315	7.24E−07	0.0318	*NA*	Chr20:24037848
cg23157501	−0.02116235	−5.051450423	8.27E−07	0.0345	*NA*	Chr1:209446585

β—represents the effect size, with '−' and '+' values indicating the direction of the effect to be hypo-methylated and hyper-methylated respectively; USCS, University of California Santa Cruz Genome Browser.

Table V Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis to identify enriched biological pathways amongst the significantly altered methylation probes when comparing IVF and ICSI offspring within the 'Growing Up Healthy Study' (GUHS) cohort adjusting for age, sex, multiple births and maternal smoking.

Description	Size	Count	*P*-value	FDR (5%)
Neuroactive ligand-receptor interaction	**334**	**43**	**4.29E−06**	**0.00048**
Signalling pathways regulating pluripotency of stem cells	142	22	0.005211	0.255127
Calcium signalling pathway	191	29	0.006834	0.255127
cAMP signalling pathway	216	30	0.009217	0.258085
Cell adhesion molecules (CAMs)	143	21	0.011636	0.260650
Hippo signalling pathway	157	24.5	0.015939	0.281647
Pathways in cancer	526	60	0.018415	0.281647
Relaxin signalling pathway	129	19.5	0.023317	0.281647
Cytokine-cytokine receptor interaction	283	22	0.024112	0.281647
Hepatocellular carcinoma	168	23	0.025376	0.281647

FDR, false discovery rate.

we would encourage a replication of this outcome in unrelated cohort to strengthen our finding.

Personal susceptibility to epigenetic modification has been explored by Ghosh *et al.* as a potential factor contributing to the altered DNA methylation signatures in ART-born offspring. Only a small proportion of individuals, 'outliers', with a particular clinical phenotype (low birth weight), appeared to be more susceptible to changes in the DNA methylation signatures due to the laboratory procedures undertaken during IVF in comparison to the naturally conceived counterparts (Ghosh *et al.*, 2016). We were unable to verify this assertion, as we do not have the neonatal epigenome profiles and only four of the GUHS adolescents appeared growth restricted at term (<2.5 kg) not allowing us to validate this in our cohort. Published meta-analysis has shown that the observed differences in DNA methylation in association with birth weight (Kupers *et al.*, 2019) and gestational age (Merid *et al.*, 2020), only marginally persist in adolescence, but not into adulthood.

Our study provided no support for a DNA methylation-based epigenetic cause for the reported increased risk of cardiovascular disorders, such as high blood pressure (Ceelen *et al.*, 2008; Guo *et al.*, 2017; Zandstra *et al.*, 2020) and premature vascular aging (Meister *et al.*, 2018), in ART-born children. Therefore, the risks may relate to their genetic predisposition or potentially other non-DNA methylation-related epigenetic influences. A potential reason that we were unable to determine any differences, between ART children and naturally

Downloaded from https://academic.oup.com/humrep/advance-article/doi/10.1093/humrep/deab078/6247390 by John Yovich on 28 April 2021

