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DNA methylation signatures in cord blood of ICSI children

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STUDY QUESTION: Does ICSI induce specific DNA methylation changes in the resulting offspring?

SUMMARY ANSWER: Although several thousand analyzed CpG sites (throughout the genome) displayed significant between-group methylation differences, both ICSI and spontaneously conceived children varied within the normal range of methylation variation.

WHAT IS KNOWN ALREADY: Children conceived by ART have increased risks for medical problems at birth and to the extent of present knowledge also in later life (i.e. impaired metabolic and cardiovascular functions). One plausible mechanism mediating these ART effects are epigenetic changes originating in the germ cells and/or early embryos and persisting during further development.

STUDY DESIGN, SIZE, DURATION: We compared the cord blood methylomes and candidate gene methylation patterns of newborns conceived through ICSI or spontaneously.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Umbilical cord bloods were obtained from healthy newborn singletons conceived spontaneously (53 samples), through ICSI (89) or IVF (34). Bisulfite-converted DNA samples of 48 ICSI and 46 control pregnancies were used for genome-wide analyses with Illumina's 450K methylation arrays. Candidate genes from the methylation screen were analyzed in all three groups by bisulfite pyrosequencing.

MAIN RESULTS AND THE ROLE OF CHANCE: Altogether, 4730 (0.11%) of 428 227 analyzed CpG sites exhibited significant between-group methylation differences, but all with small ($\beta < 10\%$) or very small ($\beta < 1\%$) effect size. ICSI children showed a significantly decreased DNA methylation age at birth, lagging approximately half a week behind the controls. ART-susceptible CpGs were enriched in CpG islands with low methylation values (0–20%) and in imprinting control regions (ICRs). Eighteen promoter regions (six in microRNA and SNORD RNA genes), four CpG islands (three in genes including one long non-coding RNA), and two ICRs contained multiple significant sites. Three differentially methylated regions were studied in more detail by bisulfite pyrosequencing. *ATG4C* and *SNORD114-9* could be validated in an independent ICSI group, following adjustment for maternal age and other confounding factors. *ATG4C* was also significant in the IVF group.

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: The observed epigenetic effects are small and there are numerous potential confounding factors such as parental age and infertility. Although our study meets current standards for epigenetic screens, sample size is still two orders of magnitude below that of genome-wide association studies.

WIDER IMPLICATIONS OF THE FINDINGS: Our study suggests an impact of ICSI on the offspring's epigenome(s), which may contribute to phenotypic variation and disease susceptibility in ART children. Epigenetic regulation of gene expression by different classes of non-coding RNAs may be a key mechanism for developmental programming through ART.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by a research grant (no. 692185) from the European Union (ERA of ART). There are no competing interests.

Key words: ART / DNA methylation / fetal cord blood / developmental origins of health and disease / ICSI

Introduction

Since the world's first test-tube baby in 1978, IVF has been used for treatment of human, in particular female-factor infertility (Kamel, 2013). ICSI, which was introduced in 1992 (Palermo *et al.*, 1992), has improved fertilization rates and also enabled treatment of severe male-factor infertility. Today, the number of IVF/ICSI children has exceeded 5 million worldwide. The latest world report on fertility treatment (Dyer *et al.*, 2016) suggests that in many countries ICSI has become the standard infertility treatment even in couples who would also get pregnant with IVF. ICSI bypasses biological processes of sperm selection and carries an additional risk of physically damaging the oocyte during fertilization (Eichenlaub-Ritter *et al.*, 2002).

ARTs involve the handling and *in vitro* culture of germ cells and preimplantation embryos at critical stages when genome-wide epigenetic reprogramming occurs, in particular during late oocyte and early embryo development (Horsthemke and Ludwig, 2005; van Montfoort *et al.*, 2012; El Hajj and Haaf, 2013). Concerns have emerged that ART-induced epigenetic changes may be transmitted to the offspring, conferring a higher risk for imprinting and other disorders. Epidemiological studies have associated ARTs with a 2–3-fold increased risk for low-birth weights (McDonald *et al.*, 2010) and a slightly increased risk for birth defects (Hansen *et al.*, 2002). Birth defects may be primarily increased in ICSI children, but not after IVF (Davies *et al.*, 2012). ART pregnancies are more likely predisposed to preterm birth and adverse perinatal outcomes (Jackson *et al.*, 2004). There appears to be an increased risk for Beckwith–Wiedemann (Maher *et al.*, 2003) and Angelman syndrome (Ludwig *et al.*, 2005) but due to the low prevalence of imprinting disorders this is difficult to prove at the population level (Lazaraviciute *et al.*, 2014). A large number of studies in rodent and large animal models have demonstrated that different ARTs, in particular ovarian stimulation and embryo culture can lead to aberrant methylation and expression patterns of developmentally important (in particular imprinted) genes in oocytes, embryos, fetuses and/or placentae (Velker *et al.*, 2012; El Hajj and Haaf, 2013; Fauque, 2013; Urrego *et al.*, 2014; Anckaert and Fair, 2015; de Waal *et al.*, 2015).

