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REVIEW
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Epigenetics of Male Infertility: The Role of DNA Methylation

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In recent years, a number of studies focused on the role of epigenetics, including DNA methylation, in spermatogenesis and male infertility. We aimed to provide an overview of the knowledge concerning the gene and genome methylation and its regulation during spermatogenesis, specifically in the context of male infertility etiopathogenesis. Overall, the findings support the hypothesis that sperm DNA methylation is associated with sperm alterations and infertility. Several genes have been found to be differentially methylated in relation to impaired spermatogenesis and/or reproductive dysfunction. Particularly, DNA methylation defects of MEST and H19 within imprinted genes and MTHFR within non-imprinted genes have been repeatedly linked with male infertility. A deep knowledge of sperm DNA methylation status in association with reduced reproductive potential could improve the development of novel diagnostic tools for this disease. Further studies are needed to better elucidate the mechanisms affecting methylation in sperm and their impact on male infertility.

Keywords: DNA methylation, sperm DNA, male infertility, imprinting, infertility, epigenomics, sperm, imprinted genes

INTRODUCTION

Male infertility affects about 15% of couples worldwide (Agarwal et al., 2015). In reproductive age, approximately 7% of males suffer from infertility (Cooper et al., 2009; Krausz, 2011; Rotondo et al., 2013). Male infertility is a multifactorial disease comprising a wide variety of disorders (Abrao et al., 2013; Stouffs et al., 2014; Contini et al., 2018; Tognon et al., 2020). Endocrine and immunological disorders, anatomical and genetic abnormalities as well as infections of the genital tract can affect the male reproductive potential (Abrao et al., 2013; Stouffs et al., 2014). Several other factors including age, stress, and lifestyle, such as obesity, smoking, and alcohol, have been associated with male infertility (Kovac et al., 2015; Craig et al., 2017; Ilacqua et al., 2018).

In recent years, a number of studies have focused on the role of epigenetics in spermatogenesis and male infertility (Seisenberger et al., 2012; Laurentino et al., 2015; Stuppia et al., 2015; Urdinguio et al., 2015; Laqqan et al., 2017b; Nasri et al., 2017; McSwiggin and O'Doherty, 2018). Epigenetics refers to the gene regulation process without changes in DNA sequence and includes DNA methylation, posttranslational histone modifications and microRNA (miRNA) regulation

Abbreviations: ARTs, assisted reproductive technologies; AT, asthenoteratozoospermia; CH, congenital hypopituitarism; CGIs, CpG islands; CTCF6, CTCF-binding site 6; DMRs, differentially methylated regions; DNMTs, DNA methyltransferases; ICEs, imprint control elements; ICRs, imprinting control regions; LINEs, long interspersed nuclear elements; OA, oligoasthenozoospermia; OT, oligoteratozoospermia; OAT, oligoasthenoteratozoospermia; PGCs, primordial germ cells; TET, ten-eleven translocation.

(Giacone et al., 2019; Stomper et al., 2021). Several specific epigenomic/epigenetic modifications are established during spermatogenesis to form highly specialized mature sperm cells, allowing significant reorganizations of sperm chromatin structure (Jenkins and Carrell, 2011). Therefore, spermatogenesis is particularly vulnerable to epigenetic alterations. Dysregulations in the DNA methylation process during spermatogenesis can result in the abnormal expression of target genes, which may lead to infertility (Cho et al., 2003; Aston et al., 2012). While many epigenetic abnormalities causing male reproductive dysfunction are still unknown, it is likely that most cases of idiopathic infertility could be accounted for underlying DNA methylation mechanisms (Rose and Klose, 2014).

This review provides an overview on gene and genome methylation and its regulation during spermatogenesis and the current knowledge of those DNA methylation defects potentially involved in the etiopathogenesis of male infertility.

METHODS

We performed an investigation of the scientific literature by searching PubMed (Medline) database until March 2021. All studies investigating the relationship between DNA methylation and male infertility published from 1987 up to March 2021 were reviewed for specific topic areas, and the most relevant reports were included. The literature search was performed using the following keywords (alone and/or in combination): epigenomics, epigenetics, gene, methylation, DNA methylation, genome, methylome, hypermethylation, hypomethylation, regulation, genomic imprinting, imprinted genes, sperm, sperm cells, spermatozoa, semen parameters, spermatogenesis, infertility, subfertility, sterility, and male infertility. The reference lists of all publications included in this review have also been considered for additional relevant works.

DNA METHYLATION IN GERM CELL DEVELOPMENT

DNA methylation is a biochemical process where a nucleotide is enzymatically methylated with a methyl group ($-CH_3$) at the five-carbon position, usually cytosine (Moore et al., 2013; Rotondo et al., 2021). Cytosine methylation predominantly occurs at the CpG dinucleotide (**Figure 1**). Regions rich in CpGs are called CpG islands (CGI) and are usually found in gene promoters, where gene expression is regulated through methylation (Greenberg and Bourc'his, 2019; Rotondo et al., 2020b). CpG methylation in the promoters of genes typically leads to gene silencing (Rotondo et al., 2016, 2018a). The suppression of gene expression can occur by DNA methylation itself that, in some cases, can prevent the binding of transcriptional factors (Moore et al., 2013; Yin et al., 2017). Methylated DNA could also be recognized and bound by the methyl-binding proteins methyl CpG binding protein 2 and methyl-CpG-binding domain protein-1, -2, and -4, which recruit the enzymes histone methyltransferases and histone deacetylases to trigger histone modifications and chromatin packing, leading to the repression of gene expression (Parry and Clarke, 2011).

DNA methylation plays a pivotal role in regulating germ cell development (Reik and Walter, 2001; Castillo et al., 2018; Hanna et al., 2018; Lobo et al., 2019; Sharma et al., 2019; Wasserzug-Pash and Klutstein, 2019). During germ cell development, the genome is widely remodeled by waves of DNA demethylation and methylation (**Figure 2**; Reik and Walter, 2001; Li, 2002; Santos and Dean, 2004; Rousseaux et al., 2005; Biermann and Steger, 2007; Marcho et al., 2019). The wave of DNA demethylation occurs in the primordial germ cells (PGCs), which are precursors of male and female germ cells. Initially, the content of DNA methylation is equally distributed between PGCs and embryo somatic cells. Upon migration of PGCs from the epiblast to the gonadal ridge, the DNA methylation marks are extensively erased (Monk et al., 1987; Santos and Dean, 2004), resulting in lower methylation levels in PGCs compared to those in embryo somatic cells (Gkountela et al., 2015; Guo et al., 2015; Tang et al., 2015; Von Meyenn and Reik, 2015). These epigenetic modifications are essential for the genome reprogramming of PGCs, allowing sex-specific germ cell development during embryo development (Reik and Walter, 2001; Yao et al., 2015). *De novo* methylation proceeds in prospermatogonia or gonocytes arrested in mitosis, and it is firstly established at the repeated DNA sequences, such as retrotransposons, and then at the imprinted genes, leading to appropriate sex-specific methylation patterns in the germ cells.

