A systematic review and meta-analysis of DNA methylation levels and imprinting disorders in children conceived by IVF/ICSI compared with children conceived spontaneously

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BACKGROUND: Increasing numbers of children are being conceived by assisted reproductive technology (ART). A number of studies have highlighted an altered epigenetic status in gametes from infertile couples and the possibility of an increased risk of imprinting defects and somatic epigenetic changes in ART conceived children, but the results have been heterogeneous. We performed a systematic review of existing studies to compare the incidence of imprinting disorders and levels of DNA methylation in key imprinted genes in children conceived through in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) with those in children conceived spontaneously.

METHODS: A detailed search strategy was used to conduct electronic literature searches (spanning 1978 to 2013) on Medline, EMBASE, the Cochrane Library and Web of Science. Abstracts of relevant conference papers were identified. As randomized trials are not feasible in this context, we included observational (cohort and case–control) studies comparing outcomes in children conceived through ART with those conceived spontaneously, irrespective of the language of publication. The outcome measures were DNA methylation and the incidence of imprinting disorders.

RESULTS: A total of 351 publications were identified by the initial search. Of these, 26 were excluded as duplicates and 241 were excluded after reviewing the abstracts, then of those remaining 66 were excluded after review of the full text. A total of 18 papers were included in the review. Apart from one case–control study, all were cohort studies. There was a degree of clinical heterogeneity in terms of the study population, type of infertility treatment, and samples obtained from exposed and unexposed children. DNA methylation levels were either presented as categorical data (haplo-, hyper- or normally methylated DNA) or continuous data (i.e. percentage of methylated DNA). The combined odds ratio (95% confidence intervals) of any imprinting disorder in children conceived through ART was 3.67 (1.39, 9.74) in comparison with spontaneously conceived children. Meta-analysis of data from relevant studies revealed that the weighted mean difference (95% confidence intervals) in methylation percent between IVF/ICSI versus spontaneously conceived children were as follows: H19: −0.46 (−1.41, 0.49), PEG1-MEST: 0.47 (−2.07, 3.01), GRB10: −0.05 (−0.43, 0.33), IGF2: −0.15 (−1.09, 0.79), SNRPN: −0.55 (−1.55, 0.46), KvDMR/KCNQ1OT1: −0.16 (−0.34, 0.02) and PEG3: −0.24 (−1.72, 1.24).

CONCLUSIONS: There was an increase in imprinting disorders in children conceived though IVF and ICSI but insufficient evidence for an association between ART and methylation in other imprinted genes. Heterogeneity in the types of fertility treatment, the imprinted regions studied, the tissues used and the methods of measurement, reduce our ability to assess the full effect of ART on DNA methylation and imprinting. More controlled studies, using standardized methodologies, in larger, better clinically defined populations are needed.

Key words: IVF / ICSI / DNA methylation / epigenetic disorders / imprinting disorders

Introduction

Over 48 000 women in the UK underwent IVF treatment in 2011 and the number continues to rise (www.hfea.org.uk). Worldwide, ART has been responsible for the birth of 5 million infants (Cedars, 2013). Children conceived through IVF now account for 2% of all births and there is considerable interest in their long-term health. A number of studies have highlighted an association between ART and birth defects (Hansen et al., 2002; Davies et al., 2012), cardiometabolic disorders and subclinical hypothyroidism (Ceelen et al., 2007, 2008; Sakka et al., 2009, 2010; Kanaka-Gantenbein et al., 2010). Changes in DNA methylation and the frequency of imprinting disorders have also been linked with ART (Manipalvira et al., 2009; Owen and Segars, 2009).

Epigenetics refers to the information in the genome over and above that contained in the DNA sequence and epigenetic regulation is central to many aspects of genome function (Haggarty and Ferguson-Smith, 2013). Epigenetic modifications include DNA methylation, histone modification, remodelling of nucleosomes, higher-order chromatin reorganization and regulation by noncoding RNAs (Sasaki and Matsui, 2008). DNA methylation is the most commonly studied epigenetic process in relation to fertility treatment and imprinting syndromes.

