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Mini-Review

Developments in reproductive biology and medicine

Could the sperm epigenome become a diagnostic tool for evaluation of the infertile man?

Ettore Caroppo [1,*] and Michael K. Skinner [2]

[1]Asl Bari, Andrology Outpatients Clinic, Bari, Italy
[2]Center for Reproductive Biology, School of Biological Sciences, Washington State University, Pullman, WA, USA

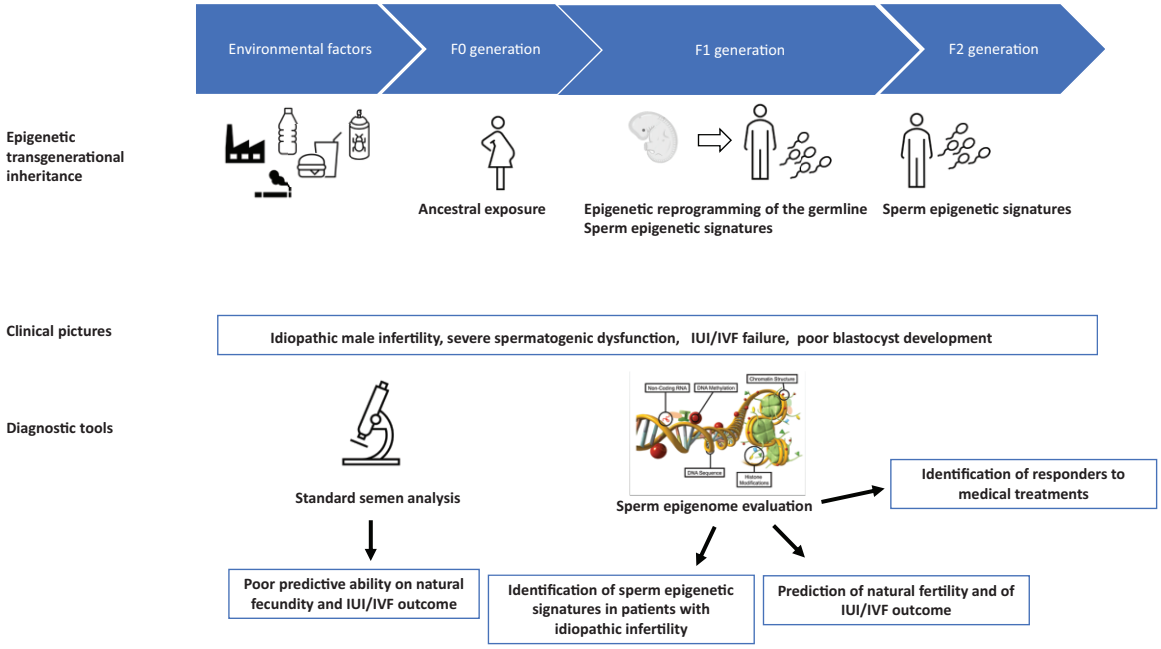
*Correspondence address. Asl Bari, Andrology Outpatients Clinic, Via De Amicis 15, 70014 Conversano (Ba), Bari, Italy. E-mail: ecaroppo@teseo.it https://orcid.org/0000-0001-8411-2736

ABSTRACT

Although male infertility is currently diagnosed when abnormal sperm parameters are found, the poor predictive ability of sperm parameters on natural fecundity and medically assisted reproduction outcome poses the need for improved diagnostic techniques for male infertility. The accumulating evidence about the role played by the sperm epigenome in modulation of the early phases of embryonic development has led researchers to focus on the epigenetic mechanisms within the sperm epigenome to find new molecular markers of male infertility. Indeed, sperm epigenome abnormalities could explain some cases of unexplained male infertility in men showing normal sperm parameters and were found to be associated with poor embryo development in IVF cycles. The present mini-review summarizes the current knowledge about this interesting topic, starting from a description of the epigenetic mechanisms of gene expression regulation (i.e. DNA methylation, histone modifications, and non-coding RNAs' activity). We also discuss possible mechanisms by which environmental factors might cause epigenetic changes in the human germline and affect embryonic development, as well as subsequent generations' phenotypes. Studies demonstrating sperm epigenome abnormalities in men with male infertility are reviewed, with particular emphasis on those with the more severe form of spermatogenic dysfunction. Observations demonstrate that the diagnostic and prognostic efficacy of sperm epigenome evaluation will help facilitate the management of men with male factor infertility.