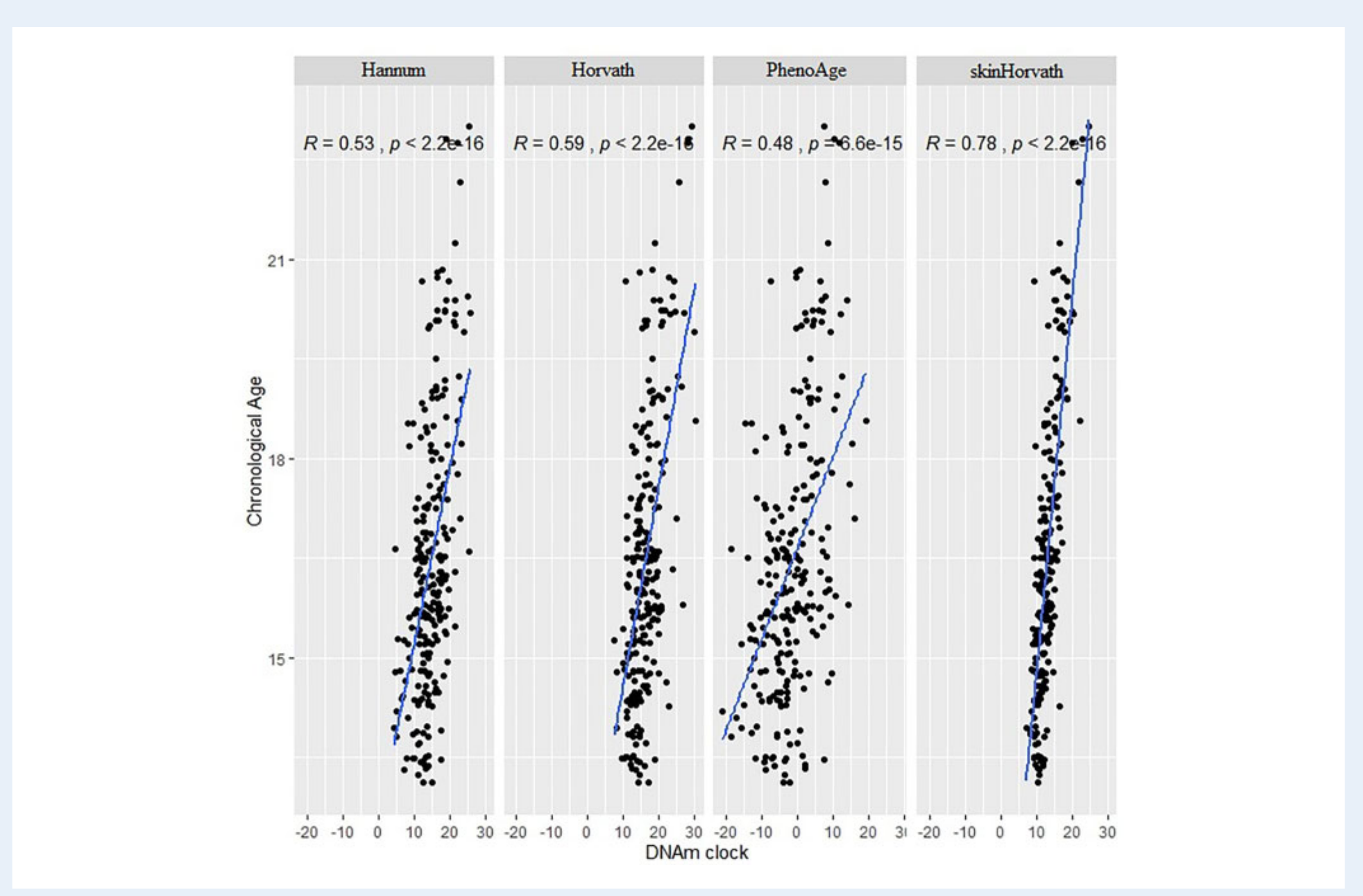

Figure 3. Moderate to high correlation of DNA methylation age (DNAmAge) with chronological age in the IVF born offspring within whole blood. Outliers show degree of accelerated aging from unknown reason, possibly preterm birth, multiple birth, BMI.

conceived children, is that in this study, embryo transfers were routinely performed on Day 2 after egg collection, consistent with practice at the time, whereas current practice favours blastocyst stage transfer. Hence, the use of extended culture may provide an opportunity for further epigenetic re-programming not detected with the short embryo culture reported in this study.

In contrast to the reassuring findings when the ART group was compared to the naturally conceived group, and comparing children born from fresh and from frozen embryos within the ART group, we demonstrated a significant difference in DNA methylation levels between children conceived with IVF and ICSI treatment. This is of particular relevance as, in contrast to the rate of ICSI when these children were conceived in the 1990s, the rate of ICSI across the world has substantially increased to 67% in Australia, in the most recent data analysis (Newman *et al.*, 2019). Consequently, it will be important to replicate this comparison in other cohorts across the world.

Links between ICSI and subfertility have also been investigated as potential contributors to the observed differences in the DNA methylation patterns at specific genomic loci in the offspring; however, the range of methylation variation was found to be no different when compared to the naturally conceived offspring (Estill *et al.*, 2016; El Hajj *et al.*, 2017). Furthermore, an aberrant methylation of the imprinted genes within spermatozoa of men with severe oligospermia, may in part contribute to a potential increase in the incidence of imprinting disorders in IVF/ICSI conceived children (Kobayashi *et al.*, 2009).

A meta-analysis confirmed the purported increased risk of imprinting disorders in IVF and ICSI conceived offspring (Lazaraviciute *et al.*, 2014). Furthermore, in a recent review by Hutanu *et al.*, the authors concluded that ICSI may indeed induce epigenetic changes that may be transmitted in the offspring (Hutanu *et al.*, 2019). However, an investigation into the epigenetic profile of developing blastocysts reported no difference in the occurrence of epigenetic errors, regardless of whether the developing blastocyst originated from an ICSI or IVF cycle as determined by genome-wide DNA methylation analysis coupled with chromatin organization in human embryos (Santos *et al.*, 2010). Whether the claimed differences in methylation patterns relate to the procedure itself, or to the intrinsic features of the subfertile couple requiring ICSI, will only be determined by further study, as it is well established that the use of ICSI has its unique indications (Practice Committees of the American Society for Reproductive and the Society for Assisted Reproductive Technology. Electronic address, 2020). Hence, it will be of great importance to replicate our findings in other cohorts across the world.