Epigenetic activity is closely linked with critical developmental steps which occur around the time of conception (Ferguson-Smith and Patti, 2011; Hackett and Surani, 2013). A key phenomenon in early development is imprinting, where genes are epigenetically regulated and expressed according to parental origin. Imprinting syndromes can result in significant pathology and, although uncommon in the general population (Odom and Segars, 2010), these conditions are thought to occur more frequently in the offspring of subfertile parents (Gosden et al., 2003).

Ovarian stimulation and retrieval of oocytes, manipulation of spermatozoa and the duration of embryo culture are all thought to influence methylation changes and imprinting disorders (Edwards, 2003) but reports of the effects of IVF on imprinting have been heterogeneous (Kanber et al., 2009; Le Bouc et al., 2010; Tierling et al., 2010; Oliver et al., 2012; Nelissen et al., 2013; Zheng et al., 2013). This is perhaps not surprising due to heterogeneity in the types of fertility treatment included, the imprinted regions studied, the tissues used, and the methods of measurement.

Our objectives were to conduct a systematic review of the literature on DNA methylation levels and the risk of epigenetic and imprinting disorders in children conceived through IVF/ICSI compared with children conceived spontaneously (objective a) and in children born spontaneously to infertile couples were compared with children born spontaneously to fertile couples (objective b).

Methods

Population, exposures and comparators

Using standard systematic review techniques, a focused review question was initially developed using the following population, exposure, comparator and outcomes (PECO). The population was children conceived through ART or spontaneously. The exposures were (a) IVF/ICSI and (b) history of infertility in parents. The comparators were (a) children conceived spontaneously by fertile or infertile couples and (b) children conceived spontaneously by fertile couples.
Outcome measures
The following outcome measures were considered: (a) subclinical alterations in DNA methylation and (b) imprinting disorders (altered gene expression in imprinted genes).

We anticipated that DNA methylation levels would be reported as either categorical (DNA is either hypo-, hyper- or normally methylated) or continuous data (i.e. percentage of methylated DNA).

Literature searches
Electronic literature searches were performed on Medline, EMBASE, the Cochrane Library and Web of Science, using a detailed search strategy (Supplementary data, Information S1) to identify all articles published between 1978 and September 2013. Two different search strategies were used for objectives (a) and (b). We did not apply any language restrictions to our search. Two review authors (M.K. and G.L.) independently conducted the literature searches and selected the studies to be included. Any differences in opinion were resolved through team discussion. We also searched abstracts from relevant conference papers. Eligible studies were included irrespective of country and language. Authors of papers which appeared to be relevant, but lacked adequate information were contacted via e-mail.

Inclusion criteria
As we did not expect many data from randomized controlled trials, we included cohort and case-control studies. For objective (a) we included studies that compared DNA methylation levels and the incidence of imprinting and epigenetic disorders in children conceived through IVF/ICSI and children conceived spontaneously. For objective (b), we only included studies reporting outcomes of spontaneous pregnancies in infertile compared with fertile couples.

Exclusion criteria
Studies were excluded if there was no control group, e.g. case series and case reports. Review papers were excluded; however, the primary research papers referenced in them were included if appropriate. Many epigenetic processes are species specific; therefore animal studies were also excluded. Studies involving samples from children (conceived spontaneously or following ART) with imprinting syndromes known to involve epigenetic changes were also excluded from meta-analyses of data on DNA methylation.

Types of tissue samples included in the review
Many imprinted genes maintain their allele-specific methylation signal in a wide range of adult human somatic tissues over decades (Sandovici et al., 2003; Coolen et al., 2011; Woodfine et al., 2011). Therefore, of all the categories of genes that could be studied, imprinted genes are probably the most amenable to general extrapolation from different tissues. We decided to include methylation data regardless of the source of the sample, e.g. peripheral blood, placenta, umbilical cord blood or buccal mucosa. Where feasible, we aggregated data from samples regardless of the tissue of origin. This decision was informed by data from a study (Byun et al., 2009) which suggested that DNA methylation from different tissues could be aggregated.