Keywords: male infertility / sperm / DNA methylation / diagnostic / epigenetic / review

GRAPHICAL ABSTRACT



Environmentally responsive sperm epigenome signatures as a diagnostic tool for male factor infertility.

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Introduction

Male infertility accounts for 30–50% of infertility cases, and its prevalence in the general population ranges between ~9 and 15%, according to the available surveys (Barratt *et al.*, 2017). The growing demand for infertility treatments due to male factor infertility is demonstrated by data of the national registries, reporting that about 30–33% of all IVF cycles included a diagnosis of male infertility (HFEA, 2014–2016; Newman *et al.*, 2022). Male infertility is largely diagnosed when semen parameters fall below the World Health Organization (WHO) reference values; however, semen parameters are not predictive of natural fecundity unless they reach values well beyond the WHO references values (Buck Louis *et al.*, 2014; Keihani *et al.*, 2021; DeVilbiss *et al.*, 2022). Semen parameters are also not predictive of IVF outcome, either in terms of the number of top-quality embryos obtained (Bartolacci *et al.*, 2018) or in terms of pregnancy and live birth rates (Bartolacci *et al.*, 2018; Mariappen *et al.*, 2018). Even the assessment of the sperm DNA quality seems not to be helpful in predicting the IVF outcome (Buck Louis *et al.*, 2014; Cissen *et al.*, 2016), unless oocytes from older women are used (West *et al.*, 2022).

The poor predictive ability of sperm parameters on male fecundity is particularly relevant in the case of men with idiopathic or unexplained infertility, who experience reproductive difficulties despite showing normal sperm parameters. The paternal contribution to embryo development is not only restricted to the delivery of the sperm genome but also has a pivotal role in modulating the gene expression regulation after embryo genome activation. A number of studies have focused on the epigenetic mechanisms within the sperm epigenome to find new molecular markers of male infertility and improve the traditional diagnostic tools in the hands of the andrologists.

The present mini-review is intended to summarize the current evidence about the potential diagnostic potentialities of the sperm epigenome evaluation in the setting of male infertility.

The sperm epigenome

Classically, the human phenotype has been considered the result of a developmental programme coded in its genotype. More recently, however, studies have demonstrated that environmental influences can drive major phenotypical changes by promoting genome modifications that do not affect the underlying genetic sequence. This ability to respond to the surrounding environment during cell differentiation by changes in gene expression and in the phenotype is called 'epigenetic developmental plasticity' (King and Skinner, 2020). Such plasticity may be advantageous for survival, however changes in the epigenetic patterns in the germline due to environmental insults may promote the epigenetic transgenerational inheritance of diseases, defined as germline-mediated inheritance of epigenetic information between generations in the absence of continued direct environmental influences that leads to phenotypic variation (Skinner, 2011). When the germline is exposed during fetal gonadal sex determination, the epigenome in the germline can be modified: these epimutations potentially escape post-fertilization methylation erasure, to allow transmission to the subsequent generations (Sadler-Riggleman *et al.*, 2019). Regulation of genomic activity independently of the DNA sequence is made possible by means of epigenetic modifications such as DNA methylation, sperm chromatin modifications and non-coding RNA (ncRNA) activity.

