DNA methylation in human sperm: a systematic review

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Abstract

Background

Studies in non-human mammals suggest that environmental factors can influence spermatozoal DNA methylation, and some research suggests that spermatozoal DNA methylation is also implicated in conditions such as subfertility and imprinting disorders in the offspring. Together with an increased availability of cost-effective methods of interrogating DNA methylation, this premise has led to an increasing number of studies investigating the DNA methylation landscape of human spermatozoa. However, how the human spermatozoal DNA methylome is influenced by environmental factors is still unclear, as is the role of human spermatozoal DNA methylation in subfertility and in influencing offspring health.

Objective and rationale

The aim of this systematic review was to critically appraise the quality of the current body of literature on DNA methylation in human spermatozoa, summarise current knowledge and generate recommendations for future research.

Search methods

A comprehensive literature search of the PubMed, Web of Science and Cochrane Library databases was conducted using the search terms "semen" OR "sperm" AND "DNA methylation". Publications from 1 January 2003 to 2 March 2020 that studied human sperm and were written in English were included. Studies that used sperm DNA methylation to develop methodologies or forensically identify semen were excluded, as were reviews, commentaries, meta-analyses or editorial texts. The Grading of Recommendations, Assessment, Development and Evaluations (GRADE) criteria were used to objectively evaluate quality of evidence in each included publication.

Outcomes

The search identified 446 records, of which 135 were included in the systematic review. These 135 studies were divided into three groups according to area of research; 56 studies investigated the influence of spermatozoal DNA methylation on male fertility and abnormal semen parameters, 20 studies investigated spermatozoal DNA methylation in pregnancy outcomes including offspring health,

and 59 studies assessed the influence of environmental factors on spermatozoal DNA methylation. Findings from studies that scored as ‘high’ and ‘moderate’ quality of evidence according to GRADE criteria were summarised. We found that male subfertility and abnormal semen parameters, in particular oligozoospermia, appear to be associated with abnormal spermatozoal DNA methylation of imprinted regions. However, no specific DNA methylation signature of either subfertility or abnormal semen parameters has been convincingly replicated in genome-scale, unbiased analyses. Further, although findings require independent replication, current evidence suggests that the spermatozoal DNA methylome is influenced by cigarette smoking, advanced age and environmental pollutants. Importantly however, from a clinical point of view, there is no convincing evidence that changes in spermatozoal DNA methylation influence pregnancy outcomes or offspring health.

Wider implications

Although it appears that the human sperm DNA methylome can be influenced by certain environmental and physiological traits, no findings have been robustly replicated between studies. We have generated a set of recommendations that would enhance the reliability and robustness of findings of future analyses of the human sperm methylome. Such studies will likely require multicentre collaborations to reach appropriate sample sizes, and should incorporate phenotype data in more complex statistical models.

Registration number

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Keywords

DNA methylation; methylome; human sperm; human spermatozoa; fertility; reproductive health; epigenetics; transgenerational inheritance; epigenetic association studies

Introduction

Adequate functioning of germ cells is imperative for fertility, embryogenesis and offspring health. Infertility, commonly defined as a failure to achieve a successful pregnancy after 12 months or more of regular unprotected intercourse, affects approximately one in seven couples in the UK, and is attributed to male factor infertility in around half of cases (American Society for Reproductive Medicine, 2013; Fields *et al.*, 2013). Epidemiologically, male factor infertility has been linked to a range of lifestyle and environmental factors, including cigarette smoking, obesity and toxicants such as organophosphates (Fields *et al.*, 2013; Mima *et al.*, 2018). Observational studies also suggest that environmental and acquired paternal traits, such as advanced age and smoking, can influence the health of his offspring, presumably via alterations to spermatozoa (Oldereid *et al.*, 2018). Investigation into spermatozoal genomic imprinting was further prompted by epidemiological reports of an association between ART and an increased risk of imprinting disorders in the offspring (Gosden *et al.*, 2003), as it was hypothesized that spermatozoa from subfertile males who conceived with the aid of

ART could harbour a greater frequency of abnormally imprinted genes. Together, these observations have generated considerable interest in the epigenetic landscape of human spermatozoa.

Epigenetics refers to mitotically or meiotically heritable changes in gene expression that do not involve changes in the underlying DNA sequence (Dupont *et al.*, 2009). The term is generally taken to include three distinct but interrelated mechanisms that alter the accessibility of chromatin towards the transcriptional machinery or regulate gene expression on a post-transcriptional level; DNA modifications, in humans most prevalently via methylation of CpG dinucleotides (forming 5-mC), post-translational modifications of histones (including methylation, acetylation, phosphorylation and sumoylation), and non-coding RNAs (Daxinger and Whitelaw, 2012; Desai *et al.*, 2015) (**Fig. 1**). Of these, DNA methylation is the most studied epigenetic mechanism to date, owing to its relative stability and to the availability of cost-effective tools for analysing DNA methylation at single base resolution. We have therefore made DNA methylation specifically the focus of this review.

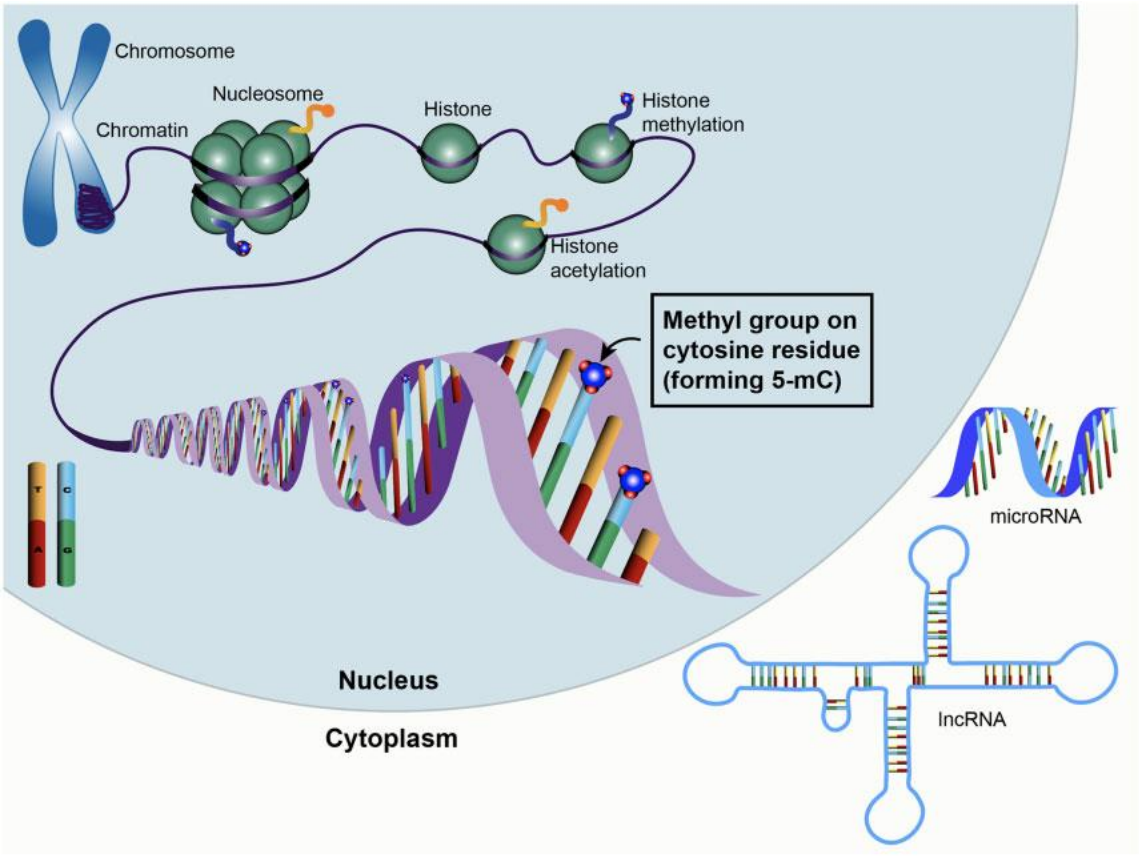

Figure 1. Overview of epigenetic mechanisms.

The epigenetic machinery includes three distinct but interrelated mechanisms; DNA modifications, post-translational modifications of histones and non-coding RNA. The most prevalent DNA modification, DNA methylation, involves the attachment of a methyl group onto cytosine residues, forming 5-mC. lncRNA, long non-coding RNA.

In non-human mammals, the sperm methylome has been shown to be responsive to environmental and physiological changes, including dietary alterations, toxicants and even psychological stress (Dias and Ressier, 2014; Radford *et al.*, 2014; Barbosa *et al.*, 2016; Youngson *et al.*, 2016; Sakai *et al.*,

2018; Watkins *et al.*, 2018). In addition, some studies suggest that acquired traits in male mice can induce epigenetic changes in sperm that influence offspring physiology (Wei *et al.*, 2014; Huypens *et al.*, 2016).

Compared to readily available somatic tissues, such as peripheral blood, our knowledge of the human sperm methylome is considerably less detailed. Nevertheless, the last two decades have seen an increasing number of studies investigating epigenetic signatures of human germ cells, fuelled in part by an increasing interest in the potential for acquired phenotypes to influence offspring physiology.
The aim of this systematic review was to critically appraise the current body of research, summarize credible findings and generate recommendations for future research in the context of investigating DNA methylation in human spermatozoa. In doing so, we interrogated the available literature to answer three specific questions:

- What is the strength of evidence for an association between spermatozoal DNA methylation and male subfertility, including abnormal semen parameters?
- What is the strength of evidence for an association between spermatozoal DNA methylation and pregnancy outcomes and offspring health?
- What is the strength of evidence for environmental influences on spermatozoal DNA methylation?

Methods

Protocol and registration

The methods employed for the systematic review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Liberati *et al.*, 2009). The protocol for the review was registered on the international prospective register for systematic reviews, PROSPERO, before data extraction was completed (CRD42019146399; https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=146399).

Search strategy

An exhaustive literature search was performed on 2 March 2020 using the PubMed electronic database and the following search criteria:

‘semen’ [Mesh] OR ‘sperm’ [Mesh] AND ‘DNA methylation’ [Mesh]

The same search criteria were thereafter applied to search the Web of Science and Cochrane databases to identify further relevant articles. Only publications that pertained to studies in humans and were written in English were included. Reference lists of identified articles and review articles were also searched for additional references. The search and filtering process were performed independently by two authors to avoid selection bias.

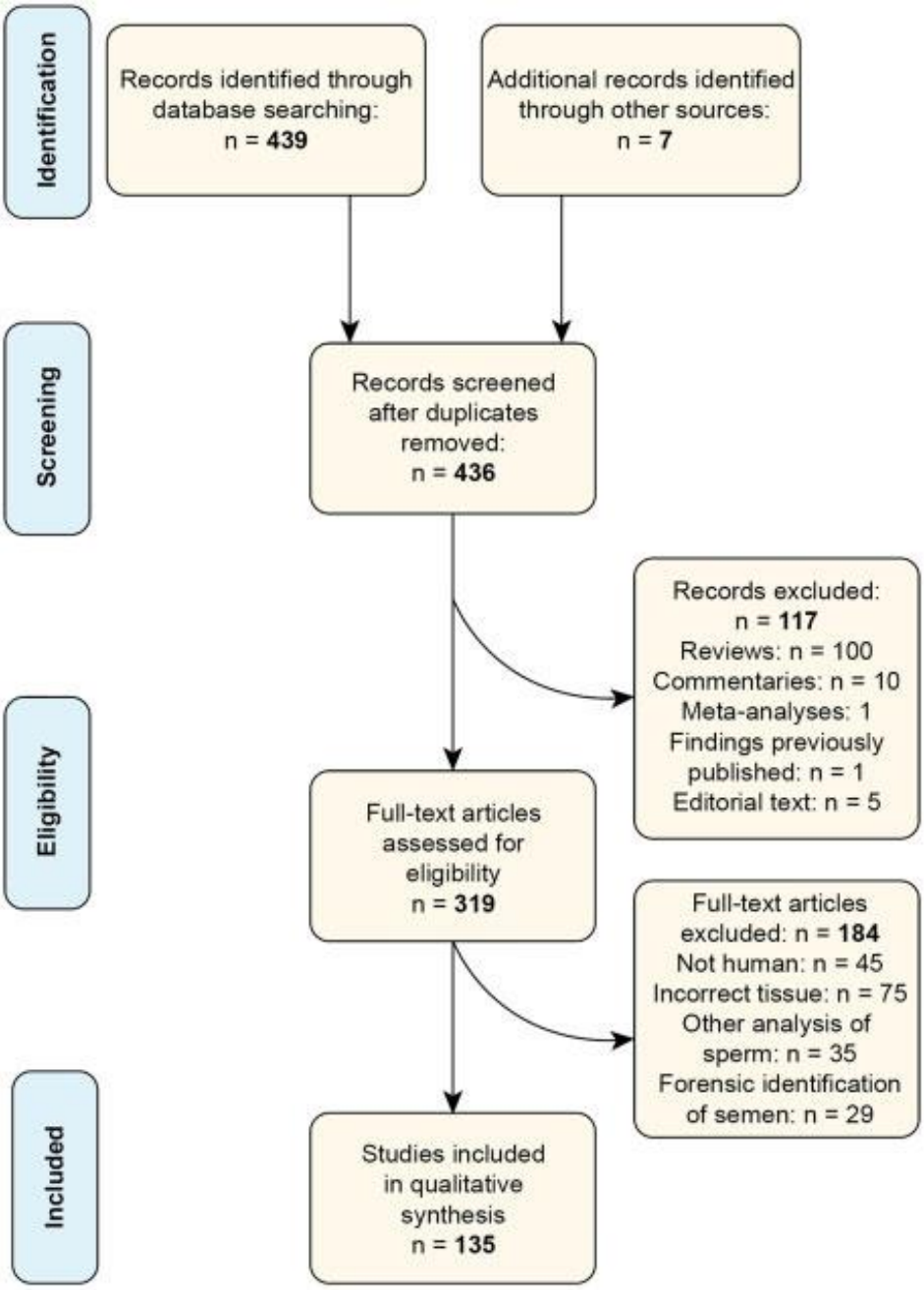

Figure 2. Flow chart of selection of records for the systematic review.

