Clinical Epigenetics

REVIEW

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How do lifestyle and environmental factors influence the sperm epigenome? Effects on sperm fertilising ability, embryo development, and offspring health

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Abstract

Recent studies support the influence of paternal lifestyle and diet before conception on the health of the offspring via epigenetic inheritance through sperm DNA methylation, histone modification, and small non-coding RNA (sncRNA) expression and regulation. Smoking may induce DNA hypermethylation in genes related to anti-oxidation and insulin resistance. Paternal diet and obesity are associated with greater risks of metabolic dysfunction in offspring via epigenetic alterations in the sperm. Metabolic changes, such as high blood glucose levels and increased body weight, are commonly observed in the offspring of fathers subjected to chronic stress, in addition to an enhanced risk of depressive-like behaviour and increased sensitivity to stress in both the F0 and F1 generations. DNA methylation is correlated with alterations in sperm quality and the ability to fertilise oocytes, possibly via a differentially regulated *MAKP81IP3* signalling pathway. Paternal exposure to toxic endocrine-disrupting chemicals (EDCs) is also linked to the transgenerational transmission of increased predisposition to disease, infertility, testicular disorders, obesity, and polycystic ovarian syndrome (PCOS) in females through epigenetic changes during gametogenesis. As the success of assisted reproductive technology (ART) is also affected by paternal diet, BMI, and alcohol consumption, its outcomes could be improved by modifying factors that are dependent on male lifestyle choices and environmental factors. This review discusses the importance of epigenetic signatures in sperm—including DNA methylation, histone retention, and sncRNA—for sperm functionality, early embryo development, and offspring health. We also discuss the mechanisms by which paternal lifestyle and environmental factors (obesity, smoking, EDCs, and stress) may impact the sperm epigenome.

Keywords Sperm, Epigenetics, Lifestyle, Environmental factors, Offspring health

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The sperm epigenome

Lifestyle and environmental factors, such as diet, smoking, physical activity, and endocrine-disrupting chemicals (EDCs), are correlated with a predisposition to type 2 diabetes, obesity, cardiovascular disease, and cancer [44]. Not only do these factors affect the health of an individual, but, as recent studies suggest, they might also increase the risk of developing chronic conditions in subsequent generations via parental effects [41, 62, 88, 113, 123, 134, 172, 191]. This is highly prominent in the context of obesity and type 2 diabetes, which are understood to be associated with the availability of diet and food during childhood and adolescence, leading to an increased risk of cardiovascular diseases in the first and second generations [48, 145]. Most significantly, paternal lifestyle and diet before conception have been reported to affect the health of the offspring via epigenetic inheritance [139, 143]. By exposing the male only to a certain environmental condition, it has been suggested that receptive sperm-borne factors might play an essential role in the functionality of the male gamete, their ability to fertilise the oocyte, and the developmental reprogramming of the offspring.

Specific evidence relating to the transgenerational inheritance of epigenetic changes is, at present, limited. Most of the established findings are related to maternal epigenetics in embryo development. Studying maternal effects on the offspring is technically challenging due to confounding factors, as well as the difficulty of discerning between maternal effects and the direct consequences of in utero exposure. Maternal effects may result in intergenerational rather than transgenerational changes, as germ cells develop into the embryonic stage. Paternal effects on epigenetic modifications may also be influenced by confounding factors, such as contamination of maternal microbiota, consequently leading to changes in the in utero environment; furthermore, at the time of mating, the seminal fluid can also have an impact on the in utero environment [165]. Genetic predisposition is a confounder factor in studying epigenetic changes, as inherited genetic variants can affect the baseline epigenetic landscape. Certain genetic polymorphisms may influence the susceptibility to environmental or lifestyle-induced epigenetic changes, making it difficult to determine if modifications are genuinely the result of lifestyle factors or are partly due to genetic background. For instance, genetic variations in genes related to DNA methylation enzymes (like DNMTs) or histone modification pathways (such as histone acetyltransferases) might lead to baseline differences in DNA methylation and histone marks, potentially affecting how sperm respond to external factors [169]. Lifestyle factors like diet, exercise, and exposure to toxins often interact in complex ways, making it challenging to attribute epigenetic changes to one specific behaviour. For example, a high-quality diet combined with regular exercise may have a different epigenetic impact than a poor diet combined with a sedentary lifestyle. In addition, lifestyle behaviours are rarely constant, and fluctuations over time add another layer of complexity. An individual's lifestyle may shift over their lifespan—such as changes in diet or physical activity levels—making it hard to pinpoint the epigenetic effects of any single factor. Controlled, longitudinal, and multidimensional models that account for these confounders are essential to address the complex interplay between genetics, behaviour, and epigenetics.

Epigenetics, defined as mitotically or meiotically heritable changes in gene expression that do not entail alterations of the DNA sequence, involves three discrete but reciprocal mechanisms that modulate the accessibility of tightly packed chromatin towards the transcriptional machinery and/or regulate gene expression at the post-translational level. These effects are achieved by DNA methylation and histone modification via methylation, acetylation, phosphorylation, sumoylation, and small non-coding RNA (sncRNA) [44]. In sperm, the epigenome (Fig. 1), which is passed along with the haploid genome during fertilisation, consists of a pattern of epigenetic signatures that is established during germ cell development in the testis, as well as along the epididymis where the male gamete matures. Mounting evidence suggests that male reproductive potential can be affected by lifestyle and environment and that this may underlie poor sperm quality and pose a hazard to the health of offspring, via, among other factors, epigenetic inheritance [195].

This review aims to summarise (i) the nature of epigenetic marks in sperm, including DNA methylation, histone retention, and sncRNA; (ii) the relevance of these epigenetic marks for sperm function, early embryo development, and offspring health; and (iii) how paternal lifestyle and environmental factors (obesity, smoking, EDCs, and stress) may affect the sperm epigenome.

Sperm DNA methylation

DNA methylation, the addition of a methyl group to the C-5 position of the cytosine ring, governs several cellular processes, including cell differentiation and embryo development, by regulating gene expression, transposon silencing, X chromosome inactivation, and genomic imprinting. DNA methylation is controlled by the action of DNA methyltransferases (DNMTs), whereas demethylation is a stepwise process orchestrated by Ten-Eleven Translocation enzymes, thymine-DNA-glycosylase, and DNA base excision repair [82] [30]. While DNA methylation occurs mostly in cytosines within the DNA sequence

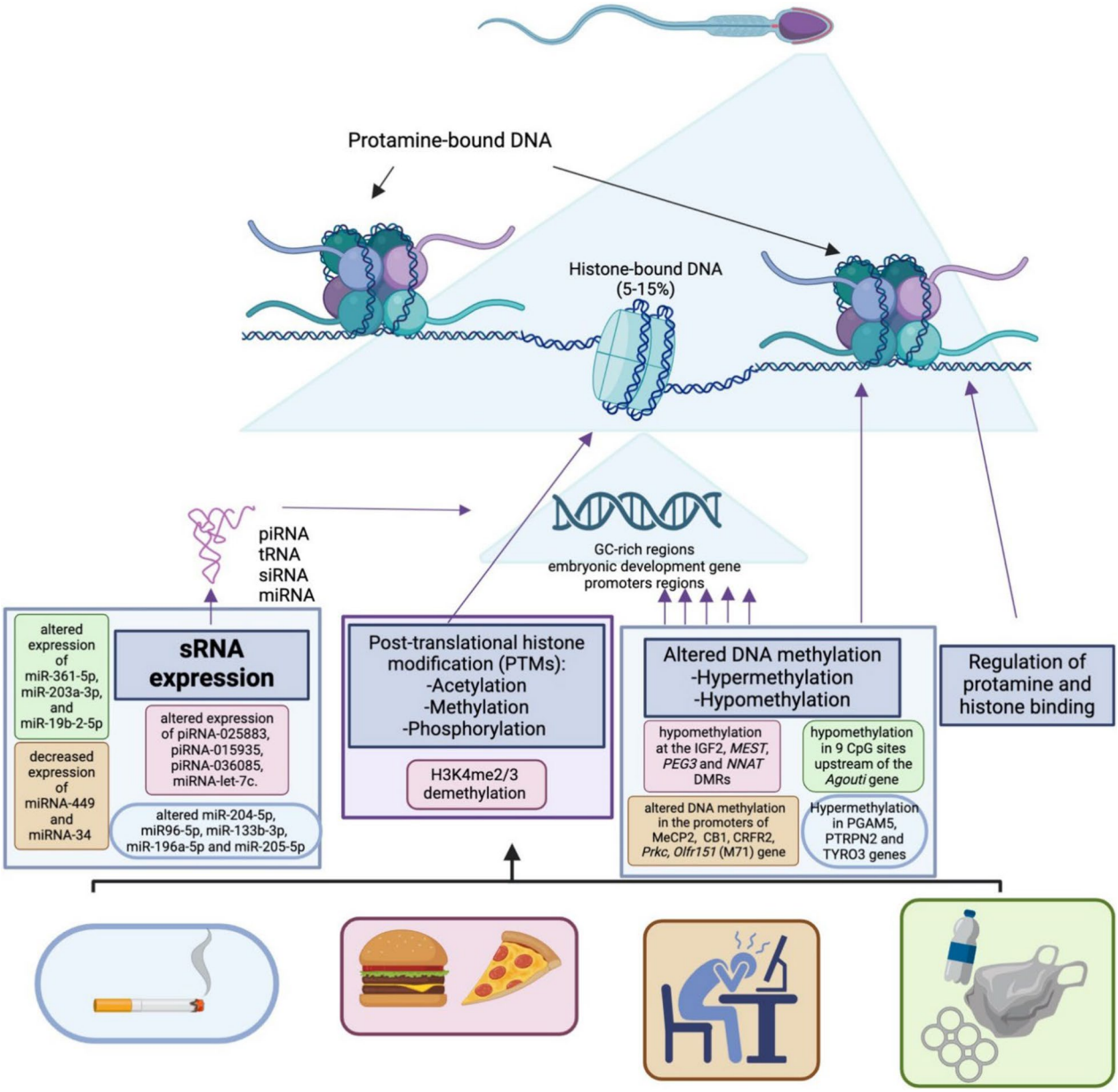

Fig. 1 Epigenetic signatures in sperm. Sperm chromatin is tightly packaged by binding to protamines and histones (< 15% of the genome). DNA methylation occurs at CG-rich, histone-bound DNA regions and repetitive sequences. Environmental factors may influence DNA methylation, histone modification, as well as the expression of small non-coding RNA, such as tsRNA, microRNA, and PIWI-interacting RNA. The figure was adapted from Donkin and Barrès [44] and created with Biorender.com

at birth, constituting CpG islands, approximately 25% of methylation can be found in non-CpG regions of the sperm genome [171]. Non-CpG methylation takes place within and around the B1 SINE transposon region in male germ cells as the result of de novo methylation followed by the loss of methylation towards the later stages of sperm maturation [79]. Methylation patterns in germ cells are set during embryo development; these patterns are characterised by dynamic de novo methylation, known as DNA reprogramming (Fig. 2) [64].