Of particular interest, we identified that the ligand–receptor interaction pathway was differentially regulated between the IVF and ICSI born children. This finding may be relevant, as this pathway is believed

Downloaded from https://academic.oup.com/humrep/advance-article/doi/10.1093/humrep/deab078/6247390 by John Yovich on 28 April 2021

to be related to addictive disorder in adulthood (Biernacka et al., 2013); this may in part explain why a preponderance of binge drinking has been reported in young adults conceived from ART (Beydoun et al., 2010).

The dynamic changes of DNA methylation levels throughout lifetime, such as global loss of DNA methylation and region-specific hypermethylation (Johnson et al., 2012), have become the new predictor of biological aging (Xiao et al., 2019). An association of 'accelerated aging', derived from a difference between the epigenetic-predicted ages and chronological age, and later life comorbidities and increased mortality risk has been proposed (Marioni et al., 2015). Hence, it is reassuring that when we analysed the 239 IVF/ICSI born participants, despite some individuals with accelerated aging, overall, there is moderate to high correlation between the chronological and epigenetic predicted age within the whole blood of IVF/ICSI conceived adolescents.

Even though our data concluded no observable difference in the overall DNA methylation levels between the ART-born and naturally conceived adolescents, we have not excluded the possibility that alternative epigenetic mechanisms, such as histone modification, imprinting and non-coding RNA regulation (El Hajj et al., 2017) may be at play.

Study limitations

While the sample size is a limiting factor in this study, our cohort of 231 participants is relatively large compared to most other ART DNA methylation studies. Due to our small sample size, we have limited statistical power to determine if our nominally significant findings represent true results.

In addition, the use of whole-blood, limits our ability to identify tissue-specific differences as DNA methylation is thought to be tissue-specific and whole-blood is a heterogeneous tissue comprised of several cell types. However, several studies have shown moderate to strong correlations between blood tissue DNA methylation and other tissue types such as subcutaneous fat (Wahl et al., 2017). To reduce the effect of heterogeneity on our study, we adjusted for estimated cell count.

The EPIC array only captures a small part of the methylome (∼3%), so there is a possibility that we may be missing some rare individual differences, or that other non-methylation-based epigenetic differences could exist that are so far undetected. The DNA-methylation profiles of the GUHS and the Raine Gen2 participants were not quantified contemporaneously and, hence, were performed using two most robustly available DNA methylation platforms at the time, the EPIC and 450 K array, respectively. Previous studies have successfully compared the DNA methylation profiles from the two platforms (Novakovic et al., 2019). Studies have previously addressed the possible problems with comparing the methylation profiles between the two platforms with reassuring findings, such as a high correlation in the overall methylation patterns, cell type proportion estimates and strong replication of differentially methylated probes (Solomon et al., 2018). Additionally, this study may not be directly relevant to current practice as routine blastocyst culture is standard, whereas in our study, Day 2 embryo culture was performed. Our findings require replication in an independent cohort with longer duration of embryo culture.

Conclusion

Our large study of adolescents and young adults conceived by ART demonstrated that there are no significant differences in the DNA methylation profiles of individuals born from ART when compared to their naturally conceived similar aged counterparts, using a conservative Bonferroni approach to account for multiple testing. Two additional analyses looking at DMRs between the cohorts and four measures of accelerated aging in the whole blood of ART conceived offspring demonstrated similar results, providing further evidence of no difference in DNA methylation between the cohorts. However, we did identify some differences in the DNA methylation profiles when IVF conceived offspring were compared to those conceived by ICSI treatment. Overall, there is a need to better understand the complex outcomes and effects of these widely used ART techniques as well as the gamete and embryo manipulation and cryopreservation on the DNA methylation profiles of adolescents. Replication in an independent cohort would strengthen our findings.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article cannot be shared publicly for ethical reasons and privacy protection of the individuals that participated in the study. The data will be shared on reasonable request to the corresponding author.

Acknowledgements

The author's would like to acknowledge the following: Families participating in GUHS; GUHS staff; Professor Jeffrey Keelan for proofreading the manuscript; Department of Health Western Australia, Midwives Notification System; PathWest Laboratory Medicine—Perth, Western Australia; Staff at the PIVET Medical Centre and Concept Fertility Centre; The Raine Study participants and their families; The Raine Study team for cohort co-ordination and data collection; NHMRC epigenetics project grant (Huang et al., ID 1059711); NHMRC fellowship Huang et al., ID 1053384; NHMRC ID 1142858; European Union H2020-SC1-2016-2017 ID 733206; The University of Western Australia; Curtin University; Women and Infants Research Foundation; Telethon Kids Institute; Edith Cowan University; Murdoch University; The University of Notre Dame Australia; The Raine Medical Research Foundation; The Pawsey Supercomputing Centre provided computation resources to carry out analyses required with funding from the Australian Government and the Government of Western Australia.