Assembled according to PRISMA guidelines (Liberati et al., 2009).

Selection criteria

Articles were selected for inclusion in the systematic review if they fulfilled the following criteria:

- Studies investigating DNA methylation in human spermatozoa.
- Any age group.
- Investigations of any type of analysis method of DNA methylation.
- Publications in English only.
- Published from 2003 onwards.

A cut-off date of 2003 was applied as this was deemed a realistic date for studies to begin using next-generation sequencing and microarray-based technologies, and so to minimize penalization of earlier studies due to an absence of the technology. Thus, articles published between 1 January 2003 and 2 March 2020 were included. Articles identified through the search, but which did not meet the inclusion criteria detailed above, were recorded along with their reason for being excluded (**Supplementary Data S1**). A flow diagram of the selection process, including the number of articles filtered at each step of the process is shown in **Fig. 2**. A description of semen sample parameters referred to throughout this systematic review is provided in **Supplementary Data S2**.

Data extraction

The following information was extracted from every identified study:

- Author.
- Year of publication.
- Rationale for study.
- Method of assaying DNA methylation.
- Area of research (fertility or semen parameters, pregnancy outcomes and offspring health, toxicants, metabolic interventions (including diet and lifestyle), studies of tissue specificity (including cancer, ageing and methodological studies comparing tissues) and other (including studies of genetic and psychiatric conditions, and methodological experiments)).
- Method of semen processing, i.e. the method of removing somatic cells. A description of different methods of semen processing relevant to this systematic review is provided in **Supplementary Data S2**.
- Global, targeted or genome-wide analysis of DNA methylation.
- Number of study participants.
- Age of study participants.
- Matching of cases and controls (where relevant).
- Main results of study.
- Statistical analyses, e.g. correction for multiple comparison.
- Covariates.
- Exclusion criteria applied in the study.

Information was recorded by the first primary author and independently verified by the second primary author. Any disagreements were resolved in discussion with the senior author. Complete records can be found in **Supplementary Data S3**.

Quality of evidence assessment

The Grading of Recommendations, Assessment, Development and Evaluations (GRADE) criteria were used to objectively evaluate the quality of evidence in every study included in the systematic review (Ryan and Hill, 2016). The criteria were adapted for relevance to the subject of the review, as summarized in **Supplementary Table SI** (**Supplementary Data 2**). No randomized controlled trials were identified in the search; therefore, quality of evidence ratings were assigned based on the scoring of the other criteria. Studies were each scored based on risk of bias (application of appropriate statistics, including correction for multiple tests, and consideration of covariates), inconsistency, indirectness, imprecision, publication bias and other upgrading factors, such as large effect sizes, large sample sizes or use of a replication cohort. Publications with GRADE ratings of ≥0 were assigned a quality score of 'high', −1 were assigned 'moderate', −2 were assigned 'low' and ≤ −3 were assigned 'very low'. Only findings of studies scoring 'high' or 'moderate' for quality of evidence were summarized.

Synthesis of results

The quality of evidence was graded for each study and is presented in **Tables I–III**. Results were summarized according to the research question they addressed. Recommendations for future DNA methylation studies in human sperm were given based on the perceived shortcomings of previous work and the gaps in the existing literature. All figures were generated in Adobe Illustrator® (Adobe, Inc., San Jose, CA, USA) and Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) specifically for this publication.

Results

Study selection

A total of 446 articles were identified by the search terms and through manual filtering of reference lists (**Fig. 2**). Ten of these were duplicate records and were therefore removed. Reviews or records that otherwise did not describe original research (n = 117) were excluded. A further 184 articles were excluded because they did not investigate humans (n = 45), did not specifically investigate DNA methylation (n = 35), investigated another tissue than sperm (n = 75) or related to forensic identification of semen (n = 29). This resulted in 135 studies that met the predetermined inclusion criteria and were included in the systematic review. To our knowledge, this represents all published studies investigating DNA methylation in human sperm that were available up to 2 March 2020.

Evaluation of studies based on GRADE criteria

To objectively evaluate the current body of research on humans spermatozoal DNA methylation and identify current knowledge gaps in the field, we adapted the GRADE criteria as described by Ryan and

Hill (2016) to specifically be applicable to the aims of this systematic review. Details of how each study was graded are available in **Supplementary Data S3**. Using the adapted criteria also allowed us to identify common technical and methodological flaws, which are discussed under their respective headings below.

Risk of bias

There is convincing evidence in both humans and animal models that age and smoking affect DNA methylation across the genome in organs other than sperm (Horvath, 2013; Joehanes *et al.*, 2016). Furthermore, there is extensive clinical evidence that advanced paternal age and cigarette smoking are associated with adverse effects on male fertility and offspring health (Sharm *et al.*, 2016; de Kluiver *et al.*, 2017; Oldereid *et al.*, 2018). Of the 135 studies identified in our literature search, 57 studies either did not report the age of participants, did not account for age in a statistical model, such as a linear regression model with covariates, or reported significant differences in the ages of cases and controls without correcting for this. Similarly, 44 studies did not consider smoking status.

There were 11 studies that did not use appropriate semen processing methods or include an analysis to assess somatic cell contamination. In such studies, one cannot disregard the possibility that somatic cells, which have considerably different DNA methylation profiles to spermatozoa, could have influenced results, particularly in the context of imprinted loci (Bartolomei and Ferguson-Smith, 2011). In seven studies, differences in semen parameters between cases and controls were not corrected for, and in two studies maternal factors were not considered in pregnancy outcomes (Benchaib *et al.*, 2003, 2005). The matching of sperm with embryos or other tissues, i.e. that spermatozoa is derived from the same person who fathers the embryo, in studies of pregnancy outcomes or in studies of tissue specificity, is important as it limits confounding of DNA methylation data due to genetic effects (Hannon *et al.*, 2015). Five studies were marked down for not using matched tissues under such circumstances. Appropriate statistical analysis is imperative, and this includes correcting for the use of multiple statistical tests to reduce the risk of identifying false positive outcomes by chance. We found that the poor use of statistics affected the conclusions in seven studies. Finally, one study was downgraded for applying different sperm isolation methods between cases and controls, and another was downgraded for using multiple embryos from the same family in analyses and not accounting for biases caused by relatedness.

Inconsistency

Studies that had inconsistent sperm processing amongst samples scored −1 for inconsistency. Only one study included a replication cohort and scored +1 in this category.

Indirectness

Few studies were downgraded for indirectness, but in two studies it was considered that the findings may not be generalizable due to the fact that participants were recruited from fertility clinics.

Imprecision

There were 18 studies that used global methods of measuring DNA methylation in sperm. Global DNA methylation refers to the total levels of DNA methylation (5-mC) across the entire genome. Due to the lack of quantitation in these methods (immunostaining or flow cytometry), all global studies were downgraded for imprecision if they did not validate their results with another method. There were 20 studies with inappropriately small sample sizes for the resolution of measurement that was being made, and these were also downgraded for imprecision. In contrast, 13 studies were upgraded for including relatively large sample sizes.

Publication bias

There were 70 candidate gene studies, in which targeted sequencing or PCR-based methods, or occasionally array-based techniques, were used to investigate-specific genes chosen based on prior knowledge. Such studies are prone to publication bias, given that the chosen genes were selected because of published positive results, and they also prohibit the replication of genes other than those selected. Studies using candidate gene approaches were therefore downgraded.

Evidence for the association between spermatozoal DNA methylation and male subfertility, including abnormal semen parameters

The most common question of studies included in this review was the association between spermatozoal DNA methylation and male subfertility, including abnormal semen parameters. Fifty-six studies investigated this topic (**Table I**). Among these, the quality of evidence was scored as high for 6, moderate for 16, low for 17 and very low for 17 studies.

There was one moderate-quality study of global spermatozoal DNA methylation in association with semen parameters: Barzideh *et al.* (2013) analysed the difference in global DNA methylation between the high-density (higher quality) portion of spermatozoa and the low-density (lower quality) portion of spermatozoa separated by a Percoll density gradient and found that the high-quality sperm portion was relatively hypomethylated.

Among moderate- or high-quality candidate gene studies of DNA methylation and subfertility or abnormal semen parameters, three studies found that oligozoospermia was associated with spermatozoal DNA hypomethylation of *H19*(Marques *et al.*, 2004, 2008; Dong *et al.*, 2017), and three studies found that oligozoospermia was associated with spermatozoal DNA hypermethylation of the paternally expressed transcript 1/mesoderm-specific transcript gene (*PEG1/MEST*) (Marques *et al.*, 2008; Poplinski *et al.*, 2010; Klaver *et al.*, 2013). Other studies, in contrast, found no association between aberrant DNA methylation of *PEG1/MEST*and oligozoospermia (Marques *et al.*, 2004). Kabartan *et al.* (2019) found no association between oligozoospermia and DNA methylation of breast cancer gene 1 and 2 (*BRCA1* and *BRCA2*). Tang *et al.* (2018) found that male subfertility, whether or not semen samples were oligozoospermic, was associated with DNA methylation defects in imprinted genes, including *H19*. One study found hypermethylation of the methylenetetrahydrofolate reductase (*MTHFR*) gene promoter in males with idiopathic subfertility and oligozoospermia, compared

with fertile males (Wu *et al.*, 2010), and another group found that DNA methylation of *MTHFR* was correlated with lower quality sperm parameters (Tian *et al.*, 2015). In contrast, in a third targeted analysis of *MTHFR* promoter DNA methylation, hypomethylation of *MTHFR* was shown to be associated with low motility spermatozoa (Botezatu *et al.*, 2014). In the context of candidate gene analyses of spermatozoal DNA methylation, it is of note that a relatively small number of genes have been investigated multiple times. Thus, as visualized in **Fig. 3**, spermatozoal DNA methylation of *H19*, *PEG1/MEST* and the small nuclear ribonucleoprotein polypeptide N gene (*SNRPN*) in relation to fertility was investigated in 20, 17 and 6 studies, respectively, whereas other genes were typically investigated in fewer than five studies, generally without resulting in replication of results.

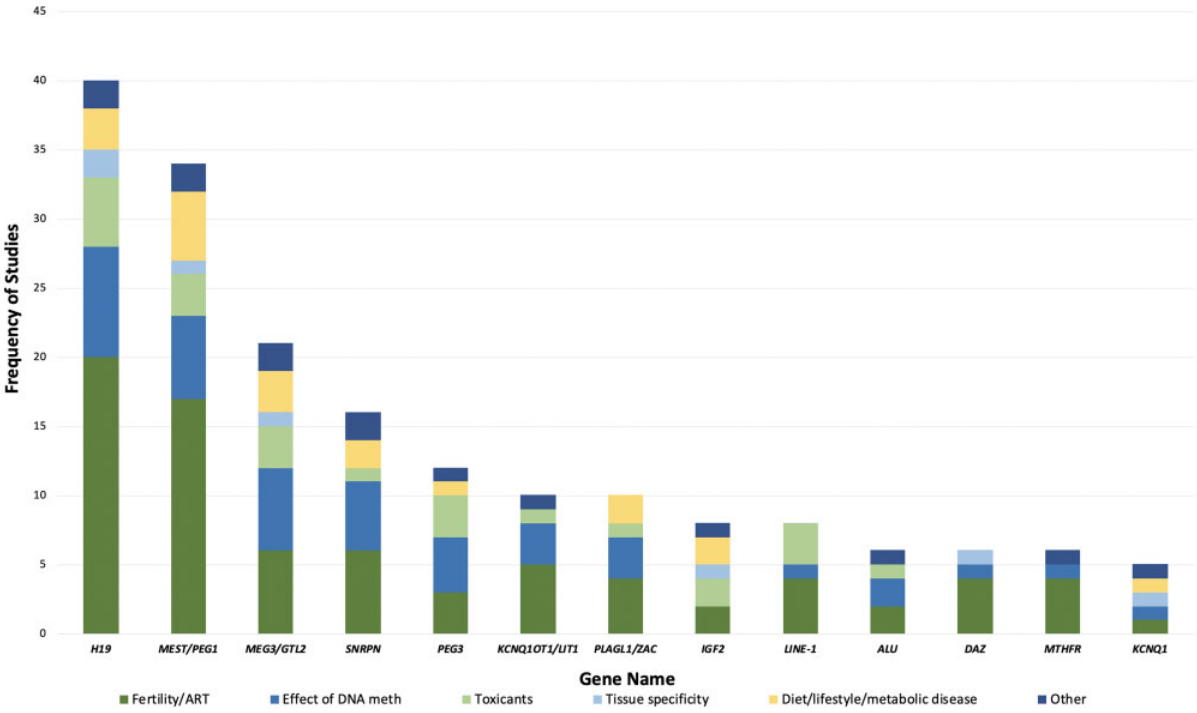

Figure 3. Frequency of specific candidate genes being investigated in candidate gene analyses of human sperm.
A total of 75 candidate gene studies of spermatozoal DNA methylation were identified in our literature search. Approximately half of these investigated either or both of the imprinted genes *H19* or *PEG1/MEST*, as presented in the diagram. *PEG3*, paternally expressed gene 3; *MEG3*, maternally expressed gene 3; *KCNQ1*, potassium voltage-gated channel subfamily Q member 1; *KCNQ1OT1*, KCNQ1 opposite strand/antisense transcript 1; *PLAGL1*, PLAG1 like zinc finger 1.