As reprograming events occur within highly critical embryonic development timeframes, any perturbations in epigenetic reprogramming may exert considerable effects on sperm function and lead to transgenerational epigenetic changes. Interestingly, alterations in sperm DNA methylation have been demonstrated to correlate

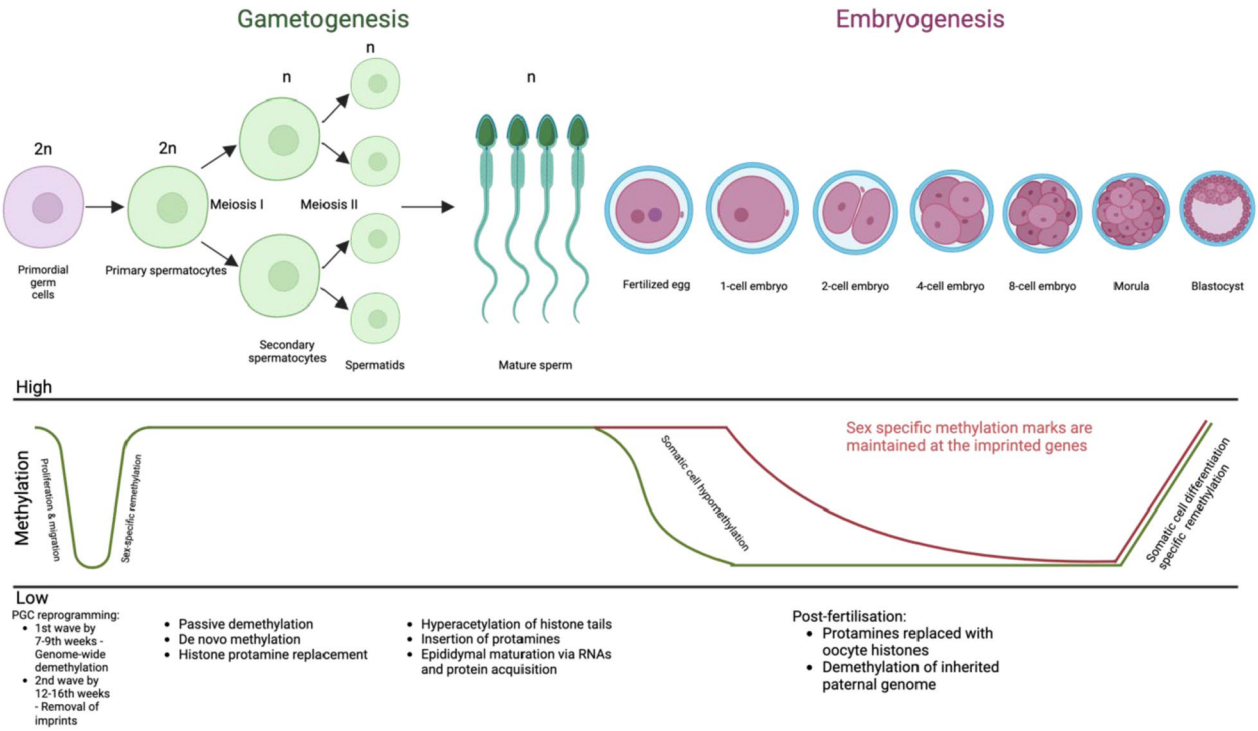

Fig. 2 Epigenetic reprogramming in sperm and embryos. In humans, the first reprogramming event occurs early in embryo development, presumably at embryonic day (E) 37, when primordial germ cells divide and migrate to the genital ridge, leading to up to 70% erasure of methyl groups and thus allowing for cell totipotency. As cells differentiate and mature, de novo DNA methylation occurs in approximately 4% of total cytosine methylation. Further spermatogenesis involves mitotic division of pro-spermatogonia to form primary spermatocytes, followed by meiotic division resulting in secondary spermatocytes. The second meiotic division produces haploid spermatids, which mature by morphological changes involving loss of cytoplasm and organelles, microtubule expansion, formation of tail and acrosome, and chromosome remodelling [133]. The figure was adapted from Nevin and Carroll [133] and created with Biorender.com

to impaired sperm concentration and motility [108, 109, 128]. The incomplete erasure of DNA methylation during epigenetic reprogramming may lead to the inheritance of environmental-associated DNA changes to the next generation. For example, the L1HS transposon escapes epigenetic reprogramming, the repercussion of which is technically hard to address as the L1s transposon family can efficiently insert itself into regions with various chromatin states [60, 107]. A previous study investigated the effect of endurance training on the methylation of somatic cell genes in humans and detected significant changes in transposon regions related to nervous system development [81]. Collectively, these findings suggest that environmental factors may lead to changes in DNA methylation, and that this can exert an impact on development.

In mammals, sexual reproduction follows the Mendelian inheritance law whereby zygotes inherit two alleles, one from each parent. Whilst most genes have two functional alleles, certain genes require the silencing of one of the alleles by DNA methylation [11, 185]. These silenced genes are referred to as imprinted genes, which result in the expression of the unmethylated allele from one parent. DNA reprogramming after fertilisation removes methyl groups from both parent-of-origin alleles, yet some imprinted regions that retain methyl groups are required for normal embryo development [190]. The mammalian genome contains approximately 200 imprinted genes in the embryo, which are diverse in expression pattern due to tissue-of-origin and stage of embryo development [13]. Imprinted genes can be located within relatively small genomic intervals and are highly conserved in structure among species. Although the specific identification of individual gene function and regulation is challenging, the altered methylation status of genes such as small nuclear ribonucleoprotein polypeptide N (SNPRN) was associated with enlarged placentomes and large offspring syndromes in mammals (LOS) [104, 119]. In humans, the LOS phenotype is observed in Beckwith–Wiedemann syndrome, which results in loss-of-imprinting at the *KCNQ1* gene associated with ART [34, 104]. Aberrant DNA methylation in paternally imprinted genes may, therefore, result in deleterious effects on embryo development.

DNA methylation changes may also affect specific paternal genes. A genome-wide study reported that paternal prediabetes affects methylation patterns in the pancreatic islets of mice offspring [33, 180]. The sequencing of immunoprecipitated methylated DNA (MeDIP-Seq) showed that paternal prediabetes altered the degree of methylation in 446 genes in the pancreatic islets involved in glucose metabolism and insulin signalling pathways [180]. Increased methylation in intragenic regions of *Pik3r1* and *Pik3ca* was also detected in sperm from the offspring, thus suggesting a transgenerational inheritance pattern that occurs via the bypassing of global demethylation at fertilisation [180].

Sperm histone modification

Unlike somatic cell chromatin, sperm harbour very dense and highly packaged chromatin, where DNA is mostly bound to protamines. This structure and organisation in the chromatin ensure that sperm DNA is well protected from external stressors. During protamination, 85–95% of histones, depending on species, are removed by transition proteins and H2A.L.2 (Fig. 3) [14].

Histones are primarily modified by hyperacetylation, the addition of an acetyl group to lysine residues within the N-terminal tail protruding from the histone core of the nucleosome, and by butyrylation [44]. The latter process prevents acetyl-dependant histone removal, thus impeding histone replacement and chromatin compaction [44]. A previous study by Goudzari et al. [63] discovered that these post-translational modifications (PTMs) at H4K5 are associated with alterations in sperm cell genome programming by preventing binding of the testis-specific gene expression-driver *Brdt* and by inhibiting histone removal during late spermatogenesis, which could have significant functional consequences for genome reorganisation in sperm [63]. In animal models, such as *Caenorhabditis elegans* and *Drosophila melanogaster*, histone PTMs have also been associated with inter- and trans-generational epigenetic inheritance [161]. Based on this body of evidence, any alteration in the histone PTMs of the sperm epigenome could exert an impact on the inheritance of epigenetic traits by offspring.

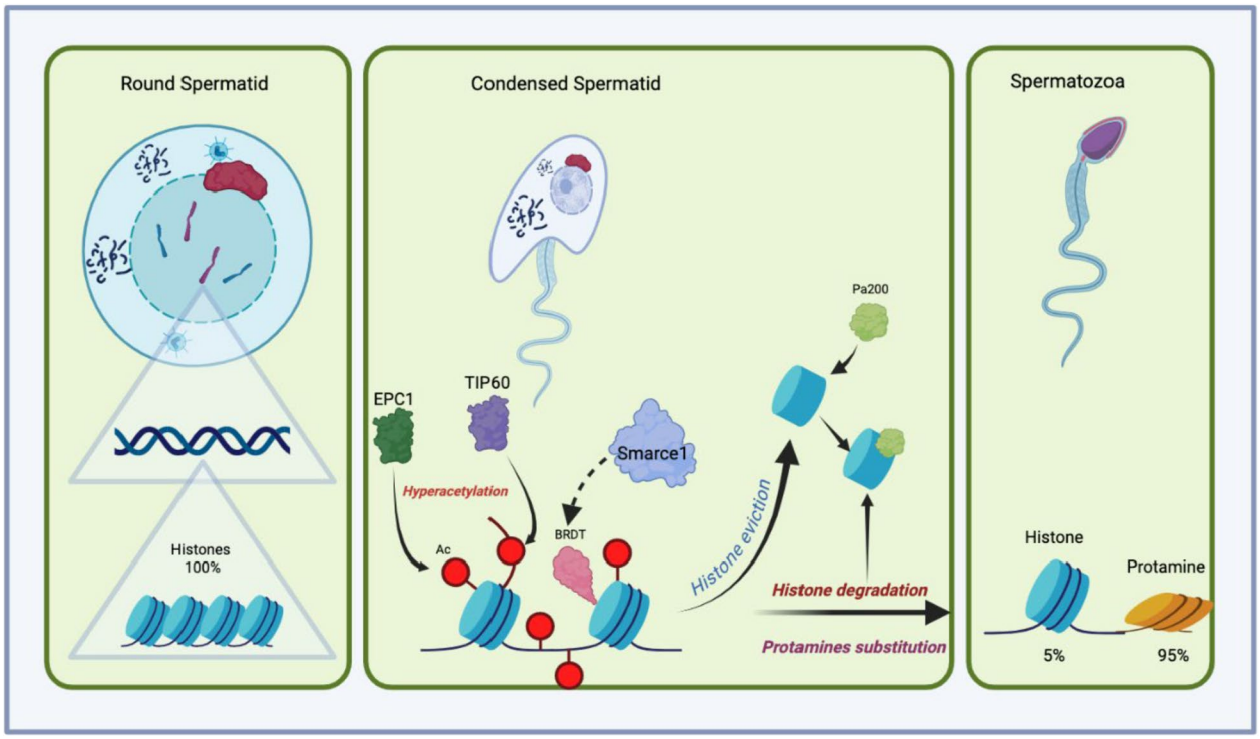

Fig. 3 Overview of the histone modification process in the mouse. The incorporation of H2A, H2B, and H3 histone variants during spermiogenesis results in an unstable nucleosome condition, which allows hyperacetylation by EPC1/TIP60 (Enhancer Of Polycomb Homolog/Tat-interactive protein, 60 kDa) or similar nucleosome acetyltransferases. The acetylation of lysine ensures further destabilisation when incorporating BRDT (testis-specific BET member protein), which in turn interacts with Smarce1, the member of SWI/SNF family proteins. This leads to histone eviction and recognition by the proteasome activator PA200, which induces the degradation of histones by proteasomes during spermiogenesis. RNF8 (E3 ubiquitin-protein ligase) is responsible for the ubiquitination of H2A, whereas PHF7 (PHD Finger Protein 7) tags H3K4me3/me2 and H2A for degradation in elongating spermatids. Figure created was adapted from Wang et al. 178 and created with Biorender.com