DNA methylation establishment in the germline is of particular importance, as failure to establish correct methylation in retrotransposons and imprinted genes has serious consequences for embryo development. Indeed, retrotransposons, such as *long interspersed nuclear elements 1* (*LINE1*), are interspersed repeated DNA sequences which are able to propagate throughout the genome. Lack of methylation in retrotransposons could allow their propagation throughout the genome, causing insertional mutagenesis and, in turn, several diseases, including male infertility (Solyom and Kazazian, 2012). Regarding the imprinted genes, methylation regulates the expression through an important process termed "genomic imprinting," which leads to the expression of either the maternal or paternal allele. When maternal/paternal alleles undergo global demethylation upon fertilization, the imprinted genes preserve the methyl marks of the parental genome, leading to parental-origin patterns of mono-allelic gene expression. This process occurs in specific sequences called differentially methylated regions (DMRs), also known as imprinting control regions (ICRs) (Habib et al., 2019). For instance, the imprinted genes *IGF2-H19* share common enhancers and ICR located downstream and upstream of the *H19* gene, respectively. Under normal circumstances, ICR and *H19* are methylated in sperm cells and unmethylated in oocytes, leading to the reciprocal expression of the maternal *H19* allele and paternal *IGF2* allele in somatic cells (**Figure 3**; Peters, 2000; Lanzillotti et al., 2021). Other paternally imprinted genes have been found to be similarly regulated in sperm cells, such as *Ras Protein Specific Guanine Nucleotide Releasing Factor 1*, *Delta Like Non-Canonical Notch Ligand 1*, and *Zinc Finger DBF-Type Containing 2* (Arnaud, 2010; Gunes et al., 2016). Contrariwise, maternally imprinted genes are

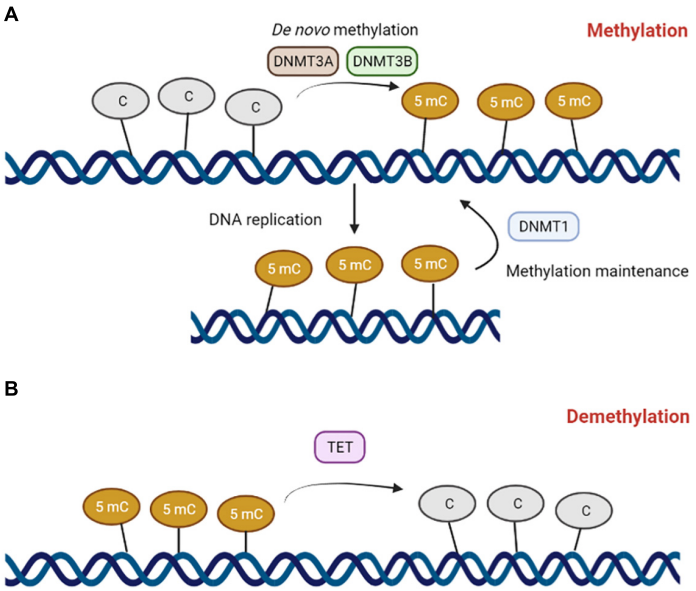

FIGURE 1 | The DNA methylation machinery. **(A)** DNA methyltranferase 3A (DNMT3A) and 3B (DNMT3B) catalyze *de novo* methylation reactions on cytosines belonging to CpG dinucleotides to form 5-methylcytosine. DNA methyltransferase 1 (DNMT1) mediates DNA methylation maintenance. **(B)** Ten-eleven translocation (TET) enzymes mediates DNA demethylation reactions.

methylated in female germ cells, while they are expressed in male germ cells, including *mesoderm-specific transcript* (*MEST*), also known as *paternally expressed gene 1* (*PEG1*), *paternally expressed 3* (*PEG3*), and *small nuclear ribonucleoprotein polypeptide N* (*SNRPN*) (Mayer et al., 2000; Higashimoto et al., 2003; Broad et al., 2009; Hammoud et al., 2010; Zheng et al., 2011; Zhao et al., 2015; Bruno et al., 2018). However, failure in the maintenance of imprinted gene methylation patterns in the germline might occur, and this has been associated with low sperm quality and pregnancy rate and impaired post-fertilization development (Rotondo et al., 2013; Kitamura et al., 2015; McSwiggin and O'Doherty, 2018).

DNA methylation is carefully coordinated by a family of enzymes named DNA methyltransferases (DNMTs). Four members of this family, DNMT1, DNMT3A, DNMT3B, and DNMT3C, are endowed with catalytic activity

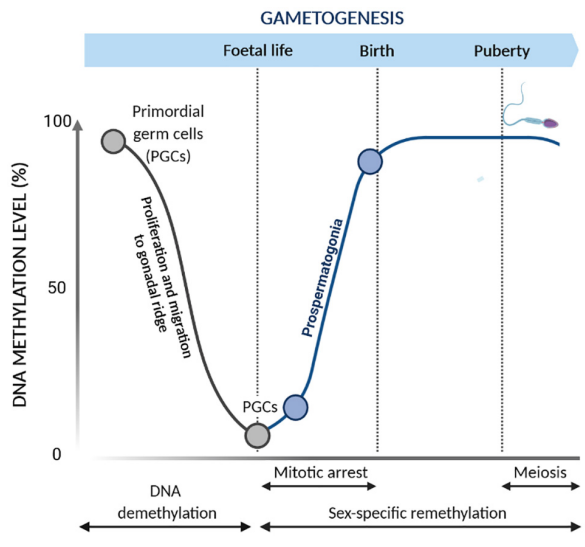

FIGURE 2 | Epigenetic reprogramming during gametogenesis. Changes of DNA methylation occur during primordial germ cell (PGC) development. During the proliferation and migration into the gonadal ridge, PGCs undergo global demethylation to remove parental imprints. Subsequently, reestablishment of the male germ cell DNA methylation patterns occurs during gametogenesis. *De novo* methylation occurs prior to meiosis in mitotically arrested cells while being completed before birth.

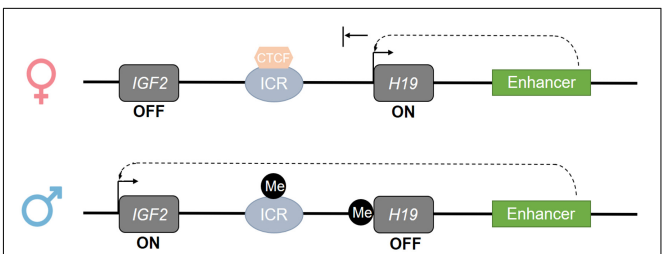

FIGURE 3 | Epigenetic regulation of the imprinted *IGF2-H19* gene cluster. The regulation of the imprinted genes is mediated by imprinting control regions (ICR) whose methylation regulates the imprinted genes located at the same gene cluster. On the maternal allele, at the *IGF2-H19* locus, the ICR is de-methylated; this allows CTCF binding and promotes the expression of *H19* and silencing of *IGF2* through down-stream enhancer activity. On the paternal allele, at the *IGF2-H19* locus, the ICR and *H19* are methylated; this prevents CTCF binding and induces the inactivation of *H19* and the expression of *IGF2*. Consistently, *IGF2* is expressed in the sperm cell (Cannarella et al., 2019).

(Lopomo et al., 2016). DNMT1, also known as the maintenance DNMT, copies pre-existing methylation marks onto the new strands following DNA replication (Baylin and Jones, 2011). DNMT3A and DNMT3B are *de novo* methyltransferases, as they are able to methylate previously unmethylated DNA sequences (Baylin and Jones, 2011). DNMT3C, along with *P-element induced wimpy testis/interacting RNA* (*PIWI/piRNA*) (Yang and Wang, 2016; Nagamori et al., 2018), is responsible for the promoter methylation of retrotransposons in the male germline (Barau et al., 2016). piRNAs are small non-coding RNAs that form RNA–protein complexes through interaction with PIWI proteins, a gonad-specific class of Argonaute proteins (Yang and Wang, 2016). These RNA–protein complexes are essential for the *LINE1* methylation processes in male germ cells (Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008). DNA methylation is a reversable chemical modification, and active demethylation processes are mediated by erasing DNA methylation mechanisms, mainly controlled by Ten-Eleven Translocation (TET) enzymes, such as TET1, -2, and -3, (Melamed et al., 2018). TET enzymes specifically convert 5mC to 5-hydroxymethylcytosine (5hmC). The TET-mediated oxidation of 5mC induces DNA demethylation by passive or active processing. As 5hmC is not recognized by DNMT1 during DNA replication, passive DNA demethylation can occur in proliferating cells (Valinluck and Sowers, 2007). In the active process, TETs convert 5hmC to 5-formylcytosine (5fC) and then 5fC into 5-carboxylcytosine (5cC). 5fC and 5cC are both recognized and excised by the base-excision DNA repair machinery and subsequently replaced with cytosine (Williams et al., 2012; Jin et al., 2015).