Ten moderate- or high-quality studies investigated the association between genome-wide spermatozoal DNA methylation and semen parameters or male subfertility (Houshdaran *et al.*, 2007; Pacheco *et al.*, 2011; Schutte *et al.*, 2013; Camprubi *et al.*, 2016, 2017; Jenkins *et al.*, 2016; Laqqan *et al.*, 2017a,b,d, 2018). Notably, none of these genome-wide analyses identified any of the frequently investigated candidate genes discussed above as significantly differentially methylated in subfertility or in association with abnormal semen parameters, such as oligozoospermia. This is despite the fact that the majority of candidate genes—all genes featured in **Fig. 3**—have 40 or more probes annotated to them on the Illumina 450K DNA methylation array (450K array), which was used across these 10

studies. There was also little overlap of findings from different genome-wide investigations of DNA methylation of subfertility performed using comparable methodologies: Laqqan *et al.* (2017b) found that subfertility was associated with differential DNA methylation at CpG sites annotated to genes encoding aldehyde dehydrogenase family 3 member B2 (*ALDH3B2*) and prostaglandin I2 receptor (*PTGIR*) using the 450K array. In a subsequent investigation using a similar approach, the same group (Laqqan *et al.*, 2017a) found that subfertility was associated with spermatozoal DNA methylation alterations in the genes encoding potassium inwardly rectifying channel subfamily J member 5 (*KCNJ5*), melanophilin (*MLPH*) and structural maintenance of chromosomes 1B (*SMC1B*). In a third study of spermatozoa from subfertile males by the same group, again using the 450K array, Laqqan *et al.* (2018) found that subfertility was associated with DNA methylation differences in the genes encoding proline-rich coiled-coil 2A (*PRRCA2*), annexin A2 (*ANXA2*) and mitogen-activated protein kinase 8 interacting protein 3 (*MAPK8IP3*). There was a similar lack of overlap of findings from studies of genome-wide DNA spermatozoal methylation in oligozoospermia. Using the 450K array, Laqqan *et al.* (2017d) found that oligozoospermia was associated with differential DNA methylation of the gene encoding ubiquitin conjugating enzyme E2 G2 (*UBE2G2*), whereas Camprubi *et al.* (2016) found a significant association between spermatozoal hypermethylation of the gene encoding amyloid P component, serum (*APCS*) and oligozoospermia using a similar approach. Jenkins *et al.* (2016) did not identify any CpG site that met the genome-wide threshold for differential methylation in teratozoospermia, but found that teratozoospermia was associated with a higher rate of DNA methylation variability. Camprubi *et al.*(2017) investigated genome-wide DNA methylation in spermatozoa from males of proven fertility and found that spermatozoa exhibit relative hypomethylation compared to somatic tissues.

Lastly, two groups (Pacheco *et al.*, 2011; Schutte *et al.*, 2013) used an earlier version of the Illumina Methylation Array, the 27K DNA Methylation Array (27K array), to investigate DNA methylation in association with sperm motility and abnormal semen parameters, respectively. Pacheco *et al.* (2011) found that unsupervised clustering of methylation data correctly differentiated the 21 sperm samples by their motility values, and Schutte *et al.* (2013) found that that unsupervised clustering of methylation data correctly separated samples according to whether they had normal or low semen parameters.

Conclusion

There is evidence to suggest that idiopathic male subfertility and abnormal semen parameters, in particular oligozoospermia, are associated with abnormal spermatozoal DNA methylation of imprinted regions. However, no specific DNA methylation signature of either subfertility or abnormal semen parameters has been convincingly replicated in genome-scale, unbiased analyses and the clinical benefits of findings to date is therefore limited. Furthermore, whilst these studies attempt to address the important question of the potential mechanism leading to subfertility, they do not tackle the arguably more pertinent question of whether DNA methylation in sperm can affect pregnancy outcomes and offspring health.

Evidence for the association between spermatozoal DNA methylation and pregnancy outcomes, including offspring health

The second question targeted by studies included in this review concerned the association between spermatozoal DNA methylation and pregnancy outcomes, including offspring health. Twenty studies addressed this question (**Table II**). Among these, the quality of evidence was scored as high for three, moderate for three, low for six and very low for eight studies.

Among moderate- or high-quality candidate gene approaches, the majority of studies focused on methylation of imprinted genes as previously discussed (**Fig. 3**). Despite this considerable research effort, there is limited and conflicting evidence as to whether aberrant methylation of imprinted loci does affect ART outcomes, and there is no evidence that it affects offspring health. El Hajj *et al.* (2011) and Camprubi *et al.* (2012) found no evidence that DNA methylation at seven selected imprinted regions had an association with the success of ART, however Kobayashi *et al.* (2009) found that spermatozoa with aberrant methylation at one or more of 22 selected imprinted loci was associated with higher rates of miscarriage and rates of live births. This contradiction may be driven by the differing number of candidate genes chosen in the respective studies. At present, there are no high-quality genome-wide studies to validate the findings.

Methylation of other genomic elements have been found to be predictors of ART outcome. El Hajj *et al.* (2011) found that methylation of Alu elements, the most abundant type of transposable element in the human genome (Deininger, 2011), correctly predicted whether pregnancy ended in spontaneous miscarriage or a live birth in 89% of couples. Using a multiple linear regression model, El Hajj *et al.*found no evidence that DNA methylation at long interspersed nuclear elements 1 (LINE-1) in sperm was correlated with live birth rates, nor were the ages of the parents, the number of retrieved oocytes, the number of fertilized oocytes, embryo quality, sperm shape, sperm count or sperm motility. Rotondo *et al.* (2013)identified spermatozoal hypermethylation of *MTHFR* as predictive of recurrent spontaneous miscarriage. Lastly, among studies of spermatozoal DNA methylation and pregnancy outcomes, Tavalaee *et al.* (2009) found that although spermatozoal DNA fragmentation was associated with global sperm DNA methylation and fertilization rates in males undergoing ART, none of the parameters assessed, including global sperm DNA methylation, were associated with pregnancy rates. Only one high-quality study looked at the effect of sperm DNA methylation on the future health of the offspring. Atsem *et al.* (2016) found that paternal age at conception was correlated with methylation of the gene encoding forkhead box K1 (*FOXK1*) in both sperm and on the paternal allele in cord blood of offspring. However, the attempt to link the methylation difference at the gene to the observation that the risk for autism spectrum disorder (ASD) in children increases with the age of the father did not result in a significant association, only a ‘trend’ between lower *FOXK1* methylation and ASD ($P = 0.07$) (Atsem *et al.*, 2016).

Conclusion

There is limited evidence that spermatozoal DNA methylation affects pregnancy outcomes or offspring health. This is largely due to a lack of high-quality genome-wide association studies which specifically address this topic. Further research is warranted, as this could readily inform clinical practice.

Evidence for the association between spermatozoal DNA methylation and environmental factors

The final research question addressed by studies summarized in this review was the association between spermatozoal DNA methylation and environmental factors (**Table III**). The 59 studies that addressed this topic were divided into four categories; toxicants, metabolic interventions (including diet and lifestyle), studies of tissue specificity (including cancer, ageing and methodological studies comparing tissues) and other (including genetic, psychiatric and other methodological experiments). These categories are discussed separately below.

Toxicants

Nineteen studies investigated the effect of various toxicants on spermatozoal DNA methylation, including six studies of cigarette smoke (Kim *et al.*, 2015; Jenkins *et al.*, 2017; Laqqan *et al.*, 2017c; Al Khaled *et al.*, 2018; Alkhaled *et al.*, 2018; Hamad *et al.*, 2018), two studies of recreational drugs (cannabis and opioids) (Chorbov *et al.*, 2011; Murphy *et al.*, 2018) and four studies of organic pollutants such as mercury, polycyclic aromatic hydrocarbons (PAHs), per- and polyfluoroalkyl substances and phthalate metabolites (Leter *et al.*, 2014; Wu *et al.*, 2017; Lu *et al.*, 2018; Ma *et al.*, 2019; Tian *et al.*, 2019). Among these, the quality of evidence was scored as high for three studies, moderate for 10, low for five and very low for one study (**Table III**). As smoking is associated with lower semen sample parameters (Sharm *et al.*, 2016), studies of the effects of cigarette smoke on spermatozoal DNA methylation were downgraded if they failed to take semen parameters into account (Jenkins *et al.*, 2017; Hamad *et al.*, 2018). The study of the effects of cannabis on spermatozoal DNA methylation was marked down as it did not account for the higher alcohol consumption reported in the cannabis user group (Murphy *et al.*, 2018).

Laqqan *et al.* (2017c), Alkhaled *et al.* (2018) and Jenkins *et al.* (2017) each published a high- or moderate-quality genome-wide association study assessing DNA methylation differences between the spermatozoa from smokers and non-smokers using the 450K array. Notably, there was no overlap of findings between studies from different groups. Laqqan *et al.* (2017c) reported differences between smokers and non-smokers in *MAPK8IP3* and TYRO3 protein tyrosine kinase (*TYRO3*) genes, and Alkhaled *et al.* (2018) reported differences in the protein tyrosine phosphatase receptor type N2 (PTPRN2) and TYRO3. Jenkins *et al.* (2017)found 141 significantly differentially methylated sites associated in smokers, however none of these were associated with the genes identified by Laqqan *et al.*(2017c) or Alkhaled *et al.* (2018). Jenkins *et al.* (2017) and Alkhaled *et al.* (2018) also reported significant decreases in several semen parameters in smokers, including semen volume, total sperm count and total progressively motile sperm count, but did not account for this in a multiple regression model or by other means in the DNA methylation analyses (which resulted in the evaluation of both

studies as moderate rather than high quality). In a study of 12 cannabis users compared with 12 non-users, Murphy *et al.* (2018) found 3979 CpG sites that differed significantly in sperm using reduced representation bisulphite sequencing (RRBS), and 46 genes which had more than 10 differentially methylated sites. One of these genes, *PTPRN2*, was also found to be altered by smoking by Alkhaled *et al.* (2018). However, Murphy *et al.* (2018) also saw significantly lower sperm concentration in cannabis users.

Several candidate gene studies of imprinted loci showed that exposure to toxicants, such as mercury (Lu *et al.*, 2018), PAHs 1-OHP and 1-OHPH (Ma *et al.*, 2019) and high levels of reactive oxygen species (ROS) in seminal plasma (Darbandi *et al.*, 2019b), was associated with *H19* hypomethylation. Soubry *et al.* (2017)found that exposure to flame retardants in men was associated with spermatozoal DNA hypermethylation of *MEG3*, necdin, melanoma antigen family member (*NDN*) and *SNRPN*, and a separate group found an association between ROS levels in seminal plasma and hypermethylation of the insulin-like growth factor 2 gene (*IGF2*) (Darbandi *et al.*, 2019a). High ROS levels were also found to be associated with lower semen parameters (Darbandi *et al.*, 2019a). Tian *et al.* (2019) found no association between high levels of urinary phthalate metabolites and DNA methylation of imprinted loci, but found an association between urinary phthalate metabolites and LINE-1 hypomethylation. Also investigating urinary phthalates, Wu *et al.* (2017) identified 131 differentially methylated regions (DMRs), which were associated with exposure. Similarly, Pilsner *et al.* (2018) identified 52 DMRs that were differentially methylated between men with high and low serum levels of the organic pollutant dioxin. Consales *et al.* (2016) found no difference in methylation of repetitive sequences in relation to exposure to the persistent organic pollutants polychlorinated biphenyls and dichlorodiphenyltrichloroethane, but did find that exposure was associated with global spermatozoal DNA hypomethylation as measured by flow cytometry. Finally, Leter *et al.* (2014) found no association between exposure to perfluoroalkyl substances and methylation of repetitive genomic sequences.

Conclusion

While several studies have identified specific spermatozoal DNA methylation signatures associated with toxicants, such as cigarette smoke, cannabis and pollutants, there are yet no robust findings that have been replicated across studies. Furthermore, it is frequently difficult to distinguish between reported DNA methylation differences and the effects the toxicants themselves have on semen parameters. Lastly, the use of widely varying research methodologies (e.g. global-, candidate gene- and genome-wide analyses) makes it difficult to compare the scale and severity of the DNA methylation differences observed under these exposures.

Metabolic interventions.

Eleven studies assessed the effect of metabolic interventions on spermatozoal DNA methylation, including exercise (Denham *et al.*, 2015; Ingerslev *et al.*, 2018), diet and supplements (Tunc and Tremellen, 2009; Aarabi *et al.*, 2015; Chan *et al.*, 2017; Salas-Huetos *et al.*, 2018), BMI (Consales *et al.*, 2014; Donkin *et al.*, 2016; Soubry *et al.*, 2016; Potabattula *et al.*, 2019) and endocrine factors

(Stoger, 2006). Among these, the quality of evidence was scored as high for one study, moderate for six, low for three and very low for one study (**Table III**).

Two studies analysed the effects of exercise on spermatozoal DNA methylation. Both of these compared samples collected before and after a 3-month (Denham *et al.*, 2015) or a 6-week (Ingerslev *et al.*, 2018) exercise intervention, using the 450K array and RRBS, respectively, to analyse spermatozoal DNA methylation. Both groups found multiple differentially methylated positions when comparing DNA methylation in sperm before and after the intervention, however there was no overlap of findings between the two studies.