Genome-wide analysis conducted in mice and infertile men has revealed that histone variants of H3K4me3 and H3K27me3 are retained in genes associated with embryo development [51, 194]. Because these help to balance the activation and repression of genes, these two marks are known as "bivalent" chromatin with antagonistic roles [17]. Immunostaining techniques demonstrated that upon gamete fusion, protamines are replaced by maternal nucleosomes, whilst paternal histones remain associated with the paternal genome [174, 179]. The retained paternal histones are located in genetic regions with high CpG density and low DNA methylation, which include housekeeping and development-regulating genes [45, 51]. Other studies suggested that the loss of H3K4me3 and H3K27me3 under the influence of lifestyle and environmental factors could represent predictive epigenetic marks for reduced fertility in men [22, 69]. Although no specific locus was found to be susceptible to chromatin packaging and/or DNA modification alterations, this does not exclude the possibility of a cumulative effect on fertility [22, 69].

Small non-coding RNA

Small non-coding RNAs (sncRNAs) are one of the epigenetic regulators of gene expression that interact with mRNAs and induce their degradation during translation. During spermatogenesis, most of the transcripts are depleted, leading to the silencing of spermatozoa at the translational level. While the remaining small portion of transcripts containing coding and non-coding RNA such as mRNAs, piwi-interacting (pi) RNAs, transfer (t) RNAs, small interfering (si) RNAs, micro (mi)-RNAs, and long non-coding (lnc) RNAs were previously considered as non-functional, they are now considered as selectively retained functional elements [121].

The most studied sncRNAs, miRNAs, play a key role during spermatogenesis and after fertilisation [4, 89, 188]. miRNAs regulate the expression of genes by targeting the 3′-UTR of mRNA for degradation or activation as part of the RNA-induced silencing complex and AGO proteins, which also include siRNAs [83, 86]. The role of siRNAs during fertilisation was studied using knockout mice, and an association between the altered profile of siRNA and the reduced developmental potential of the offspring was observed [187]. Interrogating the function of a specific miRNA poses a challenge as a single miRNA may target several mRNAs, and/or one mRNA may be targeted by multiple miRNAs. There are, nevertheless, studies that identified the function of sperm-borne miRNAs such as miR-34c, which targets *BCL2*, the gene involved in the first cleavage of zygotes [118, 188]. The BCL2 protein increases the expression of cell cycle regulator p27 and inhibits entry to the S-phase [46, 47, 118]. Embryos treated with a miR-34c inhibitor are known to show increased synthesis of p27 and failure to inhibit zygotic division [118].

tRNA-derived small RNAs, tsRNAs, which are generally scarce in the testis, become one of the most abundant types of sncRNA as sperm mature along the genital tract [157]. The maturation of tsRNAs involves the removal of the 5′ leader, the intron and the 3′ trailer, and the addition of CCA trinucleotide. Before tsRNA is transported to the cytoplasm, it undergoes post-transcriptional modification, such as enzymatic degradation into pre-tRNA-derived small RNA (pre-tsRNA), tRNA-derived fragments (tRFs), and tsRNA halves (tiRNA) [100, 102]. It has been suggested that tsRNA in mature spermatozoa are brought from the epididymis by epididymosomes (Fig. 4) [157]. These tsRNAs are derived from the 5′ end of either mature tsRNA or pre-tsRNA, consisting of 10 to 45 nucleotides. Mice consuming a low protein diet showed that tRF-Gly-GCC fragments were associated with the repression of endogenous retroelement MERVL and their respective genes, which could affect placental size or function, causing downstream effects on metabolism secondary to altered placentation [157]. In a study using a mouse model fed with HFD, Qi Chen et al. discovered that tsRNA showed altered expression and RNA modification in F0 [32]. Injecting these altered tsRNA from HFD males into normal mice resulted in metabolic disorders in the F1 offspring, regardless of the DNA methylation status in CpG-rich regions [32]. This suggests the possible role of tsRNA in the paternal inheritance of diet-induced epigenetic changes.

piRNAs are gonad-specific transcriptional regulators that silence transposons by the expression of PIWI proteins in the germline, thus contributing to the integrity of the genome and supporting fertility in animal models[135, 136, 140]. In humans, piRNAs are the most abundant sncRNA in sperm, representing approximately 17% of all sncRNAs, whereas, in rodents, tsRNAs are the most abundant subgroup [135, 136]. As sperm mature, the composition of sncRNA changes, as the absolute levels of piRNA decrease and that of tsRNA increase [168]. One of the suggested mechanisms for piRNA action is the prevention of the activity of TEs, such as SINE, LINE, MER, and LTR, at certain stages of embryogenesis by binding to DNA [98]. Unlike piRNAs in embryonic germ cells, which primarily silence transposable elements, pachytene piRNAs have broader roles [135, 136]. They are involved in regulating both non-coding and protein-coding RNAs during meiotic and post-meiotic stages and play a role in the translational control of mRNAs [135, 136]. A few studies have reported the association between altered PIWI expression and reduced semen parameters in humans, swine, and cattle [2]. Experiments studying the

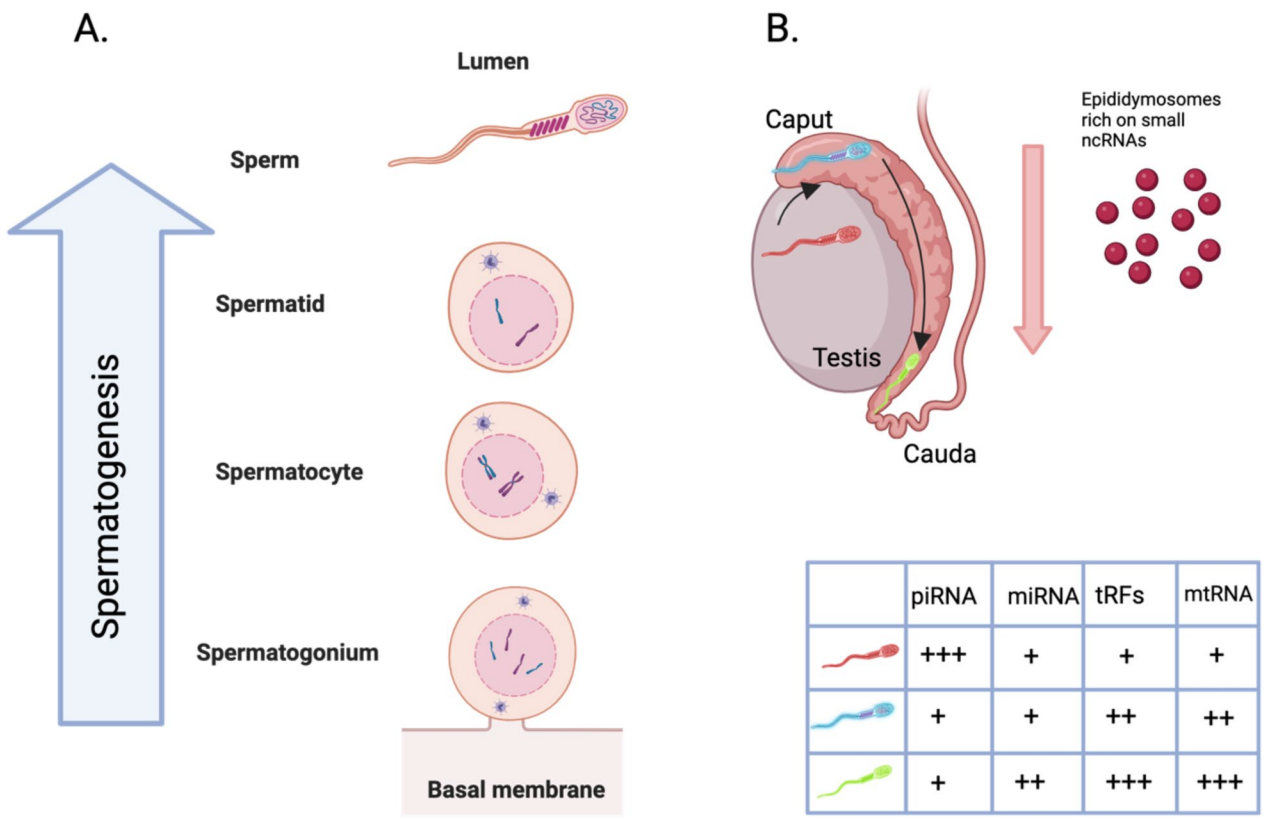

Fig. 4 sncRNA composition throughout sperm maturation. **A** Germ cells mature from spermatogonia at the basal membrane to immature spermatozoa inside the testis, which are then released from the tubules (green sperm). **B** As spermatozoa travel from caput (blue) to cauda (green), they receive input from the epididymis in the form of epididymosomes containing sncRNA. The table below shows the content of different sncRNA, during the transit of spermatozoa along the epididymis, namely decreasing levels of piRNA and increasing miRNA and tRNA-derived fragments (tRFs) down the cauda. Tomar et al. [172] recently discovered that mitochondrial-derived tRNAs (mtRNAs) are sperm-borne and enrich the spermatozoa as it travels down to cauda. Figure was adapted from Cheuquemán and Maldonado (2021) and created with Biorender.com

effects of disrupting the gene encoding three PIWI family members demonstrated that *Miwi2*-knockout mice resulted in spermatogenic arrest, the increased expression of TEs, and the loss of methylation in testis [24, 97]. In humans, Giebler et al. found in a cohort study (n = 90) that the levels of *PIWI-like 1* and *PIWI-like 2* transcripts were altered in men with a reduced sperm count and motility, whilst these two mRNAs were not associated with sperm morphology [58].

How paternal sncRNAs may impact embryogenesis remains largely unknown. As new technologies, such as transcriptomic profiling of a single or few cells, emerge, the body of knowledge relating to the role of sncRNAs has expanded accordingly. For instance, it was discovered that the action of sncRNA can be mediated via the recruitment of histone methylases and interaction with DNA methyltransferases [131, 141]. At present, studying the crosstalk between all epigenetic marks is still time-consuming and financially difficult. The following sections describe how lifestyle and environmental factors may affect sperm fertilising ability, embryo development, and offspring health, via modifications in the epigenome.