The Developmental Origins of Health and Disease or Barker hypothesis associates adverse environmental exposures during early development with increased susceptibilities for complex, in particular metabolic and cardiovascular diseases later in life (Gillman, 2005; Barker, 2007; Gluckman *et al.*, 2009). Maternal nutritional studies suggest that epigenomic plasticity is the highest during the periconceptional period and then steadily decreases during prenatal and postnatal development (Gluckman *et al.*, 2009; El Hajj *et al.*, 2014). The most dramatic change of environment in the earliest possible time window occurs during assisted reproduction. Thus, our main concern should not be whether preimplantation development is compromized or a few rare imprinting disorders are increased after ART, but disease susceptibility in later life. Indeed, higher fasting glucose concentrations, increased blood pressure and vascular dysfunction have been reported in ART children (Ceelen *et al.*, 2008; Scherrer *et al.*, 2012). In addition, ART children may have an elevated risk for some cancers, in particular leukemia and Hodgkin's lymphoma (Reigstad *et al.*, 2016). Mice generated via ART displayed hypertension, increased arterial stiffness, and a shortened life span when exposed to high-fat diet (Rexhaj *et al.*, 2013). Vascular dysfunction was associated with an increased promoter methylation of *eNOS* in the aorta, decreased vascular eNOS expression and NO synthesis. The most likely mechanism for translating ART effects into long-term disease susceptibilities is persisting epigenetic dysregulation of underlying genes and pathways.

To study the possible impact of ICSI on the epigenome of the exposed offspring and to identify susceptible loci, we compared the cord blood methylomes of healthy ICSI versus naturally conceived newborns, using 450K methylation arrays. Similar to other environmental, i.e. nutritional exposures during early development (El Hajj *et al.*, 2014), the observed methylation changes were widespread but of small effect size.

Materials and Methods

Study samples and DNA preparation

Written informed consent was obtained from all participating pregnant women. Epigenetic studies on fetal cord blood were approved by the Ethics Committee of the Medical Faculty at Würzburg University (votum no. 100/10 and 212/15). Umbilical cord bloods from newborn singletons conceived spontaneously (53 samples), through ICSI (89) or IVF (34) were collected by collaborating obstetric clinics throughout Germany. The vast majority of women were of middle European descent with a few from South-Eastern Europe and Turkey. Only newborns without medical problems at birth were included. Blood samples were stored at −80°C until further analysis. Genomic DNA was isolated with the FlexiGene Kit (Qiagen, Hilden, Germany). Amount and quality of the DNA were measured using the NanoDrop 2000c spectrophotometer (Thermo Scientific, Massachusettes, USA).

Methylation array analysis

Cord blood DNAs (500 ng each) of 48 ICSI newborns and 46 controls were bisulfite-converted with the EZ-96 DNA Methylation Kit (Zymo Research, CA, USA), whole-genome amplified, enzymatically fragmented, and hybridized to eight Illumina HumanMethylation450 (450K) BeadChips according to the manufacturer's protocol (Illumina, San Diego, CA, USA). To avoid batch effects, the 12 arrays on a chip were hybridized with matched ICSI and control samples and all chips were processed simultaneously. The arrays were scanned with an Illumina iScan. Microarray data were exported as idat files and analyzed using the statistical software package R (version 3.2.2) and the BioConductor platform (version 3.2).

Preprocessing has been performed using the infrastructure implemented in the minfi (Aryee *et al.*, 2014) and watermelon package (Pidsley *et al.*, 2013). First, sites with low signal quality (beadcount < 3 and detection P-value > 0.05) were filtered and sites overlapping known SNPs removed.

Furthermore, probes on the sex chromosomes were excluded, leaving a total number of 428 227 probes (of >485 000 CpGs on the chip covering 99% of RefSeq genes and 96% of CpG islands) for subsequent analyses. Intensity values were normalized using the dasen method as implemented in the watermelon package (Pidsley *et al.*, 2013). To account for potential probe type effects an intra-sample normalization procedure (BMIQ) has been applied which corrects for the bias of type two probes. Differential methylation analysis has been performed using the moderated *t*-test model based on β-values as implemented in the limma package (Ritchie *et al.*, 2015), including maternal age, birth mode, birth weight and sex of the child as covariates.