Two studies analysed the effect of BMI on methylation of imprinted loci in targeted analyses (Soubry *et al.*, 2016; Potabattula *et al.*, 2019). However, whilst Soubry *et al.* found that high BMI was associated with *MEG3*, *NDN*, *SNRPN*, sarcoglycan epsilon (*SGCE*) and paternally expressed gene 10 (*PEG10*) hypomethylation and *H19* hypermethylation, Potabattula *et al.* found no associations that met genome-wide significance. This discrepancy could in part be explained by the fact that Soubry *et al.* did not correct for the use of multiple statistical tests, as well as a considerably smaller study cohort (Soubry: $n = 67$; Potabattula: $n = 294$). However, Potabattula *et al.* were unable to correct for participant smoking states due to lack of information on their participants, which could have confounded results. Tunc and Tremellen (2009) found no association between spermatozoal DNA methylation and serum homocysteine levels, while Salas-Huetos *et al.* (2018) found no association between spermatozoal DNA methylation and nut consumption in the 'FERTINUTS' study. In a study including 269 participants, Consales *et al.* (2014) measured a number of different exposures, including BMI, semen parameters, sperm chromatin integrity, biomarkers of accessory gland function and the plasma concentration of reproductive hormones, but found no global differences in sperm DNA methylation. However, spermatozoal DNA methylation of LINE-1 elements separated participants by geographical location in this study.

Conclusion

There is limited and conflicting evidence on whether diet and lifestyle interventions are associated with altered spermatozoal DNA methylation. Larger-scale, longitudinal analyses with appropriate controls for potential confounding factors are required.

Tissue specificity and ageing

Eighteen studies were categorized as relating to the effect of ageing, cancer and tissue specificity of DNA methylation in spermatozoa. Among these, the quality of evidence was scored as high for one, moderate for two, low for five and very low for nine studies (**Table III**).

Of the three studies scoring high or moderate for quality of evidence, two were from the same group. These both identified the presence of multiple age-associated DMRs in sperm (Jenkins *et al.*, 2014), and showed that these regions could be used to predict chronological age at relatively high accuracy

(mean absolute error = 2.04 years) (Jenkins *et al.*, 2018). The third paper generated a genome-wide DNA methylation reference of 13 normal somatic tissues, as well as human sperm, and showed broad-scale tissue-specific differences in DNA methylation patterns (Rakyan *et al.*, 2008).

Conclusion

Ageing appears to be strongly associated with spermatozoal DNA methylation. It is unclear, however, whether and how such ageing-related changes have lasting effects on processes such as embryogenesis or offspring health. It is also clear that the DNA methylation signature of human spermatozoa differs considerably to that of many, if not all, somatic human tissues.

Other

Eleven studies that assessed the effect of a variety of environmental stimuli and exposures on sperm DNA methylation did not fit into the previous three categories. These included studies of genetics, psychiatric illness and methodological studies. Among these, the quality of evidence was scored as high for four, low for five and very low for two studies (**Table III**).

Two of the high-quality studies related to psychiatric disease. Kaminsky *et al.*(2012) showed hypomethylation of a CpG site in the HLA complex group 9 (*HCG9*) gene in patients with schizophrenia or bipolar disorder compared to healthy controls. Grouping of these two disorders was deemed justifiable due to their shared genetics and overlap in symptoms (Maggioni *et al.*, 2017). In a genome-wide analysis of patients that suffered from childhood abuse, 12 regions of DNA in sperm were identified as being differentially methylated in response to abuse (Roberts *et al.*, 2018).

The remaining two high-quality studies were methodological in nature. Flanagan *et al.* (2006) aimed to evaluate intra- and inter-individual DNA methylation variation in sperm using targeted sequencing and microarray technologies. This study showed, for the first time, that there is significant variation of DNA methylation both within and between sperm samples. Finally, Klaver *et al.* (2012)showed that there were no significant differences in DNA methylation at a series of imprinted genes and other loci, such as Alu and LINE-1 elements, before and after cryo-perseveration. This is a critical study, for both sperm DNA methylation and fertility research as cryo-preservation of sperm is ubiquitously used in ART, and represents the only high-quality methodological study of human sperm preservation techniques that was identified by our comprehensive search.

Conclusion

Although two studies suggest a possible association between psychiatric disease and spermatozoal DNA methylation, there is insufficient evidence to make conclusive statements. There appears to be little detrimental effect of cryo-preservation on sperm via spermatozoal DNA methylation.

Discussion

This systematic review represents the largest critical evaluation of studies of DNA methylation in human spermatozoa performed to date. The comprehensive nature of the review allowed us to identify common methodological limitations as well as draw overarching conclusions regarding our current knowledge of the human sperm DNA methylome with respect to three specific questions, as well as identify existing knowledge gaps.

Overall summary of findings

A currently widely held supposition in the field of human sperm epigenetics suggests that aberrant DNA methylation of imprinted loci and the promoter of *MTHFR* is causally associated with changes to sperm function and health, which in turn lead to lower pregnancy rates and poor pregnancy outcomes (Fields *et al.*, 2013). Generally, the assumption made in this hypothesis is that environmental exposures can cause changes to sperm DNA methylation at these key genes, that in turn affect fertility and pregnancy outcomes. In the existing literature (between 2003 and 2020, scoring high or moderate for quality of evidence), there is suggestive evidence that idiopathic male subfertility, in particular oligozoospermia, is associated with aberrant methylation of imprinted loci and other genes in sperm. However, results are often conflicting and have not been replicated in unbiased, genome-wide analyses. There is similarly conflicting evidence that spermatozoal DNA methylation, including aberrant DNA methylation of imprinted genomic regions, can affect pregnancy outcomes or offspring health. In sum, therefore, changes in spermatozoal DNA methylation should not, at present, be seen as an established mechanism of subfertility, poor pregnancy outcomes or offspring ill health. Spermatozoal DNA methylation is also of limited clinical utility as a biomarker for such outcomes (**Fig. 4**).

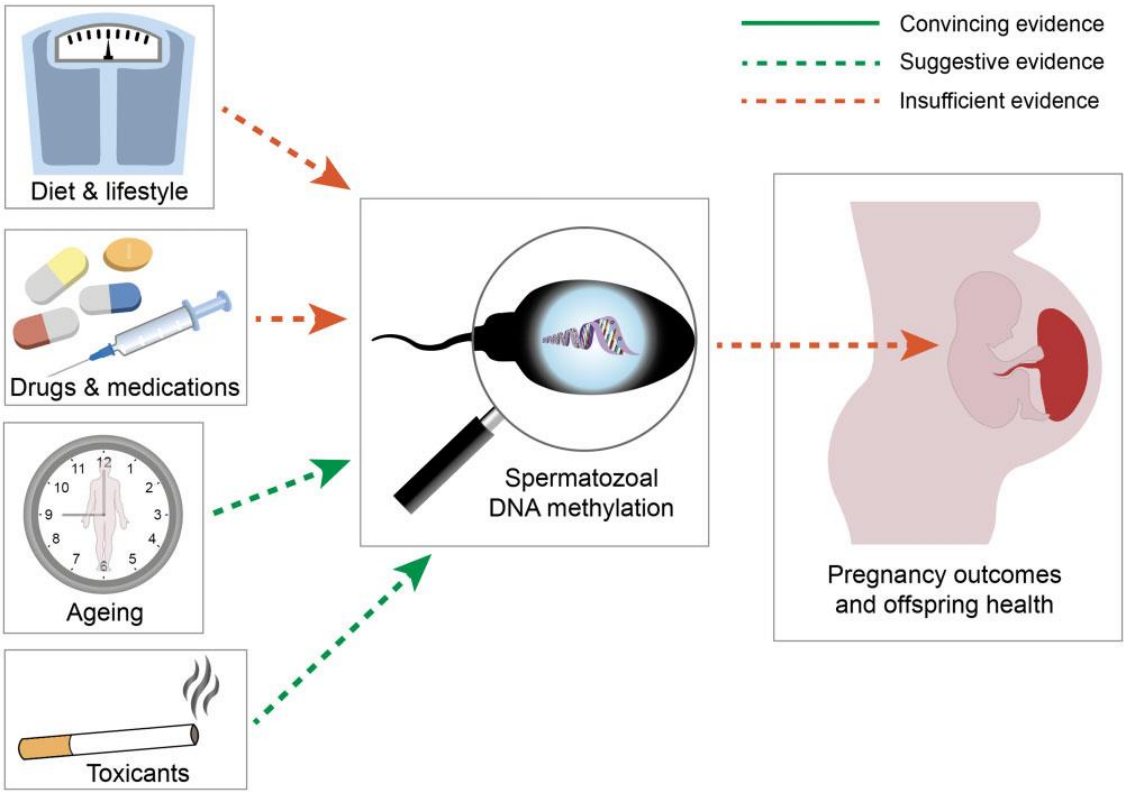

Figure 4. Overview of the quality of evidence for how selected acquired and environmental factors can influence the spermatozoal DNA methylome and offspring health.

This systematic review found suggestive evidence that ageing and toxicants, such as cigarette smoke, are associated with changes in human spermatozoal DNA methylation. However, there is yet insufficient evidence that such changes are associated with pregnancy outcomes, including success of assisted reproductive technologies and offspring health.

There is suggestive evidence that ageing and some toxicants, particularly cigarette smoke, can alter DNA methylation of sperm, but there is insufficient evidence for an effect of BMI, diet, lifestyle, psychiatric disorders, drugs or medication. In some cases, this is due to a lack of high- or moderate-quality studies. In other cases, associations have not been studied sufficiently or replicated to accumulate sufficient evidence. However, while DNA methylation in sperm may not have effects on the next generation, and may not affect fertility, it could nonetheless be used as a biomarker of exposure to certain environmental stressors, for example, to predict age and to assess exposure to pollutants. Given that human sperm is relatively accessible and composed of a comparatively homogenous cell population, DNA methylation in sperm in this context could become a useful resource clinically.

Possibly the most striking conclusion from this systematic review is the lack of overlap of findings obtained from the same or related research questions. Despite several high- and moderate-quality studies independently demonstrating associations between spermatozoal DNA methylation and fertility, smoking status, exercise interventions and exposure to some environmental pollutants, none of these studies identified DNA methylation changes at the same genomic regions. These discrepancies undermine the overall evidence for any one of these exposures being associated with sperm DNA methylation and could be explained in several ways.

First, despite sperm being a relatively homogeneous cell type, the human sperm methylome is highly heterogeneous across individuals and populations, meaning that different results could be observed between biologically different cohorts. This is supported by the finding from Consales *et al.* (2014), who showed that DNA methylation in a sample of 316 men clustered significantly by geographical location and no other investigated parameter was significantly associated with the clustering. This issue can only be overcome with larger sample sizes, preferably performed using multicentre collaborations, as well as appropriate strategies controlling for genetic backgrounds.

From a technical perspective, there are differences between studies in the application and effectiveness of sperm processing methods used to eliminate somatic cell contamination. Harmonization of research protocols would make cross-cohort comparisons and meta-analyses more feasible, increasing the robustness of findings. We also identified wide variations in the statistical models used to analyse DNA methylation data, particularly in how confounders relevant to human studies of fertility were accounted for. Different methods of analysing DNA methylation may also yield different results for technical reasons. For example, CpG sites found to be differentially methylated in candidate gene analyses may not be present on the 450K array, which captures around 1.7% of the ~28 million CpG sites in the human genome (Pidsley *et al.*, 2013), and only covers a small proportion of enhancers and other regulatory regions. The more recent Infinium MethylationEPIC array has improved coverage of enhancers and regulatory regions, and will thus make future DNA methylation analyses more comprehensive (Pidsley *et al.*, 2016), although given the unique nature of the sperm methylome a custom array may be more suitable (Chan *et al.*, 2019). Standardization of these methods is required to allow cross-study comparisons.

Finally, for individual studies with high quality of evidence, independent replication of findings is required to solidify credible evidence, and eventually inform clinical practice. For example, while Atsem *et al.* (2016) report an intriguing association between age-related spermatozoal DNA methylation and neurodevelopmental problems in offspring of older fathers, this study constitutes the only study on this topic and requires replication.

Recommendations for future research

The drawbacks and major weaknesses we identified in the current body of literature on human spermatozoal DNA methylation were common across many studies and spanned all three investigated themes. Most of them relate to general methodological considerations for epigenetic studies of human phenotypes and tissues, and include study design, experimental processes and statistical analyses, while some challenges are specific to the nature of sperm as a tissue. In light of these prevailing criticisms, we have put forward a series of recommendations for future research, which are briefly summarized in **Fig. 5** and discussed below.

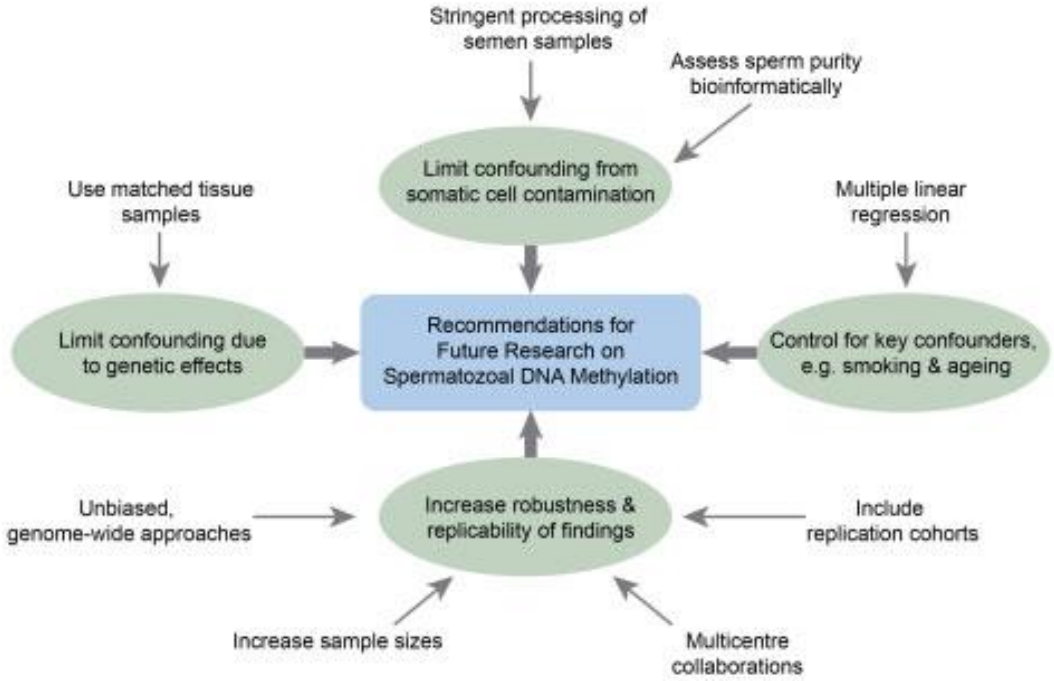

Figure 5. Recommendations for future research on spermatozoal DNA methylation.