DNA METHYLATION AND MALE INFERTILITY

Overview

DNA methylation plays a critical role during spermatogenesis (Gunes et al., 2018b; Fend-Guella et al., 2019; Franzago et al., 2019; Huang et al., 2019; Pisarska et al., 2019). The correct methylation of DNA ensures proper chromatin condensation in the sperm head, enabling sperm maturation and its ability in fertilization and post-fertilization events (Carrell, 2019). Contrariwise, incomplete or abnormal condensation of the sperm chromatin results in damaged DNA, leading to the impairment of egg cell fertilization and/or reduction in pregnancy rates (Benchaib et al., 2005; Miller et al., 2009; McSwiggin and O'Doherty, 2018). Considering these aspects, several studies have analyzed the gene and genome methylation levels of sperm DNA in association with male reproductive dysfunctions (Houshdaran et al., 2007; Rotondo et al., 2012, 2013; Das et al., 2017; Alkhaled et al., 2018). These studies have mainly, but not only, investigated associations between improper DNA methylation in spermatozoa genome and negative variations in semen parameters, such as concentration (oligozoospermia), morphology (teratozoospermia), and progressive motility (asthenozoospermia), alone or in combinations, i.e., oligoasthenozoospermia (OA), oligoteratozoospermia, asthenoteratozoospermia (AT), and oligoasthenoteratozoospermia (OAT). Semen samples are considered normal when the concentration, motility, and morphology are $\geq 15 \times 10^6$ sperm/ml, $\geq$32% (sperm progressive motility), and $\geq$4% normal, respectively (Cooper et al., 2009).

Gene Methylation Changes

Several genes carrying defective methylation have been associated with abnormalities in semen parameters (**Table 1**). One of the most highly studied genes is *methylenetetrahydrofolate reductase* (*MTHFR*) (Haggarty, 2015; Saraswathy et al., 2018; Coppedè et al., 2019; Mishra et al., 2019; Kulac et al., 2021), which is a key regulatory enzyme involved in folate metabolism and in DNA synthesis and methylation (Födinger et al., 2000). In male mice testes, the activity of MTHFR is five times higher than in other organs (Chen, 2001), whereas its inactivation results in hyperhomocysteinemia, decreased methylation capacity, and the arrest of spermatogenesis due to sperm DNA hypomethylation (Chen, 2001). In humans, a number of studies have pointed out that impairment of the *MTHFR* gene can contribute to disease, including male infertility (Stangler Herodež et al., 2013; Tara et al., 2015; Coppedè et al., 2016, 2019; Lévesque et al., 2016; Asim et al., 2017). Mutations/polymorphisms in *MTHFR* gene are broadly known causes of reduced MTHFR enzyme activity in sperm cells, resulting in reduced methionine availability and decreased DNA methylation (Gupta et al., 2011; Montjean et al., 2011; Murphy et al., 2011; Safarinejad et al., 2011; Choi et al., 2016; Poorang et al., 2018; Ullah et al., 2019). The improper methylation of *MTHFR* gene in sperm germ cells may likewise result in decreased MTHFR enzyme activity, leading to aberrant methylation of sperm DNA. Hypermethylation of *MTHFR* has been found in testes from males affected by non-obstructive azoospermia and in sperm cells from males affected by oligozoospermia and teratozoospermia as well as in idiopathic infertile males (Khazamipour et al., 2009; Wu et al., 2010; Botezatu et al., 2014; Karaca et al., 2017). In our previous studies, hypermethylation at the *MTHFR* promoter has been found to be associated with aberrant concentration, motility, and morphology in males from infertile couples affected by recurrent spontaneous abortion (Rotondo et al., 2012). In addition, we have found a correlation between *MTHFR* gene promoter hypermethylation and extensive methylation defects at the paternal imprinted gene *H19* in sperm DNAs from infertile males with both normal and abnormal semen parameters. These results have suggested that *MTHFR* hypermethylation-induced inactivation may affect methylation at the imprinted loci of sperm cells, leading to impaired fertilization (Rotondo et al., 2013). Contrariwise, no correlation was found between methylation errors at *H19* and *MEST* genes and inactivating *MTHFR* C677T single-nucleotide polymorphism (SNP) [a cytosine (C) to a thymine (T) substitution at position 677] in sperm DNA from infertile patients with defective semen parameters. However, a higher incidence trend of aberrant DNA methylation among severe oligozoospermic infertile males carrying C677T genotype was observed (Louie et al., 2016).

Germline mutations arise from replication errors, potentially resulting in reproductive health impairment (Gao et al., 2019;

TABLE 1 | Aberrant methylation of non-imprinted genes associated with abnormalities of semen parameters.