Selection of studies, data extraction and meta-analysis
Studies with relevant titles were subjected to a review of the abstract, and then, if appropriate, a full-text review (Figs 1 and 2). Data extraction was performed by G.L. using a data extraction template (Supplementary data, Information S2). All of the findings were then grouped by a specific epigenetic/imprinting disorder or a specific DNA region. We wrote to authors to seek additional data where this was necessary to obtain data in a format suitable for meta-analyses. Review Manager 5.1 software was employed to conduct meta-analyses, where feasible, using a random effects model in all analyses of mean methylation at various sites and a fixed effect model for any imprinting disorder.

Risk of bias (quality of the included studies)
The Downs and Black Checklist (Downs and Black, 1998) was used to assess the quality of included studies (Table I). This has 15 questions which can be answered in the affirmative (i.e. ‘yes’), generating a score of 1 or negative (i.e. ‘no’) producing a score of 0. A maximum of 15 points can be awarded to any study.

Results
Epigenetic and imprinting disorders in children conceived through IVF/ICSI compared with children conceived spontaneously
The electronic literature search on Medline, EMBASE, The Cochrane Library and Web of Science yielded a total of 351 articles for objective (a) (Fig. 1). Of these, 26 studies were excluded as duplicates and 241 were excluded based on the title and abstract either because they were review articles, irrelevant to our review (i.e. the title suggests an entirely different topic) studies performed on animals. Of the remaining 84 articles, 66 were excluded after a full-text review (Supplementary data, Table S1), leaving 18 relevant articles including two conference abstracts. The reason for exclusion of most of these articles was the fact that they lacked a control group or were review papers.

Of the 18 articles included, 17 were cohort studies and 1 was a case-control study (Table I). The Downs and Black Checklist was used to evaluate their quality (Table I) and the average score was 10.9.

The number of participants varied across the studies (Table I). Some included both IVF and ICSI conceptions without differentiating between the two (e.g. Turan et al., 2010; Puumala et al., 2012; Rancourt et al., 2012). Others did differentiate between IVF and ICSI and produced separate results for children conceived via IVF and for children conceived via ICSI (e.g. Tierling et al., 2010). One study (Chan Wong et al., 2011b) presented results from six different groups of children (IVF-AGA (appropriate for gestational age), IVF-SGA (small for gestational age), ICSI-AGA, ICSI-SGA, NC (naturally conceived)-AGA and NC-SGA) instead of two (IVF/ICSI and NC). Non-IVF pregnancies resulting from ovulation induction were reported by one of the studies (Rancourt et al., 2012), but we excluded data related to those 27 conceptions and only included data from 59 children conceived via IVF/ICSI. Some studies (Hiura et al., 2012) included a population of children with known imprinting disorders such as Silver–Russell syndrome or Beckwith–Wiedemann syndrome, and therefore data from these studies could not be aggregated with data from other studies on DNA methylation based on clinically normal children.

Some of the studies used one type of tissue sample for DNA extraction though the tissue varied by study, e.g. chorionic villi samples (Chan Wong et al., 2011b), umbilical cord blood samples (Li et al., 2011), peripheral blood samples (Oliver et al., 2012). Others reported methylation levels in more than one tissue type, e.g. cord blood, cord and placenta samples (Turan et al., 2010), peripheral blood, buccal...
samples (Puumala et al., 2012), umbilical cord, amnion/chorion tissue samples (Tierling et al., 2010). Results from placenta samples are included here for completeness but there may be differences in epigenetic status between the trophoblast lineages and the tissues of the offspring: The ‘conflict theory’ of imprinting (Wilkins and Haig, 2003) is based on the premise that the function of the imprinted genes is to control resource allocation to the fetus (Ferguson-Smith et al., 2006). Therefore, where placental data have been included in the forest plots, this has been highlighted.