Sample size

To generate robust, reproducible findings, sample sizes need to be sufficiently large to meet the power needs of the study design. Performing a power calculation prior to study commencement is recommended, and methods for a range of platforms and approaches have been described previously (Tsai and Bell, 2015; Saffari *et al.*, 2018; Graw *et al.*, 2019; Mansell *et al.*, 2019). As sperm is not routinely collected outside of reproductive medicine settings, it is likely that multicentre collaborations will be required to meet the need for increased sample sizes. Such collaborations should harmonize protocols for phenotyping of participants, processing samples and data analyses to limit batch effects (Leek *et al.*, 2010; Forest *et al.*, 2018).

Sperm sample processing

A range of protocols exists for selecting motile spermatozoa and cleaning semen samples from contaminating somatic cells (**Supplementary Fig. S1**, **Supplementary Data 2**). Of importance, irrespective of which protocol is employed, samples should be checked post-processing to ensure that spermatozoa used for epigenetic analyses contain a pure sperm population. Assessing somatic cell contamination visually, and ideally also using computational methods, is highly advisable.

Methodology

Unbiased, epigenome-wide association studies of biologically relevant tissues have yielded important insights into a range of common human conditions, including metabolic and neurodegenerative disease (De Jager *et al.*, 2014; Wahl *et al.*, 2017). Whole-genome bisulphite sequencing (WGBS) remains the gold standard method to profile DNA methylation across all CpG sites in the human genome. However, achieving adequate coverage at each site can be expensive. Until the price of WGBS drops

substantially, RRBS, which targets CpG rich regions, and microarray-based approaches will remain more cost-effective option for large-scale, unbiased investigations. The latest edition of human DNA methylation microarray from Illumina, the MethylationEPIC array (EPIC array), covers around 850 000 CpG sites annotated to most coding genes and regulatory regions of the human genome (Illumina, 2017). In addition, results from previous generations of arrays can largely be compared across studies. For example, the EPIC array covers >90% of CpG sites present on the 450K array, and contains ~350 000 additional CpG sites located in enhancers. Therefore, results obtained from analyses using the EPIC array can generally be checked for consistency with previous results from studies using the 450K array, provided that similar methodologies were used.

Tissue specificity

For cross-tissue comparisons, biological samples should be matched, i.e. taken from the same individual or from the individual's offspring. Because genetic variation influences the DNA methylome, not using matched tissues, especially in small sample sizes, increases the likelihood of confounding and should be avoided in future research (Bell *et al.*, 2011; McClay *et al.*, 2015; McRae *et al.*, 2018).

Multiple testing

Performing a statistical test always comes with a risk of reporting a false positive association (~5%). This is a particularly pertinent problem when multiple genomic loci are investigated, notably so in the case of array-based studies of DNA methylation, where there can be up to 850 000 individual tests. To reduce the chance of reporting a false positive, a correction for multiple tests is crucial. Standard methods, such as Bonferroni's correction or a false discovery rate correction, can be applied but more accurate genome- and array-wide *P*-value thresholds estimating the specific testing burden have been reported recently (Mansell *et al.*, 2019). Concerningly, multiple correction is most often omitted in targeted or candidate gene studies, despite multiple CpG sites across a specific genomic region being investigated. If, for instance, a candidate gene study investigates 20 CpG sites without correcting for multiple testing, the overall probability of detecting at least one false positive association is 64%.

Modelling covariates

Confounding factors, where differences in characteristics between samples could explain observed differences in DNA methylation, need to be considered when conducting epigenetic association studies. On the one hand, appropriate sampling is required to avoid complete overlap between conditions and confounding factors (e.g. when all samples in the control group are older than the case group); on the other hand, these confounding factors should be controlled for in the statistical analysis. We recommend collecting extensive phenotype data and including these as covariates in the analysis as well as using batch correcting normalization and control procedures (Pidsley *et al.*, 2013; Fortin *et al.*, 2014; Maksimovic *et al.*, 2015; Saffari *et al.*, 2018). Based on the findings of this systematic review, relevant covariates include but are not limited to: age, smoking status, BMI, alcohol consumption, exposure to environmental toxicants, medications, semen parameters, disease and exercise regimes. While associations of most of these covariates with spermatozoal DNA methylation are often still

speculative and require further evidence, it is crucial to err on the side of caution with regards to covariates and confounding. In practise this means high-quality studies should clearly demonstrate that any potential and plausible confounding factors do not affect the outcomes.

Expanding the scope of DNA methylation studies in human sperm

The majority of studies to date have focused on differential DNA methylation in relation to subfertility or abnormal semen parameters. Widening the scope in future investigations to include more research on, for example, the influence of metabolic health, toxicants and ageing on sperm epigenetics would be recommended. Such research would also motivate improved biological hypotheses about the epidemiological associations between paternal ageing, smoking and BMI on offspring health (Oldereid *et al.*, 2018). In this regard, however, other causes than changes to spermatozoa, whether genetic or epigenetic, need also to be taken into account. For example, there has long been an assumption that increased paternal age is associated with an increased risk for neurodevelopmental conditions, such as autism and schizophrenia, in the offspring due to age-related increases in the rate of *de novo* mutations in spermatozoal genomes (Gratten *et al.*, 2016). However, recent research suggests that this association is at least partly explained by the fact that psychiatric illness is associated with an increased propensity for men to father their first child at a relatively either young or old age (Gratten *et al.*, 2016). These two explanations for the association are of course not mutually exclusive, and it is possible that the increased rate of *de novo* mutations also plays a part. Nevertheless, future research exploring the association between paternal age-related changes to his (epi)genome and the propensity for his offspring to develop psychiatric disease should take these behavioural and social factors into account.

Future work should also investigate the possibility that the unique chromatin packaging in spermatozoa, wherein histones are sequentially replaced with protamines during sperm maturation (Wang *et al.*, 2019), may influence the accuracy of commonly used methodologies for interrogating DNA methylation in spermatozoa. Furthermore, chromatin packaging changes rapidly after ejaculation (Björndahl and Kvist, 2010). Therefore, the time of sperm collection after ejaculation may influence the accuracy of findings and protocols will need to be harmonized to make results comparable across studies. Overall, future research should explore the complex crosstalk between different epigenetic processes, including DNA methylation, histone modifications and small RNA species, in sperm functioning and how these may be altered by environmental and physiological change.

Strengths and limitations of the systematic review

This systematic review is an exhaustive literature search, including a large number of publications studying DNA methylation variation in human sperm from 2003 until 2020. Although this review is not a meta-analysis, a key strength is that it critically evaluates the findings of these studies with respect to the quality of the evidence they present, enabling the reader to navigate a large and often self-contradictory field. A limitation of this systematic review is the applicability of the GRADE criteria to fit its particular purpose; it was necessary to modify the criteria to account for the lack of randomized

control trials in this field. Second, potential bias is introduced in the reporting of studies by the fact that several groups publish multiple high-quality papers, potentially using the same cohorts. Finally, it would be interesting to evaluate other epigenetic modifications, such as histone modifications and small non-coding RNA, in human sperm in response to environmental stressors and their potential impact on transgenerational inheritance, but this was beyond the scope of this review.

Conclusion

This systematic review of studies investigating DNA methylation in human spermatozoa found that the reliability of results from studies thus far has frequently been hampered by inadequate sample sizes and methodological drawbacks. Nevertheless, it appears from higher quality studies that factors such as ageing, cigarette smoking and environmental pollutants are associated with differences in the DNA methylation landscape of spermatozoa. Such findings are not yet sufficiently robust or replicated to be translated to clinical practice or to inform whether, and how, paternal lifestyle could influence offspring health. To increase the likelihood of uncovering biologically and clinically meaningful findings in future research, we have outlined a set of recommendations that we advise should be taken into account when designing a study in this or related areas. Further characterization of the human sperm epigenome has the potential to improve our understanding of fertility, embryogenesis and the capacity of environmental factors to impact the next generation.

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Authors' roles

F.A. designed the study and created the figures. F.A. and A.F.D. collected the records and the data, analysed the data and wrote the manuscript. F.A., A.F.D. and S.J.M. discussed the conclusions, made the recommendations and edited the manuscript.

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Conflicts of interest

The authors declare that they have no competing interests.

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Table I. Studies assessing the effect of DNA methylation in human sperm on fertility and semen parameters.

Author, year	*Imprinting studied?*	*Method of assaying DNA methylation*	*Initial processing*	*N (cases)*	*Age in years (SD); description of cases*	*N (controls)*	*Age in years; description of controls*	*Main Results*	*Study Quality*
Laqqan et al., 2017b	No	450K array; targeted bisulfite sequencing	Gradient (50%) centri-fugation. Medium ND	70	35.89 (± 6.03); 15 for screening and 55 for validation	71	33.98 (± 5.53); 15 for screening and 56 for validation	Significant assoc. between subfertility and altered DNAm at CpGs related to *ALS2CR12*, *ALDH3B2, PRICKLE2*, and *PTGIR*	High
Laqqan et al., 2017c	No	450K array; targeted bisulfite sequencing	Somatic cell lysis and PureSperm gradient (45 and 90%)	101	36.4 (± 3.24); 20 + 81 (screening + validation) OZS	66	36.4 (± 3.24); 20 + 44 (screening + validation) proven fertility	Assoc. between OZS and DNAm of *UBE2G2* and cg04807108	High
Tian et al., 2015	Yes	Methylation specific PCR	Samples washed in PBS and sperm wash buffer	118	31.4 (± 5.1); males under evaluation for subfertility	None	N/A	*LINE-1* DNAm negatively correlated with sperm motility	High
Wu et al., 2010	No	Targeted bisulfite Sanger sequencing	Swim-up	94	29.04 (± 4.52); idiopathic subfertility	54	29.52 (± 3.72); proven fertility	Significantly ↑ DNAm of the MTHFR promoter in subfertile males cf fertile controls	High
Poplinski et al., 2010	Yes	Targeted bisulfite Sanger sequencing	Swim-up	148	35.5 (32-41); subfertile	33	37.0 (31.5-40); NZS; infertility attributed to female side	*MEST* ↓ DNAm significantly assoc. with OZS in subfertile males	High
Kabartan et al., 2019	No	Methylation-specific PCR	Somatic cell lysis	73	31.95 (± 5.5); OZS, infertile	20	27.2 (± 2.6); NZS, proven fertility	No assoc. between DNA fragmentation and DNAm of *BRCA1* or *BRCA2* in OZS males	Moderate
Tang et al., 2018	Yes	Bis-PCR	Percoll gradient (40 and 80%)	135	29.07 (± 4.25); subfertile	59	29.08 (± 3.84); proven fertility	Aberrant DNAm in imprinted genes of infertile men	Moderate
Laqqan et al., 2018	No	450K array followed by Bis-PCR-Seq	Somatic cell lysis	50	38.7 (± 6.9); subfertile males	28	38.5 (± 5.6); proven fertility	Assoc. between subfertility and DNAm of *PRRC2A, ANXA2, MAPK8IP3* and *GAA*	Moderate

Camprubi et al., 2017	No	450K array	Somatic cell lysis	19	25.95 (± 1.41); proven fertility	N/A	N/A	Promoter CpGs relatively ↓ DNAm in spermatozoa. Identified 94 genes that appear resistant to demethylation	Moderate
Dong et al., 2017	Yes	Bisulfite pyro-sequencing	Percoll gradient (45 and 90%)	155	31.13-32.17; 48 OZS, 52 AsZS, 55 TZS	50	32.22 (± 3.59); NZS	In smokers, subfertility was assoc. with ↓ DNAm of *H19* in OZS and ↑ DNAm of *SNRPN*-ICR in AsZS and TZS	Moderate
Laqqan et al., 2017a	No	450K array; targeted bisulfite sequencing	Somatic cell lysis	72	37.4 (± 6.1); 15 + 57 (screening and validation) from subfertile men (unable to conceive for at least 10 years)	36	38.5 (± 5.2); 15 + 21 (screening and validation) men with proved fertility	Assoc. between subfertility and DNAm in *KCNJ5, MLPH* and *SMC1B*	Moderate
Camprubi et al., 2016	No	450K array	Somatic cell lysis	42	38.36 (± 5.31); subfertile males	19	25.95 (± 4.80); proven fertility	Assoc. between age and DNAm of *RPS6KA2*; OZS and DNAm of *APCS*; chromosome abnormalities and DNAm of *JAM3/ NCAPD3* and between fecundity and DNAm of *ANK2*	Moderate
Jenkins et al., 2016a	No	450K array	ISolate gradient	94	32.0-36.32; classified according to semen parameters	N/A	N/A	No DNAm alterations associated with TZS; Abnormal semen had increased DNAm variability	Moderate
Jenkins et al., 2015	No	450K array	ISolate gradient (35 and 90%)	20	35.69 (± 2.0); subfertile	N/A	N/A	Higher DNAm variability in low quality sperm	Moderate
Botezatu et al., 2014	No	Methylation specific PCR	ND	27	26-41; OAT	11	24-37; NZS	↓ DNAm of *MTHFR* and *SNRPN* in samples with low sperm motility. No significant trend for other semen parameters	Moderate
Schutte et al., 2013	No	27K array followed by targeted bisulfite pyrosequencing	Swim-up procedure and PureSperm gradient (40 and 80%)	38	27-54; males under evaluation for subfertility (longitudinal)	4	Age ND; NZS	27K array data showed clustering by sperm count; no candidate genes showed significant difference	Moderate