Gene	Function	Associated sperm parameters	References
ALU YB8	Repetitive sequences	Normozoospermia	Urdinguio et al. (2015)
BCAN	Extracellular matrix formation	Oligo-/oligoasthenozoospermia	Sujit et al. (2018)
CPEB2	Tumor suppressor	Oligo-/oligoasthenozoospermia	Sujit et al. (2018)
CREM	Germline regulator	Not specified	Nanassy and Carrell (2011)
CRISPLD1	Cysteine-rich secretory proteins	Oligo-/oligoasthenozoospermia	Sujit et al. (2018)
D4Z4	Repetitive sequences	Normozoospermia	Urdinguio et al. (2015)
DAZL	Germline regulator and gametogenesis	Oligoasthenoteratozoospermia	Navarro-Costa et al. (2010)
DNMT1	DNA-methyltransferase	Non-obstructive azoospermia	Uysal et al. (2019)
DNMT3A	DNA-methyltransferase	Non-obstructive azoospermia	
DNMT3B	DNA-methyltransferase	Non-obstructive azoospermia	
EZH2	Histone methyltranferases	Oligo-/oligoasthenozoospermia	Sujit et al. (2018)
HDAC4	Histone deacetylases	Oligo-/oligoasthenozoospermia	
HLA-C	Antigen-presenting MHC I	Oligo-/oligoasthenozoospermia	
HLA-DQA1	Antigen-presenting MHC II	Oligo-/oligoasthenozoospermia	
HLA-DRB6	Antigen-presenting MHC II	Oligo-/oligoasthenozoospermia	
HRAS	GTPase	Oligoasthenoteratozoospermia	Houshdaran et al. (2007)
HSPA1L	Molecular chaperone	Normozoospermia	Jenkins et al. (2016b)
HSPA1B	Molecular chaperone	Normozoospermia	
JMJD1C	Histone demethylases	Oligo-/oligoasthenozoospermia	Sujit et al. (2018)
KDM4C	Histone demethylases	Oligo-/oligoasthenozoospermia	
LINE1	Repetitive sequences	Normozoospermia	Urdinguio et al. (2015)
		Oligoasthenoteratozoospermia	Boissonnas et al. (2010)
		Not specified	Khambata et al. (2021)
LPHN3	Member of GPCR	Oligo-/oligoasthenozoospermia	Sujit et al. (2018)
MLH1	DNA mismatch repair	Oligozoospermia	Gunes et al. (2018a)
		Oligoasthenoteratozoospermia	Hekim et al. (2021)
MT1A	Metallothionein	Oligoasthenoteratozoospermia	Houshdaran et al. (2007)
MTHFR	Methylation regulator	Non-obstructive azoospermia	Khazamipour et al. (2009)
		Normo-/oligozoospermia	Wu et al. (2010)
		Oligoasthenozoospermia	Botezatu et al. (2014)
		Oligozoospermia	Rotondo et al. (2012)
		Normozoospermia	Karaca et al. (2017)
		Non-obstructive azoospermia	Kulac et al. (2021)
		Oligozoospermia	
NBL2	Repetitive sequences	Normozoospermia	Urdinguio et al. (2015)
NTF3	Neurotrophic factor	Oligoasthenoteratozoospermia	Houshdaran et al. (2007)
P16	Tumor suppressor	Asthenozoospermia	Zhang et al. (2019)
PAX8	Transcription factor	Oligoasthenoteratozoospermia	Houshdaran et al. (2007)
PIWIL2	Endoribonuclease	Non-obstructive azoospermia/oligozoospermia	Heyn et al. (2012)
RHOXF1	Transcription factor	Oligo-/astheno/-teratozoospermia	Richardson et al. (2014)
RHOXF2	Transcription factor	Oligo-/astheno/-teratozoospermia	
rs2656927	SNP variants of *UHRF1*	Oligozoospermia	Zhu et al. (2019)
rs8103849	SNP variants of *UHRF1*	Oligozoospermia	
SAT2CHRM1	Spermine N1-acetyltransferase	Oligoasthenoteratozoospermia	Houshdaran et al. (2007)
SFN	Regulator of mitotic translation	Oligoasthenoteratozoospermia	
SOX30	Transcription factor	Non-obstructive azoospermia	Han et al. (2020)
SPATA4	Apoptosis regulator	Oligozoospermia	Sujit et al. (2020)
SPATA5	ATP-dependent chaperone	Oligozoospermia	
SPATA6	Myosin light chain binding	Oligozoospermia	
SPATA7	Microtubule cytoskeleton organization	Oligo-/oligoasthenozoospermia	Sujit et al. (2018)
SPATA16	Testis-specific protein	Oligo-/oligoasthenozoospermia	
SPATA22	Meiosis-specific protein	Oligo-/oligoasthenozoospermia	
TDRD1	Repressor of transposable elements	Non-obstructive azoospermia/oligozoospermia	Heyn et al. (2012)
TET1	Dioxygenase	Oligoasthenozoospermia	Ni et al. (2016)
TET2	Dioxygenase	Oligoasthenozoospermia	
TET3	Dioxygenase	Oligoasthenozoospermia	
VDR	Transcription factor	Oligo-/astheno/-teratozoospermia	Vladoiu et al. (2017)
XRCC1	Enzyme binding	Oligoasthenoteratozoospermia	Metin Mahmutoglu et al. (2021)

Altakroni et al., 2021). Accordingly, the genetic defects of DNA mismatch repair mechanisms have been related to impaired spermatogenesis and male infertility (Gunes et al., 2015; Hekim et al., 2021; Metin Mahmutoglu et al., 2021). A recent study has investigated the methylation profile of the DNA mismatch repair gene *MutL alpha* (*MLH1*) in sperm DNA from oligozoospermic patients and normozoospermic males enrolled as the control group (Gunes et al., 2018a). The results indicated an association between *MLH1* hypermethylation and male infertility, suggesting that this gene might be under epigenetic regulation during sperm cell development (Gunes et al., 2018a). High methylation levels of the DNA repair *X-ray repair cross-complementing protein 1* gene have been found in OAT males, thereby suggesting that the improper methylation of this gene may have a role in sperm chromatin condensation and OAT (Metin Mahmutoglu et al., 2021). Contrariwise, two additional well-known DNA repair genes, i.e., *breast cancer type 1 susceptibility protein* (*BRCA1*) and *BRCA2*, did not show defective methylation in infertile men with OAT (Kabartan et al., 2019).

Several other genes found aberrantly methylated in altered sperm samples from infertile males have been investigated at a single-study level (**Table 1**). Overall, several abnormally methylated genes have been found to be associated to altered sperm parameters, mainly oligozoospermia, indicating a potential involvement of DNA methylation in male infertility. All, but *MTHFR*, gene investigations have been carried out at a single-study level and need further studies to be confirmed. On the contrary, improper *MTHFR* promoter methylation, such as hypermethylation, has been repeatedly found in aberrant sperm samples, suggesting the methylation-induced *MTHFR* dysregulation as a potential causal factor of abnormal sperm changes. Interestingly, *MTHFR* hypermethylation has been found in normal sperm samples of males from idiopathic infertile couples. This may indicate that *MTHFR* dysfunction could play a role in changing sperm function and health, possibly by lowering CpG methylation content in imprinted genes, which, in turn, may affect embryo development and pregnancy outcome (Rotondo et al., 2013). In view of finding gene methylation hallmarks in male infertility, these data are interesting and will deserve further investigations. For instance, large studies of different infertile populations along with a functional analysis of hypermethylation are needed in order to elucidate the role and the mechanisms of the *MTHFR* gene in human spermatogenesis and the related pathologies.

Gene Methylation Changes in *LINE*s

The methylation status of *LINE1* in sperm DNA from infertile males was initially evaluated by Kobayashi et al. (2007) who reported no correlation between *LINE1* methylation and male reproductive function. No correlation has also been found in sperm DNA from idiopathic infertile males belonging to recurrent spontaneous miscarriage couples (Ankolkar et al., 2013). Low methylation levels at *LINE1* have been shown in asthenospermic and OAT patients in an additional study, although no statistical significance was found between cases and controls (Boissonnas et al., 2010; Tian et al., 2014). A meta-analysis in 291 infertile patients and 198 fertile individuals from seven published works found no significant correlation with *LINE1* methylation levels (Santi et al., 2017). Conversely, DNA methylation marks at *LINE1* and the repetitive sequences *ALU YB8*, *NBL2*, and *D4Z4* have been found to be significantly lower in spermatozoa from idiopathic infertile males than in somatic cells (Urdinguio et al., 2015). A significant decrease in global 5mC levels and *LINE1* promoter methylation in spermatozoa of males from couples experiencing recurrent pregnancy loss has also been described (Khambata et al., 2021). Similarly, *LINE1* hypomethylation along with promoter hypermethylation-associated silencing of *PIWIL2* and *Tudor Domain Containing 1* genes, two *PIWI/piRNA* pathway-related genes, has been found in sperm DNA from infertile males affected by spermatogenic disorders (Heyn et al., 2012). Animal model studies have reported similar results with other *PIWI/piRNA* pathway-related genes and satellite DNA regions (Capra et al., 2019; Zhang et al., 2020).

LINE1 methylation levels evaluated through sperm genome methylation analyses might be a good indicator of genome-wide alterations. Indeed, these repetitive sequences have frequently been considered as surrogate markers for global methylation changes (Pinto-Medel et al., 2017). However, the role of *LINE1* methylation in male infertility is far to be elucidated. Few studies are currently available and often reporting contradictory data, probably due to the lack of homogeneity in experimental design and data processing among studies.