Authors' roles

B.PV. was involved in data quality control, analyses, interpretation of results and discussion and wrote the draft manuscript. P.E.M. designed the DNA methylation modelling, performed the statistical analyses and reviewed the interpretation of results. R.C.H. provided expert opinion.

Downloaded from https://academic.oup.com/humrep/advance-article/doi/10.1093/humrep/deab078/6247390 by John Yovich on 28 April 2021

J.L.Y. and P.B. provided the necessary IVF clinical data. L.A.W. assisted with data clean up. R.J.H. played vital role in the inception and study design, interpretation of results and contributed to the critical discussion. All authors reviewed and agreed to the final version of the manuscript.

Funding

This project was funded by NHMRC project grant number 1042269 and R.J.H. has received funding support from Ferring Pharmaceuticals.

Conflict of interest

R.J.H. is the Medical Director of Fertility Specialists of Western Australia and a shareholder in Western IVF. He has received educational sponsorship from Merck Sharp & Dohme Corp.-Australia, Merck-Serono Australia Pty Ltd and Ferring Pharmaceuticals Pty Ltd. P.B. is the Scientific Director of Concept Fertility Centre, Subiaco, Western Australia. J.L.Y. is the Medical Director of PIVET Medical Centre, Perth, Western Australia. The other authors have no conflicts of interest.

References

Adkins RM, Thomas F, Tylavsky FA, Krushkal J. Parental ages and levels of DNA methylation in the newborn are correlated. *BMC Med Genet* 2011;**12**:47.

Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, Irizarry RA. Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. *Bioinformatics* 2014;**30**:1363–1369.

Assenov Y, Muller F, Lutsik P, Walter J, Lengauer T, Bock C. Comprehensive analysis of DNA methylation data with RnBeads. *Nat Methods* 2014;**11**:1138–1140.

Bellver J, Donnez J. Introduction: infertility etiology and offspring health. *Fertil Steril* 2019;**111**:1033–1035.

Bellver J, Mariani G. Impact of parental over- and underweight on the health of offspring. *Fertil Steril* 2019;**111**:1054–1064.

Bergh C, Pinborg A, Wennerholm UB. Parental age and child outcomes. *Fertil Steril* 2019;**111**:1036–1046.

Beydoun HA, Sicignano N, Beydoun MA, Matson DO, Bocca S, Stadtmauer L, Oehninger S. A cross-sectional evaluation of the first cohort of young adults conceived by in vitro fertilization in the United States. *Fertil Steril* 2010;**94**:2043–2049.

Biernacka JM, Geske J, Jenkins GD, Colby C, Rider DN, Karpyak VM, Choi DS, Fridley BL. Genome-wide gene-set analysis for identification of pathways associated with alcohol dependence. *Int J Neuropsychopharmacol* 2013;**16**:271–278.

Brezinka C, Khanjani S. Assisted reproduction techniques (ART) and fetal growth. In Lees C, Visser G, Hecher K (eds). *Placental-Fetal Growth Restriction*. Cambridge, UK: Cambridge University Press, 2018, 44–47.

Cantone I, Fisher AG. Epigenetic programming and reprogramming during development. *Nat Struct Mol Biol* 2013;**20**:282–289.

Castillo-Fernandez JE, Loke YJ, Bass-Stringer S, Gao F, Xia Y, Wu H, Lu H, Liu Y, Wang J, Spector TD *et al.* DNA methylation changes at infertility genes in newborn twins conceived by in vitro fertilisation. *Genome Med* 2017;**9**:28.

Catford SR, McLachlan RI, O'Bryan MK, Halliday JL. Long-term follow-up of ICSI-conceived offspring compared with spontaneously conceived offspring: a systematic review of health outcomes beyond the neonatal period. *Andrology* 2018;**6**:635–653.

Cavalli G, Heard E. Advances in epigenetics link genetics to the environment and disease. *Nature* 2019;**571**:489–499.

Ceelen M, van Weissenbruch MM, Vermeiden JP, van Leeuwen FE, Delemarre-van de Waal HA. Cardiometabolic differences in children born after in vitro fertilization: follow-up study. *J Clin Endocrinol Metab* 2008;**93**:1682–1688.

Choufani S, Turinsky AL, Melamed N, Greenblatt E, Brudno M, Berard A, Fraser WD, Weksberg R, Trasler J, Monnier P, 3D Cohort Study Group. Impact of assisted reproduction, infertility, sex and paternal factors on the placental DNA methylome. *Hum Mol Genet* 2019;**28**:372–385.