Kläver et al., 2013	Yes	Bisulfite pyro-sequencing	Swim-up	212	Age ND; males under evaluation for subfertility	41	34.5 (± 7.2); 31 NZS samples. Longitudinal.	*MEST* meth assoc. with OZS. DNAm levels at selected genes in NZS males were stable for up to 951 days.	Moderate
Barzideh et al., 2013	No	HPLC for DNAm; flow cytometry	Percoll gradient	16	20-23; NZS	N/A	N/A	DNAm ↓ in high-density (high quality) portion of spermatozoa than in low-density portion	Moderate
Pacheco et al., 2011	No	27K array	Percoll gradient (50%)	21	Age ND; males under evaluation for subfertility	None	N/A	Assoc. between 9,189 CpGs and low sperm motility	Moderate
Marques et al., 2008	Yes	Bisulfite sequencing (Sanger)	Suprasperm gradient (55, 80 and 90%) and swim-up	20	Age ND; 6 OZS; 14 AsZS; all abnormal morphology	ND	Age ND; NZS	*H19* and *MEST* abnormally imprinted in severely OZS group	Moderate
Houshdaran et al., 2007	No	MethyLight; Illumina bead array of 1421 CpG sites	ISolate gradient	65	22-49; partners of females undergoing fertility investigation	None	N/A	Assoc. between sperm parameters and DNAm of *HRAS, NTF3, MT1A, PAX8, DIRAS3, PLAGL1, SFN, SAT2CHRM1* and *MEST*	Moderate
Marques et al., 2004	Yes	Bisulfite sequencing	Gradient centrifugation and swim-up	96	Age ND; OZS	27	Age ND; NZS	*H19* abnormally imprinted in OZS group	Moderate
Cheng et al., 2017	No	Agilent custom 1M Promoter-CpG island microarray; targeted bisulfite pyrosequencing	Enzymatic digestion and countercurrent centrifugal elutriation	17	33.2 (± 0.5); 1 sample from patient with moderate HS; candidate genes in 16 patients	9	ND; 9 NZS males with obstructive AZS, 1 selected for whole genome methylation profiling	HS assoc. with DNAm of *BOLL, DDX4, HORMAD1*, and *MAEL*	Low
Du et al., 2016	No	Liquid hybridization (promoter capture) capture-based bisulfite sequencing	Percoll gradient (47.5, 57, 76 and 95%)	7	31.7 (± 4.0); AsZS	8	29.8 (± 3.2); NZS	No significant assoc. between DNAm or DNAm variability and AsZS	Low

Jenkins et al., 2016b	No	450K array	Somatic cell lysis	29	27.55 (± 0.71); subfertile	27	29.74 (± 0.71); proven fertility	DNAm of *HSPA1L* and *HSPA1B* significantly assoc. with pregnancy rates	Low
Louie et al., 2016	Yes	Methylation-specific PCR	Swim-up for high concen-tration samples. Handpicked spermatozoa for low concentration samples	44	32.9-35.7; OAT	ND	34.1 (± 2.4); NZS	No significant association between DNAm in selected imprinted genes, severe OAT and the *MTHFR* C677T SNP	Low
Cassuto et al., 2016	No	5-mC immuno	Sperm separation medium gradient (45 and 90%)	10	37-50; 448 S6 spermatozoa compared to 428 S0 spermatozoa	N/A	N/A	↓ DNAm in morphologically higher scoring spermatozoa compared to morphologically lower scoring spermatozoa	Low
Laurentino et al., 2015	Yes	Bisulfite pyroseq and deep targeted bis-seq	Swim-up	26	34.0-39.33; males with abnormal sperm parameters	19	33.68 (± 1.58); proven fertility	Significantly ↑ variation in the DNAm values of the maternally methylated gene *KCNQ1OT1* in samples with abnormal sperm parameters	Low
Zhou et al., 2015	No	Methylation specific PCR and bisulfite sequencing PCR	Percoll gradient (50%)	48	30.13 (± 5.8); AsZS	42	29.0 (± 4.8); NZS	No DNAm of the *CRISP2* promoter; expression likely regulated by miR-27b	Low
Urdinguio et al., 2015	No	450K array; targeted bisulfite pyrosequencing; immunostaining for DNAm	PureSperm gradient (65 and 90%)	7	30-55; 29 subfertile NZS (7 included in array analysis)	5	22-49; 17 NZS fertile males (5 included in array analysis)	A number of CpG sites significantly differentially methylated between subfertile cases and fertile controls	Low
Tavalaee et al., 2015	No	5-mC immuno	Samples washed in PBS	23	31.3 (± 4.3); grade II and III varicocelectom y (longitudinal)	None	N/A	Varicocele surgery associated with improved sperm parameters, but no significant change in DNAm	Low
Montjean et al., 2015	Yes	Global DNAm assayed by immunostaining	Percoll gradient	30	38.3 (± 6); OAT	62	38.5 (± 5.3); NZS	Significant association between *H19* DNAm and sperm parameters	Low

		for DNAm, targeted bisulfite sequencing						
Rotondo et al., 2013	Yes	Methylation specific PCR and combined bisulfite restriction analysis	PureSperm gradient (40 and 80%)	10	36.1 (± 1.2); MTHFR promoter hypermethyl-ation. 5 with normal and 5 with abnormal semen parameters	10	Age ND; normal MTHFR promoter meth; 5 with normal and 5 with abnormal semen parameters	For NZS samples, *H19* meth was assoc. with *MTHFR* DNAm	Low
Sato et al., 2011	Yes	COBRA and methylation-specific PCR with Luminex technology	Swim-up	128	Age ND; OZS	209	Age ND; NZS	Correlation between DNAm analysis methods. Higher levels of imprinting errors in OZS.	Low
Boissonnas et al., 2010	Yes	Targeted pyro-sequencing	Percoll gradient (45, 60 and 90%)	41	36.6 (± 5.7); divided into TZS, OZS and AsZS groups	17	Age ND; NZS	↓ DNAm of various CpG positions in the *H19-IGF2* DMR in cases cf controls	Low
Hammoud et al., 2010	Yes	Targeted Sanger sequencing	ND	20	Age ND; 10 with abnormal P1/P2 ratio and 10 with OZS	ND	Age ND; proven fertility	↑ DNAm of *LIT1, SNRPN, MEST, ZAC, PEG3* in OZS and abnormal P1/P2 ratio samples cf NZS samples	Low
Marques et al., 2010	Yes	Bisulfite sequencing	Testicular biopsies washed in sperm prepar-ation medium and somatic cells removed with erythro-cyte lysing buffer	24	22-44; AZS (sperm obtained from testicular biopsies)	ND	Age ND; NZS	*H19* DNAm errors identified in AZS samples obtained via testicular biopsy	Low
Navarro-Costa et al., 2010	No	Bisulfite sequencing	PureSperm gradient (40 and 80%)	5	39.4 (± 7.2); OZS	5	39.2 (± 7.3); NZS	↑ DNAm of the *DAZL* (but not *DAZ*) promoter more prevalent in OZS samples compared to NZS controls	Low

Aoki et al., 2006	No	Quantitative immune-fluorescence microscopy	ND	195	Age ND; subfertile (3 groups based on P1/P2 ratios)	None	N/A	No significant differences in DNAm between P1/P2 groups	Low
Sujit et al., 2018	No	450K array; targeted bisulfite sequencing	Somatic cell lysis for cases and swim-up procedure for controls	38	Age ND; subfertile; OZS	26	Age ND; NZS	1436 probes had ↑ DNAm and 244 had ↓ DNAm in subfertile OZS cases relative to fertile NZS controls	Very low
Gunes et al., 2018	No	Methylation specific PCR	Somatic cell lysis	10	Age ND; OAT	29	Age ND; NZS	In OAT men, *MLH1* was ↑ DNAm. Seminal ROS levels were assoc. with *MLH1* DNAm	Very low
Olszewska et al., 2017	No	TLC and immuno-fluorescence	Samples washed in F10 medium	9	30.7; subfertile carriers of chromosomal abnormalities	14	28.5; healthy volunteers	Chromosomal abnormalities not assoc. with DNAm	Very low
Marques et al., 2017	Yes	Methylation specific PCR	Micro-manipulation from testicular biopsy	15	Age ND; AsZS	ND	Age ND; NZS males unable to produce semen due to spinal cord injuries	No significant association between *H19* and *MEST* DNAm and AsZS	Very low
Nasri et al., 2017	Yes	Combined bisulfite restriction analysis	PureSperm gradient (40 and 80%)	23	Age ND; samples with abnormal sperm parameters	11	Age ND; NZS	No significant difference between *H19* DNAm and sperm parameters	Very low
Xu et al., 2016a	No	Methylation specific PCR	ND	27	28; AsZS	25	28; NZS	AsZS significantly assoc. with abnormal DNAm of the *VDAC2* promoter	Very low
Xu et al., 2016b	Yes	Targeted DNAm quantification using the MassArray Epityper	Percoll gradient (50%)	46	31.95 (± 3.1); AZS	49	32.16 (± 3.26); NZS	↓ DNAm in *MEST, GNAS, FAM50B, H19, LINE-1* and *P16* in AsZS males	Very low
Uppangala et al., 2016	No	5-mC immuno	Swim-up	19	Age ND; proven fertility. 1, 3, 5 and 7	N/A	N/A	No significant association between DNAm and length of ejaculatory abstinence	Very low

					days of abstinence				
Bahreinian et al., 2015	No	Flow cytometric immuno-detection for DNAm	Samples washed in PBS	44	31.1 (± 0.6); Patients with grades II and III varicocele	15	37.8 (± 2.0); proven fertility	Varicocele associated with ↓ DNAm and poorer sperm parameters	Very low
Kuhtz et al., 2014	Yes	Bisulfite sequencing	Swim-up and PureSperm gradient (40 and 80%)	27	Age ND; OAT	27	Age ND; NZS	No significant differences in the occurrence of abnormal methylation imprints between sperm with and without morphological abnormalities	Very low
Richardson et al., 2014	No	Bisulfite pyro-sequencing	Swim-up	95	32-39; abnormal semen parameters	45	34-40; NZS	The *RHOX* homeobox gene cluster is regulated by DNAm and *RHOX* gene cluster ↑ DNAm is significantly assoc. with semen abnormalities	Very low
Camprubi et al., 2013	Yes	Bisulfite pyro-sequencing	Swim-up	6	Age ND; Subfertile males showing hypo-methylation of the *H19-ICR* locus	None	N/A	No significant association between *CTCFL* mutations and *H19-ICR* sperm DNAm	Very low
Li et al., 2013	Yes	Methylation specific PCR	Gradient (45, 60 and 90%) gradient centri-fugation. Both Percoll and PureSperm mentioned	40	Age ND; 20 OZS and 20 AsZS men	20	Age ND; NZS, proven fertility	No significant association between *H19* and *DAZL* DNAm and AsZS.	Very low
Minor et al., 2011	Yes	Bisulfite sequencing	Swim-up	18	37.8 (± 6.9); AZS (sperm obtained from testicular biopsies)	9	34.1 (± 2.4); proven fertility (from ejaculate)	↓ DNAm of *H19* in AZS males	Very low
Nanassy and Carrell, 2011a	No	Bisulfite pyro-sequencing	ND	92	28-33; 60 with abnormal P1/P2 ratio, 32 with OZS	40	32-33; NZS, proven fertility, normal P1/P2 ratio	↑ freq. *CREM* promoter methylation in males with abnormal P1/P2 ratio and in OZS cf fertile, NZS controls	Very low

Nanassy and Carrell, 2011b	No	Bisulfite sequencing	ND	10	Age ND; abnormal P1/P2 ratio	10	Age ND; NZS	No significant DNAm differences in the selected genes between samples with an abnormal P1/P2 ratio and NZS controls	Very low
Li et al., 2006	No	Bisulfite sequencing	Samples washed in PBS	4	Age ND; proven fertility	N/A	N/A	*DAZ1* promoter methylated in leukocytes but unmethylated in sperm	Very low

'5-mC immuno' = immunostaining for 5-methylCytosine; Bis-PCR = bisulfite-specific PCR; TLC = thin-layer chromotography; HPLC = high-performance liquid chromatography; NZS=normozoospermic; ND = not disclosed; OAT = oligoasthenoteratozoospermia; TZS = teratozoospermia; DNAm = DNA methylation levels; AsZS = asthenozoospermia; OZS = oligozoospermia; assoc. = association; ↑ = increased or hypermethylation; ↓ decreased or hypomethylation ; P1/P2 = protamine 1/ protamine 2; HS = hypospermatogenesis.