High-Throughput Techniques for DNA Methylome Investigation

Over the years, with the development of molecular technology/methods (Park et al., 2012; Tognon et al., 2015; Rotondo et al., 2018b, 2020a; Mazzoni et al., 2020; Preti et al., 2020; Oton-Gonzalez et al., 2021), many studies have investigated the relationship between DNA methylation and male infertility on a genome-wide scale by employing high-throughput techniques (Houshdaran et al., 2007; Ferfouri et al., 2013; Schütte et al., 2013; Camprubí et al., 2016; Du et al., 2016; Jenkins et al., 2016a,b; Laqqan et al., 2017a; Lee et al., 2019). The first array-wide analysis was performed by Houshdaran et al. (2007) highlighting improper DNA methylation at *repetitive element satellite 2*, *H-RAS*, *neurotrophin* 3, *paired-box gene 8*, and *Stratifin*, *3′-nucleotidase*, and *metallothionein 1A* genes in semen samples with abnormal sperm concentration, motility, and morphology. DNA methylation variations at over 9K CpGs spread throughout the testicular genome have been detected between obstructive and non-obstructive azoospermic patients (Ferfouri et al., 2013). The human methylation 450k array analysis in semen samples from couples who conceived and couples who did not showed significantly decreased and increased methylation in two and three genomic regions, respectively, in the "failure-to-conceive" group. Interestingly, the two sites with low methylation content were at closely related genes known to be expressed in the sperm, i.e., *HSPA1L* and *HSPA1B* (Jenkins et al., 2016b). A recent methylome and transcriptome study in euploid blastocysts derived from couples with OAT males reported significant alterations in approximately 1.1K CpGs compared to the

cryopreserved euploid blastocysts used as controls (Denomme et al., 2018). A pathway analysis elucidated the genes involved in the regulation of cellular metabolic process as universally affected. This epigenetic dysregulation provided an explanation for the reduced reproductive potential in OAT patients despite euploid blastocyst transfers (Denomme et al., 2018).

Additional array-wide studies have been reported to show the following: (i) changes in the methylation of testis/epididymis-specific genes in the testis and epididymis of vasectomized males (Wu et al., 2013), (ii) loss of methylation in inflammation- and immune response-related genes in sperm samples from males belonging to infertile couples (Schütte et al., 2013), and (iii) gain of methylation in spermatogenesis-related genes in sperm samples derived from individuals undergoing assisted reproduction (Schütte et al., 2013).

In normospermic males, the DNA methylome of the sperm cell differs between infertile and fertile, and this difference may be predictive of embryo quality during *in vitro* fertilization (Aston et al., 2015). Nevertheless, differences in sperm epigenome within healthy subjects have been also detected, indicating intra-sample heterogeneity in DNA methylation patterns (Jenkins et al., 2014). Furthermore, additional studies have underlined potential epigenetic heterogeneity among, and within, sperm cells, spermatogonia, and gonocytes (Kuhtz et al., 2014; Laurentino et al., 2015, 2016).

The impairment of both sperm epigenome and gene expression in relation to male reproductive dysfunction was recently explored using high-throughput techniques. Hypermethylation-induced silencing at *SRY-Box Transcription Factor 30* gene has been detected in association with non-obstructive azoospermia (Han et al., 2020). Two array-wide studies have described inverse correlations between DNA methylation and the mRNA expression of genes involved in different pathways, such as the response to hormone stimulus, activation of protein kinase activity, apoptosis, and reproduction, in sperm from both severe oligozoospermic and azoospermic patients (Li et al., 2017; Wu et al., 2020).

As high-throughput approaches are able to detect extensive variations in epigenetic marks, DNA methylation changes could potentially be used as a diagnostic tool to predict the risk of both male infertility and male infertility-related diseases (Aston et al., 2015; Fang et al., 2019; Patel et al., 2020). For instance, an array-based DNA methylation profile conducted on peripheral blood has revealed variations in DNA methylation patterns in infertile males compared to normozoospermic fertile controls, suggesting that epigenome-based blood markers can be used for diagnostic purposes (Sarkar et al., 2019). This has also been suggested by the results of another high-throughput study focused on congenital hypopituitarism (CH), which is a pituitary gland hormone deficiency that leads to metabolic disorders and male reproductive dysfunction. This study identified methylation at CpG sites on two spermatogenesis/testicular development-related genes in whole blood DNA isolated from a cohort of CH patients (Fang et al., 2019). In this context, the clinical application of these highly sensitive techniques cannot be ruled out. As high-throughput approaches can potentially support work on defining the forms of male infertility, these methods will deserve attention in andrological clinical practice (Ferlin and Foresta, 2014).

DNA Methylating and Demethylating Enzymes

DNA methyltransferases and *TET* are two key regulative gene families involved in DNA methylation and demethylation pathways, respectively, during both embryogenesis and spermatogenesis (Nettersheim et al., 2013; Barišić et al., 2017; Li et al., 2019). Despite their critical role in sperm methylation, the *DNMT* and *TET* genes have been poorly investigated in the context of male infertility (Uysal et al., 2016, 2019; Tang et al., 2017, 2018). Decreased expression of DNMT1, -3A, and -3B enzymes in association with global DNA methylation changes has been detected in the testes of non-obstructive azoospermic patients, suggesting that the methylation-induced *DNMT* inactivation may be involved in male reproductive dysfunction (Uysal et al., 2019). The presence of *DNMT1* SNP variant rs4804490 was found to be associated with an increased risk of idiopathic infertility in males with abnormal semen parameters (Tang et al., 2017). In the same study, no associations were afterward determined between additional four DNMT variants, i.e., *DNMT1* (rs4804490), *DNMT3A* (rs1550117), *DNMT3B* (rs2424909), and *DNMT3L* (rs7354779), and aberrant DNA methylation at several imprinted genes, including *H19*, *GNAS complex locus* (*GNAS*), and *GTP-binding protein Di-Ras3* (*DIRAS3*) (Tang et al., 2018). Conversely, two SNP variants, rs2656927/rs8103849, of *ubiquitin-like, containing PHD and RING finger domains 1* (*UHRF1*) gene, which is tightly related to the *DNMT1* pathway, have been identified in the blood of oligozoospermic infertile males (Zhu et al., 2019). Other recent evidence indicated that *UHRF1* inactivation may have a negative impact on male fertility potential, as the conditional loss of this gene in germ cells leads to DNA hypomethylation, upregulation of retrotransposons, and DNA damage response activation (Dong et al., 2019). *UHRF1* also cooperates with the *PIWI/piRNA* pathway during spermatogenesis, suggesting a molecular link between DNA methylation and the *PIWI/piRNA* pathway in the germline (Dong et al., 2019).

Ten-eleven translocation *1*, *-2*, and *-3* genes have been found to be consecutively expressed at different stages of spermatogenesis, and their expression levels have been positively correlated to male reproductive potential and pregnancy (Ni et al., 2016). Indeed sperms from oligozoospermic and/or asthenozoospermia males have significantly reduced TET1, -2 and -3 mRNAs compared to fertile donors, but not to normozoospermic males (Ni et al., 2016). TET1 protein deficiency in spermatogonia decreases the 5hmC levels and downregulates genes that are involved in several pathways, such as cell cycle, germ cell differentiation, meiosis, and reproduction, resulting in the reduction of spermatogonia stem cells and germ cell differentiation (Huang et al., 2020).

When taken together, these studies suggest that *DNMT* and *TET* impairment in sperm cells may represent a potential risk factor for male infertility. Therefore, further investigations are

needed to assess the role of DNMT and TET enzymes in the male infertility phenotype.