Effects of obesity and diet

Effects of paternal obesity on the offspring

It is widely acknowledged that the health of a newborn' is greatly affected by the mother's periconceptional undernutrition via epigenetic changes at imprinted genes, leading to coronary artery disease, obesity, and hypertension later in adulthood [92, 156]. Interestingly, the Newborn Epigenetics Study (NEST) revealed that paternal diet and BMI might also influence the epigenetics of the offspring, reducing methylation at differentially methylated regions of the *IGF2* gene established during gametogenesis [164]. Hypomethylation of *IGF2* is associated with an increased risk of cancer development, namely Wilms tumour, colorectal and ovarian cancers, and Silver–Russell Syndrome in neonates [29, 65, 78, 91]. Furthermore, it was discovered that hypomethylation at *IGF2* DMR is associated with an increased level of circulating IGF2,

which is responsible for the production of insulin-like growth factor 2 that enhances cell survival and proliferation by binding to the IGF1 receptor (IGF1R) [16]. Based on these findings, further research found out that not only in utero exposure but pre-conceptual obesity of the father may lead to hypomethylation at the *MEST*, *PEG3,* and *NNAT* DMRs, genes associated with rhabdomyosarcoma, glioma, and childhood obesity [158, 163]. Similarly, paternal and offspring sperm from mice fed with a high-fat diet were observed to undergo insufficient erasure of DNA methylation in the imprinted genes responsible for cellular transport, localisation, and metabolic patterns, thus altering insulin secretion and glucose metabolism [25]. Moreover, the presence of H3K27me3 in the monoamine oxidase A (*Maoa*) and elongation factor Tu GTP-binding domain containing 1 (*Eftud1a*) genes was found to be increased in the sperm of mice fed with a low-protein diet for three months [25].

During spermiation, H3K4me2/3 and H3K27me3 marks are lost in most sperm genes. The relevance of retained paternal H3K4me2/3 and H3K27me3 for embryo development was previously investigated in Xenopus. Immature oocytes were first injected with mRNA encoding H3K4me/2/3 demethylase (KDM5B), which eliminates H3K4me/2/3 and H3K27me3, and in vitro-matured into eggs. ICSI with sperm or spermatids were performed to study the embryos at the gastrulation stage using RNA sequencing [170]. The result was the down-regulation of 68% and 80% of all differentially expressed genes in sperm and spermatid-derived embryos, respectively. There was only limited overlap between genes down-regulated in sperm-derived embryos when compared to genes derived from spermatid-derived embryos, thus supporting the role of H3K4me2/3 demethylation of paternal chromatin on the regulation of gene expression in the embryo [170]. These findings were in line with those reported by Siklenka et al., who demonstrated the transgenerational inheritance of epigenetic changes in histone demethylation during the process of spermiogenesis in mice, thus identifying an important regulator of gene expression in embryos [159]. Specifically, sperm from transgenic mice overexpressing histone H3 lysine 4 (H3K4) demethylase LSD1/KDM1A during spermatogenesis were observed to exhibit reduced H3K4 methylation [115, 147, 159]. Related to this, it is also worth mentioning that the paternal overexpression of *KDM1A* induces major developmental defects and low survival in offspring for three generations, even in the absence of germ-line *KDM1A* expression [115, 159].

In humans, it was discovered that obesity is associated with downregulation of *ANKRD26* and hypermethylation of its promoter region [166]. This epigenetic change and its inheritance pattern were studied in mice, whereby researchers introduced stable DNA methylation at targeted promoter-associated CpG islands without genetic alterations [166]. This DNA methylation of the *Ankrd26* CpG islands was inherited by the F3 offspring, showing the obese phenotype in all previous generations [166]. Bodden et al. also showed long-term transgenerational effects of HFD during paternal adolescence, resulting in higher body weights, increased Actinobacteria abundance in gut microbiota, and a food preference for HFD in the F1 generation [19]. These findings are significant for understanding how paternal factors contribute to transgenerational inheritance and influence offspring health and vulnerability to diseases.

Effects relating to the paternal intake of folate

In a previous study, Lambrot et al. found that male mice with folate deficiency (FD) were less capable of fathering pups as they were associated with a significantly reduced pregnancy rate when compared to a folate sufficient group (52.38% vs. 85%) [106]. Furthermore, offspring from FD fathers were observed to have developmental abnormalities [106]. Noticeably, these effects were related to DNA methylation, as folate intake significantly reduced methylation at CpG locations of genes such as *Rfwd2, Sfi1, Kdm3b, Gm52, Rbks*, which are crucial for embryo development and metabolic processes. Folate metabolism is mediated by methylenetetrahydrofolate reductase (MTHFR), which converts 5,10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate; this provides methyl groups for the synthesis of methionine (Fig. 6). MTHFR is one of the main regulatory enzymes involved in folate metabolism, DNA synthesis, and methylation, which is highly expressed in testes due to increased DNA methylation levels during spermatogenesis and epigenetic reprogramming [1]. Perturbations in *MTHFR* expression can therefore lead to a decreased pool of methyl groups altering the DNA methylation process [1]. Folate is vital for paternal reproductive function; abolishing the expression of *Mthfr*, a key enzyme involved in the metabolism of folate and expressed at high levels in the testes, is known to cause infertility in mice [106]. Furthermore, a greater level of *MTHFR* hypermethylation was observed in the sperm DNA of 94 idiopathic infertile men when compared to 54 normal fertile men (45% vs. 15%) [184]. These results support the findings of Khazamipour et al., whereby hypermethylation of *MTHFR* was observed in the testes of infertile men, suggesting the association between disruptive spermatogenesis and epigenetic silencing of *MTHFR* [93]. These authors also investigated methylation patterns in infertile men by comparing normozoospermic and oligozoospermic patients. Interestingly, the degree of *MTHFR* methylation was found to be greater in men

with oligozoospermia, thus confirming the significance of folate on the quality of sperm and fertility [184].

Folate intake could have a future clinical implication in addressing the offspring's health issues. Interestingly, a previous study revealed that treating rat dams with folate improved glucose metabolism in the female progeny [112]. This effect was likely due, in part, to the folate's role in preserving beta cells in the offspring and restoring the normal hepatic connective tissue density [112]. Additionally, folate treatment corrected the abnormal expression of the *Ppara*, *Lcn2*, and *Tmcc2* genes while restoring normal liver total DNA methylation levels in these animals [112].

Effects of a paternal high-fat diet

The chronic consumption of a high-fat diet (HFD) is highly likely to cause metabolic diseases, which could potentially be transmitted to the offspring by epigenetic changes in sperm [37, 172]. A previous study demonstrated that F1 and F2 females born from F0 fathers that were fed with an HFD exhibited dysfunctional glucose metabolism when compared to those born from fathers that were given a control diet [16]. While these metabolic changes were only observed in female offspring, they were suggested to be potentially mediated by specific sperm sncRNAs: three piRNAs (piRNA-025883, piRNA-015935, piRNA-036085) and one miRNA (miRNA-let-7c). These metabolic changes in the offspring were caused by altered peripheral glucose metabolism rather than insulin secretion [16]. The sexual dimorphism observed in these results could be potentially explained by adipose tissue plasticity, which was increased in females [16]. On the other hand, as miRNA-let-7c is the most abundant class of sncRNA in sperm [146], further research is required to determine the role of miRNA-let-7c in mediating these metabolic changes. A study by Crisotomo et al. also discovered alterations in glucose homeostasis, serum hormones, and antioxidant enzyme activities in HFD-fed mice and their offspring [35]. Namely, the F1 generation of HFD-fed mice showed impaired choline metabolism, mitochondrial function, and antioxidant defence, whereas the F2 generation showed an increased pro-inflammatory profile with a shift of testicular ω3/ω6 ratio and increased 3-hydroxybutyrate levels [35]. Interestingly, HFD-fed mice displayed decreased sperm count, regardless of the time of exposure to HFD [35]. In a following study, researchers discovered the sperm sncRNA content in HDF-fed mice and controls, which led to the discovery of altered expression of tiRNAs of mitochondrial origin. This finding supported the authors' hypothesis that reversion of diet from HFD to chow diet induces the mobilisation of fatty acids that are used as energy substrates by testicular mitochondria [35]. The most recent study by Pastore et al. [144] discovered that HFD-fed male mice produced male progeny with a higher predisposition to hypoandrogenism and diabetes and female progeny with increased glucose tolerance [144]. HFD was associated with altered methylation and the preferential expression of a specific isoforms of *cyp19a1* hnRNAs [144]. In particular, this promoter region contains a binding site for Oestrogen Receptor and for the transcription factors Jun and Fos, which was proven to affect the activity of the enzyme in offspring testis and promote hypoandrogenism [144]. Further research should interrogate if these findings, obtained from a mouse model, could be extrapolated into humans; if this were the case, these sncRNAs could represent novel biomarkers for metabolic diseases.

The most recent data by Tomar et al. [172] showed that epididymal spermatozoa, but not developing germ cells, are susceptible to environmental perturbations via dynamic regulations of mitochondrial-encoded tRNAs (mt-tRNAs) and their fragments (mt-tsRNAs) in the sperm. The study included two groups of 6-week-old male mice either fed with HFD or a low-fat diet for two weeks before mating. Although an HFD for two weeks did not result in significant changes in the offspring body weight and composition, an approximately 30%-penetrant glucose intolerance was observed in male offspring [172]. Male offspring were divided into HFD-tolerant (HFDt) and HFD-intolerant (HFDi) groups and studied further. Interestingly, gene expression analysis and the Leipzig Adipose Tissue Childhood genome-wide association study data revealed that 30% of the HFDi signature is also expressed in human adipocytes and associated with childhood obesity [172]. Differential expression analysis of cauda spermatozoa sncRNA sequencing from HFD-fed male mice (F0) showed up to 30% of mt-tRNAs sequences reaching statistically significant upregulation. The sperm-borne mitochondrial-derived RNA, mtRNAs, were found to be transferred to the oocyte at fertilisation [172]. Using the International Mouse Phenotyping Consortium (IMPC), they evaluated the relative glucose intolerance (measured as AUCipGTT) in wild-type (WT) offspring, specifically focusing on genes essential for mitochondrial function. Cryopreserved sperm samples from these genes were analysed to explore small non-coding RNA (sncRNA) biotypes present in spermatozoa [172]. Their findings revealed significant alterations in the distribution of 5′ n-tsRNAs and 5′ mt-tsRNAs in mutant spermatozoa compared to controls. Notably, mutants such as *Mrpl23* and *Ndufb8* exhibited a marked accumulation of 5′ mt-tsRNAs, whereas *Tsfm* did not, serving as a negative control [172]. These results underscore the role of mitochondrial dysfunction in reprogramming offspring metabolism, resembling paternal HFD exposure

effects. This supports the hypothesis that alterations in sperm-borne mtRNAs contribute to transgenerational transmission of metabolic traits, emphasising the potential impact of paternal mitochondrial health on offspring health outcomes.