The blood cell composition was estimated on the basis of methylation profiles of cell-type-specific CpGs (Jaffe and Irizarry, 2014). Cell compositions between the ICSI and the control group were compared by the Wilcoxon-Mann–Whitney test for each cell-type and multiple testing was corrected by the Benjamini–Hochberg method. To study the effect of variation in cell composition on array CpG methylation, Pearson's correlation between methylation levels and predicted cell counts was calculated for each CpG site and subsequently tested for overall significance of correlation with the observed methylation differences between the ICSI and the control group. To estimate the effect of male infertility on methylation, a model including sperm concentration and sex of the child as covariates was fitted on the group of ICSI samples. Multiple testing corrections were performed with the Benjamini–Hochberg method. The epigenetic clock is defined as a weighted average across 148 selected age-associated CpG sites (Knight *et al.*, 2016). The resulting age estimate (in units of gestational weeks) is referred to as DNA methylation or epigenetic age.

Bisulfite pyrosequencing

Bisulfite conversion with the EpiTect Fast 96 Bisulfite Conversion Kit (Qiagen) and PCR reactions were performed in 96-well plates containing DNA samples (1 µg each) from ICSI, IVF and control cord bloods. The PyroMark Assay Design 2.0 software (Qiagen) was used for primer design (see Supplementary data, Table SI). The accuracy of the pyrosequencing assays was tested using standard DNAs with 0, 25, 50, 75 and 100% methylation. PCR reactions were performed in a total volume of 25 µl consisting of 2.5 µl 10× PCR buffer (with $MgCl_2$), 0.5 µl dNTPs, 1.25 µl (10 pmol/ml) of each forward and reverse primer, 0.2 µl FastStart Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany) and 1 µl (~25 ng) bisulfite-converted DNA. Bisulfite pyrosequencing was performed on a PyroMark Q96 MD system using the PyroMark Gold Q96 CDT reagent kit (Qiagen). The Pyro Q CpG software (Qiagen) was used to quantify methylation percentages. In our experience, the average methylation difference between technical replicates (including bisulfite conversion, PCR and pyrosequencing) is ~1–2% points. Each pyrosequencing run included artificially methylated and unmethylated DNA standards (Qiagen) as controls.

To adjust for potential confounding factors, multivariate linear regression models were used for the analysis of the pyrosequencing data. Potential confounders were selected based on known and observed factors potentially influencing DNA methylation. The regression coefficients of the final model were adjusted for maternal age, mode of birth, gestational week, birth weight and sex of the child. *Post hoc* analysis of pair-wise comparisons between the groups (ICSI, IVF and control) have been performed using Tukey's honest significance method.

Statistical analysis

Statistical analyses were performed with the statistical software package R (version 3.2.2) and IBM SPSS statistics 23. Power analyses were based on the observed effect sizes (methylation differences of 1 and 0.5%, respectively) and variability of the data (standard deviation of 5 and 2%) in this study. To detect methylation differences of 1% (0.5%) with a standard deviation of 5% (2%) sample sizes of 393 and 252, respectively, would be required in each group to achieve a power of 80%. With the sample sizes in the range of 40 as available in our cohorts, this would translate into an observed power of ~20% for this study.

Results

The cord blood methylation patterns of 48 ICSI and 46 naturally conceived singleton pregnancies were compared, using Illumina 450K arrays. We did not include IVF newborns in our methylation screen, assuming that ICSI represents the more adverse environmental exposure and, therefore, may be associated with larger epigenetic effects. A three group comparison would require larger sample size or reduce power. ICSI and control samples were matched for possible confounding factors including mode of birth, gestational age, birth weight and sex of the child (Table I). Only maternal age differed significantly between groups: ICSI mothers were on average 4 years older than controls. To exclude possible effects of variation in blood cell composition, we investigated the correlation of array CpG methylation values and predicted cell counts. As expected, many sites showed a significant correlation with predicted cell counts (see Supplementary data, Fig. S1). However, no significant correlation between the site-wise correlations with cell counts and the observed methylation differences between ICSI and the control group could be observed for any of the investigated cell-types. To assess the effect of male infertility on cord blood methylation, we analyzed the correlation of array CpG methylation and sperm concentration (1–243 million/ml), ranging from severe oligozoospermia to normozoospermia, in the ICSI group. For the spontaneously conceived controls, paternal semen parameters were not available. None of the 428 227 sites tested reached genome-wide significance after multiple testing correction, indicating that sperm concentration has no substantial effect on cord blood methylation levels (see Supplementary data, Fig. S2).