We used a data extraction template (Supplementary data, Information S2) for the studies included in the review. We were only able to combine data from studies that presented mean (SD) percentages of methylated DNA or where the authors were able to provide these data on request. We were unable to combine data from studies that used other methylation measures, e.g. a study (Tierling et al., 2010) which used a parameter called Methylation Index, which is not equivalent to the exact percentage of methylated DNA. We were also unable to include one of the papers (Turan et al., 2010) in the meta-analysis as the outcomes were presented as the ratio between DNA methylation levels on maternal and paternal alleles. Where data were presented as the mean methylation and standard error of the mean (SEM), we converted SEM into standard deviation (SD) using the formula SEM = SD/sqrt(N), where N is the number of observations.

**Figure 1** Flowchart of included/excluded studies for objective (a).

### DNA methylation at specific regions

#### H19

Ten papers reported on H19 (Supplementary data, Table SII). Tissues examined included peripheral blood, placenta, umbilical cord blood, buccal swabs and amnion/chorion. The studies varied in their methods of selecting the sample population and presenting the outcomes. Two studies (Chan Wong et al., 2011a; Hiura et al., 2012) analysed samples from IVF/ICSI conceived children as well as those who were spontaneously conceived; one (Hiura et al., 2012) included children...
with Silver–Russell syndrome (SRS), whereas another (Chan Wong et al., 2011b) reported on clinically normal children born either small for gestational age or appropriate for gestational age.

Some studies presented DNA methylation results as mean methylation (with or without standard deviation or variance), median or range methylation percentages or mean methylation index. Methylation status was reported as median % methylation (Li et al., 2011; Rancourt et al., 2012), mean and variance, mean (standard deviation) methylation index (Hiura et al., 2012) or methylation index (maternal/paternal ratio) (Tierling et al., 2010; Turan et al., 2010).

The mean methylation percentage in IVF/ICSI children ranged from 38.07 (Puumala et al., 2012) to 51.29% (Oliver et al., 2012), whereas mean methylation in spontaneously conceived children ranged from 35.15 (Puumala et al., 2012) to 51.51% (Oliver et al., 2012).

A majority of studies (i.e. Kanber et al., 2009; Chan Wong et al., 2011a; Hiura et al., 2012; Oliver et al., 2012; Rancourt et al., 2012) expressed H19 methylation in terms of the mean (standard deviation) percentage which allowed us to include them in a meta-analysis (Fig. 3A). We excluded data from one of the studies (Hiura et al., 2012) from the meta-analysis as all samples were obtained from children with Silver–Russell syndrome or Beckwith–Wiedemann syndrome, i.e. children with a diagnosed imprinting defect. Another study (Chan Wong et al., 2011b) included different groups of children: IVF-SGA, IVF-AGA, ICSI-AGA, ICSI-SGA, NC-SGA and NC-AGA (in this study, SGA was defined as birthweight below the 10th percentile for gestational age). We attempted meta-analyses by grouping data in different ways. First we combined results from two studies (Kanber et al., 2009; Oliver et al., 2012), with results from IVF-AGA, ICSI-AGA and NC-AGA (Chan Wong et al., 2011a, b). Next, we tried combining the two former studies with IVF-AGA, IVF-SGA, ICSI-AGA, ICSI-SGA, NC-AGA and NC-SGA (Chan Wong et al., 2011a, b). The results were similar irrespective of whether or not we included children born small for gestational age and we decided to include all six groups (Chan Wong et al., 2011a, b) in our meta-analysis. This yielded a

Figure 2 Flowchart of included/excluded studies for objective (b).
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Weighted mean difference (95% CI) of $-0.22\% (-0.44\%; -0.01\%)$ in terms of DNA methylation in spontaneously conceived children compared with those conceived by IVF or ICSI (Fig. 3A). It is worth noting that the children in one of the studies (Kanber et al., 2009) were appropriate for gestational age in the natural conception group but born small for gestational age in the IVF/ICSI group. Since these children were otherwise healthy, we decided to include them. Overall, the pooled percentage mean methylation difference (95% confidence intervals) for H19 was $-0.46 (-1.41, 0.49)$, suggesting no evidence of a statistically significant difference (Fig. 3A).

