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Themed Issue: Role of Epigenetics in Mediating Programming Effects.

ORIGINAL ARTICLE

Association of *in vitro* fertilization with global and *IGF2/H19* methylation variation in newborn twins

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In vitro fertilization (IVF) and its subset intracytoplasmic sperm injection (ICSI), are widely used medical treatments for conception. There has been controversy over whether IVF is associated with adverse short- and long-term health outcomes of offspring. As with other prenatal factors, epigenetic change is thought to be a molecular mediator of any *in utero* programming effects. Most studies focused on DNA methylation at gene-specific and genomic level, with only a few on associations between DNA methylation and IVF. Using buccal epithelium from 208 twin pairs from the Peri/Postnatal Epigenetic Twin Study (PETS), we investigated associations between IVF and DNA methylation on a global level, using the proxies of Alu and LINE-1 interspersed repeats in addition to two locus-specific regulatory regions within *IGF2/H19*, controlling for 13 potentially confounding factors. Using multiple correction testing, we found strong evidence that IVF-conceived twins have lower DNA methylation in Alu, and weak evidence of lower methylation in one of the two *IGF2/H19* regulatory regions and LINE-1, compared with naturally conceived twins. Weak evidence of a relationship between ICSI and DNA methylation within *IGF2/H19* regulatory region was found, suggesting that one or more of the processes associated with IVF/ICSI may contribute to these methylation differences. Lower within- and between-pair DNA methylation variation was also found in IVF-conceived twins for LINE-1, Alu and one *IGF2/H19* regulatory region. Although larger sample sizes are needed, our results provide additional insight to the possible influence of IVF and ICSI on DNA methylation. To our knowledge, this is the largest study to date investigating the association of IVF and DNA methylation.

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Key words: DNA methylation, early life environment, *in vitro* fertilization (IVF), twins

Introduction

There is mounting evidence that maternal factors during pregnancy influence prenatal and postnatal health. Epigenetics, recently defined as 'the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states'[1] has been extensively studied as a potential mediating mechanism of such influences. The most widely studied epigenetic mechanism in somatic cells is methylation of DNA at the CpG dinucleotide. Dynamic changes in DNA methylation occur during germ cell and pre-implantation development,[2,3] which has led to studies investigating the effect of gestational factors that may affect early life development and latent disease risk via changes to DNA methylation. *In vitro* fertilization (IVF) is a medical treatment used to help couples to conceive. It involves fertilization of the oocyte by sperm outside of the body in a culture medium, after which, viable embryos are transferred into the uterus to establish pregnancy.[4] In subsets of cases in which sperm motility is affected, intracytoplasmic sperm injection (ICSI) is performed, in which sperm is microscopically injected into the oocyte. Clearly, the initial early life experiences of IVF/ICSI offspring are different from the naturally occurring situation. The question of paramount importance is whether this critical difference of early life environment alters epigenetic profile, including DNA methylation, during development.

A number of studies have attempted to look for associations between IVF/ICSI and DNA methylation[5–10] but most have not controlled for other maternal factors, which may have contributed to the lack of consistency of previous reports. We, and others working with humans and mice, have found evidence that maternal factors such as smoking,[11–14] vitamin B12,[14] folate,[15,16] alcohol,[17] stress,[18] macronutrients,[14,19–21] placenta weight,[14] cord insertion[14] and gestational diabetes[14] each associate with DNA methylation in infants. In addition, consumption of excess folic acid but with vitamin B12 deficiency resulted in reduced global methylation in mouse placental tissue[22] and possibly affects fetal growth.[23] Therefore, we have also analyzed possible associations between DNA methylation and the ratio of serum vitamin B12 to serum folate. Therefore, it is vital to control for these maternal factors that are possibly confounding the effect of IVF on DNA methylation in neonates.

We aimed to investigate associations of IVF with global and imprinted gene DNA methylation, controlling for a wide range of maternal factors. As surrogates for global methylation, we used the interspersed repeats LINE-1 and Alu, which account

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for 17 and 11% of the genome, respectively.[24] Levels of transcription of imprinted genes are regulated in a parent-of-origin-specific manner by differentially methylated regions (DMRs), a subset of which, imprinting control regions (ICRs) control the expression of multiple genes within a single locus.[25] The locus containing the genes *IGF2* and *H19* is the most common imprinted locus studied in relation to the influence of maternal factors on DNA methylation because the genes play important roles in fetal growth and embryonic development.[26,27] Previous animal and human studies have identified the *IGF2/H19* ICR and *H19* promoter DMR as both being associated with gene regulation within the locus,[28] and methylation levels at both loci have been previously associated with IVF.[8,9,29]