DNA methylation is catalyzed by a family of DNA methyltransferases that transfers a methyl group from S-adenyl methionine to the fifth carbon of a cytosine residue to form 5 m: the protrusion of the methyl group from the DNA interferes with the binding of transcription factors, with resulting inhibition of transcriptional activity and gene silencing (Xavier *et al.*, 2019). DNA methylation is implicated in regulation of promoters, about 50% of which overlap with clusters of CpG dinucleotides (CpG islands), and repetitive DNA sequences (Xavier *et al.*, 2019). DNA methylation is generally stable, however during critical windows of development this process is more dynamic. This occurs during the early stages of embryogenesis, immediately after fertilization, and during gametogenesis. Such epigenetic reprogramming is intended to erase most epigenetic alterations accumulated during the parents' adult life, to minimize their potential harmful effects in the offspring (Xavier *et al.*, 2019), and is critical to obtain a pluripotent stem cell state during development (King and Skinner, 2020). However, although the global demethylation observed in primordial germ cells (PGCs) results in a dramatic loss of almost all DNA methylation at CpG islands, the loss of CpG methylation does not correlate with changes in gene expression in the human germline (von Meyenn and Reik, 2015). In addition, specific genomic areas (sub-telomeric and retrotransposon regions in PGCs, imprinted loci in embryonic cells, and lower density CpG regions) resist demethylation erasure (Ben Maamar *et al.*, 2023). About 70% of DNA in human sperm is in a methylated state, and this widespread DNA methylation is critical for progression of spermatogenesis (Greenberg and Bourc'his, 2019) and for offspring viability (Lismer and Kimmins, 2023).

During spermiogenesis, the sperm chromatin undergoes extensive remodeling, with somatic histones being replaced by testis-specific histone variants, which changes the stability of the nucleosomes and increases the accessibility of DNA for binding factors, facilitates the transition of nucleosomes to transition proteins and then to protamines (Meyer *et al.*, 2017), and allows the packaging of the paternal genome into a highly condensed sperm nucleus, rendering the paternal genome transcriptionally inactive. Some histone variants are present from early meiotic spermatocytes through the late elongated spermatids, while some are exclusively present in the haploid spermatids (Bao and Bedford, 2016). Histones are subject to dynamic post translational modifications, that are crucial for the control of gene expression during spermatogenesis and may serve also as epigenetic marks that may be transmitted to the offspring (Guerrero-Bosagna and Skinner, 2014). Approximately 10–15% of the genome remains wrapped around histones when the histone-to-protamine transitions are completed in human sperm (Meyer *et al.*, 2017; Ben Maamar *et al.*, 2020). Such retained histones are not randomly located but retained at important regulatory genes involved in spermatogenesis and embryo development (Hammoud *et al.*, 2009; Brykczynska *et al.*, 2010; Lambrot *et al.*, 2021). Disruption of the sperm retained histones landscape is associated with severe developmental defects in the offspring (Siklenka *et al.*, 2015).

Sperm contain ncRNA molecules to act as epigenetic factors. Indeed, injection of sperm ncRNAs into fertilized mouse eggs mediates epigenetic phenotypic variation in the offspring (Rassoulzadegan *et al.*, 2006; Wagner *et al.*, 2008). The ncRNAs appear to be required for normal embryo development (Liu *et al.*, 2012; Sendler *et al.*, 2013; Conine *et al.*, 2018). The sperm RNA profile is highly complex, comprising long ncRNAs (lncRNAs) and small non-coding RNAs (sncRNAs), the latter including microRNAs (miRNAs), piwi-interacting RNAs (piRNAs), tRNA-