**PEG1/MEST**

Seven articles in total studied methylation of PEG1/MEST in a number of tissues including peripheral blood, placenta, umbilical cord blood, buccal swabs and amnion/chorion tissue. Of studies which focused on children with imprinting defects, one (Hiura et al., 2012) found evidence of hypermethylation in four children in total (three IVF/ICSI and one spontaneously conceived), whereas another (Rossignol et al., 2006) identified three naturally conceived children who had hypomethylation of the region. Results of a meta-analysis presented in Fig. 3B do not show altered DNA methylation in ART children. The pooled percentage mean methylation difference (95% confidence intervals) was $0.47 (-2.07, 3.01)$.

**GRB10**

Three papers studied GRB10 methylation (Supplementary data, Table SII). Tissues studied included umbilical cord blood, amnion/chorion tissue, placenta, cord blood, buccal smears and peripheral blood. One study (Hiura et al., 2012) found that two children with SRS
conceived through IVF/ICSI had hypomethylation at the GRB10 region. Meta-analysis of data from two different sets of samples from the only eligible study (Rancourt et al., 2012) failed to demonstrate altered DNA methylation in samples from ART conceived children with a pooled percentage mean methylation difference (95% confidence intervals) of $-0.05\ (-0.43, 0.33)$ (Fig. 3C).

IGF2

Four papers reported findings on IGF2 (Oliver et al., 2012; Puumala et al., 2012; Rancourt et al., 2012; Nelissen et al., 2013) (Supplementary data, Table SII). The first paper (Rancourt et al., 2012) reported methylation data on placenta and cord blood samples of both IVF and spontaneously conceived children; the second (Puumala et al., 2012) reported mean

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**Figure 3** Forest plot analyses for weighted mean difference (95% confidence intervals) in methylation percent between IVF/ICSI versus spontaneously conceived children for (A) H19, (B) PEG1/MEST, (C) GRB10, (D) IGF2, (E) SNRPN, (F) KvDMR/KCNQ10T1 and (G) PEG3.
methylation (no standard deviation reported) in buccal and peripheral blood samples in their original paper but provided further data on request. The third (Nelissen et al., 2013) examined placental tissue, while the last (Oliver et al., 2012) reported mean (SD) percentage methylation from peripheral blood samples. Results of a meta-analysis shown in Fig. 3D did not show any significant difference in mean percentage methylation between ART and spontaneously conceived children with a pooled percentage mean methylation difference (95% confidence intervals) of 0.15% (−0.89, 0.79).

Two studies analysed the site of the IGF2R gene using peripheral blood and buccal samples (Rossignol et al., 2006; Puumala et al., 2012). One of them (Rossignol et al., 2006), not included in the meta-analysis, included children with Beckwith–Wiedemann syndrome and identified six with hypomethylation within IGF2R (two IVF/ICSI and four conceived spontaneously).

**SNRPN**

Six groups studied methylation of the SNRPN region (Supplementary data, Table SII). One study (Rossignol et al., 2006) reported SNRPN hypomethylation in ART conceived children with Beckwith–Wiedemann syndrome. Tissues studied included peripheral blood, placenta, umbilical cord blood, buccal swabs and amnion/chorion tissue. As Fig. 3D shows, results of a meta-analysis suggested increased DNA methylation in children conceived by IVF/ICSI with a pooled percentage mean methylation difference (95% confidence intervals) of −0.55% (−1.55%, 0.46%) although the results did not reach statistical significance.

**KvDMR/KCNQ1OT1**

The KvDMR, KCNQ1OT1 and KCNQ1 regions are found together within one of the imprinting control domains in chromosome 11p15.5 and have therefore been considered jointly in the meta-analysis and the forest plot.

Of the six articles reporting data on the KvDMR region (Supplementary data, Table SII), three reported hypomethylation of the region in both IVF/ICSI and spontaneously conceived children (Gomes et al., 2009; Li et al., 2011; Oliver et al., 2012). The mean percentage methylation in IVF/ICSI children ranged from 45.4% (Kanber et al., 2009) to 53.46% (Puumala et al., 2012), whereas mean methylation in spontaneously conceived children ranged from 46 (Oliver et al., 2012) to 52.6% (Puumala et al., 2012). One of the studies (Li et al., 2011) reported methylation status using median methylation percentages and another
(Tierling et al., 2010) presented their output as mean methylation index (with standard deviation). Tissues used when studying the KvDMR region included peripheral blood, placenta, umbilical cord blood, buccal swabs and amnion/chorion.