Gene Methylation Changes in Imprinted Genes

Imbalances in the methylation patterns of imprinted genes could potentially impair spermatogenesis and/or favor the male infertility phenotype (Hartmann et al., 2006; Rotondo et al., 2012, 2013; Das et al., 2017; Laurentino et al., 2019; Åsenius et al., 2020a; Pohl et al., 2021). The methylation status of a number of imprinted genes has been investigated in relation to impaired spermatogenesis and/or reproductive dysfunction (**Table 2**). DNA methylation defects of three maternally imprinted genes, i.e., *PLAGL1*, *MEST*, and *DIRAS3*, have been identified in sperm DNAs from infertile males with a low semen concentration (Houshdaran et al., 2007). One of these genes, *MEST*, has been repeatedly found as hypermethylated in association with the male infertility phenotype (Marques et al., 2004; Hammoud et al., 2010; El Hajj et al., 2011; Montjean et al., 2013; Xu et al., 2016). Specifically, the improper DNA methylation at *MEST* has been (i) linked to low sperm concentration and motility as well as poor sperm morphology in idiopathic infertile males (Marques et al., 2008; Poplinski et al., 2010; Kläver et al., 2013), (ii) detected in primary spermatocytes from azoospermic patients presenting complete or incomplete maturation arrest (Marques et al., 2017), (iii) associated with decreased bi-testicular volume and increased follicle-stimulating hormone levels (Kläver et al., 2013), and (iv) associated with abnormal protamine ratio in oligozoospermic patients (Hammoud et al., 2010). These data have been further confirmed by a meta-analytic study (Santi et al., 2017). Recently, *MEST* hypermethylation has been found in the spermatozoa of male partners from couples experiencing recurrent pregnancy loss (Khambata et al., 2021).

Altered methylation in *H19* has been broadly documented to be associated with infertile males affected by oligozoospermia, asthenozoospermia, teratozoospermia, OA, AT, and OAT (Marques et al., 2004, 2008, 2010; Kobayashi et al., 2007; Boissonnas et al., 2010; Minor et al., 2011; Rotondo et al., 2013; Li et al., 2016; Dong et al., 2017; Bruno et al., 2018; Peng et al., 2018). Idiopathic infertile males with normal semen parameters have also been found to carry methylation defects at *H19* in their sperm (Poplinski et al., 2010; Ankolkar et al., 2013; Tang et al., 2018). Imprinting errors at *H19* gene were detected in primary spermatocytes and elongated spermatids/spermatozoa with incomplete maturation arrest from two azoospermic patients, although the differences in *H19* methylation levels between patients and controls were not statistically significant (Marques et al., 2017). In addition, abnormal methylation at *H19* has been shown at the regulatory region CTCF-binding site 6 (CTCF6), located within the DMR of *IGF2-H19*, in sperm DNA from infertile males (Rotondo et al., 2013). Therefore, sperm cells carrying methylation defects at the *IGF2-H19* CTCF6 region are at a high risk of causing biallelic inactivation of the *IGF2* gene, which, in turn, could negatively impact embryo development and/or pregnancy outcome (Boissonnas et al., 2010; Santi et al., 2017; Bruno et al., 2018). Similarly, methylation defects at *IGF2-H19* have been detected in placenta tissues from offspring conceived by assisted reproductive technologies (ART) (Pinborg et al., 2016; Hattori et al., 2019; Lou et al., 2019). Thus, these studies have pointed out that ART procedures may account for the epigenetic defects in the embryo.

Altered DNA methylation patterns at *overlapping transcript 1* (*LIT1*) and *SNRPN* have been found in infertile males tested with abnormal protamine ratio in sperm cells (Hammoud et al., 2010). Aberrant methylation at the *SNRPN* gene has also been associated with altered sperm motility and morphology as well as with OA and AT (Botezatu et al., 2014; Peng et al., 2018). Interestingly, imprinting errors at the *SNRPN* gene and *IGF2-H19* have also been found in spermatozoa from asthenozoospermia patients and in ART-conceived human fetuses (Lou et al., 2019). Hypermethylation at *SNRPN*, alongside other maternally imprinted genes involved in embryonic germ cell development, such as *MEG3*, *PEG1* (also known as *MEST*), *PEG3*, *LIT1*, and *PLAG1 like zinc finger 1* (*ZAC*), as well as loss of methylation at the paternal imprinted gene *GTL2* has been detected in sperm DNA derived from patients affected by moderate/severe oligozoospermia (Kobayashi et al., 2007). Hypermethylation of *PEG1*, *PEG3*, *PEG10*, and *ZAC* genes has been shown in spermatozoa from male partners from couples experiencing recurrent pregnancy loss (Khambata et al., 2021). Contrariwise, the lack of altered methylation has been reported for *PEG1*, *ZAC*, and *GTL2* in the sperm of male partners from recurrent spontaneous miscarriages (Ankolkar et al., 2013). Lastly, the maternally imprinted gene *SNRPN upstream reading frame protein*, which is related to the *SNRPN* pathway, has been detected as hypermethylated in association with oligozoospermia (Bruno et al., 2018).

An additional imprinted gene possibly involved in male reproductive dysfunction is *zinc-finger CCHC-type containing 13* (*ZCCHC13*). *ZCCHC13* is a novel imprinted gene involved in an epigenetic mechanism known as X-chromosome inactivation and has been detected both hypermethylated and down-regulated in testis biopsies isolated from non-obstructive azoospermia patients (Li et al., 2018). Low methylation at *GNAS* and *DIRAS3* imprinted genes has also been found to be more prevalent in idiopathic infertile males, especially in oligozoospermic males, than in fertile males (Bruno et al., 2018; Tang et al., 2018). The evidence that improper DNA methylation at the *GNAS* gene is linked to male infertility is also supported by animal models (Congras et al., 2014).

Imprinting errors could also be related to DNA fragmentation in sperm cells. Indeed, the improper methylation of the paternally imprinted gene *Potassium Voltage-Gated Channel Subfamily Q Member 1* (*KCNQ1*), along with that of *IGF2*, has been found to be associated with a high level of DNA fragmentation in semen samples (Ni et al., 2019). In addition, the centromeric (domain 2) differentially methylated region (KvDMR) located in exon 10 of *KCNQ1OT1* has been found to be hypermethylated in the spermatozoa of male partners from couples experiencing recurrent pregnancy loss (Khambata et al., 2021).

A recent array-wide methylation analysis conducted on sperm DNA from a cohort of males identified a number of genes

TABLE 2 | Aberrant methylation of imprinted genes associated with abnormalities of semen parameters.