Following correction for multiple testing and adjustment for possible confounding factors (maternal age, birth mode, birth weight and sex of the child), 4730 (0.11%) of 428 227 analyzed CpG sites exhibited a significant (FDR-adjusted $P < 0.05$) methylation difference between ICSI and control samples. The number of hypermethylated sites (2743) was significantly (Fisher's exact test; $P = 2.2e\text{-}16$) higher than that of hypomethylated (1987) sites. Enrichment analysis (Table II) showed an overrepresentation of significant sites in the low (0–20%) methylation range and in CpG islands. There was no association with enhancers, DNAse hypersensitive sites, and differentially methylated regions (DMRs). An epigenetic clock based on cord blood methylation of 148 CpGs (Knight *et al.*, 2016) accurately estimated gestational age across all samples (Pearson's $r = 0.624$; $P = 2.92e\text{-}11$). Compared to controls, the ICSI group showed a significant ($P = 0.008$) decrease in DNA methylation age at birth (Fig. 1). ICSI newborns were lagging approximately half a week behind the controls. No significant between-group difference in age acceleration could be detected, which may be due to the relatively low range of gestational age (38–42 weeks).

To identify candidate genes which are susceptible to ART reprogramming, we focused our further analysis on promoter regions. A total of 15 490 promoters were interrogated by at least two array CpGs. Altogether, 57 CpGs in 18 genes displayed a methylation difference of $\beta > \pm 0.03$ and an adjusted *P*-value < 0.05 (Table III). Differentially methylated promoters were enriched for both micro RNAs (χ^2 test, $P = 0.01$) and small nucleolar RNAs, C/D box (*SNORD*) genes ($P = 0.0002$).

Table I Clinical parameters of analyzed cord blood samples.

Array cohorts	Controls	ICSI	*P*-value
Sample size (*N*)	46	48	
Comorbidities (*N*)[a]	1 H, 1 T	None	
Maternal age (years; mean ± SD)	30.3 ± 5.8	34.2 ± 3.7	<0.001
Mode of birth[b]	74% UVB, 6% VVB, 20% CS	56% UVB, 15% VVB, 29% CS	0.19
Gestational week (mean ± SD)	39.5 ± 1.4	39.8 ± 1.2	0.90
Sex of child	52% male, 48% female	50% male, 50% female	0.84
Birth weight (g; mean ± SD)	3391 ± 576	3363 ± 469	0.35
Weight for gestational age[c]	4% SGA, 96% AGA	100% AGA	0.50
Blood pH at birth (Mean ± SD)	7.29 ± 0.09	7.27 ± 0.08	0.31

Pyrosequencing cohorts	Controls	ICSI	IVF	*P*-value
Sample size	53	41	34	
Comorbidities (*N*)[a]	1 H, 2 T	5 D, 1 H, 1 T	1 D, 1 T	
Maternal age (years; mean ± SD)	30.2 ± 5.9	34.0 ± 3.9	34.3 ± 4.5	<0.001
Mode of birth[b]	73% UVB, 6% VVB, 21% CS	69% UVB, 8% VVB, 23% CS	33% UVB, 7% VVB, 60% CS	0.002
Gestational week (mean ± SD)	39.5 ± 1.4	40.1 ± 1.4	39.7 ± 1.6	0.23
Sex of child	53% male, 47% female	59% male, 41% female	43% male, 57% female	0.46
Birth weight (g; Mean ± SD)	3344 ± 480	3357 ± 593	3221 ± 549	0.66
Weight for gestational age[c]	4% SGA, 96% AGA	13% SGA, 87% AGA	12% SGA, 88% AGA	0.23
Blood pH at birth (Mean ± SD)	7.30 ± 0.09	7.30 ± 0.07	7.29 ± 0.07	0.95

[a]D, diabetes mellitus; H, hypertension; P, preeclampsia; T, thyroid dysfunction.
[b]CS, cesarean section; UVB, unassisted vaginal birth; VVB, ventouse-assisted vaginal birth.
[c]SGA, small for gestational age (<third percentile); AGA, appropriate for gestational age (3rd–97th percentile), no babies were large for gestational age (>97th percentile).

The *SNORD114-9* gene is located in the imprinted *DLK1-DIO3* cluster and reminiscent of the *SNORD* cluster in the Prader-Willi/Angelman region (Cavaille *et al.*, 2002). The top-ranked ($\beta = 3.8\%$; $P = 0.004$) gene was *BAZ2B*, whereas the *ATG4C* promoter was endowed with the highest number ($N = 9$) of significant CpG sites.