derived small RNAs (tsRNAs), and rRNA-derived small RNAs (rsRNAs). Their function is not to code for a protein, but to regulate gene expression epigenetically (Nilsson *et al.*, 2018). The function of sperm lncRNAs is poorly understood, but they have been proposed to have a role in the regulation of gene expression by causing epigenetic modification in early embryo development (Santiago *et al.*, 2021). The quantity and composition of sncRNAs cargo vary during spermatogenesis. Different miRNA clusters have been found to be expressed by PGCs, spermatogonia, spermatocytes, spermatids and mature sperm following transit through the epididymis (Yang *et al.*, 2023). The miRNAs regulate gene expression by inhibiting or activating translation or by targeting mRNA for degradation (Santiago *et al.*, 2021). They are implicated in the regulation of spermatogenesis, and altered miRNA expression has been found to lead to sperm abnormalities, spermatogenic arrest and male infertility (Santiago *et al.*, 2021). In addition, miRNAs may play a role in the regulation of early histone replacement, gene expression and epigenetic modification in the early phases of embryo development (Santiago *et al.*, 2021). The piRNAs are highly expressed in germ cells but are lost before the maturation into mature sperm. There is a wide consensus about their role in transposon silencing and genome stability during spermatogenesis (Santiago *et al.*, 2021; Yang *et al.*, 2023). The tsRNA mechanism of action is poorly understood but has been implicated in the intergenerational inheritance of an acquired metabolic disorder (Chen *et al.*, 2016; Schuster *et al.*, 2016) and in the control of early embryo cleavage (Chen *et al.*, 2020) by regulating embryonic genome activation (Chen *et al.*, 2021). The tsRNAs have been shown to be involved in epigenetic transgenerational inheritance (Skinner *et al.*, 2018; Guerrero-Bosagna *et al.*, 2018).

RNA modifications have also been demonstrated to play a role in the epigenetic programming of spermatogenesis. The most common RNA modification is N6-methyladenosine (m6A), which is a reversible modification catalyzed by the RNA methyltransferase complex Mettl3, Mettl14 and Wilms' tumor 1-associating protein. This can be erased by demethylases, fat-mass and obesity-associated protein, and is involved in mRNA splicing, translation efficiency and microRNA processing. During testicular development in adulthood, RNA m6A regulators are expressed in almost all types of testicular cells. Experiments in Mettl3 knockout mice demonstrated that Mettl3 regulates spermatogonial differentiation and meiosis and is essential for male fertility and spermatogenesis (Xu *et al.*, 2017). Dysregulated RNA m6A regulators and the resultant imbalanced RNA m6A have also been found in infertile men: however, it is currently unknown how RNA m6A dysregulation results in spermatogenic dysfunction (Cai *et al.*, 2021).

Idiopathic male infertility: role of epigenetic transgenerational inheritance

The dramatic decline in human total sperm count (62.3% overall decline among unselected men) over the past 50 years corresponds to an increase in male infertility (Levine *et al.*, 2023) and trends in a range of male reproductive problems, including testicular cancer, disorders of sex development, cryptorchidism, hypospadias, low testosterone levels, and poor semen quality (Skakkebaek *et al.*, 2016), have suggested the possible role of environmental exposure in the pathogenesis of spermatogenic dysfunction and male infertility. Indeed, the link between environmental exposure and male infertility has been experimentally proven in studies in rats, a useful animal model because they reach sexual maturity early and demonstrate an abbreviated gestational period, such that several generations can be analyzed within a relatively short timeframe. The experimental exposure of F0 generation gestating female rats to the fungicide vinclozolin or the pesticide dichlorodiphenyltrichloroethane was shown to promote the epigenetic transgenerational inheritance of testis pathology (atrophy of seminiferous tubules, vacuoles in the seminiferous epithelium, and sloughed spermatogenic cells in the tubule lumens) in 45% of non-exposed F3 generation male population compared to 8% in the control population, similar to what was observed in other studies, as induced by other toxicants (Sadler-Riggleman *et al.*, 2019). Ancestral exposure promoted the epigenetic transgenerational inheritance of disease susceptibility in the testis through molecular alterations in the Sertoli cell epigenome and transcriptome (Sadler-Riggleman *et al.*, 2019).