Gene	Imprinted allele	Function	Associated sperm parameters	References
CTNNA3	Paternal	Alpha catenin	Oligo-/oligoasthenozoospermia	Sujit et al. (2018)
DIRAS3	Maternal	GTPase	Oligozoospermia	Houshdaran et al. (2007)
DLGAP2	Maternal	Membrane-associated protein	Not specified	Schrott et al. (2020)
			Oligo-/oligoasthenozoospermia	Sujit et al. (2018)
GATA3	Maternal	Transcriptional activator	Oligo-/oligoasthenozoospermia	Sujit et al. (2018)
GNAS	Maternal/Paternal	G-protein alpha subunit	Oligozoospermia	Tang et al. (2018)
			Oligoasthenozoospermia	Zhang et al. (2019)
GTL2	Paternal	Transcription regulator (lncRNA)	Oligozoospermia	Kobayashi et al. (2007)
H19	Paternal	Tumor suppressor (lncRNA)	Oligozoospermia	Tang et al. (2018)
			Oligozoospermia	Marques et al. (2008)
			Oligozoospermia	Li et al. (2016)
			Oligoasthenozoospermia	Dong et al. (2017)
			Oligoastheno-/astenoteratozoospermia	Peng et al. (2018)
			Oligoasthenoteratozoospermia	Boissonnas et al. (2010)
			Oligozoospermia	Marques et al. (2004)
IGF2	Maternal	Growth factor	Not specified	Poplinski et al. (2010)
			Not specified	Ni et al. (2019)
IGF2-H19	Maternal/paternal		Oligo-/asteno-/terato-/oligoteratozoospermia	Rotondo et al. (2013)
			Oligozoospermia	Bruno et al. (2018)
			Asthenozoospermia	Lou et al. (2019)
KCNQ1	Maternal	Potassium channel	Not specified	Ni et al. (2019)
LIT1	Maternal	Transcription regulator (lncRNA)	Oligozoospermia	Hammoud et al. (2010)
			Oligozoospermia	Kobayashi et al. (2007)
MAGI2	Paternal	Membrane-associated protein	Oligo-/oligoasthenozoospermia	Sujit et al. (2018)
MEG3	Paternal	Transcription regulator (lncRNA)	Oligozoospermia	Bruno et al. (2018)
MEST	Maternal	Hydrolase	Oligozoospermia	Houshdaran et al. (2007)
			Oligozoospermia	Kläver et al. (2013)
			Oligozoospermia	Marques et al. (2008)
			Oligozoospermia	Marques et al. (2004)
			Oligozoospermia	Kobayashi et al. (2007)
			Oligozoospermia	Hammoud et al. (2010)
			Oligoasthenozoospermia	Zhang et al. (2019)
			Not specified	Poplinski et al. (2010)
			Not specified	Khambata et al. (2021)
PEG3	Maternal	Zinc finger	Oligozoospermia	Kobayashi et al. (2007)
			Not specified	Khambata et al. (2021)
PEG10	Maternal	Zinc finger	Not specified	Khambata et al. (2021)
SNRPN	Maternal	Small nuclear ribonucleoprotein	Oligozoospermia	Hammoud et al. (2010)
			Oligoasthenozoospermia	Botezatu et al. (2014)
			Oligoastheno-/astenoteratozoospermia	Peng et al. (2018)
			Astheno-/teratozoospermia	Dong et al. (2017)
SNURF	Maternal	SNRPN upstream reading frame	Oligozoospermia	Bruno et al. (2018)
TP73	Paternal	Tumor protein	Oligo-/oligoasthenozoospermia	Sujit et al. (2018)
ZAC	Maternal	Zinc finger	Oligozoospermia	Kobayashi et al. (2007)
			Not specified	Khambata et al. (2021)
ZCCHC13	Maternal	Zinc finger	Non-obstructive azoospermia	Li et al. (2018)

associated with infertility, including the maternally imprinted genes *DLG associated protein 2* (*DLGAP2*) and *GATA binding protein 3* as well as the paternally imprinted genes *catenin alpha 3*, *membrane-associated guanylate kinase*, and *tumor protein 73* (Sujit et al., 2018). An additional gene, *brevican*, which is a non-imprinted gene involved in brain extracellular matrix formation, has been identified in this study. All these genes might be considered as novel potential candidates accounting for male infertility phenotype, and their roles in spermatogenesis need to be further investigated (Sujit et al., 2018).

In conclusion, many imprinted genes have been investigated for methylation in aberrant semen samples, such as oligozoospermia, asthenozoospermia, and teratozoospermia. H19 and MEST were the most investigated genes in aberrant sperm samples, showing repetitively aberrant loss or gain of methylation, respectively, which, in turn, may be causal factors in the development of male infertility. Of note is the fact that aberrant methylations have also been found in infertile males with normal sperm parameters. This aspect may have important implications in the diagnosis of male infertility, which, to date,

lacks explanation in 30% of infertile males. It is also hoped that further studies will shed light into the etiology of these aberrant imprinting patterns in paternally or maternally imprinted genes in idiopathic infertility cases. Indeed the loss of methylation of paternally imprinted genes may be due to a deficiency of DNMTs, whereas hypermethylation of maternally imprinted genes might result from erroneous *de novo* methylation or a failure to erase maternal imprint in the male germ cell genomes (Barlow and Bartolomei, 2014; Uysal et al., 2019).

Lifestyle Factors

Habits and lifestyle factors, such as smoking, alcohol consumption, and diet, have been investigated in an effort to gain insight into the causes of aberrant sperm DNA methylation in relation to male infertility (El Khoury et al., 2019). A recent pilot study conducted on sperm DNA from a cohort of infertile males who underwent a supervised yoga practice regimen reported DNA methylation changes at nearly 400 genes, suggesting a link between positive lifestyle practices and male reproductive health (Bisht et al., 2020).

Tobacco smoke has a strong negative impact on sperm DNA methylation in a number of genes, also demonstrated in animal models (Xu et al., 2013; Dai et al., 2016; Dong et al., 2017; Laqqan et al., 2017a; Hamad et al., 2018; Wyck et al., 2018; Fragou et al., 2019). The imprinted gene *SNRPN* has been found to be aberrantly methylated in asthenozoospermia and teratozoospermic smokers (Dong et al., 2017). Other studies on humans and/or animal models have reported modifications to sperm DNA methylation following cannabis use (Murphy et al., 2018; Reece and Hulse, 2019). Improper *DLGAP2* methylation has recently been reported in sperm from cannabis users, thereby suggesting the potentially negative effect of cannabis use prior to conception on imprinting marks (Schrott et al., 2020). In this context, the impact of parental drug addiction on sperm DNA methylation in drug-sired offspring needs to be more deeply explored (Jarred et al., 2018; Nieto and Kosten, 2019). Moreover, both alcohol and nicotine consumption seem to impair DNA methylation in several genes and genomic regions, leading to male infertility. Indeed data on DNA methylation changes at *cyclin-dependent kinase inhibitor 2A* and *LINE1* and an increased risk of male reproductive dysfunction have been reported (Zhang et al., 2019). More recently, evidence has also indicated that DNA methylation defects at *MEST* and *GNAS* genes, along with altered sperm motility, morphology, and concentration, could be linked to alcohol and nicotine exposure (Zhang et al., 2019).