Because imprinted genes are frequently used as a model for studying the epigenetic risks of ARTs (Denomme and Mann, 2012), we performed an in-depth analysis of imprinted control regions (ICRs). A set of 227 array CpGs has recently been mapped to imprinted iDMRs (Pidsley *et al.*, 2013). Eight (3.5%) of these 227 imprinted CpGs displayed significant (adjusted $P < 0.05$) methylation differences between the ICSI and control group (Table IV). Considering that overall only 0.11% array CpGs are differentially methylated, this is a significant (Fisher's exact test; $P = 0.003$) enrichment. Three significant CpGs are located in the ICR of the *NAP1L5-HERC3* locus on chromosome 4 and two in *L3MBTL* on chromosome 20. The ICRs in the *HM13* and *GNAS* loci, which are also on chromosome 20, are endowed with one significant CpG each. In addition, there is one significant imprinted CpG in *SGCE-PEG10* on chromosome 7.

Similarly, we looked for CpG islands (CGI) which are differentially methylated between ICSI and control group. Four of the 26 490 interrogated CGIs displayed a methylation difference of $\beta > \pm 0.03$ and an adjusted $P < 0.05$ (Table V). The top-ranked CGI (with nine significant CpGs) was located within the *WRB* gene on chromosome 21. In addition, the *ELL2P1* gene on chromosome 1 and the non-protein-coding RNA *LINC00273* on chromosome 16 contained differentially methylated CGIs. The fourth CGI is located in an intergenic region between CTD-2522B17.8 and CTD-2522B17.5, ~25 kb upstream of the CGI in *LINC00273*.

Three genes with differentially methylated array CpGs in their promoter regions, *ATG4C*, *BAZ2B* and *SNORD114-9*, were selected for validation with bisulfite pyrosequencing of cord bloods from 41 ICSI, 34 IVF and 53 naturally conceived children (Table I). Only ICSI samples that had not been on the array were selected. The same was true for IVF. The 53 controls included the 46 array samples. Two pyrosequencing assays were designed for *ATG4C*, one covering 17 CpGs (including five significant array CpGs) and another one with five CpGs (including four array CpGs) (see Supplementary data, Table SI). The *BAZ2B* assay targeted four CpGs including one of the two significant array CpGs. Two pyrosequencing assays for *SNORD114-9* targeted three CpGs (including one array CpG) each. When using multivariate regression analysis to adjust for the confounding factors maternal age, mode of birth, gestational age, birth weight and sex of the child, *ATG4C* assay 1 revealed a small but highly significant hypermethylation in both the ICSI ($P < 0.001$) and IVF group ($P = 0.002$), compared to controls (see Supplementary data, Table SII). The *SNORD114-9* assay 1 revealed a trend difference ($P = 0.083$) between ICSI and control group, but not between IVF and controls. In a multivariate regression analysis of individual array CpGs, CpGs 1 and 15 (cg18129198 and cg16739396) of *ATG4C* assay 1 showed a significant difference between ICSI and controls ($P = 0.007$ and 0.002) and a trend difference between IVF and controls $P = 0.002$ and 0.069 (see Supplementary data, Table SIII). CpG 3 (cg26435481) of *SNORD114-9* assay 1 differed significantly between ICSI and controls ($P = 0.010$), but not between IVF and controls.

Table II Methylation level and genomic distribution of significant (differentially methylated) sites.

Genomic region/feature		Non-significant sites (*N*)[a]	Significant sites (*N*)[a]	Significant sites (%)[a]	*P*-value[b]
Methylation level	0–20%	162 464	3074	1.86	<0.001
	21–80%	83 042	739	0.88	
	81–100%	177 991	917	0.51	
Relation to CpG islands	CpG island	134 154	2256	1.65	<0.001
	North shelf	20 987	153	0.72	
	North shore	55 865	542	0.96	
	South shelf	187 17	169	0.89	
	South shore	43 632	472	1.07	
	Open sea	150 142	1138	0.75	
Enhancer	No	328 388	3950	1.19	<0.001
	Yes	95 109	780	0.81	
DMRs[c]	DMR	16 522	331	1.96	
	rDMR	11 362	101	0.88	
	cDMR	5984	57	0.94	
	iDMR	219	8	3.5	<0.05
DNase I hypersensitive	No	370 086	3906	1.04	<0.001
	Yes	53 411	824	1.52	

[a]Significant sites are differentially methylated (after multiple testing correction) between ICSI and control group, whereas non-significant sites display comparable methylation values in both groups.
[b]For enrichment of genome-wide significant sites in a given CpG subgroup.
[c]cDMR: cancer-specific DMR, rDMR: reprogramming-specific DMR, iDMR imprinted DMR. Array CpGs for DMRs, rDMRs and cDMRs were annotated by Illumina, array CpGs for iDMRs by Pidsley *et al.* (2013).