Although the same study design cannot be replicated in human subjects, it has been demonstrated that environmental factors may induce abnormal epigenetic changes in the human germ cell epigenome and affect the phenotype of subsequent generations. Hamad and coworkers evaluated the effect of cigarette smoke on the sperm epigenome of a well-selected cohort of infertile men showing a normal sperm count (>20 million/ml) and without known confounding factors (history of testes surgery or pathology, acute or chronic urinary tract infection, and chronic illness). The two groups were divided according to their smoking habits (19 non-smokers and 35 heavy smokers) and found that heavy smokers had abnormalities in the histone-to-protamine transition (Hamad *et al.*, 2014). Wu *et al.* (2017) found that urinary phthalates were associated with 131 sperm differentially DNA-methylated regions (DMRs). Many DMRs were associated with metabolites whose parent compounds had known anti-androgenic effects and were enriched in genes related to growth and development. Greeson *et al.* (2020) demonstrated that exposure to a mixture of polybrominated biphenyl compounds led to sperm epigenome alterations. Shnorhavorian *et al.* (2017) found that 19- to 30-year-old male survivors of osteosarcoma, who were treated with cisplatin-based chemotherapy regimens when they were 14–20 years of age, had sperm epimutations present on all chromosomes, with a number of statistically over-represented clusters of DMRs (epimutation signature) in CpG deserts and none identified in CpG islands, and this genomic feature was similar to those previously identified in other species using a variety of different exposures.

Epigenetic abnormalities in infertile men

A recent systematic review demonstrated that studies evaluating the correlation between male infertility and sperm epimutations have provided conflicting results, mostly due to differences in the study design, different methods of analyzing DNA methylation, different populations studied, and wide variation in the statistical models used to analyze DNA methylation data (Asenius *et al.*, 2020). In addition, while early studies demonstrated aberrant methylation of imprinted genes in infertile men, such results were not replicated by further studies. The reason has been clarified by well-designed studies: imprinted genes are not regulated but stable, so they do not respond to environmental factors and are, therefore, not relevant to environmental induced epigenetic transgenerational inheritance. The imprinted sites are critical, epigenetic and inherited, but not in the same class of epigenetic sites that are environmentally responsive or altered during most cellular differentiation (Ben Maamar *et al.*, 2023). Although imprinted gene expression may be altered, the

differential methylation of the imprinted site has not been shown to be altered (Ma *et al.*, 2015; Costa-Júnior *et al.*, 2021).

Even if, as pointed out by the authors of the systematic review (Asenius *et al.*, 2020), no specific signature of male infertility has been convincingly replicated in genome-scale, unbiased analysis, this does not imply that the sperm epigenome analysis is worthless in infertile men.

There is quite a consensus about the finding that the increase in DNA methylation (hypermethylation) may be an aspect of the male infertility molecular disease etiology occurring during early gametogenesis and/or spermatogenesis (Camprubi *et al.*, 2016; Jenkins *et al.*, 2016; Sujit *et al.*, 2018, 2020; Luján *et al.*, 2019). Gene promoters in testicular germ cells are usually hypomethylated; therefore, hypermethylation of promoters may results in silencing of genes that play crucial roles in spermatogenesis, which may affect sperm production and patients' fertility. Interestingly, studies evaluating the most severe form of spermatogenic dysfunction (non-obstructive azoospermia: NOA) concur, to demonstrate that the testicular DNA methylation pattern differs significantly in these patients compared to men with obstructive azoospermia (OA), in whom spermatogenesis is not compromised. Ferfouri *et al.* (2013) evaluated the testis samples of 96 patients with azoospermia, 29 with OA and 67 with NOA, and found that the methylation profile of over 9000 of the 27 578 screened CpG sites differed significantly among the two groups. In addition, among patients with NOA, those with Sertoli-cell only syndrome (SCO: germ cell aplasia) differed significantly in terms of methylation profile compared to those with pachytene maturation arrest (spermatogonia and spermatocytes are present). Wu *et al.* (2020) found clear dissimilar epigenetic patterns between patients with NOA and OA, which involved gene ontology showing epigenetic modification related to metabolic pathways and hormone responses. Li *et al.* (2017), who evaluated the testicular tissue of men with severe oligozoospermia compared to men with OA, found 960 CpG sites related to 1440 different genes, which included several genes with known functions in male infertility, with significant differences of DNA methylation. Finally, Di Persio *et al.* (2021) investigated men with cryptozoospermia compared to patients with OA, applying a range filter for methylation values between samples of the same group to identify 271 DMRs associated with 132 genes, with 61 of them regulated during spermatogenesis and relevant at several stages. Notably, it has been demonstrated that the epigenetic signatures change significantly only during the early stages of stem cell differentiation (from PGCs to spermatogonia), while later stages of germline development show less variability between developmental stages in terms of epigenetic traits (Galan *et al.*, 2021; Ben Maamar *et al.*, 2022). This means that the different testicular cell composition in patients with distinct histopathological patterns (i.e. those with maturation arrest or mixed atrophy compared to patients with hypospermatogenesis) does not affect the epigenetic analysis.