Choux C, Binquet C, Carmignac V, Bruno C, Chapusot C, Barberet J, Lamotte M, Sagot P, Bourc'his D, Fauque P. The epigenetic control of transposable elements and imprinted genes in newborns is affected by the mode of conception: ART versus spontaneous conception without underlying infertility. *Hum Reprod* 2018;**33**:331–340.

Davies MJ. Infertility treatment at the edge: discovery and risk converge at the limits of knowledge. *Arch Dis Child* 2013;**98**:89–90.

Doherty DA, Newnham JP, Bower C, Hart R. Implications of polycystic ovary syndrome for pregnancy and for the health of offspring. *Obstet Gynecol* 2015;**125**:1397–1406.

Dontje ML, Eastwood P, Straker L. Western Australian pregnancy cohort (Raine) Study: Generation 1. *BMJ Open* 2019;**9**:e026276.

El Hajj N, Haaf T. Epigenetic disturbances in in vitro cultured gametes and embryos: implications for human assisted reproduction. *Fertil Steril* 2013;**99**:632–641.

El Hajj N, Haertle L, Dittrich M, Denk S, Lehnen H, Hahn T, Schorsch M, Haaf T. DNA methylation signatures in cord blood of ICSI children. *Hum Reprod* 2017;**32**:1761–1769.

Estill MS, Bolnick JM, Waterland RA, Bolnick AD, Diamond MP, Krawetz SA. Assisted reproductive technology alters deoxyribonucleic acid methylation profiles in bloodspots of newborn infants. *Fertil Steril* 2016;**106**:629–639.

Fauser BC. Towards the global coverage of a unified registry of IVF outcomes. *Reprod Biomed Online* 2019;**38**:133–137.

Feng C, Tian S, Zhang Y, He J, Zhu XM, Zhang D, Sheng JZ, Huang HF. General imprinting status is stable in assisted reproduction-conceived offspring. *Fertil Steril* 2011;**96**:1417–1423.

Fijorek K, Sokoowski A. Separation-resistant and bias-reduced logistic regression: STATISTICA Macro. *J Stat Soft* 2012;**47**:1–12.

Firth D. Bias reduction of maximum likelihood estimates. *Biometrika* 1993;**80**:27–38.

Fransquet PD, Wrigglesworth J, Woods RL, Ernst ME, Ryan J. The epigenetic clock as a predictor of disease and mortality risk: a systematic review and meta-analysis. *Clin Epigenet* 2019;**11**:62.

Gentilini D, Somigliana E, Pagliardini L, Rabellotti E, Garagnani P, Bernardinelli L, Papaleo E, Candiani M, Di Blasio AM, Viganò P. Multifactorial analysis of the stochastic epigenetic variability in cord blood confirmed an impact of common behavioral and environmental factors but not of in vitro conception. *Clin Epigenet* 2018;**10**:77.

Downloaded from https://academic.oup.com/humrep/advance-article/doi/10.1093/humrep/deab078/6247390 by John Yovich on 28 April 2021

Ghosh J, Coutifaris C, Sapienza C, Mainigi M. Global DNA methylation levels are altered by modifiable clinical manipulations in assisted reproductive technologies. *Clin Epigenet* 2017;**9**:14.

Ghosh J, Mainigi M, Coutifaris C, Sapienza C. Outlier DNA methylation levels as an indicator of environmental exposure and risk of undesirable birth outcome. *Hum Mol Genet* 2016;**25**:123–129.

Guo XY, Liu XM, Jin L, Wang TT, Ullah K, Sheng JZ, Huang HF. Cardiovascular and metabolic profiles of offspring conceived by assisted reproductive technologies: a systematic review and meta-analysis. *Fertil Steril* 2017;**107**:622–631.

Halliday J, Lewis S, Kennedy J, Burgner DP, Juonala M, Hammarberg K, Amor DJ, Doyle LW, Saffery R, Ranganathan S. *et al.* Health of adults aged 22 to 35 years conceived by assisted reproductive technology. *Fertil Steril* 2019;**112**:130–139.

Han L, Zhang H, Kaushal A, Rezwan FI, Kadalayil L, Karmaus W, Henderson AJ, Relton CL, Ring S, Arshad SH. *et al.* Changes in DNA methylation from pre- to post-adolescence are associated with pubertal exposures. *Clin Epigenet* 2019;**11**:176.

Hann M, Roberts SA, D'Souza SW, Clayton P, Macklon N, Brison DR. The growth of assisted reproductive treatment-conceived children from birth to 5 years: a national cohort study. *BMC Med* 2018;**16**:224.