Most studies investigating associations of IVF/ICSI and other maternal factors with DNA methylation have been on singletons.[5,8,9] Twin studies are powerful because they have the ability to separate out genetic and environmental factors, which include shared factors specific to mothers and non-shared factors associated with each twin. Previous studies showed that there were higher inter-individual and intra-pair variation in *IGF2/H19* DMR methylation in IVF singletons[8] and twins.[8,30] For the former, this could be due to between-clinic or between-individual heterogeneity in IVF timing and methodologies and for the latter, such effects could represent interactions between IVF and twin-specific factors such as the nutrient supply line to each twin and/or genetic differences between dizygotic twins. To investigate such interactions, we compared the within- and between-pair differences in DNA methylation between IVF and naturally conceived twins using DNA from buccal epithelium. We hypothesized that IVF will associate with differential methylation levels within *IGF2/H19* DMRs and on a global scale and that there will be larger within-pair methylation discordance within IVF-conceived twin pairs compared with naturally conceived twins. We used cells of the buccal epithelium because they represent a homogenous cell type that can be collected non-invasively.

Materials and methods

Study cohort

A subgroup of 208 twin pairs from the Peri/Postnatal Epigenetic Twin Study (PETS) cohort[31,32] were included in this study. Mothers were recruited half way through their second trimester and details of IVF procedures were obtained via questionnaire. Twins from mothers who said no to 'did you need medical help to conceive these twins' were classified as 'naturally-conceived twins,' and twins from mothers who said yes to IVF or ICSI were classified as IVF-conceived twins. Also collected at recruitment were details of smoking, alcohol and supplementary folate intake, and with details of maternal health including gestational diabetes.[32] The same information was collected at 24 and 36 weeks of pregnancy and additional details of maternal nutritional intake were collected via questionnaire and from blood-derived serum collected at 28 weeks' gestation. Our study was carried out with appropriate human Ethics approval from the Royal Women's Hospital (06/21), Mercy Hospital for Women (R06/30) and Monash Medical Centre (06117C), Melbourne.

Tissue collection and cell processing

Cells from the buccal epithelium were collected with Catch-all Sample Collection Swabs (EPICENTRE Biotechnologies, Madison, WI, USA) within 2 weeks of delivery and stored immediately at −20°C until DNA extraction.

DNA extraction, bisulphite conversion, methylation analysis

Genomic DNA (gDNA) was extracted from buccal epithelium through salt extraction as described previously.[33] Five hundred nanograms of gDNA were bisulphite converted using the MethylEasy Exceed Rapid Bisulphite Modification Kit (Human Genetic Signatures, North Ryde, NSW, Australia). The *IGF2/H19* ICR and *H19* promoter DMR regions were the same regions analyzed in our previous study.[14] Alu primers were designed in-house using EpiDesigner (Sequenom Inc., Herston, QLD, Australia), and primers for LINE-1 were obtained from a previous study.[34] Primer sequences and PCR conditions are outlined in Supplementary Table 1. DNA methylation data were obtained by using MassARRAY Epi-TYPER (Sequenom Inc.). The genomic coordinates for CpG units analyzed for *IGF2/H19* ICR and *H19* promoter DMR are detailed in Supplementary Table 2. Data for at least two technical replicates per amplicon were obtained for analysis. Data cleaning and removal of CpG sites overlapping with SNPs that abolish CpG site in one or two alleles were done as outlined previously.[33] Batch correction was performed in a few steps. First, samples that overlapped (samples that had methylation data for both batches) were examined on scatterplots to investigate the nature of the batch effect. It was judged that a linear translation of the values of the second sample would suffice to remove the effect. A 'constant value' was calculated using these overlapped samples, by subtracting 'mean of second batch' from 'mean of first batch.' This was done separately for each assay. Second, the 'constant values' were added to the methylation values from the second batch.

Statistical analysis

Multiple linear regression was used to assess associations between DNA methylation and IVF as a whole or its ICSI and non-ICSI subsets, taking account of twin pairs sharing the same mother. 'Macronutrient' level was derived from measures of protein, energy and carbohydrate intake, as outlined in our previous study.[14] All continuous variables and methylation data were converted to *z*-scores for comparison of effect sizes across all variables. Within-pair methylation discordance values were calculated by the absolute methylation difference between twin 1 and twin 2 and were also converted to *z*-scores. Within-pair methylation variances for IVF/ICSI and naturally

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conceived twins were compared using the *F*-test, which compares the variances between two populations. Regression coefficients were converted to percentage difference in mean DNA methylation by multiplying each regression coefficient by the average standard deviation across CpG units. All 95% confidence intervals are provided in the tables. Bonferroni testing[35] was used to calculate a stricter *P*-value threshold to reduce the number of false positives, leading to an adjusted *P*-value threshold of 0.003. Unadjusted *P*-values between 0.01 to <0.05 were considered as 'weak evidence,' *P*-values between 0.003 and <0.01 were considered as 'moderate evidence' and *P*-values <0.003 were considered as 'strong evidence' against the null hypothesis of no differences in DNA methylation. Multiple linear regression analyses and *F*-tests were performed using STATA 11, and box plots were produced using GraphPad Prism 5 software.