Paternal diet is able to alter sperm epigenome and has been associated with negative pregnancy outcomes in mice (Lambrot et al., 2013). In addition, sperm from mice under a folate-deficient diet showed differential DNA methylation marks at genes implicated in development, diabetes, autism, and schizophrenia (Lambrot et al., 2013). In humans, several studies have reported on the relationship between paternal and/or maternal diet and sperm DNA methylation in male infertility (Shukla et al., 2014; Hoek et al., 2020; Toschi et al., 2020). A recent study conducted on infertile males who underwent oral supplementation with micronutrients in support of folate, B vitamins, zinc, and cysteines reported an increase in DNA methylation levels, resulting in improved sperm nuclear maturation and antioxidant defenses, with a possible positive effect on reproductive function (Bassiri et al., 2020). Furthermore, evidences on sperm DNA from infertile males indicate a high methylation of *vitamin D receptor* promoter in vitamin D-deficient conditions, thus suggesting the role of diet in epigenetic modifications and male reproductive dysfunction (Vladoiu et al., 2017). Similarly, one investigation indicated that parental diet can modulate the methylation levels of several genes, including liver-specific genes involved in cholesterol/lipid metabolism, whereas no effect of paternal diet on sperm DNA methylation was found in other studies (Carone et al., 2010; Rando and Simmons, 2015; Shea et al., 2015; Whitelaw, 2015). Similarly, parental pre-conceptional obesity could potentially impact on the establishment of imprinting marks during embryogenesis (Soubry et al., 2015; Ou et al., 2019; Åsenius et al., 2020b; Keyhan et al., 2021). Changes in methylation in two imprinted genes, *PLAGL1* and *MEG3*, have been detected in umbilical cord blood leukocytes in a group of newborns from obese mothers, making a link between maternal overnutrition and the impairment of imprinting marks in the offspring (Soubry et al., 2015). In males, imprinted genes involved in growth and development, including *PEG3* and *neuronatin* alongside *MEST*, have been found to be hypomethylated in DNA derived from the blood of children of obese fathers. The relationship between parental obesity and the methylation status of the offspring clearly indicates that spermatogenesis may be susceptible to environmental factors from an epigenetic point of view (Soubry et al., 2015). A recent animal model study supported and extended this conclusion by reporting data about environmental toxicant exposure and transgenerational inheritance of epigenetic marks (Sadler-Riggleman et al., 2019). Additional animal models have shown similar results, reporting that epigenetic alterations at *PEG3* and *H19* can occur in sperm cells in offspring from diabetic and/or obese mice (Ge et al., 2014). These studies, when taken together, highlight that both defective DNA methylation and imprinting marks may be transferred through generations, potentially affecting the health of adult offspring (Grossniklaus et al., 2013; Ge et al., 2014; Soubry et al., 2015; Blanco Rodríguez and Camprubí Sánchez, 2019). However, this assumption has been questioned in a recent meta-analysis of coordinated epigenome-wide association studies of paternal prenatal body mass index (BMI) in relation to DNA methylation (Sharp et al., 2021). The study conclusions do not support the hypothesis that paternal BMI around the time of pregnancy is linked with offspring-blood DNA methylation, even at the imprinted regions (Sharp et al., 2021). Considering the data available, more research is needed to address the role of paternal diet on sperm DNA methylation in infertile males.

FUTURE PERSPECTIVES

This review provides an overview of gene and genome methylation, its regulation during the spermatogenesis process, and the current knowledge on those DNA methylation defects which are known to be involved in male reproductive dysfunctions.

Most of the aforementioned studies have added sufficient strength to the hypothesis that sperm methylation is associated with sperm alterations and infertility. In spite of these reports, the causative role of improper DNA methylation marks in inducing male infertility remains poorly characterized, particularly due to the lack of studies on the mechanisms involving DNA methylation in sperm cells.

It is convincible that aberrations in methylation at specific target genes may reflect whole methylome defects due to altered DNMT and TET activities during sperm cell development and spermatogenesis. On the other hand, several risk factors, such as lifestyle, drugs, hormones, and diet, may account for the gain or loss of methylation in key genes in sperm cells. New functional studies on this topic are needed to better elucidate the mechanisms affecting methylation in sperm. Nevertheless, extensive knowledge of sperm DNA methylation status in association with reduced reproductive capability is useful in developing novel diagnostic tools for male infertility (Balasubramanian et al., 2019; Sarkar et al., 2019). The current primary diagnostic protocol for identifying infertile males relies on the assessment of sperm number, motility, and morphology. This diagnostic approach has several limitations in differentiating fertile and infertile males, as abnormalities causing male reproductive dysfunction are still unknown in a fraction of males. In addition, this approach also provides limited information on male reproductive potential and cannot be employed as a prognostic tool in predicting pregnancy success and possible outcome (Kläver and Gromoll, 2014). When taken together, these drawbacks in the current protocols make DNA methylation evaluation a putative useful tool in clinical practice (Sarkar et al., 2019). A large number of studies have already demonstrated that aberrant DNA methylation in spermatozoa is linked to defective sperm parameters and male infertility phenotype as well as a negative pregnancy outcome. In this context, sperm DNA methylation could become a novel diagnostic and prognostic parameter for assessing male infertility and pregnancy outcome, respectively (Kläver and Gromoll, 2014). This approach has also been reported in monitoring other diseases (Luján et al., 2019). Thanks to high-throughput technology, which is rapidly increasing in epigenetic studies, methylome analyses may soon allow the methylation differences between infertile and fertile males to be characterized, thereby greatly improving the current knowledge on the relationship between sperm DNA methylation and male infertility (Ferlin and Foresta, 2014). Hopefully, the translation of this technology into clinical andrological practice will improve the diagnostic and prognostic assessment of infertile males (Kläver and Gromoll, 2014).

CONCLUSION

The role of DNA methylation in spermatogenesis, sperm function, and male infertility is an important research area that deserves attention. Although recent evidence seems to indicate a possible overestimation of aberrant DNA methylation in male infertility (Camprubí et al., 2012; Leitão et al., 2020; Åsenius et al., 2020b), the aforementioned studies have provided significant associations between improper DNA methylation in sperm and infertile males.

A number of genes have been found to be differentially methylated in relation to impaired spermatogenesis and/or reproductive dysfunction. DNA methylation defects in genes, including *MEST* and *H19* within imprinted genes and *MTHFR* within non-imprinted genes, have been previously identified in a wide number of studies, and their defective marks could be potentially employed as useful tools in clinical practice for assessing male infertility (Marques et al., 2004, 2008, 2010; Kobayashi et al., 2007; Boissonnas et al., 2010; Hammoud et al., 2010; El Hajj et al., 2011; Minor et al., 2011; Rotondo et al., 2012, 2013; Montjean et al., 2013; Haggarty, 2015; Dai et al., 2016; Xu et al., 2016; Dong et al., 2017; Li et al., 2017; Bruno et al., 2018; Saraswathy et al., 2018; Coppedè et al., 2019; Mishra et al., 2019; Sarkar et al., 2019).

The lack of studies for several of the additional genes reported herein and/or the conflicting results for others make the use of defective DNA methylation marks as a diagnostic tool still unlikely. Further studies are necessary to identify novel genes that could potentially be employed as tools in clinical practice.

As suggested in several works, the dysregulation of epigenetic mechanisms, including the aberrant methylation of DNA, may play an important role in the development of infertility with unknown etiology in a high fraction of males. However, further research is needed to epigenetically explore the etiology of this disease. Indeed the use of DNA methylation as a marker or cause of sperm abnormalities in the field of fertility is only beginning to be explored thoroughly and is currently not completely clarified. Moreover, the use of methylation changes in DNA as a marker to identify the male infertility factor is difficult, as these changes may have little or even no significant biological impact or multiple different changes may be necessary to establish infertile phenotypes. In this effort, methylation signatures in normal sperm from fertile individuals should provide useful knowledge for elucidating these topics. Moreover, as aging is correlated with changes in DNA methylation, investigating sperm DNA methylation in age-stratified normal fertile individuals should also be taken into account as well as to improve our knowledge in this field (Johnson et al., 2012). Another point deserving attention in the context of methodological approaches is the potential contamination by somatic cells during epigenetic analyses, such as blood, white, and epithelial cells, as a result of incorrect sperm cell isolation/processing (Jenkins et al., 2017). Standardized and detailed protocols for processing sperm samples should be used in studying DNA methylation in male infertility, as even only a few contaminated somatic cells might alter the epigenetic signatures of sperm cells. Research into sperm DNA methylation in male infertility might be an important future area of study. We thus encourage further studies focused on the role of DNA methylation in spermatogenesis and male reproductive potential as well as embryo development and pregnancy outcome. Such newly acquired data could improve the diagnostic and prognostic setup of male infertility phenotypes and pregnancy outcomes, respectively, with high health benefits for humans.

AUTHOR CONTRIBUTIONS

JR, MT, and FM contributed to conceptualization and writing—review and editing. JR contributed to writing—original draft preparation. CM and CL contributed to visualization. MT and FM contributed to supervision, contributed to funding acquisition, and project administration. All the authors have read and agreed to the published version of the manuscript.

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