Another study found that the offspring of male rats fed with a HFD diet presented with metabolic alterations, including reductions in body mass and pancreatic beta cell mass, and glucose intolerance; furthermore, these alterations were passed onto the F2 generation [54]. Similar to the findings of de Castro Barbosa et al., females of the F1 offspring, as well as those of the F2 generation that came from F1 males originating from F0 HFD fathers, developed obesity. The analysis of testes from HFD fathers revealed 23 altered miRNAs. Four of these miRNAs were deregulated in sperm, targeting 50 mRNAs involved in lipid metabolism, metabolic disorders, spermatogenesis, and embryonic development according to cell pathway analyses [54].

Effects of a paternal high-sugar diet

Nätt et al. found that the sncRNA composition of human sperm was altered after individuals were administered a high-sugar diet (HSD) for one week in a controlled clinical trial [132]. In particular, an HSD was associated with increased levels of mitochondrial tsRNAs, a specific subset referred to as nitRNA. This increase in tsRNAs was positively linked to sperm motility, mitochondrial activity, and ROS levels. As mature human sperm use glucose and fructose as energy sources, this change in tsRNA levels could be attributed to sugar-dependent gene transcription. A reasonable hypothesis to explain the rapid responses to diet in human sperm could be due to the activation of latent factors present within sperm, by sensing the dietary molecules present in seminal plasma and activating the intercellular signalling by receptor–ligand cascade reactions [132]. As tsRNAs are involved in the control of retrotransposons and regulatory genes affected by retrotransposons during reprogramming to a pluripotent stage of embryo development, the transgenerational inheritance of paternal metabolic changes could be driven by tsRNA.

Effects of smoking

Despite all regulations and increased awareness of the consequences of smoking on health, figures released by the World Health Organization indicate that, in 2020, 22.3% of the global population (36.7% of men, 7.8% of women) still smoked tobacco [182]. Maternal tobacco consumption is related to foetal growth retardation, sudden infant death, immunological disorders, obesity, cardiovascular diseases, and even the development of addictive behaviour [3, 181, 196]. Interestingly, exposure to maternal cigarette smoking also increases the circulating levels of oestrogens levels in foetuses. Chemicals in smoke activate the aryl hydrocarbon receptor, which dysregulates genes important for ovarian development, thus reducing germ cell proliferation [52]. Based on this, prenatal exposure to cigarette smoke could detrimentally affect the fecundity of female offspring. Apart from these effects in females, smoking also impacts male gametes, and its detrimental repercussions may be passed on to the offspring [61].

The effects of smoking on semen parameters

Smoking tobacco has consistently been shown to reduce semen volume, sperm concentration, morphology, motility, and viability and increase the proportion of sperm with abnormal morphology [71, 101, 108, 109, 149]. Smoking has also been associated with a poorer ability of sperm to penetrate and fertilise the oocyte; this may directly or indirectly lead to reduced embryo implantation [149, 162]. These deleterious effects are due to the carbon monoxide, nicotine, cotinine, and cadmium present in tobacco. For example, high levels of cadmium have been reported to reduce the availability of zinc in seminal plasma and disrupt spermatogenesis, thus manifesting as a low sperm count, impaired motility, abnormal morphology, and plasma membrane instability [15, 116]. Furthermore, sperm from smokers have also been reported to retain greater levels of nuclear histone H2B, which is important during early chromatin remodelling at fertilisation, and higher ratios of P1/P2 compared to sperm from non-smokers, potentially leading to hypertranscription or transcriptional arrest during spermatogenesis [67].

Alterations in DNA methylation associated with smoking

The consequences of cigarette consumption on sperm quality could be partially explained by the sperm DNA methylation changes induced by smoke [5, 7, 66, 95, 189] [5, 9]. In support of this hypothesis, a study involving 28 sperm samples (14 from fertile smokers and 14 from fertile non-smokers) found that smoking tobacco increased DNA methylation in *PGAM5* (cg00648582) and *PTPRN2* (cg23841288) genes, and decreased that of three CpG sites in the *TYRO3* gene [6, 8]. *PGAM5*, which encodes for two mitochondrial protein isoforms (PGAM5s and PGMA5L), found in the testis and played a significant role in mitophagy and the antioxidation process [31, 186]. In mice, the *Ptprn2* gene is involved in the development of insulin-dependent diabetes mellitus, which may, in turn, lead to a reduction in semen parameters [8, 99, 103]. The *TYRO3* gene plays a crucial role in spermatogenesis.

The possible mechanism by which smoking tobacco affects sperm DNA methylation could be related to the

recruitment of DNMTs, which occurs after DNA damage by inducing double-stranded breaks due to the toxicity of nicotine and carbon monoxide, as shown in early mouse embryos in vitro (Fig. 5) [76, 77, 111]. Huang et al. showed that cigarette smoke condensate, which simulated acute and chronic smoke effects, induced high levels of telomere shortening and double-stranded DNA breaks based on immunostaining results, which coincided with compromised embryo development in vitro [76, 77]. Nicotine injected into mice at a concentration found in the blood of a compulsive smoker (0.5–0.6 μM) was observed to reduce the transcript and protein levels of DNMT1 in cortical layers and hippocampal GABAergic interneurons [153]. DNA methylation could also be modulated by the expression and activity of DNA-binding factors that, like SP1, are upregulated in lung epithelial cells in response to cigarette smoke [40, 191]. Specifically, SP1 is a transcription factor that binds to GC-rich motifs in gene promoters and prevents de novo methylation during early embryogenesis [59, 70]. Moreover, cigarette smoke may also affect DNA methylation by inducing hypoxia and the upregulation of *MAT2a* (Methionine adenosyltransferase 2A), a vital methyl donor [117].

A genome-wide study analysed semen samples of 78 smokers vs. 78 non-smokers and reported an association between the DNA methylation of *SNRPN* and *H19* imprinting regions and male infertility [85]. Their results identified 141 differentially methylated CpG islands and total methylation status associated with smoking, which were linked to an increased health risk of the offspring [85]. Similar results were reported by Laqqan et al. who showed that differentially methylated CpG sites were located on the *MAPK8IP3* and *TKR* genes [108, 109]. Although the exact functions of

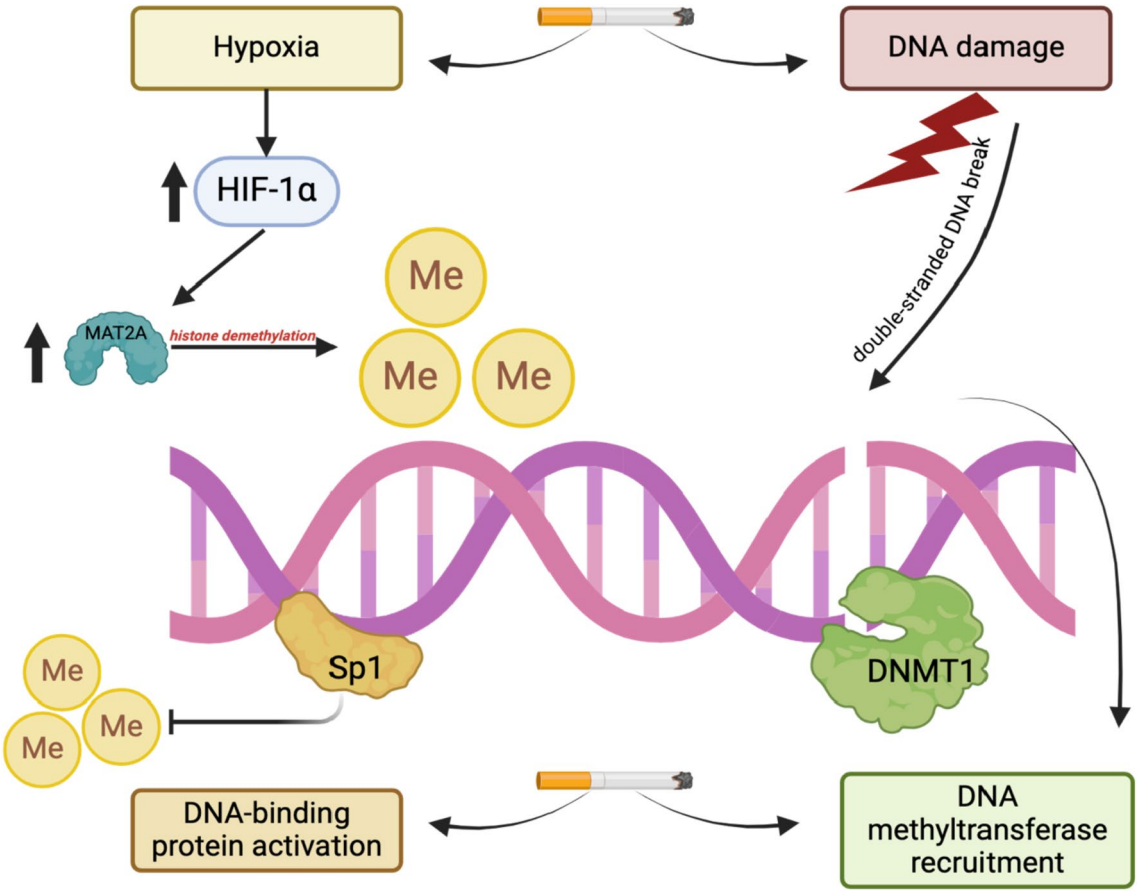

Fig. 5 Effects of tobacco smoking on sperm DNA methylation. Cigarette smoking is associated with alterations in the expression of *DNMT1*, at both the mRNA and protein levels. Smoking results in double-stranded DNA breaks, resulting in the recruitment of DNMT1 for repair, and the activation of DNA-binding proteins that protect DNA from de novo methylation. Moreover, hypoxia induced by cigarette smoke alters the availability of methyl groups during early embryonic development. Hypoxia upregulates HIF-1α-dependent MAT2a, which synthesises S-adenosylmethionine, a methyl donor for DNA methylation. Finally, tobacco smoke may also alter DNA methylation by differential expression and activity of DNA binding factors such as SP1, a transcription factor involved in early embryogenesis. Figure was adapted from Lee and Pausova [111] and created with Biorender.com

these genes are not completely understood, previous studies reported that *MAPK8IP3* may be involved in the regulation of signalling pathways involved in sperm capacitation [38]. Differential DNA methylation exhibits a transgenerational inheritance pattern as observed in the cord blood of human F1 offspring from smoking parents [110]. The most recent epigenome-wide association study revealed that the pubertal onset of father's smoking is associated with hypermethylation of 19 CpGs (FDR < 0.05) mapped to promoter regions of 14 genes (*TLR9*, *DNTT*, *FAM53B*, *NCAPG2*, *PSTPIP2*, *MBIP*, *DRC1*, *NTRK2*, *DNAJC14*, *CDO1*, *PRAP1*, *TPCN1*, *IRS1,* and *CSF1R*) in their offspring [94]. In particular, these sites were associated with phenotypic changes in the offspring, including asthma (NTRK2), wheezing (DNAJC14, TPCN1), increased weight (FAM53B, NTRK2), and increased BMI (FAM53B, NTRK2) [94].