ALS2CR12 = amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 12; ALDH3B2 = aldehyde dehydrogenase family 3 member B2; PRICKLE2 = prickle planar cell polarity protein 2; PTGIR = prostaglandin I2 receptor; UBE2G2 = ubiquitin-conjugating enzyme E2 G2; LINE-1 = long interspersed nuclear element 1; MTHFR = methylenetetrahydrofolate reductase; MEST = mesoderm specific transcript; BRCA = breast cancer; PRRC2A = proline rich coiled-coil 2A; ANXA2 = annexin A2; MAPK8IP3 = mitogen-activated protein kinase 8 interacting protein 3; GAA = glucosidase alpha, acid; H19 = H19, imprinted maternally expressed transcript; SNRPN = small nuclear ribonucleoprotein polypeptide N; KCNJ5 = potassium voltage-gated channel subfamily J member 5; MLPH = melanophilin; SMC1B = structural maintenance of chromosomes 1B; RPS6KA2 = ribosomal protein S6 kinase A2; APCS = amyloid P component, serum; JAM3 = junctional adhesion molecule 3; NCAPD3 = non-SMC condensin II complex subunit D3; ANK2 = ankyrin 2; HRAS = HRas proto-oncogene, GTPase; NTF3 = neurotrophin 3; MT1A = metallothionein 1A; PAX8 = paired box 8; DIRAS3 = DIRAS family GTPase 3; PLAGL1 = PLAG1 like zinc finger 1; SFN = stratifin; SAT2CHRM1 = spermidine/spermine N1-acetyltransferase family member 2; BOLL = boule homolog, RNA binding protein (member of the DAZ gene family); DDX4 = DEAD-box helicase 4; HORMAD1 = HORMA domain containing 1; *MAEL = maelstrom spermatogenic transposon silencer; HSPA1L = heat shock protein family A (Hsp70) member 1 like; heat shock protein family A (Hsp70) member 1B; KCNQ1OT1 = KCNQ1 opposite strand/antisense transcript 1; CRISP2 = cysteine rich secretory protein 2; IGF2 = insulin like growth factor 2; PEG3 = paternally expressed 3; LIT1 (more commonly known as KCNQ1OT1) = KCNQ1 opposite strand/antisense transcript 1; ZAC (more commonly known as PLAGL1) = PLAG1 like zinc finger 1; DAZL = deleted in azoospermia like (member of the DAZ gene family); DAZ = deleted in azoospermia (group of 4 genes on chromosome Y: DAZ1-DAZ4, members of the DAZ gene family); MLH1 = mutL homolog 1; voltage dependent anion channel 2; GNAS = GNAS complex locus; FAM50B = family with sequence similarity 50 member B; P16 (more commonly known as CDKN2A) = cyclin dependent kinase inhibitor 2A; RHOX = reproductive homeobox on the X chromosome; CTCFL = CCCTC-binding factor like; CREM = CAMP responsive element modulator; DAZ1 = deleted in azoospermia 1 (member of the DAZ gene family)*

Table II. Studies assessing the effect of DNA methylation in human sperm on pregnancy outcomes and offspring health.

Author, year	*Outcome studied*	*Method of assaying DNA methylation*	*Initial processing*	*N (cases)*	*Age in years (SD); description of cases*	*N (controls)*	*Age in years; description of controls*	*Main Results*	*Study Quality*
Atsem et al. 2016	Offspring cord blood DNAm	Bisulfite pyro-sequencing	Swim-up and PureSperm gradient (40 and 80%)	350	Age ND; 162 samples that had led to the birth of a child (cohort 1), 188 samples without pregnancy (replication cohort)	None	N/A	DNAm of *FOXK1* assoc. with paternal age in sperm and cord blood of offspring	High
Camprubi et al. 2012	ART success	Bisulfite pyro-sequencing	Swim-up	107	36 (± 5.5); subfertile	30	26 (± 6.15); proven fertility	No significant association between rates of imprinting errors and outcome of ART	High
El Hajj et al. 2011	ART success	Bisulfite pyro-sequencing	PureSperm gradient (40 and 80%)	106	Age ND; subfertile	28	ND	Significant association between DNAm of *Alu* and outcome of ART	High
Kobayashi et al. 2017	Pregnancy outcomes	Combined bisulfite restriction analysis	Swim-up	70	36.5 (± 6.1); 40 males with moderate OZS and 30 males with severe OZS	151	35.4 (± 5.4); NZS	OZS assoc. with smoking and high consumption of carbonated drinks. Adverse pregnancy outcomes assoc. with sperm DNAm errors	Moderate
Rotondo et al. 2012	Spontaneous pregnancy loss	Bisulfite-PCR sequencing	PureSperm gradient (40 and 80%)	20	35 (± 5.4); couples with recurrent spontaneous pregnancy loss	167	35.3 (± 4.4); 20 males with proven fertility, 147 couples not affected by recurrent spontaneous pregnancy loss	*MTHFR* ↑ DNAm more common in semen from couples affected by recurrent spontaneous pregnancy loss	Moderate
Tavalaee, Razavi, and Nasr-Esfahani 2009	ART success	5-mC immuno	PureSperm gradient (40 and 80%)	61	36.2 (± 5.2); under-going ART	None	N/A	DNAm negatively correlated with DNA fragmentation. No assoc. with ART success	Moderate

Denomme et al. 2017	Embryo quality	450K array on a subset of sperm samples (12 out of 40). Beta value distribution examined for histone-retained regions	PureSperm gradient (45 and 90%), swim-up and somatic cell lysis	20	40.3 (± 1.3); NZS (20 that led to 'good' embryogenesis)	20	42.0 (± 1.1); NZS (20 that led to 'poor' embryogenesis)	Significant assoc. between DNAm in 1634 CpG sites and embryo quality	Low
Feinberg et al. 2015	Autism in offspring	CHARM 3.0 array; 30 samples also run on a 450K array	No sperm selection method employed	44	27-51.2; fathers in a cohort enriched for autistic children	None	N/A	193 DMRs in paternal sperm associated with performance on the Autism Observational Scale for Infants at 12 mo of age in offspring	Low
Aston et al. 2012	Abnormal embryo-genesis	27K array followed by targeted bisulfite pyrosequencing	ND	28	31.0 (± 1.9); 15 males with ab-normal chromatin packaging + 13 males with poor embryo outcomes	15	37.1(± 2.0); NZS	No significant CpG methylation differences identified	Low
Ankolkar et al. 2012	Spontaneous pregnancy loss	Bisulfite Sanger sequencing	HiSep solution gradient	26	35.4; couples with recurrent spontaneous miscarriage	26	31.3; proven fertility	*H19* ↓ DNAm in sperm from recurrent spontaneous miscarriage group	Low
Kobayashi et al. 2009	Conceptus DNAm	Combined bisulfite PCR restriction analysis and bisulfite sequencing	ND	78	Age ND; samples paired with aborted conceptuses conceived via ART	38	Age ND; samples paired with aborted conceptuses not conceived via ART	Imprinting errors ↑ in OZS samples. Imprinting errors in sperm also evident in the resulting conceptuses in 7 out of 17 cases.	Low
Benchaib et al. 2005	ART success	5-mC immuno	PureSperm gradient (50, 70 and 90%)	63	35.4 (± 5.5); under-going ART	None	N/A	↑ DNAm assoc. with ↑ rates of pregnancy and embryo development, not with fertilisation rates	Low
Spinelli et al., 2019	No	Bisulfite pyro-sequencing	ND	4	ND	N/A	N/A	*IDO1* ↑ in sperm cf. placenta; no DNAm change in oocytes	Very low

Aston et al. 2015	Embryo quality and ART success	450K array	PureSperm gradient (45 and 90%); some analyses performed on whole ejaculates	127	Age ND; 54 with successful pregnancy outcomes and 72 with poor embryogenesis	54	ND; NZS, proven fertility	DNAm may be predictive of embryo quality, but not of IVF outcome	Very low
Montjean et al. 2013	ART success	Bisulfite targeted Sanger sequencing	Percoll gradient (45 and 90%)	175	Age ND; OZS	119	Age ND; NZS	Abnormal DNAm of selected genes more prevalent in OZS. No association between DNAm and outcome of ART	Very low
Al-Khtib et al. 2012	Oocyte DNAm	Bisulfite pyro-sequencing	PureSperm gradient (50, 70 and 90%)	5	Age ND; proven fertility	None	N/A	↑ DNAm of *OCT4* and *NANOG* in sperm but ↓ DNAm in oocytes	Very low
Ibala-Romdhane et al. 2011	Oocyte DNAm and embryo quality	Targeted bisulfite Sanger sequencing	Gradient centri-fugation (medium not specified)	11	Age ND; abnormal semen parameters (OZS, AsZS, TZS)	ND	Age ND; NZS, proven fertility	Embryos with developmental failure more likely to have abnormal imprinting at *H19* but all corresponding sperm had normal *H19* imprinting	Very low
Geuns et al. 2007	Oocyte, embryo and offspring DNAm	Methylation specific PCR	Percoll gradient (45 and 90%)	ND	ND	None	N/A	Intergenic DMR of *DLK1-GTL2* ↑ DNAm in sperm cf somatic tissues (e.g. oocytes and preimplantation embryos)	Very low
Kobayashi et al. 2007	ART success	Combined bisulfite PCR restriction analysis and bisulfite sequencing	Swim-up method	18	27-50; OZS	79	Age ND; NZS	DNAm errors ↑ in OZS. No significant association between imprinting errors and ART outcome.	Very low
Benchaib et al. 2003	Pregnancy outcomes	5-mC immuno	PureSperm gradient (50, 70 and 90%)	23	35 (median); undergoing ART	None	N/A	DNAm ↓ in TZS than non-TZS. No assoc. with pregnancy rate.	Very Low

FOXK1 = forkhead box K1; IDO1 = indoleamine 2,3-dioxygenase 1; OCT4 = octamer-binding transcription factor 4; NANOG = Nanog homeobox; DLK1 = delta like non-canonical notch ligand 1; GTL2 (more commonly known as MEG3) = maternally expressed 3

Table III. Studies assessing the effect of environmental exposures on DNA methylation in human sperm.

Author, year	*Type of exposure*	*Method of assaying DNA methylation*	*Initial processing*	*N (cases)*	*Age in years (SD); description of cases*	*N (controls)*	*Age in years (SD); description of controls*	*Main Results*	*Study Quality*
Tian et al. 2019	Toxicant	High-melting resolution PCR	ND	86	31.6; Males under evaluation for subfertility	N/A	N/A	High urinary levels of selected phthalate metabolites assoc. with ↓ *LINE-1* promoter DNAm and low sperm parameters	High
Lu et al. 2018	Toxicant	Bisulfite pyro-sequencing	Samples centrifuged at 200 ×g for 15 min. Sperm selection ND.	243	22-59; Exposed to varying levels of mercury	N/A	N/A	Negative correlation between sperm DNAm levels of *H19* and urinary mercury concentrations	High
Laqqan et al. 2017	Toxicant	450K array; targeted bisulfite sequencing	Sperm separation medium gradient (45 and 90%) and somatic cell lysis buffer	36	40.39 (± 7.32); smokers (15 for screening, 36 for validation)	42	38.38 (± 8.27); non-smokers (15 for screening, 42 for validation)	Smoking assoc. with DNAm of *MAPK8IP3* and *TKR*	High
Darbandi et al. 2019	Toxicant	Methylation specific PCR	Centrifuged at 300 xg for 5 min. Sperm selection method ND	43	33-35; NZS males with high ROS levels in seminal plasma	108	33-35; NZS males with low to moderate ROS levels in seminal plasma	High ROS levels assoc. with DNAm of *H19* and *IGF2*, and with lower semen parameters	Moderate
Ma et al. 2019	Toxicant	Bisulfite pyro-sequencing	Samples centrifuged for 15 min. Sperm selection ND.	219	24-53; males under evaluation for subfertility	N/A	N/A	DNAm of *H19* assoc. with urinary levels of 1-OHPH and 1-OHP (PAHs)	Moderate
Al Khaled et al. 2018	Toxicant	450K array; validation with bisulfite sequencing	PureSperm gradient (45 and 90%)	92	25-50; fertile smokers. 14 for discovery cohort and 78 for validation	14	25-50; fertile non-smokers	Smoking assoc. with DNAm in *PGAM5, PTPRN2* and *TYRO3*	Moderate
Murphy et al. 2018	Toxicant	RRBS	ND	12	21.8 (± 3.8); cannabis users	12	25.8 (± 6.7); non-cannabis users	Cannabis use associated with DNAm at 3,979 CpG sites	Moderate

Pilsner et al. 2018	Toxicant	WGBS	ISolate gradient (50 and 90%)	4	18-19; high serum dioxin concentration	4	18-19; low serum dioxin concentration	Serum dioxin concentration assoc. with DNAm at 52 DMRs	Moderate
Wu et al. 2017	Toxicant	450K array	Gradient (90%) centrifugation. Medium ND	48	18-55; males under evaluation for subfertility	None	N/A	131 DMRs were associated with at least one urinary phthalate metabolite	Moderate
Soubry et al. 2017	Toxicant	Bisulfite pyro-sequencing	ISolate gradient (50 and 90%)	67	18-35; from TIEGER cohort	N/A	N/A	Mono-isopropylphenyl diphenyl phosphate assoc. with *MEG3, NDN, SNRPN* DNAm. Tris(1,3-dichloro-2-propyl) phosphate exposure assoc. with DNAm at *MEG3* and *H19*	Moderate
Jenkins et al. 2017	Toxicant	450K array	Somatic cell lysis	78	32.4 (± 0.9); smokers	78	31.2 (± 0.6); non-smokers	Smoking associated with DNAm in 141 CpG sites	Moderate
Consales et al. 2016	Toxicant	Flow cytometric immuno-detection of DNAm; PCR pyrosequencing	Samples washed in PBS	269	29.22 (± 3.2); 75 samples from Greenland, 97 from Poland and 97 from Ukraine	N/A	N/A	Flow cytometry suggested assoc. between persistent organic pollutant (PCBs or DDT) exposure and ↓ DNAm	Moderate
Leter et al. 2014	Toxicant	Bis-PCR and flow cytometric immuno-detection of DNAm	ND	262	29.2 (± 0.3); 71 from Greenland, 96 from Poland and 95 from Ukraine	N/A	N/A	No consistent associations between internal PFASs concen-trations and any of the methylation biomarkers	Moderate
Hamad et al. 2018	Toxicant	MethylFlash (ELISA-based)	PureSperm gradient (45 and 90%), centri-fugation, somatic cell lysis	55	42.66 (± 8.38); Smokers (>20 cigarettes/day)	54	40.07 (± 6.49); Non-smokers	↑ global DNAm in smokers; smokers had ↓ sperm count	Low
Darbandi et al. 2019	Toxicant	Methylation specific PCR	ND	151	33.09 (± 8.74); divided by seminal ROS level	N/A	N/A	No assoc. between seminal ROS levels and DNAm of *KEAP1*	Low
Al Khaled et al. 2018	Toxicant	450K array; validation with bisulfite sequencing	PureSperm gradient (45 and 90%)	19	Age ND; Smokers	20	Age ND, non-smokers	No differences in DNAm between smokers and non-smokers	Low