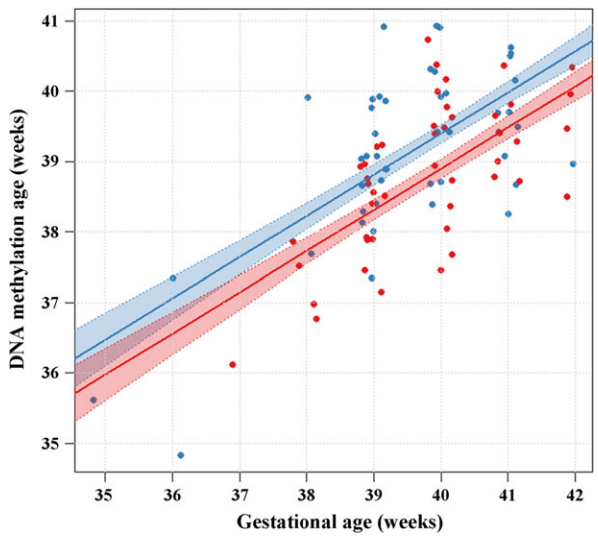

Figure 1 DNA methylation age in fetal cord blood. ICSI samples are indicated by red dots and controls by blue dots. The gestational age of all samples is positively correlated with DNA methylation age. The regression lines (standard errors are shaded in red and blue, respectively) indicate that at the same gestational age ICSI samples display a slightly lower epigenetic age than controls.

Discussion

Several candidate gene studies have associated specific methylation changes, i.e. in the imprinted *LIT1* (Gomes *et al.*, 2009; Zechner *et al.*, 2010), *SNRPN* (Whitelaw *et al.*, 2014) and *PLAG1* (Vincent *et al.*, 2016) loci with ART, whereas other studies did not find an increase of abnormal methylation patterns in ART (Tierling *et al.*, 2010; Oliver *et al.*, 2012) or abnormalities were restricted to a single sample (Kanber *et al.*, 2009; Feng *et al.*, 2011). Two genome-wide studies (Katari *et al.*, 2009; Melamed *et al.*, 2015), assessing ~1500 and 27 000 CpG sites, respectively, identified a number of differentially methylated genes in IVF children, however, due to small sample size (10 IVF children versus 13 and 8 controls, respectively) these results have to be interpreted with caution. A recent more comprehensive 450K methylation array study (Estill *et al.*, 2016) on air-dried blood spots (Guthrie cards) from 76 children conceived by ICSI, 18 by intrauterine insemination, and 43 controls revealed numerous differences, in particular at metastable epialleles between ICSI and spontaneously conceived children. A single genome-wide significant DMR (upstream of *TNP1*) between 47 IVF and 60 control twins was detected using methylated cord blood DNA immunoprecipitation (Castillo-Fernandez *et al.*, 2017).

Here, we performed a 450K array study using cord blood samples of 48 ICSI and 46 control newborns. Consistent with previous array screens, a relatively large number (0.11%) of analyzed CpG sites exhibited genome-wide significant between-group methylation differences. However, in this context it is important to emphasize that although

epigenetic changes were widespread, none of them had a large effect size. This is an inherent problem of methylation array studies assessing the epigenetic effects of environmental exposures. Considering the enormous methylation variation between individuals, there was an extensive overlap in methylation levels between ART and control samples. The observed methylation patterns in both the ICSI and the control group were within the normal range of methylation variation. In this context, it is noteworthy that our ICSI array cohort consisted of healthy newborns without reported imprinting disorders, intrauterine growth retardation, congenital malformations, delivery and postnatal complications. We cannot exclude larger effects in the epigenomes of newborns (conceived by ICSI or spontaneously) with severe medical problems. Nevertheless, it is plausible to assume that the observed minor ART-induced epigenetic changes in healthy ICSI newborns contribute to their phenotypic variation. Consistent with a multifactorial model, it may be the sum of subtle epigenetic changes modulating the phenotype and disease risk in ART children.

Table III Differentially methylated promoters in ICSI versus control cord blood samples.

Gene	Methylation β difference[a]	Adjusted *P*-value	Number of CpGs
BAZ2B	0.038	0.004	2
EIF4E2	0.041	0.006	2
C21orf128	0.080	0.017	3
ELL2P1	0.039	0.024	5
EMR4P	−0.044	0.025	2
MIRLET7C	−0.034	0.025	4
STX19	−0.047	0.026	2
MIR1296	−0.032	0.029	3
SNORD114-9	−0.048	0.032	2
ACTRT3	0.033	0.032	2
GUSBP6	−0.060	0.032	3
LINC00189	0.054	0.033	2
MTHFD2P1	−0.033	0.039	2
ATG4C	0.031	0.043	9
SNORD11	−0.031	0.043	2
C17orf98	0.031	0.044	8
MIR215	−0.031	0.044	2
KBTBD4	0.037	0.048	2

[a]Positive β difference indicates hypermethylation and negative β hypomethylation in the ICSI group.