Alterations in mRNA and miRNA associated with smoking

Smoking also affects sperm mRNA content. A previous study found that the sperm (n = 4) transcript levels of 781 genes, including protamine 2 (*PRM2*), differed between smokers and non-smokers [114]. Although pathway analysis of the differentially expressed genes was not informative due to the potential degradation of mRNA in sperm, the upstream sequence analysis of genes suggested the involvement of transcription factors related to stress response regulations, including NF-kB, FOX and YY1 in smokers [114]. As sperm mRNAs are delivered to the oocyte at fertilisation, their alterations due to paternal smoking could be passed onto the offspring and predispose them to chronic diseases, such as asthma [88, 134]. With regard to non-coding RNAs, other research with mice found that exposure to increasing doses of cigarette smoke upregulated 13 miRNAs and downregulated 32 miRNAs in sperm when compared to controls [68]. Related to this, it is worth mentioning that miR-204-5p and miR96-5p, the expression of which is altered after exposure of male mice to cigarette smoke, are associated with Hippo and oestrogen signalling pathways which are involved in lung and early embryo development, respectively [39, 176]. In addition, the dysregulations of miR-133b-3p, miR-196a-5p and miR-205-5p in sperm, which were also detected in response to exposure of male mice to cigarette smoke in the study of [68], also occur when male mice eat an HFD, thus suggesting that they may also be involved in the transmission of obesity and insulin resistance to the offspring [54].

Effect of endocrine-disrupting chemicals

Endocrine-disrupting chemicals (EDCs) are exogenous substances that interfere with the endocrine system, disrupting homeostasis and the regulation of developmental processes. EDCs mimic endogenous hormones due to similarities in structure, leading to either the activation or inhibition of receptors involved in neurological, cardiovascular, immunologic, and reproductive disorders [90]. Since the 1950s, evidence has accumulated regarding the deleterious effects of the EDCs present in human-made mass, including plastics, plasticisers, surfactants, pesticides, and herbicides. Accordingly, these EDCs may underlie disorders such as cryptorchidism, hypospadias, and testicular cancer and can also reduce sperm quality and function [90]. Metal toxicity, particularly from transition metal ions such as iron, copper, cobalt, and vanadium, is mediated by the generation of reactive oxygen species and their interaction with structural proteins and DNA [130]. A systematic review and meta-analysis by Pizzol et al. [148] showed that exposure to occupational and environmental pollutants affects sperm quality, causing motility impairment, by altering the plasma membrane fluidity and electrochemical potential, disrupting mitochondrial function, and increasing oxidative stress [148].

Previous research showed that the effects of some of these EDCs could be passed onto the offspring, thereby increasing their predisposition to suffer from infertility, testicular disorders, obesity, and PCOS in females. Specifically, it has been reported that EDCs could alter epigenetic marks during gametogenesis, as primordial germ cells are highly sensitive to environmental factors [122, 138, 160]. In rats, for example, the intraperitoneal administration of plastic-derived compounds to gestating females, such as bisphenol-A (BPA), bis (2-ethylhexyl) phthalate (DEHP), and dibutyl phthalate (DBP), was observed to result in changes in 197 differentially DNA methylated regions, which could underlie the pubertal abnormalities, testis dysfunction, obesity, primary ovarian insufficiency, and polycystic ovaries observed in the F3 generation of male and female rats [123]. Other studies focusing on BPA, which is commonly found in domestic products, demonstrated that BPA acts as an obesogenic, thyroid hormone modulator, and anti-androgenic and oestrogen agonist and can alter spermatogenesis by affecting the sperm epigenome and by reducing motility in human, mouse, chicken, fish, and bovine sperm [26, 124]. Consistent with these observations, Ho et al. found that exposing rats to low doses of BPA led to hypomethylation of the gene promoter region coding for phosphodiesterase type 4 variant 4 and increased protein synthesis [72]. These findings also concur with Dolinoy et al., who observed a change in the coat colour of Agouti mice

when exposed to BPA, likely due to hypomethylation in nine CpG sites upstream of the *Agouti* gene [43]. In rats, maternal exposure to BPA has also been linked to alterations in the expression of miR-361-5p, miR-203a-3p, and miR-19b-2-5p in the testis, whose predicted target genes are related to the RNA polymerase II promoter, an essential gene regulator [120]. These alterations, together with the mitochondrial swelling observed with an increase in BPA dose, could underlie the significant weight gain of the offspring of these BPA-exposed mothers, as well as impaired spermatogenesis in sexually mature offspring as mitochondria are essential for energy supply to the testis [120] (Fig. 6).

Humans are commonly exposed to phthalates and phthalate esters, such as DEHP, a form of EDC that is commonly used in food processing and packaging. In humans, greater concentrations of paternal urinary DEHP were found to be associated with an increased rate of infertility treatment failure [12, 126]. In mice, the sperm of males exposed to DEHP during spermatogenesis exhibited 704 differentially methylated regions (DMRs); these genes were involved in biological pathways associated with development ("epidermal cell differentiation", "cell–cell adhesion", "head, cardiovascular, sensory and skeletal development", and "embryonic organ development") [137]. These alterations were potentially transferred to the offspring, as mating DEHP-exposed males and non-exposed females prior to conception was reported to lead to alterations in 1716 DMRs in embryonic and 3181 DMRs in extraembryonic tissues, and 29 DMRs overlapped between sperm and F1 tissues [137]. Specifically, altered methylation profiles were mostly observed in the Pax (paired box) gene family in F1 tissues, which encode transcription factors that regulate cellular differentiation and embryonic tissue development related to the thymus, lymphocytes, ear, kidneys, CNS, and eye [18]. Another study found that the detrimental effects of exposing mice to DEHP on fertility, testicular steroidogenic capacity, and spermatogenesis of F3 males were more severe in the paternal lineage than in the maternal lineage [12]. This greater impact was suggested to be associated with changes in the testicular transcriptome of F3 males of the paternal lineage, with a reduction in the expression of Dynein Light Chain LC8-Type 1 (*Dynlt1a*) and an increase in the expression of zinc finger protein (*Zfp*) [12] (Fig. 6).

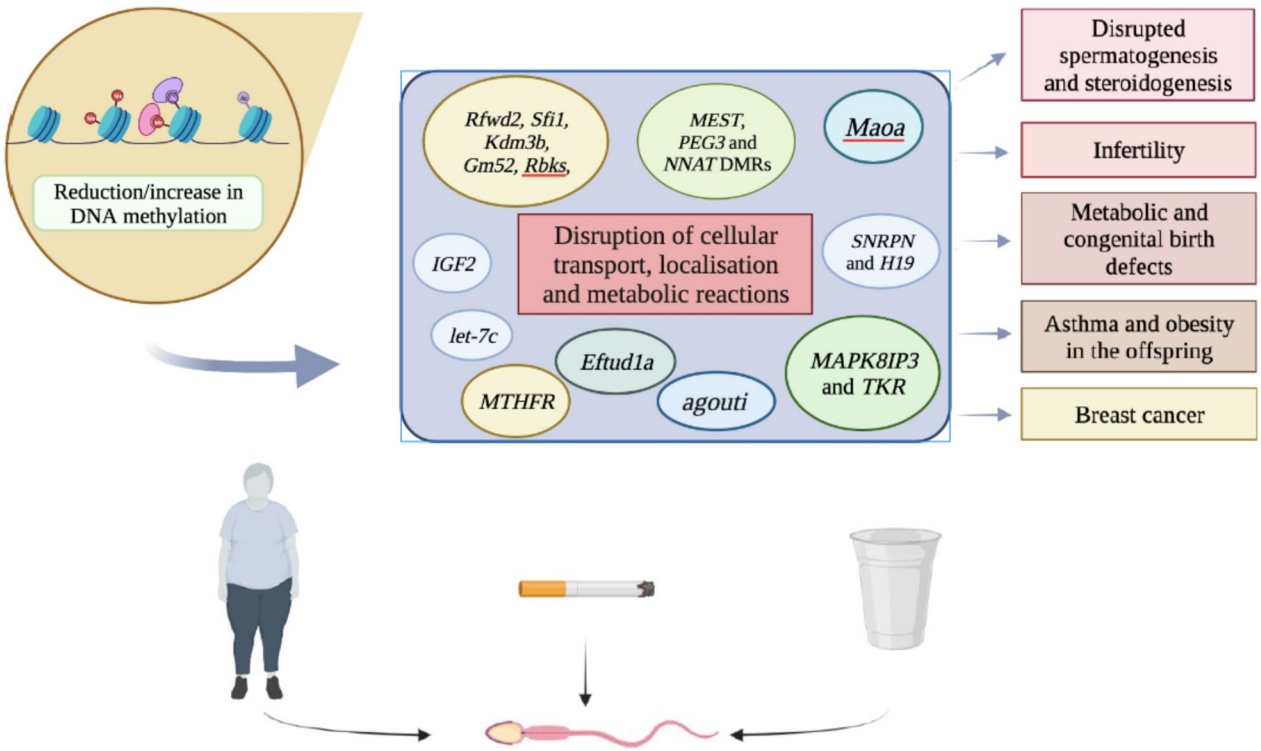

Fig. 6 Altered genes as a result of paternal obesity, smoking, and EDCs. Several studies have demonstrated that paternal lifestyle and environmental factors can influence the expression of a number of genes and transcriptional factors, which are responsible for cellular transport, localisation, and metabolism during spermatogenesis and embryo development, by hypo- or hypermethylation DNA sequence. This may, in turn, result in deleterious effects both in fathers, namely disrupted production of sperm and steroid sex hormones, resulting in male infertility, and in the offspring, predisposing to metabolic, structural defects, and cancer formation. Figure was created with Biorender.com

Related to this, it is worth mentioning that alterations in the expression of *Dynlt1a* have been associated with defective spermatogenesis in mice and *Drosophila* and that deficiencies in the expression of *Zfp* in pachytene spermatocytes are associated with sperm differentiation failure and decreased fertility [50, 74, 80, 198]. Collectively, these findings suggest that the Y chromosome could play a relevant role in transferring the effects of DEHP exposure to gene expression regulation and reproductive health in offspring.

Effects of stress

If the above-mentioned factors are mostly modifiable by lifestyle changes, then stress and psychological trauma are less agile. There is evidence that the offspring of Holocaust survivors, Vietnamese refugees, and those involved in war military action, have an increased risk of mental health issues [154, 173]. While maternal exposure to stress factors has been studied at great length, relatively little is known about the effects of paternal stress on offspring health.