Hannum G, Guinney J, Zhao L, Zhang L, Hughes G, Sadda S, Klotzle B, Bibikova M, Fan JB, Gao Y. *et al.* Genome-wide methylation profiles reveal quantitative views of human aging rates. *Mol Cell* 2013;**49**:359–367.

Hansen M, Greenop KR, Bourke J, Baynam G, Hart RJ, Leonard H. Intellectual disability in children conceived using assisted reproductive technology. *Pediatrics* 2018;**142**:e20181269.

Hansen M, Kurinczuk JJ, Milne E, de Klerk N, Bower C. Assisted reproductive technology and birth defects: a systematic review and meta-analysis. *Hum Reprod Update* 2013;**19**:330–353.

Hart R, Norman RJ. The longer-term health outcomes for children born as a result of IVF treatment: Part I-General health outcomes. *Hum Reprod Update* 2013a;**19**:232–243.

Hart R, Norman RJ. The longer-term health outcomes for children born as a result of IVF treatment. Part II-Mental health and development outcomes. *Hum Reprod Update* 2013b;**19**:244–250.

Heiss JA, Brennan KJ, Baccarelli AA, Tellez-Rojo MM, Estrada-Gutierrez G, Wright RO, Just AC. Battle of epigenetic proportions: comparing Illumina's EPIC methylation microarrays and TruSeq targeted bisulfite sequencing. *Epigenetics* 2020;**15**:174–182.

Holman CD, Bass AJ, Rouse IL, Hobbs MS. Population-based linkage of health records in Western Australia: development of a health services research linked database. *Aust N Z J Public Health* 1999; **23**:453–459.

Horvath S. DNA methylation age of human tissues and cell types. *Genome Biol* 2013;**10**:e1001551.

Horvath S, Oshima J, Martin GM, Lu AT, Quach A, Cohen H, Felton S, Matsuyama M, Lowe D, Kabacik S. *et al.* Epigenetic clock for skin and blood cells applied to Hutchinson Gilford Progeria Syndrome and ex vivo studies. *AGING (Albany NY)* 2018;**10**:1758–1775.

Huntriss J, Balen AH, Sinclair KD, Brison DR, Picton HM, Royal College of Obstetricians Gynaecologists. Epigenetics and reproductive medicine: Scientific Impact Paper No. 57. *Bjog* 2018;**125**: e43–e54.

Hutanu D, Bechir M, Popescu R. Epigenetics, assisted reproduction, and intracytoplasmic sperm injection: a review of the current data. *Eur Med J* 2019;**4**:36–42.

Jiang Z, Wang Y, Lin J, Xu J, Ding G, Huang H. Genetic and epigenetic risks of assisted reproduction. *Best Pract Res Clin Obstet Gynaecol* 2017;**44**:90–104.

Johnson AA, Akman K, Calimport SR, Wuttke D, Stolzing A, de Magalhaes JP. The role of DNA methylation in aging, rejuvenation, and age-related disease. *Rejuvenation Res* 2012;**15**:483–494.

Johnson WE, Li C, Rabinovic A. Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics* 2007; **8**:118–127.

Joubert BR, Felix JF, Yousefi P, Bakulski KM, Just AC, Breton C, Reese SE, Markunas CA, Richmond RC, Xu CJ. *et al.* DNA methylation in newborns and maternal smoking in pregnancy: genome-wide consortium meta-analysis. *Am J Hum Genet* 2016;**98**:680–696.

Kallen B, Finnstrom O, Nygren K, Olausson PO. Asthma in Swedish children conceived by in vitrofertilisation. *Arch Dis Child* 2013;**98**: 92–96.

Katari S, Turan N, Bibikova M, Erinle O, Chalian R, Foster M, Gaughan JP, Coutifaris C, Sapienza C. DNA methylation and gene expression differences in children conceived in vitro or in vivo. *Hum Mol Genet* 2009;**18**:3769–3778.

Kobayashi H, Hiura H, John RM, Sato A, Otsu E, Kobayashi N, Suzuki R, Suzuki F, Hayashi C, Utsunomiya T. *et al.* DNA methylation errors at imprinted loci after assisted conception originate in the parental sperm. *Eur J Hum Genet* 2009;**17**:1582–1591.

Kupers LK, Monnereau C, Sharp GC, Yousefi P, Salas LA, Ghantous A, Page CM, Reese SE, Wilcox AJ, Czamara D. *et al.* Meta-analysis of epigenome-wide association studies in neonates reveals widespread differential DNA methylation associated with birthweight. *Nat Commun* 2019;**10**:1893.