One study (Kanber et al., 2009) compared ICSI children who were born small for gestational age with naturally conceived normal children while another (Oliver et al., 2012) compared children conceived through assisted reproduction who were taller, had increased high-density lipoprotein levels, decreased triglycerides and increased IGF2 and IGFBP-I levels with a comparison group of naturally conceived normal children. However, as all of the children were otherwise clinically normal and healthy, we decided that it was reasonable to combine their KvDMR methylation percentages in our meta-analysis. The weighted pooled percentage mean difference (95% CI) in methylation between spontaneously conceived children and those conceived through ART was $-0.16 \ (95\% \ CI \ -0.34, \ 0.02)$ (Fig. 3F). Data from one of the relevant studies (Tierling et al., 2010) were not included in the meta-analysis as results were presented in a format (mean methylation index) which was inconsistent with results from the other studies.

**PEG3**

Two groups (Kanber et al., 2009; Hiura et al., 2012) reported PEG3 methylation in buccal swabs and peripheral blood, while one (Nelissen et al., 2013) studied placental samples (Supplementary data, Table SII). As Fig. 3G shows, meta-analysis of data from the two eligible studies which did not restrict their population to children with imprinting disorders showed no increase in methylation in IVF/ICSI children with a pooled percentage mean methylation difference (95% confidence intervals) of $-0.24 \ (-1.72, \ 1.24)$.

**Other genes**

One group (Feng et al., 2011) studied methylation of L3MBTL in nine ART conceived children and six spontaneously conceived children (Table I). The authors showed evidence of hypomethylation in ART-derived offspring.

A few authors studied a number of individual genes not listed in Supplementary data, Table SII. One paper (Hiura et al., 2012) reported on LIT1, RB1 and NAP1L5. Another two (Kanber et al., 2009; Rancourt et al., 2012) also analysed genes other than the ones previously noted; however, since these were featured in very few studies, it was difficult to be confident about the results and meta-analysis was not feasible.

One study (Tierling et al., 2010) reported on DLK1, MEG3, IG-DMR, GNAS, NEXP55, GNAS NESPas, GNAS XL- alpha-s, GNAS Exon1A using Methylation Index as the outcome and concluded that ART conceived children did not appear to be at a higher risk of imprinting disorders.

Another (Melamed et al., 2013) compared DNA methylation in cord blood samples from 10 IVF and 8 control offspring using a genome-wide approach. The results were suggestive of a genome-wide variation in methylation but this abstract contained insufficient data for aggregation of data in a meta-analysis or for a more definitive conclusion to be drawn.

**Overall risk of imprinting disorders in children conceived through IVF/ICSI compared with children conceived spontaneously**

Out of the 18 articles that were included in our systematic review, 4 focused on imprinting disorders (Halliday et al., 2004; Lidegaard et al., 2005; Sanchez-Albisua et al., 2007; King et al., 2010). One article (Lidegaard et al., 2005) included Prader–Willi syndrome, Angelman syndrome, retinoblastoma and kidney cancer in their list of imprinting disorders. Another (King et al., 2010) investigated skewed X inactivation, whereas a third (Sanchez-Albisua et al., 2007) concentrated on Angelman syndrome and the fourth (Halliday et al., 2004) presented results on Beckwith–Wiedemann syndrome. We decided to combine the results from all the studies regardless of the type of the imprinting disorder in order to investigate whether there is an increased risk of any imprinting disorder in children conceived via IVF/ICSI when compared with children conceived spontaneously. The combined odds ratio (95% confidence intervals) for any imprinting disorder in spontaneously conceived children was 3.67 (1.39, 9.74) (Fig. 4) suggesting an increased risk following ART. We elected to use the fixed effects model for this particular meta-analysis as the statistical heterogeneity was low ($I^2 = 12\%$).