Differentially methylated CpGs (ART versus controls) were enriched in CpG islands and usually displayed low-blood methylation values (0–20%). By applying strict criteria, we identified 18 promoter regions (six in non-coding RNA genes), four CpG islands (two in protein-coding genes and two in or close to a long non-coding RNA), and two imprinted DMRs containing multiple differentially methylated sites. In a previous 450K methylation array study (Estill *et al.*, 2016), imprinted genes including *HERC3-NAP1L5*, *PEG10* and *L3MBTL* were also found to be enriched with differentially methylated sites (male and/or female ICSI versus controls, respectively). The loci identified here are primary candidates by which ART may transmit effects to the exposed offspring and need to be followed up. We propose that epigenetic regulation of gene expression by non-coding RNAs and other regulatory elements plays a major role in mediating interactions between genes and ART environment.

Interestingly, ICSI was associated with a slightly decreased DNA methylation age at birth. Accumulating evidence suggests that epigenetic age is at least a passive biomarker of biological age. Blood DNA methylation age estimates can be used as a predictor for all cause mortality (Marioni *et al.*, 2015a; Perna *et al.*, 2016), cognitive and physical functioning (Marioni *et al.*, 2015b), and progeroid syndromes (Maierhofer *et al.*, 2017). However, whether and to which degree the observed changes in epigenetic age are causes or consequences of the associated phenotypes remains to be elucidated.

In individual cells, most importantly in the oocyte and the sperm that are combined at fertilization (through ART or spontaneously), a given CpG is either methylated or not. The observed small ICSI-associated methylation changes in blood (and, by extrapolation, in other tissues) imply that ICSI is associated with loss or gain of methylation at this CpG in a fraction of cells. Accumulating evidence from animal models and humans (Velker *et al.*, 2012; El Hajj and Haaf, 2013; Fauque, 2013; Urrego *et al.*, 2014; Anckaert and Fair, 2015; de Waal *et al.*, 2015) suggests that most ART-related epigenetic changes occur after fertilization, most likely due to impaired maintenance of the germ-line

Table IV Differentially methylated CpGs in imprinting control regions (iDMRs).

Array CpG	Methylation β difference[a]	Average methylation	Adjusted *P*-value	Location	Gene(s)
cg06617468	0.022	0.600	0.034	Chr4: 89,619,023	*NAP1L5;HERC3*
cg18607468	0.027	0.619	0.032	Chr4: 89,619,030	*NAP1L5;HERC3*
cg27150681	0.019	0.620	0.046	Chr4: 89,618,861	*NAP1L5;HERC3*
cg11562309	0.036	0.514	0.010	Chr7: 94,286,473	*SGCE;PEG10*
cg17840843	0.017	0.366	0.028	Chr20: 30,135,158	*HM13*
cg01071811	0.044	0.567	0.024	Chr20: 42,143,080	*L3MBTL*
cg02611863	0.045	0.549	0.045	Chr20: 42,143,096	*L3MBTL*
cg01355739	−0.024	0.570	0.014	Chr20: 57,416,888	*GNAS*

[a]Positive β difference indicates hypermethylation and negative β hypomethylation in the ICSI group.

Table V Differentially methylated CpG islands (CGIs) in ICSI versus control cord blood samples.

Location	Methylation β difference[a]	Average methylation	Adjusted *P*-value	Upstream gene	Distance to CGI	Gene with CGI	Downstream gene	Distance to CGI	Number of CpGs
Chr21: 40,760,626–40, 760,829	−0.038	0.212	0.006	*RNF6P1*	−11 633	*WRB*	*LCA5L*	16 940	9
Chr1: 158,147,433-158,147,854	0.053	0.608	0.010		−37 003	*ELL2P1*	*CD1D*	1882	4
Chr16: 33,961, 237-33,962,487	0.040	0.718	0.031	*AC136932.2*	−15 215	*LINC00273*	*RNA5–8SP2*	2938	2
Chr16: 33,936,400–33,936,681	0.036	0.778	0.017	*CTD-2522B17.8*	−13 255		*CTD-2522B17.5*	1521	3

[a]Positive β difference indicates hypermethylation and negative β hypomethylation in the ICSI group.

methylation patterns (i.e. of imprinted genes) or the appropriate methylation patterns for somatic development. Maternal nutritional studies, most importantly the Dutch famine birth cohort have convincingly shown that periconceptional exposure to an adverse environment can lead to persistent changes (malprogramming) of the epigenome and lifelong increased disease risks (Roseboom *et al.*, 2006; El Hajj *et al.*, 2014). We propose that similar to maternal under/overnutrition, different ARTs may represent an adverse environmental exposure, which increase the probability of methylation changes at specific loci during embryogenesis and somatic differentiation.