Lazaraviciute G, Kauser M, Bhattacharya S, Haggarty P, Bhattacharya S. A systematic review and meta-analysis of DNA methylation levels and imprinting disorders in children conceived by IVF/ICSI compared with children conceived spontaneously. *Hum Reprod Update* 2014;**20**:840–852.

Levine ME, Lu TA, Quach A, Chen BH, Assimes TL, Bandinelli S, Hou L, Baccarelli AA, Stewart JD, Li Y. *et al.* An epigenetic biomarker of aging for lifespan and healthspan. *Aging (Albany NY)* 2018;**10**:573–591.

Lewis S, Kennedy J, Burgner D, McLachlan R, Ranganathan S, Hammarberg K, Saffery R, Amor DJ, Cheung MMH, Doyle LW. *et al.* Clinical review of 24-35 year olds conceived with and without in vitro fertilization: study protocol. *Reprod Health* 2017;**14**:117.

Lu YH, Wang N, Jin F. Long-term follow-up of children conceived through assisted reproductive technology. *J Zhejiang Univ Sci B* 2013;**14**:359–371.

Lucas E. Epigenetic effects on the embryo as a result of periconceptional environment and assisted reproduction technology. *Reprod Biomed Online* 2013;**27**:477–485.

Maher ER, Afnan M, Barratt CL. Epigenetic risks related to assisted reproductive technologies: epigenetics, imprinting, ART and icebergs? *Hum Reprod* 2003;**18**:2508–2511.

Manning M, Lissens W, Bonduelle M, Camus M, De Rijcke M, Liebaers I, Van Steirteghem A. Study of DNA-methylation patterns

Downloaded from https://academic.oup.com/humrep/advance-article/doi/10.1093/humrep/deab078/6247390 by John Yovich on 28 April 2021

at chromosome 15q11-q13 in children born after ICSI reveals no imprinting defects. *Mol Hum Reprod* 2000;**6**:1049–1053.

Marioni RE, Shah S, McRae AF, Chen BH, Colicino E, Harris SE, Gibson J, Henders AK, Redmond P, Cox SR. *et al.* DNA methylation age of blood predicts all-cause mortality in later life. *Genome Biol* 2015;**16**:25.

Meister TA, Rimoldi SF, Soria R, von Arx R, Messerli FH, Sartori C, Scherrer U, Rexhaj E. Association of assisted reproductive technologies with arterial hypertension during adolescence. *J Am Coll Cardiol* 2018;**72**:1267–1274.

Melamed N, Choufani S, Wilkins-Haug LE, Koren G, Weksberg R. Comparison of genome-wide and gene-specific DNA methylation between ART and naturally conceived pregnancies. *Epigenetics* 2015;**10**:474–483.

Merid SK, Novoloaca A, Sharp GC, Kupers LK, Kho AT, Roy R, Gao L, Annesi-Maesano I, Jain P, Plusquin M. *et al.* Epigenome-wide meta-analysis of blood DNA methylation in newborns and children identifies numerous loci related to gestational age. *Genome Med* 2020;**12**:25.

Moore LD, Le T, Fan G. DNA methylation and its basic function. *Neuropsychopharmacology* 2013;**38**:23–38.

Newman JE, Fitzgerald O, Paul RC, Chambers GM. Assisted reproductive technology in Australia and New Zealand 2017. *National Perinatal Epidemiology and Statistics Unit, the University of New South Wales Sydney* 2019.

Newnham JP, O'Dea MR-A, Reid KP, Diepeveen DA. Doppler flow velocity waveform analysis in high risk pregnancies: a randomized controlled trial. *Br J Obstet Gynaecol* 1991;**98**:956–963.

Novakovic B, Lewis S, Halliday J, Kennedy J, Burgner DP, Czajko A, Kim B, Sexton-Oates A, Juonala M, Hammarberg K. *et al.* Assisted reproductive technologies are associated with limited epigenetic variation at birth that largely resolves by adulthood. *Nat Commun* 2019;**10**:3922.

Oliver VF, Miles HL, Cutfield WS, Hofman PL, Ludgate JL, Morison IM. Defects in imprinting and genome-wide DNA methylation are not common in the in vitro fertilization population. *Fertil Steril* 2012;**97**:147–153.

Practice Committees of the American Society for Reproductive M, the Society for Assisted Reproductive Technology. Electronic address aao. Intracytoplasmic sperm injection (ICSI) for non-male factor indications: a committee opinion. *Fertil Steril* 2020;**114**: 239–245.

Puhr R, Heinze G, Nold M, Lusa L, Geroldinger A. Firth's logistic regression with rare events: accurate effect estimates and predictions. *Stat Med* 2017;**36**:2302–2317.