Results

Sample characteristics and maternal data used for this study are listed in Table 1 and Supplementary Table 3, respectively. There was no evidence of differences in maternal characteristics between natural *v.* all-IVF groups and ICSI *v.* non-ICSI groups apart from zygosity and chorionicity ($P<0.003$ for natural *v.* all IVF groups), preconceptional folate supplement ($P<0.001$ for natural *v.* all IVF groups; $P = 0.024$ for IVCI *v.* non-ICSI groups) and maternal age ($P<0.001$ for natural *v.* all IVF group). Multiple linear regression analysis was used to assess associations between IVF and DNA methylation in Alu, LINE-1 and the *IGF2/H19* ICR and *H19* promoter DMR, adjusting for peri/preconceptional folic acid intake, 28-week maternal serum folate, periconceptional alcohol intake, periconceptional smoking, maternal stress, maternal macronutrients, ratio of serum vitamin B12 to serum folate, serum vitamin B12, placental weight, umbilical cord insertion site (central *v.* peripheral), gestational diabetes, gestational age, maternal age and infant sex.

Newborn IVF-conceived twins have lower global and IGF2/H19 *ICR methylation*

We found strong evidence of lower Alu methylation (−2.9%, $P<0.001$, Table 2) and weak evidence of lower LINE-1

Table 1. *Newborn twins and maternal characteristics*

	Natural (174 pairs)	All IVF (34 pairs)		ICSI (20 pairs)	Non-ICSI (14 pairs)	
Variable[a]	Mean (S.D.) or %	Mean (S.D.) or %	*P*-value	Mean (S.D.) or %	Mean (S.D.) or %	*P*-value
Newborn twins						
Gestational age at birth (weeks)	35.8 (2.3)	35.9 (2.8)	0.805	35.3 (3.3)	36.8 (1.6)	0.103
Birth weight (g)	2458 (524)	2451 (542)	0.915	2357 (631)	2585 (349)	0.061
Both males	38.3%	44.1%	0.342	40.0%	50.0%	0.810
Both females	39.4%	26.5%		30.0%	21.4%	
Male/female	22.3%	29.4%		30.0%	28.6%	
MZMC	32.4%	14.7%	0.003	20.0%	7.1%	0.378
MZDC	16.2%	2.9%		5.0%	0.0%	
DZDC	51.5%	82.4%		75.0%	92.9%	
Placental weight (g)	430 (100)	446 (77)	0.175	446 (75)	446 (81)	0.985
Central cord insertion	39.1%	45.6%	0.321	40.0%	53.6%	0.269
Cord insertion discordance	36.5%	32.1%	0.660	40.0%	23.1%	0.339
Mother						
Preconceptional folate supp	52.6%	90.9%	<0.001	100%	76.9%	0.024
Periconceptional folate supp	89.7%	94.1%	0.424	100%	85.7%	0.081
Periconceptional folate supp	45.7%	47.1%	0.901	35.0%	64.3%	0.092
Periconceptional smoking	21.8%	29.4%	0.581	35.0%	21.4%	0.393
Gestational diabetes	12.2%	8.8%	0.574	10.0%	7.1%	0.773
Third trim. vitamin B12 (pmol/l)	181.9 (76.5)	187.1 (88.9)	0.754	171.1 (58.6)	208.8 (117.6)	0.285
Third trimester folate (nmol/l)	35.6 (30.0)	35.6 (13.9)	0.989	35.3 (13.5)	36.2 (15.0)	0.870
1-2 trim carbohydrate intake (g/day)	229.5 (79.2)	202.2 (76.2)	0.071	193.5 (66.0)	213.3 (88.9)	0.495
1-2 trim protein intake (g/day	96.5 (39.1)	83.8 (31.9)	0.053	82.2 (28.1)	85.9 (37.3)	0.756
1-2 trim energy intake (kJ/day)	8645 (2989)	7762 (2749)	0.107	7534 (2578)	8056 (3027)	0.611
Maternal stress score	22.1 (7.78)	22.6 (6.86)	0.742	22.4 (6.4)	22.8 (7.6)	0.877
Maternal age	32.2 (4.9)	36.3 (4.9)	<0.001	36.0 (4.9)	36.8 (5.1)	0.653

IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection; MZMC, monozygotic monochorionic; MZDC, monozygotic dichorionic; DZDC, dizygotic dichorionic; supp, supplementation.
[a]A full description of variables can be found in Supplementary Table 3.