In addition to sperm methylation pattern, patients with NOA seem also to have alterations in the expression of ncRNAs compared to men with intact spermatogenesis. A study evaluating 20 patients with OA and 60 patients with NOA, both undergoing microdissection testicular sperm extraction, demonstrated by means of RNA deep sequencing that the miRNA profiles differed significantly among the two groups. Some differentially expressed miRNAs targeted genes involved in the division and differentiation of spermatogonia (fibroblast growth factor receptor 3, SMAD family member 3, Sp1 transcription factor, V-Akt murine thymoma viral oncogene homolog 3 (AKT3)) or in the regulation of meiosis (transforming growth factor beta receptor 1) (Yao *et al.*, 2017).

The sperm epigenome as a diagnostic tool for male infertility

Since bulk sperm parameters are not predictive of natural conception or of the outcome of medically assisted reproduction, and given the key role played by the sperm epigenome in regulation of early embryonic developments sperm epigenetic assessment has been proposed as an alternative strategy to improve clinical diagnostics for male infertility. The studies designed to date to address this question have provided encouraging results.

Men with unexplained infertility are unable to achieve pregnancy despite displaying normal sperm parameters. Urdinguio *et al.* (2015) evaluated 17 fertile men and 29 men with idiopathic infertility, both showing normal sperm parameters, and found almost 3000 CpGs that displayed aberrant methylation in the infertile compared to fertile subjects. These alterations were associated with regions of sperm-specific methylation and specific histone marks such as H3K4me3. Salas-Huetos *et al.* (2016) found that infertile men with normal sperm parameters, and with no female contribution to infertility, had a distinct miRNA cargo compared to men with proven fertility. A total of 57 miRNAs were found to be differentially expressed in the infertile men compared to the fertile. Consequent dysregulation of the expression of genes involved in essential biological processes, including embryogenesis (namely embryonic morphogenesis and chromatin modifications) was observed (Salas-Huetos *et al.*, 2016). These results suggest that the sperm epigenome alterations may explain some cases of unexplained male infertility.

Studies have shown that the sperm epigenome may also be predictive of natural conception. A study evaluating 96 men with normal sperm parameters and idiopathic infertility identified sperm RNA elements (SREs) reflective of fecundity status. The absence of those SREs dramatically reduced the probability to achieve live birth by timed intercourse or IUI. A total of 30% of men with idiopathic infertility displayed an incomplete set of SREs, while when all the required SREs were present a female factor was identified (Jodar *et al.*, 2015). The evaluation of the sperm epigenetic clock, a metric previously used in other clinical fields, is considered as a biomarker of aging, and displays good predictive ability for cancer, cardiovascular and all-cause mortality (Perna *et al.*, 2016). A prospective pregnancy cohort (LIFE study) comprising 379 couples demonstrated an inverse relation between sperm epigenetic aging (SEA) and time to pregnancy (TTP), with 17% longer TTP for every 1-year increase in SEA (Pilsner *et al.*, 2022).