Rakyan VK, Down TA, Balding DJ, Beck S. Epigenome-wide association studies for common human diseases. *Nat Rev Genet* 2011;**12**: 529–541.

Rauschert S, Melton PE, Burdge G, Craig JM, Godfrey KM, Holbrook JD, Lillycrop K, Mori TA, Beilin LJ, Oddy WH. *et al.* Maternal smoking during pregnancy induces persistent epigenetic changes into adolescence, independent of postnatal smoke exposure and is associated with cardiometabolic risk. *Front Genet* 2019;**10**:770.

Reik W, Dean W, Walter J. Epigenetic reprogramming in mammalian development. *Science* 2001;**293**:1089–1093.

Ren X, Kuan PF. methylGSA: a Bioconductor package and Shiny app for DNA methylation data length bias adjustment in gene set testing. *Bioinformatics* 2019;**35**:1958–1959.

Sakka SD, Malamitsi-Puchner A, Loutradis D, Chrousos GP, Kanaka-Gantenbein C. Euthyroid hyperthyrotropinemia in children born after in vitro fertilization. *J Clin Endocrinol Metab* 2009;**94**: 1338–1341.

Santos F, Hyslop L, Stojkovic P, Leary C, Murdoch A, Reik W, Stojkovic M, Herbert M, Dean W. Evaluation of epigenetic marks in human embryos derived from IVF and ICSI. *Hum Reprod* 2010; **25**:2387–2395.

Shankaran S. Outcomes from infancy to adulthood after assisted reproductive technology. *Fertil Steril* 2014;**101**:1217–1221.

Solomon O, MacIsaac J, Quach H, Tindula G, Kobor MS, Huen K, Meaney MJ, Eskenazi B, Barcellos LF, Holland N. Comparison of DNA methylation measured by Illumina 450K and EPIC BeadChips in blood of newborns and 14-year-old children. *Epigenetics* 2018; **13**:655–664.

Song S, Ghosh J, Mainigi M, Turan N, Weinerman R, Truongcao M, Coutifaris C, Sapienza C. DNA methylation differences between in vitro- and in vivo-conceived children are associated with ART procedures rather than infertility. *Clin Epigenet* 2015;**7**:41.

Straker L, Mountain J, Jacques A, White S, Smith A, Landau L, Stanley F, Newnham J, Pennell C, Eastwood P. Cohort profile: the Western Australian Pregnancy Cohort (Raine) Study-Generation 2. *Int J Epidemiol* 2017;**46**:1384–1385.

Suderman M, Staley J, French R, Arathimos R, Simpkin A, Tilling K. dmrff: identifying differentially methylated regions efficiently with power and control; 2018. bioRxiv preprint.

Teschendorff AE, Marabita F, Lechner M, Bartlett T, Tegner J, Gomez-Cabrero D, Beck S. A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data. *Bioinformatics* 2013;**29**:189–196.

Tierling S, Souren NY, Gries J, Loporto C, Groth M, Lutsik P, Neitzel H, Utz-Billing I, Gillessen-Kaesbach G, Kentenich H. *et al.* Assisted reproductive technologies do not enhance the variability of DNA methylation imprints in human. *J Med Genet* 2010;**47**:371–376.

Waddington CH. The epigenotype. *Int J Epidemiol* 2012;**41**:10–13.

Wahl S, Drong A, Lehne B, Loh M, Scott WR, Kunze S, Tsai PC, Ried JS, Zhang W, Yang Y. *et al.* Epigenome-wide association study of body mass index, and the adverse outcomes of adiposity. *Nature* 2017;**541**:81–86.

Weinrauch LA, Gerhard-Herman MD, Mendelson MM. Epigenetics: Is the mode of conception a marker for future cardiovascular risk? *J Am Coll Cardiol* 2018;**72**:1275–1277.

Whitelaw N, Bhattacharya S, Hoad G, Horgan GW, Hamilton M, Haggarty P. Epigenetic status in the offspring of spontaneous and assisted conception. *Hum Reprod* 2014;**29**:1452–1458.

Xiao FH, Wang HT, Kong QP. Dynamic DNA methylation during aging: a "Prophet" of age-related outcomes. *Front Genet* 2019;**10**:107.

Zandstra H, van Montfoort APA, Dumoulin JCM, Zimmermann LJI, Touwslager RNM. Increased blood pressure and impaired endothelial function after accelerated growth in IVF/ICSI children. *Hum Reprod Open* 2020;**2020**:hoz037.

Zhang G, Pradhan S. Mammalian epigenetic mechanisms. *IUBMB Life* 2014;**66**:240–256.

Downloaded from https://academic.oup.com/humrep/advance-article/doi/10.1093/humrep/deab078/6247390 by John Yovich on 28 April 2021