A previous study conducted in mice found that exposure to chronic stress through different conditions (36 h of constant light, 15 min of exposure to fox odour, and cage changes) led to alterations in the hypothalamic–pituitary–adrenal (HPA) axis of the offspring, thus suggesting an effect on germ cell epigenetic reprogramming [150]. The reduced responsivity of the HPA axis, which was more obvious in the offspring of stressed fathers, was found to be related to changes in the expression of glucocorticoid-responsive genes in the paraventricular nucleus (PVN) and bed nucleus of stria terminalis (BNST), which are stress-regulating areas in the brain [150]. Specifically, nine miRNAs (miR-193-5p, miR-204, miR-29c, miR-30a, miR-30c, miR-32, miR-375, miR-532–3p, and miR-698) were found to be upregulated in the offspring of these fathers exposed to stress before conception [150]. The suggested mRNA targets of these nine miRNAs were DNMT3a, a regulator of de novo DNA methylation, trinucleotide repeat containing 6b *(Tnrc6b)*, and metadherin (*Mtdh*), which are all involved in miRNA processing in sperm [125, 151]. Other research found that F0 mice experiencing odour fear prior to conception produced F1 and F2 generations with an increased sensitivity to the specific odour [41]. In effect, the olfactory neuroanatomy had been altered for the conditioned odour, thus suggesting that this arose from epigenetic inheritance. Accordingly, not only male mice (F0) exposed to acetophenone, which stimulates the odorant receptor OLFR151, but also their offspring (F1) showed hypomethylation at the *Olfr151* (M71) gene. On the other hand, Wang et al. exposed male mice to several stressors for five weeks and observed that the levels of 19 miRNAs and 24 piRNAs were altered in their sperm; these effects were inherited by the F1 offspring. This epigenetic inheritance was posited to explain that mice born from males presenting a depression-like behaviour were more likely to develop such a behaviour later in their lives [27, 179]. In humans, chronic stress induced by abusive and/or dysfunctional family behaviour diminishes the levels of miRNA-449 and miRNA-34 family members in sperm, which are known to be associated with poor sperm quality and reduced fertility [42]. Further experiments in mice reported the same stress-induced changes in sperm miRNA of F1 males [42].

The influence of stress on sperm DNA methylation was also investigated by subjecting mice to unpredictable maternal separation and stress (MSUS). In the sperm of F1 males, MSUS modified the degree of DNA methylation in the promoters of different genes, including methyl CpG-binding protein 2 (MeCP2), a transcriptional regulator that binds methylated DNA; cannabinoid receptor-1 (CB1); and corticotrophin-releasing factor receptor 2 (CRFR2), a stress hormone receptor [53] (Table 1). These changes were functionally significant as they resulted in the reduced transcription of these genes. Furthermore, postnatal traumatic stress in males can also affect brain plasticity and cognitive functions of the offspring, possibly due to altered DNA methylation at the promoter region of the brain-specific gamma isoform of protein kinase C (*Prkcc*) that was observed in the father's sperm and also in the hippocampus of offspring [20]. Furthermore, a genome-wide study discovered that the sperm of mice exposed to chronic restrain stress in a tube for 2 h/day exhibited 24,427 DMRs in the F0 generation with 11.36% of inter-generational inheritance and 0.48% of trans-generational inheritance [197] (Table 1). The genes related to the epigenetically inherited DMRs included *Rhobtb3* expressed in the testis, which is associated with the development of the male reproductive system, and *Il12rb1,* which is associated with mouse adipose tissue metabolism and development [197].

Paternal stress has also been associated with alterations in sperm RNA content. A study conducted by Gapp et al. subjected male mice to postnatal MSUS (F1) and observed behavioural changes, including risk-taking behaviour, despair, and insulin hypersensitivity [57]. The sperm of these F1 males exhibited differentially expressed levels of miRNAs and piRNAs. Interestingly, when long non-coding RNAs and miRNAs were extracted from F1 sperm and injected in naïve zygotes, the resulting F2 generation partially exhibited this pattern of behaviour. The role of these miRNAs was confirmed when antisense miRNAs injected into the zygote reversed the phenotype [179] (Table 1).

Table 1 Effect of paternal stress on paternal and offspring's behaviour and metabolism. Previous research showed that paternal stress in the form of MSUS, chronic restraint, chronic variable stress, and social instability might alter offspring behaviour, predisposing them to increased depressive-like behaviour and increased stress sensitivity. Furthermore, increased blood glucose levels and increased body weight of fathers may also influence the offspring. As literature supports, the sperm epigenome (DNA methylation, miRNA, lncRNA, etc.) accounts for the transgenerational inheritance of these behavioural and metabolic features

Study	Stressor	Epigenetic changes in sperm	Epigenetic changes in the offspring	Paternal phenotype		Offspring phenotype	
				Observed behavioural changes	Observed metabolic changes	Observed behavioural changes	Observed metabolic changes
[53]	MSUS	DNA methylation	DNA methylation	Increased depressive-like behaviour and increased anxiety	NA	Increased depressive-like behaviour and increased anxiety	NA
[55]	MSUS	miRNA, tRF, piRNA, DNA methylation	miRNA	Reduced escape response, resistance to aversive conditions	Increased GR expression, decreased methylation of GR promoter in the hippocampus	Reduced escape response, resistance to aversive conditions	Increased GR expression, decreased methylation of GR promoter in the hippocampus
[183]	Chronic restraint	DNA methylation	DNA methylation in the brain	NA	Increased DNA methylation in the *Sfmbt2* promoter in the F0 sperm	NA	Hyperglycemia, hepatic overproduction of PEPCK, increased DNA methylation in the *Sfmbt2* promoter in the F1
[57]	MSUS	lncRNA	lncRNA (zygote)	Increased risk-taking behaviours	NA	Increased risk-taking behaviours	Increased food consumption, altered glucose clearance
[56]	Dexamethasone	miRNA, tRF, rRNA, circRNA	tRF	NA	NA	NA	Higher BMI and aberrant glucose metabolism
[36]	Chronic social defeat stress	lncRNA	NA	Increased stress sensitivity	NA	Increased stress sensitivity, increased anxiety-like behaviour	NA
[179]	Chronic variable stress	miRNA, piRNA	N/A	Increased depression-like behaviour	Decreased body weight gain	Increased depression-like behaviours, enhance HPA axis activity	Increased plasma corticosterone levels
[197]	Chronic restraint	DNA methylation	DNA methylation in embryo	Lower anxiety, higher risk-taking behaviour	Increased body weight, increased blood glucose level	Lower anxiety, higher risk-taking behaviour	Developmental retardation, increased blood glucose level

Table 2 Comparative effects of lifestyle factors on epigenetic markers in the sperm epigenome. The table summarises how obesity, high-fat diet, smoking, stress, and exposure to endocrine-disrupting chemicals (EDCs) influence three key epigenetic mechanisms: DNA methylation**,** histone modifications, and small non-coding RNAs (sncRNAs). Observed effects include disruptions in metabolism, sperm quality, and developmental processes in offspring, highlighting the significance of these factors in reproductive health and transgenerational inheritance

Lifestyle factor	DNA methylation	Histone modifications	sncRNA (miRNA, piRNA, tsRNA)
Obesity	Hypomethylation of IGF2, MEST, PEG3, and NNAT associated with altered metabolism and cancer risks in offspring	Retention of H3K27me3 in certain genes affects gene regulation during embryonic development	Altered expression of miRNAs and tsRNAs, linked to metabolic disorders in offspring
High-fat diet	Altered methylation of imprinted genes affects insulin secretion and glucose metabolism	Increased H3K27me3 in metabolic and growth-related genes in sperm	Dysregulation of tsRNAs and miRNAs linked to metabolic changes and testicular function
Smoking	Hyper- and hypomethylation of specific genes (e.g. TYRO3, PGAM5) linked to infertility and metabolic diseases	Increased retention of histone H2B, affecting chromatin remodelling during fertilisation	Altered miRNA expression (e.g. miR-204-5p), linked to reduced sperm quality
Stress	DNA methylation changes in stress-response genes (e.g. MeCP2, CB1) associated with behavioral changes	Not well documented for stress but may involve loss of key histone marks during sperm formation	Alterations in miRNA and piRNA levels, linked to behavioral traits and HPA axis function
Endocrine-disrupting chemicals (EDCs)	Hypomethylation of DNA regions regulating developmental and metabolic genes in offspring	Altered histone modifications affecting gene regulation and embryonic development	Dysregulation of miRNAs involved in spermatogenesis and embryogenesis

Collectively, these findings support the fact that stress in males and females prior to conception induces changes in the epigenome, and that these effects can be, in the case of males, trans-generationally inherited via sperm (Table 2).

Sperm epigenome, male fertility, and assisted reproductive technology (ART)

The number of infertile couples seeking treatment with ART is steadily growing. Epidemiological studies support that around 30% of infertility cases are due to a male factor, and mounting evidence indicates that the number of patients with reduced sperm quality is increasing [28, 75]. The outcome of ART largely depends on embryo quality, which is undoubtedly influenced by maternal and paternal factors [142]. For this reason, identifying the paternal factors that may affect embryo quality is important, especially if one bears in mind that ICSI, which is widely used for the treatment of male infertility, may increase the odds of vertical transmission of paternal genetic and epigenetic marks due to bypassing the natural selection process. Furthermore, it is worth noting that increased paternal age (> 51 years) can negatively influence sperm quality and ART outcomes, which may be significantly influenced by alterations in epigenetic marks [28, 129].

The diagnosis of male infertility is mainly based on a conventional spermiogram, which includes the assessment of semen volume, total sperm count, total motility, progressive motility, viability, and morphology [21]. In spite of this, sperm count, morphology, and concentration are not always correlated with pregnancy rates; thus, semen analysis may not be a good predictor for pregnancy, particularly in patients suffering from idiopathic infertility [84] (Table 3). Interestingly, the sperm of infertile patients exhibits an altered pattern of DNA methylation that affects, among others, the promoter region of the *HSPA1L* gene, which encodes for a testis-specific heat shock protein and could be responsible for a reduced pregnancy rate [84]. While these altered patterns of DNA methylation are observed in 54% of couples who fail to conceive, these epigenetic marks may still be influential to male fertility and ART outcomes despite the lack of independent causation [84, 105, 177]. In effect, another retrospective cohort study showed that sperm DNA methylation patterns could be highly predictive of fertility and, to a lesser extent, of IVF embryo quality [10]. In this previous study, patients undergoing IVF treatment were split into two groups: (1) patients with good embryogenesis and a positive pregnancy outcome and (2) patients with poor embryogenesis and both positive and negative pregnancies. The genome-wide sperm DNA methylation of the two groups identified 8,500 differentially methylated regions, which were associated with genes regulating cell adhesion, a critical process in embryogenesis and sperm–oocyte fusion [10].

sncRNA in sperm have also been suggested to predict ART outcomes. In effect, up to 648 sperm RNA elements (SREs) appear to be essential for the success of IVF treatment, as the lack of a single element can underlie a significant reduction in the success rate [87]. Nine of these 648 SREs are located in intergenic regions, 12 are found in sperm-specific intronic elements, and 42 reside in non-coding regulatory RNA regions [87]. Most of these SREs are located in the exonic regions of 262 different genes, which are associated with spermatogenesis, sperm function, fertility, and early embryogenesis [87].