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Table 2. *Multiple linear regression of global methylation in buccal epithelium*

	Alu				LINE-1			
Maternal factors	Coefficient	*P*-values	95% Confident Interval		Coefficient	*P*-values	95% confident interval	
IVF	−2.9%**	<0.001 (<0.014)	−4.3%	−1.5%	−1.3%*	0.049	−2.6%	0.0%
Periconceptional folate	−1.5%	0.214	−3.9%	0.9%	0.3%	0.695	−1.2%	1.9%
Preconceptional folate	0.7%	0.377	−0.9%	2.4%	−0.2%	0.723	−1.4%	1.0%
Serum folate (*z*-score)	0.0%	0.913	−0.8%	0.9%	0.3%	0.372	−0.3%	0.9%
Periconceptional alcohol	−0.2%	0.795	−1.5%	1.2%	−0.7%	0.175	−1.7%	0.3%
Periconceptional smoking	1.7%*	0.047	0.0%	3.5%	1.1%*	0.035	0.1%	2.2%
Maternal stress (*z*-score)	−0.2%	0.407	−0.7%	0.3%	−0.3%	0.222	−0.8%	0.2%
Maternal macronutrient (*z*-score)	0.5%	0.079	−0.1%	1.1%	−0.3%	0.214	−0.8%	0.2%
Ratio of serum vitamin B12 over serum folate (*z*-score)	−0.3%	0.544	−1.4%	0.7%	0.4%	0.385	−0.5%	1.3%
Serum vitamin B12 (*z*-score)	0.0%	0.923	−0.8%	0.8%	−0.3%	0.224	−0.9%	0.2%
Placenta weight (*z*-score)	0.1%	0.85	−0.6%	0.8%	0.1%	0.69	−0.4%	0.6%
Central cord insertion (MC twins)	1.1%	0.25	−0.8%	2.9%	1.2%	0.178	−0.6%	3.0%
Central cord insertion (DC twins)	0.1%	0.868	−1.2%	1.4%	0.4%	0.392	−0.5%	1.2%
Gestational diabetes	−0.8%	0.607	−3.6%	2.1%	2.2%**	0.001 (0.014)	0.9%	3.5%

Coefficients for maternal stress, maternal macronutrients, maternal serum vitamin B12 and placenta weight represent percentage difference in mean methylation for one standard deviation unit increase in the corresponding factor. Coefficients with *P*-values <0.05 are identified by a single asterisk. *P*-values in parentheses are adjusted *P*-values (using Bonferroni), and are only listed if the unadjusted *P*-values are <0.003 (adjusted *P*-value threshold after Bonferroni correction), and accompanied by double asterisks.

methylation in IVF newborn twins compared with naturally conceived twins (−1.3%, $P = 0.049$, Table 2). We found weak evidence of lower *IGF2/H19* ICR methylation in IVF-conceived twins compared with naturally conceived twins (−3.5%, $P = 0.048$, Table 3) but no evidence of a similar relationship in the *H19* promoter DMR ($P = 0.664$, Table 3).

Methylation differences in the IGF2/H19 *ICR between IVF-conceived twins with and without ICSI*

To investigate whether the global and *IGF2/H19* methylation differences between IVF and naturally conceived twins were driven by ICSI, we further stratified IVF individuals to with or without ICSI. Linear regression was used to test for any methylation differences among these three groups, adjusting for maternal age, sex and birth weight. We found no evidence for a difference in DNA methylation of Alu and LINE-1 between twins conceived by ICSI and those conceived by IVF without ICSI (Fig. 1a and 1b) and weak evidence for lower methylation of *IGF2/H19* ICR in twins conceived by ICSI compared with IVF without ICSI (−4.8%, $P = 0.01$, Table 4, Fig. 1c). As we found weak evidence for lower *IGF2/H19* ICR methylation in ICSI twins compared with naturally conceived twins (−3.8%, $P = 0.026$, Table 4, Fig. 1c), but no evidence for differences between IVF without ICSI and naturally conceived twins, we conclude that lower methylation in IVF-conceived twins is driven by ICSI within the *IGF2/H19* ICR.

Association between IVF and within-pair and between-pair variation in DNA methylation

To test our hypothesis that IVF is associated with greater within-pair and between-pair variation in DNA methylation we used two different methods. We first used regression analysis and found no evidence for an association between IVF and within-pair methylation discordance for Alu, *IGF2/H19* ICR and the *H19* promoter DMR ($P > 0.05$, Supplementary Table 4). However, we found weak evidence of a slight reduction in within-pair LINE-1 methylation discordance within IVF-conceived twin pairs compared with naturally conceived pairs (−0.4%, $P = 0.016$, Supplementary Table 4). This evidence remained even after adjusting for chorionicity and zygosity (data not shown). Second, for between-pair variation analysis, variation of DNA methylation within IVF-conceived and naturally conceived twin pairs was analyzed using *F*-tests. We found evidence for smaller variance in Alu, LINE-1 and *H19* promoter DMR methylation in IVF-conceived twins as compared with naturally conceived twins ($P = 0.006$, $P < 0.001$, $P = 0.029$, respectively, Supplementary Table 5).