A recent study performed in a large sample of infertile men (1344 compared to 43 fertile sperm donors) demonstrated that altered methylation of promoters involved in the regulation of peptidases resulted in lower pregnancy rates in IUI cycles; since 77.8% of these men showed normal sperm parameters, such epigenetic signatures identified men with low fertility potential that would have missed by semen analysis alone (Miller *et al.*, 2023).

The evaluation of the sperm epigenome could also be predictive of IVF outcome. Denomme and coworkers evaluated 40 normozoospermic infertile men undergoing donor oocyte IVF cycles divided into two equally sized groups according to the blastocyst quality. Men in the good quality embryo group had at least 20% embryos with D5 grade 'AA' blastocysts and 60% embryos of transferable quality (grade ≥'3BB'), while the poor-quality embryo group had <10% embryos with D5 grade 'AA' blastocysts

and 40% of embryos of transferable quality were available. Sperm methylation array data showed that 1634 CpG probe sites were statistically different between the two groups at retained histones, with patients of the 'good group' showing a predominantly hypermethylated profile, while men of the 'poor group' displayed a statistically significant shift away from expected methylation levels. Gene ontology analysis revealed an enrichment of genes involved in the regulation of sperm-oocyte fusion, embryonic genome activation, implantation potential and embryonic differentiation. In addition, altered sperm methylation in the poor group correlated with altered paternal miRNA profiles with corresponding alterations to target genes involved in embryonic genome activation, blastocyst implantation and DNA methylation (Denomme *et al.*, 2017). Hua *et al.* evaluated 87 normozoospermic patients undergoing IVF divided into two groups according to the rate of good quality embryos (high rate ≥75%, N = 23 and low rate ≤25%, N = 64). The two groups were homogeneous for maternal age and for the number of mature oocytes retrieved. Ten differentially expressed tsRNAs, seven rsRNAs and five miRNAs were found between the two groups and a principal component analysis based on tsRNAs, rsRNAs and miRNAs could classify the semen samples in the two groups (high versus low quality group) with a good prognostic ability (AUC = 0.8716, 0.85, and 0.7 for tsRNAs, rsRNAS, and miRNAs, respectively) (Hua *et al.*, 2019). Aston and coworkers evaluated 127 men undergoing IVF, divided into two groups according to good embryogenesis and positive pregnancy (N = 55) or poor embryogenesis (N = 72) who achieved pregnancy (N = 42) or not (N = 30), and 54 normozoospermic men of proven fertility served as control group. Sperm DNA methylation patterns were very stable across samples from different individuals: when comparing fertile patients with patients undergoing IVF, >8500 CpGs were found to have significantly different methylation. Predictive models built using the differentially methylated CpGs were able to identify IVF patients with a sensitivity of 82%, AUC 0.93 and models built to identify patients with good versus poor embryo quality were able to classify 46% of patients in the poor embryo quality group with high specificity and positive predictive value, AUC 0.73. On the other hand, neither semen parameters nor male or female age were predictive of embryo quality and IVF outcome (Aston *et al.*, 2015). Finally, a study evaluating 47 male partners of couples with recurrent pregnancy loss and 39 men of proven fertility found 9497 differentially methylated CpGs among groups and functional annotation revealed 117 differentially methylated genes involved in embryo development and 13 involved in placenta development (Irani *et al.*, 2023).