Due to legal and ethical restrictions, it is not possible to study the relevant tissues for developmental reprogramming of metabolic or cardiovascular phenotypes. Although it is well known that the epigenomes differ between cell-types/tissues, analysis is generally restricted to easily accessible tissues such as blood. Although the number of ART and control samples analyzed here meets current standards for epigenetic screens, sample size is two orders of magnitude below that of genome-wide association studies for complex phenotypes. ART and control samples were matched for some known confounders (i.e. sex, gestational age and birth weight), however, this was not possible for maternal age. ART patients are older than the average couple wishing to have a child. Moreover, male and/or female infertility are only present in the study group. Thus, we cannot distinguish which epigenetic changes in ART children are due to parental infertility and which to the ART procedure itself. It is well known that sperm samples with poor semen parameters (which is the main indication for ICSI) display increased epimutation rates which may be transmitted to the next generation (Marques *et al.*, 2008; Kobayashi *et al.*, 2009; Kuhtz *et al.*, 2014; Atsem *et al.*, 2016). In our array study, none of the analyzed CpGs in ICSI children displayed a significant correlation with sperm concentration. This argues in favor of the notion that the observed effects are not primarily caused by male infertility.

Because existing ART data sets are likely still polluted with false positives and false negatives, we analyzed three candidate genes from our methylation screen by bisulfite pyrosequencing in ICSI, IVF and control samples. At the single CpG level, two of nine significant array CpGs in *ATG4C* and one of two in *SNORD114-9* could be validated. However, the pyrosequencing assays targeted a larger number of contiguous CpGs (22 for *ATG4C* and 6 for *SNORD114-9*) than the arrays and, therefore, allowed more accurate measurements of the regional methylation levels. Because, it is usually the density of CpG methylation in a cis-regulatory region rather than individual CpGs that turns a gene 'on' or 'off' (Weber *et al.*, 2007), average methylation of a target region is more telling than single CpG methylation. Using average methylation of all CpGs in a given assay as a measure and following adjustment for the confounding factors maternal age, mode of birth, gestational age, birth weight and sex of the child, one candidate, *ATG4C* was validated. *ATG4C* assay 1 displayed a significant methylation difference between ICSI and control as well as between IVF and control group. *SNORD114-9* (assay 1) showed a trend difference between ICSI and control group. The autophagy related 4C cysteine peptidase (*ATG4C*) gene plays an essential role in autophagy related processes (Marino *et al.*, 2003). Two tandem arrays of 9 *SNORD113* and 31 *SNORD114* copies are located in the imprinted *DLK1-DIO3* locus (Cavaille *et al.*, 2002; Girardot *et al.*, 2012). Since both downregulation of *atg4c*-mediated autophagic activity (Marino *et al.*, 2007) and upregulation of the *SNORD114* cluster (Liuksiala *et al.*, 2014) have been implicated in cancer development, it is interesting to speculate that increased *ATG4C* and decreased *SNORD114-9* methylation in ICSI children may modulate cancer susceptibility.

Conclusions

Although genome-wide methylation array studies can detect epigenetic signatures of ART in the offspring, affecting ~0.1% of CpG sites, overall the epigenomes of ART and spontaneously conceived children are very similar, displaying a normal range of methylation variation. In our cohort of healthy ART newborns there are no changes of large effect size detectable. Similar to hits in GWAS, the observed minor but significant between-group methylation differences may help to identify genes, pathways, and/or mechanisms (i.e. gene-regulation by non-coding RNAs) which are susceptible to reprogramming by ART. To separate the effects of ARTs and numerous confounding factors, we will need much larger numbers of samples and better clinical information. Only meta-analyses combining genome-wide data sets generated in different laboratories with different cohorts may reveal a more complete picture. To develop predictive markers for the long-term disease risk and possible therapeutic interventions, it will be important to systematically follow-up ART children. In this light, the Dutch famine birth cohort studies (Roseboom *et al.*, 2006) can serve as a model.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Authors' roles

N.E.H. and T.Hf. designed the study and wrote the manuscript, N.E.H., L.H. and S.D. performed the experiments, N.E.H., and M.D. analyzed the data, T.Hn., H.L. and M.S. contributed materials. All authors read and approved the final manuscript.

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Conflict of interest

None declared.

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