Associations of other maternal nutritional and lifestyle factors with global and IGF2/H19 *methylation in buccal epithelial cells*

Of the other maternal factors, we analyzed in the same manner as mode of conception (controlling for all other factors) we found weak evidence that periconceptional smoking is

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Table 3. *Multiple linear regression of* H19 *promoter DMR and* IGF2/H19 *ICR methylation in buccal epithelium*

	H19 promoter DMR				*IGF2/H19* ICR			
Maternal factors	Coefficient	*P*-values	95% confidence interval		Coefficient	*P*-values	95% confidence interval	
IVF	−0.7%	0.664	−3.8%	2.4%	−3.5%*	0.048	−7.0%	0.0%
Periconceptional folate	0.9%	0.623	−2.8%	4.7%	−0.7%	0.714	−4.4%	3.0%
Preconceptional folate	−4.7%**	0.001 (0.014)	−7.6%	−1.9%	−0.4%	0.768	−3.4%	2.5%
Serum folate (*z*-score)	2.6%*	0.007	0.7%	4.5%	0.8%	0.683	−3.2%	4.8%
Periconceptional alcohol	−1.9%	0.382	−6.4%	2.5%	−1.2%	0.371	−3.9%	1.5%
Periconceptional smoking	6.5%*	0.008	1.6%	11.4%	1.8%	0.178	−0.8%	4.4%
Maternal stress (*z*-score)	0.9%	0.184	−0.4%	2.1%	0.3%	0.651	−0.9%	1.4%
Maternal macronutrient (*z*-score)	1.6%*	0.005	0.5%	2.8%	0.3%	0.552	−0.7%	1.3%
Ratio of serum vitamin B12 over serum folate (*z*-score)	2.3%	0.125	−0.7%	5.3%	−0.1%	0.907	−1.9%	1.7%
Serum vitamin B12 (*z*-score)	−2.0%*	0.044	−3.9%	−0.1%	−0.5%	0.547	−1.9%	1.0%
Placenta weight (*z*-score)	0.4%	0.461	−0.7%	1.5%	0.2%	0.737	−1.2%	1.7%
Central cord insertion (MC twins)	0.2%	0.902	−2.9%	3.3%	1.7%	0.528	−3.5%	6.8%
Central cord insertion (DC twins)	0.5%	0.69	−1.9%	2.8%	0.3%	0.731	−1.7%	2.4%
Gestational diabetes	−1.4%	0.396	−4.6%	1.8%	−1.3%	0.492	−5.1%	2.5%

DMR, differentially methylated region; ICR, imprinting control region; IVF, *in vitro* fertilization.

Coefficients for maternal stress, maternal macronutrients, maternal serum vitamin B12 and placenta weight represent percentage difference in mean methylation for one standard deviation unit increase in the corresponding factor. Coefficients with *P*-values <0.05 are identified by a single asterisk. *P*-values in parentheses are adjusted *P*-values (using Bonferroni), and are only listed if the unadjusted *P*-values are <0.003 (adjusted *P*-value threshold after Bonferroni correction), and accompanied by double asterisks.

associated with Alu and LINE-1 methylation (1.7%, $P = 0.047$; 1.1%, $P = 0.035$, Table 2). We found strong evidence that gestational diabetes is associated with LINE-1 DNA methylation level (2.2%, $P = 0.001$, Table 2) but not Alu methylation. We found strong evidence of association of preconceptional but not periconceptional folic acid supplementation on DNA methylation of the *H19* promoter DMR (−4.7%, $P = 0.001$, adjusted $P = 0.014$, Table 3), moderate evidence for association of serum folate (2.6%, $P = 0.007$, Table 3), periconceptional smoking (6.5%, $P = 0.008$, Table 3) and macronutrient score (1.6%, $P = 0.005$, Table 3) with DNA methylation at the *H19* promoter DMR and weak evidence for association of serum vitamin B12 (−2.0%, $P = 0.044$, Table 3) in the same region. No significant associations were found between maternal factors other than IVF with *IGF2/H19* DMR methylation (Table 3) or between supply line factors of placental weight or cord insertion and DNA methylation in any of the regions studied (Tables 2 and 3).

Discussion

Comparison of IVF-conceived twins and naturally conceived twins

Our aims were to investigate associations between IVF/ICSI and DNA methylation on a global scale, using proxies of Alu and LINE-1, and the locus-specific scale, within the *IGF2/H19* imprinted region, controlling for other maternal and gestational factors.