**DNA methylation levels and the risk of epigenetic and imprinting disorders in children born spontaneously to infertile couples with children born spontaneously to fertile couples**

The electronic literature search on Medline, EMBASE, The Cochrane Library and Web of Science yielded a total of 141 articles for objective (b) (Fig. 2). Of these articles, 10 were excluded as duplicates and 120 were excluded on the basis of the title and abstract, mainly because they were irrelevant to our review or because they were animal studies. The remaining 11 papers were excluded after a full-text
review, due to lack of a control group or because the outcomes were not relevant to this review (e.g. spermatogenesis/oogenesis).

Discussion

Principal findings

Our results suggest that the risk of any imprinting disorder in children conceived through IVF or ICSI appears to be higher than that in spontaneously conceived children. There was no evidence of significantly altered DNA methylation associated with IVF in the regions, KvDMR/ KCNQ1OT1, PEG1/MEST, IGF2, GRB10, PEG3, H19, or SNRPN, but in many cases the number of studies was very small and the tissues, methods, and populations studied were heterogeneous.

Strengths and weaknesses

To our knowledge this is the first systematic review on this topic. All previous reviews on this topic, including a recent publication (Vermeiden and Bernardus, 2013), have been narrative in nature and none has attempted to perform a meta-analysis.

Robust searches of the literature were performed in our study, with explicit *a priori* search strategies and inclusion/exclusion criteria. Data extraction was meticulous, as each of the included studies underwent a rigorous data extraction process with results being categorized into different sections (DNA region, imprinting disorder, sample type, etc.).

Even though 18 studies were found to be relevant for our systematic review, much of the data were difficult to interpret and aggregate due to significant clinical heterogeneity, variations in the tissues sampled and methods of analysis and differences in the way results were expressed. We used a liberal approach in terms of aggregation of methylation data from different types of tissue samples although there is a case for considering placental imprinting methylation separately. We were conservative in terms of the meta-analyses we performed and used a random rather than a fixed methods approach in the presence of significant statistical heterogeneity ($I^2 > 50\%$) which was the case for all meta-analyses on DNA methylation but not the one for any imprinting disorder. The studies included in our review reported similar methylation levels in a particular DNA region in both groups of children (IVF and spontaneously conceived) regardless of the source of the tissue samples.

In the absence of a comparison group comprising couples with infertility who conceived naturally, it is difficult to be certain as to whether an effect is due to ART or to the infertility per se. Unfortunately our analysis of children born spontaneously to infertile couples compared with children born spontaneously to fertile couples yielded no studies suitable for analysis.

Comparison with the literature

Studies published in the last few years indicate that assisted reproduction can lead to alterations in DNA methylation in imprinted genes (Guo et al., 2008; Gomes et al., 2009; Zechner et al., 2009; Fukuda et al., 2010; Turan et al., 2010) as well as in the entire genome in offspring who appear to be otherwise normal (Katari et al., 2009; Zechner et al., 2009).

Intrinsic errors in imprinting have also been documented in sperm from men with suboptimal fertility (Hammoud et al., 2010) and in oocytes from women undergoing ovulation induction (Sato et al., 2007). Thus this is a population already at risk, in whom the stresses of ART may accentuate the potential for any further errors (Batcheller et al., 2011).

Meaning of the study

Our meta-analysis of aggregated data suggests a link between ART and imprinting disorders. This is consistent with results of individual studies and previous narrative reviews including one which attempted to generate a combined risk ratio (Vermeiden and Bernardus, 2013) for imprinting errors. Imprinting syndromes are associated with fairly profound genetic rearrangements or methylation changes but the logic often used is that the small changes, such as those reported in relation to ART, indicate a predisposition to genetic/epigenetic change at the key loci involved in the syndromes that may be more significant in a proportion of the affected population. The region associated with Beckwith–Wiedemann syndrome (11p15.5) encompasses several imprinted genes including $H19$. The region 15q11–q13 is associated with Prader–Willi and Angelman syndromes and includes SNRPN. The SNRPN gene codes for a small nuclear ribonucleoprotein-associated protein. Alternative splicing or deletion in this paternally-expressed region has been thought to be responsible for Prader–Willi syndrome (White et al., 2006).