Kim et al. 2015	Toxicant	5-mC immuno	Swim-up	18	32.0 (± 5.5); Smokers	32	Age ND; non-smokers	DNAm ↓ in swim-up fraction of spermatozoa; DNAm ↑ in non-smokers	Low
Miao et al. 2014	Toxicant	Methylation specific PCR	ND	77	22-50; exposed to BPA	72	22-50; not exposed to BPA	Sperm *LINE-1* methylation level ↓ in BPA exposed cases cf unexposed controls	Low
Chorbov et al. 2011	Toxicant	Targeted bisulfite pyro-sequencing	ND	13	42.5; males with opioid addiction	21	29.3; healthy volunteers	Significant correlation between *OPRM1* methylation and opioid dependence in blood but not in sperm	Very low
Denham et al. 2015	Metabolic	450K array and DNAm ELISA assay kit	PureSperm gradient (40 and 80%)	12	24.4 (± 5.19); randomly assigned to exercise intervention	12	22.45 (± 4.75); randomly assigned to not exercise during a 3 mo period	Global and genome-wide alterations in DNAm following 3 months of exercise	High
Potabattula et al. 2019	Metabolic	Bisulfite pyro-sequencing	Swim-up then PureSperm gradient (40 and 80%)	294	38.95 (± 5.9); males undergoing IVF	N/A	N/A	No association between DNAm of imprinted genes in sperm and BMI	Moderate
Salas-Huetos et al. 2018	Metabolic	MethylFlash (ELISA-based)	Somatic cell lysis	49	24 (± 4.6); healthy; consumed 60g nuts/day	49	25 (± 4.7); healthy; consumed no nuts	No assoc. between nut consumption and global DNAm	Moderate
Ingerslev et al. 2018	Metabolic	RRBS	Swim-up	12	18-28; endurance athletes providing 3 samples each	None	N/A	330 DMRs after training and 303 DMRs after the detraining period	Moderate
Soubry et al. 2016	Metabolic	Bisulfite pyro-sequencing	ISolate gradient (50 and 90%)	23	18-35; Males with an overweight/ obese BMI	44	18-35; lean males	Relative ↓ DNAm of *MEG3, NDN, SNRPN* and *SGCE/PEG10* DMRs in sperm from obese/ overweight males	Moderate
Consales et al. 2014	Metabolic	Bis-PCR and flow cytometric immuno-detection of DNAm	ND	316	29.3 (± 0.3); 116 from Greenland, 100 from Poland and 100 from Ukraine	N/A	N/A	Geographical location (Greenland versus European towns) identified as the main determinant of DNAm in repetitive sequences	Moderate
Tunc and Tremellen 2009	Metabolic	5-mC immuno	Gradient centrifugation	45	Age ND; subfertile (longitudinal)	12	Age ND; proven fertility	3 mo. antioxidant supplementation assoc. with ↓ seminal ROS levels and ↓ sperm DNA fragmentation, and ↑ sperm DNAm	Moderate

Chan et al. 2017	Metabolic	450K array; MassArray Epityper and other techniques	Gradient centri-fugation at 9400 xg for 20 mins	18	26-36; 10 samples from men taking 400 μg folate/day for 90 days. Additional cohort of 8 exposed to long-term folate fortification	9	33 (± 2); males taking placebo rather than folate	No significant assoc. found between DNAm and folate supplementation	Low
Donkin et al. 2016	Metabolic	RRBS	Swim-up	16	24-40; 10 obese and 6 obese males undergoing bariatric surgery	13	30-39; lean males	No DNAm difference met genome-wide significance	Low
Aarabi et al. 2015	Metabolic	RRBS and targeted bisulfite pyrosequencing	ND	30	37.9 (± 1.3); subfertile NZS	None	N/A	No significant difference in DNAm of imprinted loci after folate supplementation	Low
Stöger et al. 2006	Metabolic	Hairpin-bisulfite PCR	ND	1	ND	N/A	N/A	LEP CpG island ↓ meth in human and mouse sperm cf somatic tissues	Very low
Jenkins et al. 2018	Ageing	450K array data from previous studies.	ND	329	23-56; pooled data from previous studies. Mix of subfertile patients, sperm donors, and healthy volunteers	10	ND; 60 samples from 10 donors (validation cohort)	Model capable of predicting age with an R_2 of 0.89, a mean absolute error of 2.04 years, and a mean absolute percent error of 6.28%	High
	Ageing	450K array and bisulfite pyro-sequencing of LINE (surrogate for global levels of DNAm)	No sperm selection method employed	17	23-56; proven fertility. Two samples taken 9-19 years apart	N/A	N/A	Identified 147 regions where DNAm was significantly assoc. with age	Moderate
Rakyan et al. 2008	Tissue specificity	MeDIP-Seq	ND	4	Age ND; NZS	N/A	N/A	Generated a reference DNA methylome for human spermatozoa	Moderate
Bruno et al. 2018	Cancer	Bisulfite pyro-sequencing	PureSperm gradient (45 and 90%)	31	32.2 (± 6.5); seminoma patients	61	37.1 (± 5.7); healthy	No difference in DNAm of imprinted genes between seminoma cases and healthy controls	Low

Shnorhavorian et al. 2017	Cancer	MeDIP-Seq. Validation using minimal read depth bisulfite sequencing	ND	9	19.12-29.86; males with previous cisplatin-based treatment for osteosarcoma	9	27.5-44.4; healthy volunteers	A signature of significant DMRs identified in chemotherapy-exposed sperm	Low
Fukuda et al. 2017	Tissue specificity	Whole-genome bisulfite sequencing (WGBS)	ND	4	25-30; publicly available methylomes	N/A	N/A	Sperm methylomes contained more ↓ DNAm domains than the somatic methylomes. Hypometh domains human-specific cf. chimpanzees	Low
Zeng et al. 2014	Tissue specificity	Whole-genome bisulfite sequencing (WGBS)	Silica-based gradient centri-fugation	2	Age ND; from previously published study; anonymous donors	N/A	N/A	CpG islands relatively ↓ DNAm in sperm compared to somatic tissues	Low
Molaro et al. 2011	Tissue specificity	Whole-genome bisulfite sequencing	ND	2	Age ND; healthy males	N/A	N/A	Features that determine DNAm patterns differ between male germ cells and somatic cells; diverged between humans and chimpanzees	Low
Buckley et al. 2016	Tissue specificity	RRBS and DNase I hypersensitive site profiles	ND	ND	Age ND; publicly available methylomes	N/A	N/A	Testis and sperm ↓ DNAm of *BHMG1* and *RSPH6A* assoc. with testis-specific expression	Very low
Zhang et al. 2016	Tissue specificity	Methylation specific PCR	Percoll gradient (40 and 80%)	15	23-34; OAT	10	23-39; NZS	Promoter DNAm correlates with tissue-specific expression of *BOULE* and *DAZL*	Very low
Zhang et al. 2015	Cancer	Bisulfite pyro-sequencing	ND	43	Age ND; adenocarcinoma of prostate	40	Age ND; benign prostatic hypertrophy	No significant difference in the detection of *RARb2* promoter DNAm when comparing bis-seq in ejaculates to bis-seq of prostatic tissue	Very low
Guardiola et al. 2014	Tissue specificity	450K array	ND	1	ND	N/A	N/A	*APOA1/C3/A4/A5* cluster ↓ DNAm in liver, ↑ DNAm in sperm and other tissues	Very low
Kim et al. 2013	Tissue specificity	27K array	ND	21	Age ND; publicly available methylomes	N/A	N/A	↓ DNAm of *CTA* regulatory regions in cancer tissue and sperm compared to healthy somatic tissue	Very low

Berthaut et al. 2013	Cancer	Bisulfite pyro-sequencing	Centrifugation at 700 xg for 10 minutes	1	27; male undergoing temozolomide treatment. Longitudinal	ND	Age ND; proven fertility	Temozolomide treatment assoc. with lower sperm count (although remained normal) and *H19* ↓ DNAm	Very low
Jenkins et al. 2013	Ageing	5-mC immuno; 450K array of paired n=2	Somatic cell lysis	67	15 for DNAm and ageing study (2-3 longitudinal samples); 52 for blood-sperm comparison	N/A	N/A	↑ DNAm levels with age	Very low
Nettersheim et al. 2011	Cancer	Bisulfite Sanger sequencing	PureSperm gradient (40 and 80%)	ND	ND	N/A	N/A	*NANOG* promoter ↓ DNAm in spermatogonia and in several germ cell tumours, but ↑ DNAm in adult sperm	Very low
Fan and Zhang, 2009	Tissue specificity	Bisulfite sequencing of PCR amplicons (the Human Epigenome Project)	ND	1	24; from the Human Epigenome Project	N/A	N/A	CpG island methylation patterns more similar between somatic tissues than between somatic tissues and sperm	Very low
Grunau et al. 2005	Tissue specificity	Methylation specific PCR	ND	4	ND	N/A	N/A	↓ DNAm of *BAGE* in spermatozoa and malignant tissues cf normal, somatic tissues	Very low
Roberts et al. 2018	Psychiatry	450K array; pyrosequencing	Somatic cell lysis. Confirmed purity of sperm samples	22	23-38; Exposed to childhood abuse (5 moderate & 17 high)	12	23-29; Not exposed to childhood abuse	12 DNA regions (64 probes) differentially methylated by childhood abuse identified. Also identified probes predictive of childhood abuse	High
Klaver et al. 2012	Methodology	Bisulfite pyro-sequencing	Swim-up	10	Age ND; NZS	N/A	N/A	No significant differences in DNAm in selected genes between cryopreserved and non-cryopreserved samples	High
Kaminsky et al. 2012	Neurological disease	Bisulfite pyro-sequencing	ND	43	40.2 (± 11.6); 29 males with bipolar disorder, 14 males with schizophrenia	30	37.7 (± 10.3); healthy volunteers	↓ DNAm of one *HCG9* CpG in sperm from individuals with bipolar disorder/schizophrenia	High

Flanagan et al. 2006	Methodology	Methylation-specific PCR; microarray-based genome-wide CpG profiling	ND (from cryo-storage)	46	Cohort #1 22-35; cohort #2 24-56; healthy volunteers	N/A	N/A	Significant variation between DNAm levels of selected genes both within and between samples	High
Ni et al. 2019	Genetics	MSRE-qPCR	Somatic cell lysis	31	36.77 (± 1.15); subfertile; DFI > 25%	35	33.65 (± 1.44); subfertile; DFI < 25%	Negative assoc. between *IGF2* DNAm and DFI	Low
Dere et al. 2016	Methodology	450K array	Somatic cell lysis	12	34 (± 7); longitudinal	N/A	N/A	Intra-individual DNAm between successive samples correlated with one another more strongly than inter-individual DNAm	Low
Yu et al. 2015	Methodology	MeDIP-chip assay and immunostaining for DNAm	v/v medium gradient (40 and 80%)	54	32.4 (± 5.2); AsZS or OZS	39	33.6 (± 4.8); NZS	DNAm ↓ in motile sperm selected by density centrifugation compared to the less motile sperm	Low
Li et al. 2012	Genetics	Whole-genome bisulfite sequencing (WGBS)	ND	4	Age ND; healthy volunteers (2 from previously published research)	N/A	N/A	Assoc. between regions of ↓ DNAm and genomic instability	Low
Zeschnigk et al. 2009	Methodology	Restriction digestion of DNA followed by massive parallel bisulfite sequencing of CG-rich DNA fragments	ND	4	Age ND; pooled sperm samples	N/A	N/A	Identified 824 and 482 fully methylated autosomal CGIs in blood and sperm DNA respectively	Low
Wu et al. 2016	Methodology	MethyLight	ND	68	22-47; AZS	24	22-47; NZS	DNAm of the five selected testis-specific promoters was correlated between testicular DNA and paired cell-free seminal DNA	Very low

Hammoud et al. 2009	Genetics	MeDIP followed by promoter arrays. Validation by bisulfite-sequencing of identified sites	Gradient centri-fugation followed by somatic cell lysis	4	ND	N/A	N/A		Overall ↓ DNAm of developmental promoters compared to fibroblasts	Very low

CGIs = CpG islands; DFI = DNA fragmentation index.

TKR = tyrosine kinase receptor (gene family); PGAM5 = PGAM family member 5, mitochondrial serine/threonine protein phosphatase; PTPRN2 = protein tyrosine phosphatase receptor type N2; TYRO3 = TYRO3 protein tyrosine kinase (member of the TKR gene family); MEG3 = maternally expressed 3; NDN = necdin, MAGE family member; KEAP1 = Kelch like ECH associated protein 1; OPRM1 = opioid receptor mu 1; SGCE = sarcoglycan epsilon; PEG10 = paternally expressed gene 10; LEP = leptin; BHMG1 = basic helix-loop-helix and HMG-box containing 1; radial spoke head 6 homolog A; BOULE (more commonly known as BOLL) = boule homolog, RNA binding protein (member of the DAZ gene family); APOA1 = apolipoprotein A1 (C3, A4 and A5 are other members of the apolipoprotein gene family); CTA (more commonly known as CERNA3) = competing endogenous lncRNA 3 for miR-645; BAGE = B melanoma antigen; HCG9 = HLA complex group 9