Both global and *IGF2/H19* ICR DNA methylation showed evidence of lower DNA methylation in IVF-conceived twins compared with naturally conceived twins, with strongest evidence seen for Alu repeats. This is in contrast to a previous study[10] in which no significant differences in Alu and LINE-1 methylation were found between IVF- and naturally conceived singleton, 6–9 week-old aborted conceptuses. However, as our studies differ in tissue type and gestational age, they are difficult to compare. Whitelaw *et al.*[36] performed a similar analysis in three groups of children (natural conception, IVF non-ICSI and ICSI) and although they found no evidence of LINE-1 methylation differences in buccal cell DNA between these groups, there was a trend toward lower methylation (~ −1%) in the IVF only group, which agrees with our findings.

Reduced global methylation is found in most cancers,[37,38] where it may be associated with prognosis.[39,40] Outside cancer, global methylation is negatively correlated with exposure to traffic particles[40] and adiposity[41] in children, and heart disease, stroke,[42] inflammation[43] and levels of blood lipids[44,45] in adults, although a positive correlation between global methylation and insulin resistance was found in adults.[46] Of note, higher levels of multiple cardiometabolic phenotypes have previously been found in IVF-conceived offspring.[47] Although reduced global methylation in cancer is associated with genome instability,[48,49] no such evidence exists outside cancer, possibly because effect sizes are generally smaller (<5% for non-cancer studies[44,45] compared with >10% in cancer studies[48,50,51]). Nevertheless, taken together with our data, these findings

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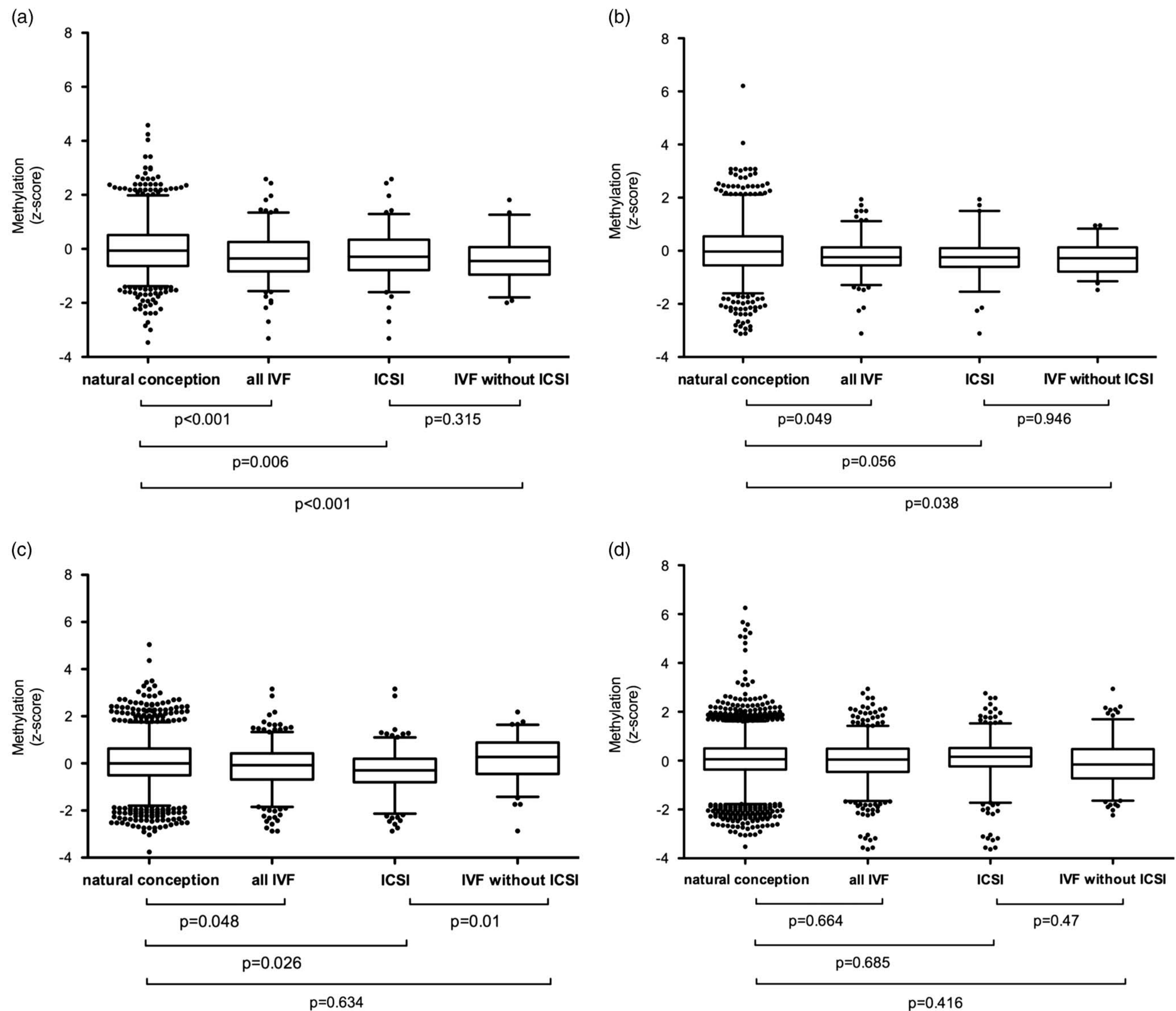