The results in relation to methylation within individual imprinted genes were inconclusive, largely because of the small numbers of studies and their heterogeneity. Individual studies have reported significant effects but there was considerable heterogeneity in terms of subject characteristics, tissues samples, and laboratory methodologies used. What is clear though is that, if there are changes in imprinting epigenetic status between spontaneous and ART conceived children, the effects are small in magnitude. Detecting these is likely to be demanding and will require highly controlled study designs.

All decisions regarding medical interventions need to be informed by a thorough evaluation of risks and benefits. However there is a trend towards more liberal use of IVF (Kamphuis et al., 2014). Until further definitive data are available, a degree of caution should be exercised to ensure that IVF/ICSI is only performed where indicated, as the risk of birth defects in these children has been highlighted previously (Davies et al., 2012; Hansen et al., 2012, 2013). In order to achieve the best results, more research on larger populations is needed, ensuring consistency of samples, laboratory techniques and ways of reporting outcomes. It is also important to select populations of children of couples with subfertility in both ART as well as non-ART (exposed and unexposed) groups in order to explore the influence of fertility problems per se as opposed to ART on epigenetic changes in the offspring. Meanwhile, clinicians and patients should continue to be conscious of the need to limit the use of ART only to situations where it is necessary and be aware of the degree of uncertainty about the risks of IVF/ICSI and the need for long-term follow-up of ART-conceived children.

Conclusion

There is an association between ART and imprinting syndromes but no evidence of generalized changes in DNA methylation of selected genes. However, it should be acknowledged that the data have been difficult to interpret. More controlled studies with standardized methodologies, in larger, better clinically defined, populations, are needed. In particular, we would make the following suggestions for future protocols in this field.
Study group

Match spontaneous and ART pregnancies as closely as possible in terms of the factors that are known, or have the potential, to be linked to epigenetic states. These include: birthweight, length of gestation, and maternal and paternal age. For the children, it is important to match for (and record) age, weight and socioeconomic status at sampling. Use only singleton births following ART. Define the type of infertility in the ART group as closely as possible, and distinguish in particular between IVF and ICSI.

Sample collection

Interpretation of placental methylation data is complicated; therefore it is better to avoid this tissue when the aim is to investigate methylation changes in the offspring. Cord blood may provide useful information but in terms of assessing long-term health, it is better to measure methylation status in children. Sampling buccal cells may be preferable in children but it is important to use robust protocols for the collection and storage of buccal samples. Where possible it is useful to also sample DNA from a second tissue (most likely blood) to confirm the soma-wide generalizability of the findings.

Laboratory analysis

Wherever possible use standard approaches, such as pyrosequencing, for methylation analysis. Most of the work in this area has been carried out on methylation changes but other epigenetic mechanisms and approaches are also worthy of study. Where possible it would be useful to prepare and retain samples in a form that would render them amenable to study using other methodologies, e.g. immunoprecipitation, used in epigenetic studies. Where ethically acceptable, retain the samples for possible reanalysis in future studies.

Data reporting

Report absolute percent methylation, rather than methylation indices or other derived values, together with standard deviations and numbers of individuals. Report all positive and negative results. Apply appropriate adjustment in regression models where full matching of spontaneous and ART offspring on the above parameters has not been possible.

Supplementary data

Supplementary data are available at http://humupd.oxfordjournals.org/.

Authors’ roles

S.B. conceived the project and supervised G.L. and M.K. who undertook the systematic search of the literature. G.L. collated the data, generated the tables and performed the initial meta-analyses and wrote the first draft. S.o.B. advised on the methods for the meta-analyses and undertook a repeat meta-analysis at the revision stage of the paper. G.L., S.o.B., S.B. and P.H. interpreted the data and drafted subsequent versions of the paper. All authors had input into the final version.

Conflict of interest

None declared.

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