Expression of ncRNAs in testicular tissue and in seminal plasma has been evaluated as a candidate biomarker of residual spermatogenesis in men with NOA, to predict the chance of successful sperm retrieval in these patients before surgery. Indeed, alteration in the expression of specific miRNA clusters has been found to correlate with severe spermatogenic dysfunction. Simultaneous inactivation of two functionally related miRNA clusters (miR-34b/c and miR-449) encoding five miRNAs (miR-34b, miR-34c, miR-449a, miR-449b, and miR-449c) was found to lead to defective meiotic progression and severe germ cell depletion (Wu *et al.*, 2014). Such results were confirmed by a study evaluating the expression of testicular miRNA in men with normal spermatogenesis (NS) and in men with NOA due to maturation arrest (MA) or SCO. Different miRNA expression profiles were found in the three groups, with miR-34b/c and miR-449 expression showing the greatest fold-change reduction in the SCO group, while miR-34b and miR-449a expression was also significantly reduced in the MA group compared to the NS group (Munoz *et al.*, 2015). Interestingly, miR-34c-5p expression in seminal plasma has been found to be significantly reduced in men with SCO (Zhang *et al.*, 2021) and in patients with NOA with unsuccessful sperm retrieval compared to those with successful sperm retrieval (Fang *et al.*, 2019), which lays the foundations for its use in the clinic for diagnostic and prognostic purposes.

Finally, evaluating the sperm epigenome may be of help in identifying the potential responders to treatment, similarly to what has been attempted in other fields of medicine (Duruisseaux *et al.*, 2018). A promising medical strategy to address male factor infertility involves the use of FSH treatment to improve seminal parameters and reproductive capacity of infertile men; however, not all patients respond to treatment (Caroppo and Niederberger, 2023). To identify epigenetic signatures that could identify possible responders to FSH treatment among patients with idiopathic male infertility, we developed a molecular analysis using genome-wide alterations in sperm DNA methylation in semen samples of a homogeneous cohort of infertile men with oligozoospermia and of a control group of fertile donors (Luján *et al.*, 2019). The infertile group received FSH treatment while the control group did not receive any treatment. Responders to treatment were identified after 3 months of treatment if they showed a two-to threefold increase in sperm parameters. Two hundred and seventeen DMRs were identified at a $P < 1e{-}05$ from the comparison of infertile and fertile subjects, with an efficient separation between the patient population with versus without infertility, with minimal overlap. Confirmation was achieved with a validation test performed in some fertile and infertile patients not used in the initial test owing to exclusions (mostly because of smoking and drinking habits). The infertility signature of DMRs was found in all the infertile patients' sperm samples, showing the efficiency of the molecular biomarkers. Responders to FSH treatment showed distinct epigenetic biomarkers (56 DMRs at $P < 1e{-}05$) compared to non-responders with an equal distribution of hypermethylation and hypomethylation changes. In contrast to the infertility diagnostic, where hypermethylation was prevalent, no overlap was observed between the infertility DMRs and FSH responder DMRs, suggesting a distinct set of epigenetic alterations (Luján *et al.*, 2019). These results, if confirmed by further studies, would be helpful in terms of developing therapeutic epigenetic biomarkers.

Conclusion

A growing body of evidence suggests an association between sperm epigenome abnormalities and male infertility. As discussed in the present review, epigenetic signatures could potentially be used to enhance the diagnosis and management of infertile men and to build models to predict the individual probability of natural conception or IVF outcome. At present, however, there still remains insufficient evidence to reach final conclusions on the diagnostic properties of sperm epigenome evaluation in infertile men. Further research should be performed to clarify the precise causative relation between sperm epigenome abnormalities and male infertility, and to identify specific epigenetic signatures to improve the current therapeutic options. There are several key aspects that need to be taken into consideration when designing further research: the studied cohort should be homogeneous for age, ethnicity and exposure to known environmental pollutants; sperm samples need to undergo sonication to destroy contaminating somatic cells; the genome-wide

approach should be considered instead of looking at specific genetic traits, since epigenetic regulation is not only a site-specific event but also spans long stretches of chromosome regions consisting of clusters of contiguous CpG islands; and the methods and bioinformatics used should allow the investigation of low density CpG regions of the epigenome, which constitute >90% of the genome. It is essential to take such an approach to resolve the conflicting results that have been reported to date.

Data availability

No new data were generated or analyzed in support of this research.

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Authors' roles

Ettore Caroppo: study conception, manuscript draft. Michael K Skinner: manuscript draft.

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Conflict of interest

None to declare.

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