Fig. 1. Box plots and *P*-values of (a) Alu, (b) LINE-1, (c) *IGF2/H19* ICR and (d) *H19* promoter DMR methylation stratified according to mode of conception. The solid horizontal lines in the boxes represent the median, the box represent data within the 25th and 75th percentile range. Whiskers represent data within 5th and 95th percentile range, and the dots represent outliers.

support the continued study of global methylation as a potential biomarker for prenatal environment and clinical outcomes associated with IVF and ICSI.

The lower levels of DNA methylation we observed in Alu, LINE-1 and *IGF2/H19* in IVF-conceived twins could be due to a number of factors including fertility of either parent and culture conditions for IVF. We found weak evidence that ICSI is driving the difference only at the *IGF2/H19* ICR, which suggests that ICSI *per se* could affect DNA methylation in a locus-specific manner. One other study performed a similar analysis on buccal cell DNA from offspring, between birth and 7 years of age, on DNA methylation within the *IGF2/H19 locus*, albeit in a DMR within the *IGF2* gene.[36] No evidence was found for such an association (n = 29 IVF no ICSI, n = 20 ICSI, n = 86 natural). The discrepancy between the two studies is likely to be due to difference in DMRs analyzed. We suggest that ICSI and/or IVF procedures affect DNA methylation in a locus-specific and tissue-specific manner. It is worth noting that within the *IGF2/H19* locus, methylation changes in the ICR are likely to be correlated with expression changes.[52,53] Such studies have shown that ICR methylation is positively correlated with expression of *IGF2*. If we assume that perinatal DNA methylation state reflects embryonic DNA methylation state, we would expect lower ICR methylation to associate with lower *IGF2* expression and reduced fetal growth. It is therefore of note that ICSI-conceived twins were 228 g lighter than no-ICSI IVF-conceived twins (P = 0.061). However, larger studies of methylation and studies of

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Table 4. *DNA methylation differences between naturally conceived twins, IVF without ICSI and ICSI newborn twins*

Region	Comparison	Coefficient	*P*-value	95% Confidence interval	
Alu	Natural conception *v.* ICSI	−1.9%*	0.006	−3.2%	−0.6%
	Natural conception *v.* IVF without ICSI	−1.5%**	<0.001 (<0.014)	−2.3%	−0.8%
	ICSI *v.* IVF without ICSI	−0.9%	0.315	−2.9%	1.0%
LINE-1	Natural conception *v.* ICSI	−1.2%	0.056	−2.4%	0.0%
	Natural conception *v.* IVF without ICSI	−0.6%*	0.038	−1.1%	0.0%
	ICSI *v.* IVF without ICSI	0.0%	0.946	−1.5%	1.4%
IGF2/H19 ICR	Natural conception *v.* ICSI	−3.8%*	0.026	−7.1%	−0.4%
	Natural conception *v.* IVF without ICSI	0.3%	0.634	−1.0%	1.7%
	ICSI *v.* IVF without ICSI	4.8%*	0.010	1.3%	8.4%
H19 promoter DMR	Natural conception *v.* ICSI	0.7%	0.685	−2.8%	4.2%
	Natural conception *v.* IVF without ICSI	−1.0%	0.416	−3.5%	1.5%
	ICSI *v.* IVF without ICSI	−2.1%	0.47	−7.9%	3.7%

IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection; ICR, imprinting control region; DMR, differentially methylated region.
Coefficients with *P*-values <0.05 are indicated by a single asterisk. *P*-values in parentheses are adjusted *P*-values (using Bonferroni correction), and are only listed if the unadjusted *P*-values are <0.003 (adjusted *P*-value threshold after Bonferroni correction), and are accompanied by double asterisks.

expression are needed to further investigate this relationship. Another possibility is that the DNA methylation differences we found in the *IGF2/H19* ICR in ICSI-conceived offspring could be explained by male infertility, although we do not have the data to investigate this. This hypothesis was extrapolated from the findings of a previous study,[10] which found that *H19* promoter methylation error, defined as having a range of methylation beyond ±2 s.d. of the mean methylation in natural conception, in IVF fetuses was also present in the fathers' sperm.