As discussed earlier, epigenetic marks, which are in turn affected by lifestyle and environmental factors, may influence both sperm quality and function. One can thus hypothesise that ART outcomes might rely upon the lifestyle choices of males and environmental factors that can be largely preventable. For instance, weight loss could affect the sperm DNA methylome [167]. Indeed, bariatric surgery in obese men was shown to modify, as early as one week after the intervention, the sperm DNA methylation profiles of 1,509 genes, including master regulators of appetite control, including melanocortin-4 receptor (*MC4R*; brain-derived neurotrophic factor (*BDNF*); neuropeptide Y (*NPY*); cannabinoid receptor type 1 (*CR1*); and genes related to obesity and metabolism, such as fat mass and obesity associated (*FTO*), carbohydrate sulfotransferase 8 (*CHST8*), and SH2 binding domain-containing protein 1 (*SH2B1*) [45]. A systematic review and meta-analysis by ElGendy et al. [49] showed that bariatric surgery could increase the methylation of *PDK4* loci in skeletal muscle and blood and reported that twelve studies found improved metabolic and inflammatory profiles consistent with the reversibility of DNA methylation [49]. As the effects of bariatric surgery on DNA methylation exhibited high levels of inconsistency, more research in this realm is warranted [49].

On the other hand, specific food items and social habits may influence sperm concentration, motility, and fertilising ability, as revealed by an observational study in 250 male patients undergoing ICSI [23]. Specifically, sperm concentration was negatively influenced by BMI and alcohol consumption and positively influenced by cereal consumption. The same observational study (n = 250) observed a negative impact for the paternal consumption of alcohol and coffee on ICSI outcomes [23]. A randomised controlled trial showed that doing physical activity and eating a healthy Mediterranean diet for a short period improve sperm concentration, total and progressive motility, and proportion of normal morphology cells [127]. Although this study did not interrogate the effects of lifestyle factors on the sperm epigenome,

Table 3 Studies investigating the effect of epigenetic marks and lifestyle and environmental factors on sperm parameters and ART outcomes. The studies published from 2012 to 2022 showed the effect of epigenetic changes, lifestyle and environmental factors, or both on the outcomes of ART and sperm parameters such as sperm concentration, motility, and morphology

Study	Study population	Epigenetic marks	Lifestyle and environmental factors	Sperm parameters change	Impact on ART
[152]	245 couples who underwent ART cycles between 2007 and 2020	NA	8 dietary patterns based on healthy dietary scores	No association between diet and semen parameters	No association between any dietary pattern and ART outcomes of IR, PR, and LBR
[23]	250 men undergoing ICSI	NA	Food intake and social habits (smoking, alcohol consumption, exercising)	The SC was negatively affected by BMI and alcohol consumption, whilst positively affected by cereal consumption and the number of meals per day. The SMot was negatively altered by BMI, alcohol intake and smoking, and positively influenced by fruits and cereals intake	FR was negatively associated with alcohol consumption, whereas red meat and weight loss diet affected the IR and PR
[86]	27 couples who conceived within 2 months, 29 couples that could not achieve a pregnancy within 12 months	Sperm DNA methylation	BMI	No association between male BMI, SC, and SM	Male BMI did not affect the pregnancy outcomes Hypomethylation of HSPA1L and HSPA1B was significantly associated with decreased PR
[10]	127 men undergoing IVF treatment due to male factor infertility, 54 normozoospermic, fertile men	Genome-wide sperm DNA methylation	NA	NA	Differential methylation between normozoospermic donors and infertile IVF patients is consistently observed. Altered global methylation is indicative of poor embryo quality
[87]	96 couples with idiopathic infertility seeking ART treatment	Sperm RNA elements (SREs)	NA	No correlation between sperm parameters and LBR. SREs within the context of sperm quality was not investigated	Absence of SREs was associated with lower LBR in timed intercourse and IUI, but not in IVF and/or ICSI
[45]	23 Caucasian male patients (20–40 years): 13 males with BMI of 20–25 and 10 obese (BMI > 29.7) patients	Histone position was unaltered between lean and obese patients, whilst 37 piRNAs were found altered in obese patients' sperm sample Weight loss surgery induced changes in the methylation status of 1509 genes a week after, and 3910 a year after the surgery	Obesity and the effects of surgery-induced weight loss	NA	NA
[155]	2370 couples undergoing infertility treatment	NA	Dietary supplements of either 5 mg of folic acid and 30 mg of elemental zinc or placebo daily for 6 months	Sperm parameters were not significantly different between two groups	LBR was not associated with the dietary intake of supplements

NA, not applicable; SC, sperm concentration; SMot, sperm motility; SM, sperm morphology; FR, fertilisation rate; IR, implantation rate; PR, pregnancy rate; LBR, live birth rates

these above-mentioned alterations in DNA methylation, RNA content, and subsequent changes in gene expression could possibly explain these phenotypic findings.

Salas-Huetos et al. showed that there was no association between dietary patterns and ART outcomes in a prospective cohort study (n = 245) [152]. The discrepancies with previous studies can be explained by the change in dietary patterns of sub-fertile couples when compared to the general population blinded to their semen quality [152]. A randomised clinical trial by Schisterman et al. presented similar results in that no significant difference was detected between a group taking daily folic acid and zinc supplements and a placebo group [155]. These findings do not concur with the findings of Wu et al., who reported that a group with oligozoospermia had higher methylation patterns of *MTHFR* in a case–control study (n = 561), thus indicating the significance of folate on the quality of sperm and fertility [184]. Due to these inconsistent data, the effects of dietary patterns on male fertility require further studies among men seeking fertility treatment. Considering the body of literature reporting the effects of diet and lifestyle on epigenetic marks in sperm DNA, which may potentially predict the success of ART, this subject should be studied further with a particular focus on the sperm epigenome [175].

Similar to the way alterations in sperm epigenetic marks can influence ART outcomes, ART might negatively affect sperm epigenetic marks and, subsequently, embryo quality and offspring health. Couples undergoing ART treatment often already have epigenetic alterations, such as approximately 41% of individuals seeking fertility treatment have altered methylation patterns in their sperm [96]. These epigenetic marks can, therefore, be transferred to the embryo during IVF and/or ICSI, potentially affecting the embryo development. Moreover, ART manipulations of gametes and embryos require exposure to various environmental factors such as oxygen levels, culture media composition, and temperature, which could impact sperm epigenetics and epigenetic reprogramming in embryo [121]. Hence, addressing the effects of paternal lifestyle choices on sperm epigenetics and its transgenerational inheritance, as well as the possible ways of reversing the epigenetic changes in sperm, is significant in relation to ART, which can potentially reduce risk of transmitting altered epigenetic marks during ART treatment.

Conclusions

The sperm epigenome, a highly unique and complex record of changes to DNA methylation, histone modifications, and the expression of sncRNA, offers a potential tool for studying sperm quality and function. Sperm epigenetics and the influence of lifestyle and environmental factors have been the subject of increasing interest, as the exact mechanism and effect of epigenetic changes remain largely unknown. Nevertheless, the sensitivity of these epigenetic marks to lifestyle and environmental changes, including smoking, obesity, endocrine-disrupting chemicals, and paternal stress, has been associated with such conditions as oligozoospermia, asthenozoospermia, testicular dysgenesis syndrome, and infertility in men. Most importantly, alterations in the sperm epigenome could be trans-generationally inherited in the offspring, both in human and animal models, thus increasing the predisposition to various chronic diseases, metabolic disorders, and obesity. The significance of such reviews and research into the epigenetic marks in sperm, such as DNA methylation, histone modification, and sncRNA expression, underscores how paternal lifestyle and environmental factors can impact fertility outcomes and the offspring's health. This can have potential clinical implications on avoiding certain "unhealthy" behaviours or integrating healthy habits such as diet and physical activity to reverse epigenetic changes in sperm, which might subsequently improve sperm quality and decrease the predisposition to certain metabolic diseases in fathers and future generations. Moreover, recent studies have suggested that paternal genetic variants, such as eNOS deficiency, can alter the sperm epigenome without transmitting the genetic defect itself and predisposing offspring to metabolic changes through decreased DNA methylation of GR and PGC1A gene promoters [73, 192, 193]. The paternal impact on the epigenetic markers in the sperm and its transgenerational effect are, therefore, being increasingly studied from different perspectives. Further studies should aim to encompass the impact of paternal genetic variants on early epigenetic modifications in sperm, including alterations in microRNAs, tRNA-derived small RNAs (tsRNAs), and DNA methylation patterns.

Targeting lifestyle changes has several clinical applications to mitigate the preconceptual adverse epigenetic changes. Including folate in the diet, avoiding high-fat and high-sugar diets, smoking cessation could improve DNA methylation and reduce DNA and/or RNA changes, thus positively affecting sperm parameters and embryo quality. Weight management in fathers has a potential to decrease the risk of metabolic diseases in the offspring. Hence, preconception health counselling can offer lifestyle modification programs that might address the epigenetic alterations due to stress, diet, physical activity and EDCs.

Although current evidence suggests a consistent association between epigenetic marks, which are influenced by lifestyle and environmental factors, and sperm quality and ART outcomes, some limitations of these

studies should be addressed. First of all, the techniques and approaches used to investigate the sperm epigenome have evolved rapidly over recent years, which leads one to anticipate that further studies using these techniques can be useful. Secondly, sperm quality and epigenetics are known to be significantly influenced by ageing, thus isolating the sole effect of epigenetic marks and lifestyle factors, which tend to accumulate over time and may thus pose a challenge to scientists. In addition, studies on the association between the sperm epigenome and ART outcomes are mostly undertaken with patients seeking infertility treatment; this may influence the results as patients are more likely to present with an underlying health condition, alterations in lifestyle before ART treatment, and recall bias. The lack of data, interrogating the dose-dependency of these lifestyle and environmental factors, explains the vast discrepancies in research relating to sperm epigenetic marks. Yet, the advancement of RNA sequencing and methylation arrays, such as the MethylationEPIC array with over 850,000 methylation site probes, provides promising opportunities for further research. Future studies addressing the causal relationship between lifestyle factors and sperm epigenetic factors should ideally include longitudinal, randomised-controlled trials with the use of advanced technological tools. Although there are special sperm selection techniques currently in use in ART clinics, such as density gradient centrifugation and swim-up, the future use of epigenetic marks as tools for sperm and embryo quality assessment could open the door to the future use of 'epigenotyping', the personalised treatment of male infertility, and epigenetically transferrable chronic conditions in the next generations.

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A.A. wrote the Manuscript. M.Y. supervised the work and critically revised the Manuscript. C.J. and K.C. made a critical revision of the Manuscript. All authors approved the final version.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

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The authors declare no competing interests.

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