Smaller within-pair and between-pair variation in DNA methylation in IVF-conceived twins

We observed a smaller variance in both within- and between-pair methylation in IVF-conceived twins. This does not support our hypothesis that IVF is associated with higher within-pair and between-pair variation in DNA methylation. This also disagrees with previous studies showing larger intra- and inter-individual variation in methylation in IVF offspring in a human study,[8] and larger differences in placenta/fetal ratio in an mouse IVF study.[54,55] Though these studies were analysing methylation of cord[8] and cord blood,[8] and placental and embryo sizes of mice,[54,55] which are different from our study (buccal epithelium), our result is an important finding because it does not support the idea that IVF pregnancies are somehow more variable in outcome than non-IVF pregnancies. We have previously shown that central cord insertion can influence DNA methylation.[14] Thus, one possible reason we speculate for lower methylation variation in IVF-conceived twins could be the lower discordance in cord insertion (central *v.* peripheral) within IVF-conceived twins compared with naturally conceived twins (32.1 *v.* 36.5%, $P = 0.660$, Table 1). However, larger studies of multiple loci and tissues are needed to further investigate these relationships.

Comparison with our previous study

Of the four maternal factors (periconceptional smoking, maternal macronutrient, serum vitamin B12, and central cord insertion in DC twins) we previously found to be associated with *H19* promoter DMR methylation in the same tissue,[14] only two, maternal macronutrient and maternal serum vitamin B12, were replicated in the larger data set analyzed in the current report (Supplementary Table 6). None of the maternal factors with evidence of associations in the *IGF2/H19* ICR from our previous study were replicated in our current, larger data set. We accept the possibility of false positives in our earlier study and/or a batch effect between studies, despite our attempt to correct this using statistical methods. Clearly, multiple, independent studies are required to achieve reliable biomarkers of early life environment.

Associations of folic acid supplement intake with H19 *promoter DMR methylation may be timing-specific*

We found evidence of a negative association of preconceptional but not periconceptional folic acid supplement intake (as assessed by questionnaire) with *H19* promoter DMR methylation with an effect size of 4.7%, which is relatively large for studies of the associations between prenatal factors and neonatal DNA methylation. Hoyo *et al.*[15] observed moderate evidence for a negative association of both pre- and periconceptional folic acid supplementation on DNA methylation in a region close to the *IGF2/H19* ICR in umbilical cord DNA (−2.8%, $P = 0.04$ and −4.9%, $P = 0.05$, respectively). Although further studies are required to confirm these relationships, our studies point to the preconception as an understudied time period that may, though the vulnerability of the oocyte, represent a vital window for the developmental origins of health and disease.

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We found a positive association between 28-week (third trimester) serum folate levels and DNA methylation at the *H19* promoter DMR, contrary to the negative association with preconceptional folic acid supplement intake. However, the two measures are not directly comparable and further studies are needed to compare effect of folate intake across gestation.

Strengths and limitations

To our knowledge, this is the first study that has controlled for multiple maternal factors when analysing the association between IVF/ICSI and DNA methylation. Also, to our knowledge, this is the largest sample size used for analysing methylation differences between IVF/ICSI and naturally conceived twins. Correction for multiple testing was also performed through stringent Bonferroni corrections to reduce the possibility of false positives.

One of the limitations of this study is that the data obtained for maternal factors (apart for 28 weeks serum folate and serum vitamin B12) was solely based on the answers given by mothers and it is difficult to know for sure whether recall was accurate. However, mothers were recruited during their second trimester, thus minimizing recall bias. Due to the time taken to collect biological samples from our cohort, which took two and a half years to recruit, our data was generated in batches, which may contribute to a batch effect, despite our batch correction. We also lacked information on parental fertility so it was not possible to adjust for this variable. While we are not able to completely rule out postnatal effects on the observed methylation differences, such effects are likely to be negligible as buccal swabs were collected within 72 h of birth and we do not expect postnatal environment to be influenced by IVF-related effects.

Conclusions

We have presented strong evidence associating IVF with lower levels of Alu methylation and weak evidence of lower methylation in LINE-1 and within a region controlling expression within the *IGF2*/*H19* imprinted gene locus involved in fetal growth. Our findings have also indicated potential evidence of ICSI being the driving factor in the methylation differences in *IGF2*/*H19* ICR. Further studies are needed to discover whether these findings are locus- and tissue-specific and whether they are related to gene expression, genome stability and health outcomes in IVF-conceived children.

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Conflicts of Interest

None.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the Australian guidelines on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008, and has been approved by the institutional committees as indicated in the manuscript.

Supplementary materials

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S2040174415000161

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